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Prolyl isomerases are important determinants of intracellular pH homeostasis in *Arabidopsis thaliana*

TESIS DOCTORAL

by

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CERTIFICAN

Que Don Gaetano Bissoli, licenciado en Biotecnologías, ha realizado bajo nuestra supervisión la tesis doctoral titulada "Prolyl isomerases are important determinants of of intracellular pH homeostasis in *Arabidopsis thaliana"*

Y para que así conste, firmamos la presente en Valencia, a 5 de Enero 2013

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Abstract

Our previous work in *Arabidopsis* has demonstrated that the presence of acetic acid, a weak organic acid, induces the expression of ROF2, an FKBP immunophilin protein. FKBPs are peptidyl prolyl *cis-trans* isomerases (PPIase EC 5.1.2.8) that catalize the isomerization of peptidyl prolyl bonds between the *cis* and *trans* configuration. FKBP are ubiquitous proteins that can be found either as a single catalytic domain proteins or being part of more complex proteins. To assess the implication of FKBP proteins in weak acid tolerance in plants we have generated plant lines overexpressing *Arabidopsis* FKBP65 (ROF2). In presence of acetic acid, *35S::ROF2* grew better than wild type plants. On the other hand a *rof1 rof2* mutant line showed weak organic acid sensitivity whereas the single *rof1* and *rof2* knock-out mutant have a similar behaviour than the wild type. Our results confirm that over-expression of *ROF2* confers tolerance to intracellular acidification by increasing proton extrusion from cells. The phenotype can be explained by indirect activation of plasma membrane proton pump (H^+-ATPase) because ROF2 activate K^+ uptake and this causes depolarization of the plasma membrane and activation of the electrogenic pump. In absence of stress, in *35S::ROF2* transgenic plants we have observed floration delay, apical dominance and increased seed size. We have also found that *35S::ROF2* plants has an enhanced response to IAA. Furthermore the roots of *rof1 rof2* mutants have a reduced number of lateral roots and exogenous application of IAA was able to revert this phenotype. Our hypothesis is that ROF2 is a positive regulator of either auxin biosynthesis or perception.

Resumen

Nuestro trabajo previo en *Arabidopsis* ha demostrado que la presencia de ácido acético, un ácido orgánico débil, induce la expresión de *ROF2*, una proteína que pertenece a las inmunofilinas de tipo FKBP. FKBPs son peptidil prolil *cis-trans* isomerasas (PPIase CE 5.1.2.8) que catalizan la isomerización de los enlaces peptídicos de las prolinas entre la configuración *cis* y *trans*. FKBP son proteínas ubicuas que se pueden encontrar como un solo dominio catalíticos en la proteína o el dominio es parte de proteínas más complejas. Al fin de evaluar la implicación de las proteínas FKBP en la tolerancia al ácido débil en las plantas, hemos generado líneas de *Arabidopsis* que sobre-expresan FKBP65 (ROF2). En presencia de ácido acético, las líneas *35S::ROF2* crecieron mejor que las plantas de tipo silvestre. Por el otro lado la línea doble mutante *rof1 rof2* mostró una sensibilidad a ese ácido orgánico débil, mientras que los mutantes individuales de pérdida de función *rof1* y *rof2* tienen un comportamiento similar a la de tipo silvestre. Los resultados confirman que la sobre-expresión de ROF2 confiere tolerancia a la acidificación intracelular mediante el aumento de extrusión de protones de las células. El fenotipo puede explicarse por la activación indirecta de la membrana plasmática de la bomba de protones (H^+-ATPasa) porque ROF2 activa la toma de K^+ y la absorción de este facilita despolarización de la membrana plasmática y la activación de la bomba electrogénica. En ausencia de estrés, en plantas transgénicas *35S::ROF2* hemos observado retraso en la floración, un aumento de la dominancia apical y un mayor tamaño de la semillas. También se ha encontrado que las plantas *35S::ROF2* tienen una mayor respuesta a IAA. Además las raíces de los mutantes *rof1 rof2* tienen un reducido número de raíces laterales y la aplicación exógena de IAA fue capaz de revertir este fenotipo. Nuestra hipótesis es que ROF2 es un regulador positivo o de la biosíntesis de auxinas o de su percepción.

Resum

El nostre treball previ en Arabidopsis ha demostrat que la presència d'àcid acètic, un àcid orgànic feble, indueix l'expressió de *ROF2*, una proteïna que pertany a les immunofilines de tipus FKBP. Les FKBPS son peptidil prolil cis-trans isomerase (PPIASE CE 5.1.2.8) que catalitzen l'isomerització dels enllaços peptídics de les prolines entre la configuració cis i trans. FKBP son proteïnes ubiqües que es poden trobar com un únic domini catalític en la proteïna o el domini es part de proteïnes mes complexes. A fi d'avaluar la implicació de les proteïnes FKBP en la tolerancia a l'àcid feble en les plantes, hem generat línies d'Arabidopsis que sobre-expressen FKBP65 (*ROF2*). En presencia d'àcid acètic, les línies *35S::ROF2* creixeren millor que les plantes de tipus silvestre. Per l'atra banda la línia doble mutant *rof1 rof2* mostrà sensibilitat a àcid orgànic feble, mentre que els mutants individuals de perdua de funció *rof1* i *rof2* tenen un comportament similar a la de tipus silvestre. Els resultats confirmen que la sobre expressió de *ROF2* confereix tolerància a l'acidificació intracelular mitjançant l'augment de la extrussió de protons de les cél.lules. El fenotipus pot explicar-se per l'activació indirecta de la membrana plasmàtica de la bomba de protons (H^+-ATPASA) perque *ROF2* activa l'entrada de K^+ i l'absorció d'aquest facilita la despolarització de la membrana plasmàtica i l'activació de la bomba electrogènica. En absencia d'estrés, en plantes transgéniques *35S::ROF2* hem observat retard en la floració, una pujada de la dominància apical i una major grandària de les llavors. També s'ha trobat que les plantes *35S::ROF2* tenen una major resposta a IAA. A més a més ls arrels dels mutants *rof1 rof2* tenen un reduït nombre d'arrels laterals i l'aplicacio exogena d'IAA fou capaç de revertir aquest fenotipus. La nostra hipòtesi es que ROF2 es un regulador positiu o de la biosíntesi de les auxines o de la seva percepció.

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SYMBOLS AND ABREVIATIONS

4-Cl-IAA: 4-ChloroIndole-3-acetic acid
ABA: Abscisic Acid
ADP: Adenosine 5'-diphosphate
AHA: *Arabidopsis* H^+-ATPase
AMP: Adenosine 5'-monophosphate
Amp^r: ampicilin resistance
ARF: Auxin Response Factor
Atm: Atmosphere
ATP: Adenosin 5'-Tri-Phosphate
BLAST: Basic Local Alignment Search Tool
CaM: CalModulin motif
CBL: Clacineurin B-like Protein
cDNA: Complementary DNA
CIPK: CBL-Interacting Protein Kinase
CO1: Constant 1 (gene)
dNTP: deoxyriboNucleotide TriPhosphate
DR5: synthetic auxin response element
dw: dry weight
EDTA: Ethylen Diamin Tetra Acetic acid
Em: Electrical membrane potential
ER: Endoplasmic Reticulum
FKBP: FK506 Binding Protein
FPR: FK506-sensitive Proline Rotamase
fw: fresh weight
GFP: Green Fluorescent Protein
GNGC: Cyclic Nucleotide Gated Channel
GORK: Guard cell Outward Rectified K^+ channel
GUS: GUS: β-glucuronidase
HAc: Acetic Acid
HAK: High Affinity K^+ transporter
HSP: Heat Shock Protein
IAA: Indol-3-Acetic Acid
IBA: Indole 3-Butyric Acid
Kan^r: Kanamicyn resistance
KAT: K^+ *Arabidopsis thaliana*
KC1: *Arabidopsis thaliana* K^+ rectifying Channel 1
KCO: K^+ channal Ca^+ -activated Outward rectifying
KT: K^+ transporter
KUP: K^+ Uptake Permease
LB: Left Border
LB: Luria-Bertani broth
MAE: MOPS Na^+ Acetate EDTA buffer
MAPK: Mitogen Activated Protein Kinase
MCP: Misfolded Cytosolic Protein
MCS: Multi Cloning Site
MES: 2-(N-morpholino)ethanesulfonic acid
MOPS: 3-(N-morpholino) propanesulfonic acid
MS: Murashige-Skoog (medium)
M-MuLV: Moloney Murine Leukemia Virus
mV: milli Volts
NHX: Na^+/H^+ eXchanger
OAA: ossalacetate ion
OLB: Oligo Labeling Buffer
ORK: Outward Rectifying K^+ channel
PAA: Phenyl Acetic Acid
PBS: Phoshate-buffered saline
PCI: Phenol/Chloroform/Isoamylic alcohol
PCR: Polymerace Chain Reaction
PDR12: Pleiotropic Drug resistance 12
PEP: Phophoenolpyruvate
PGP12: P-GlycoProtein 12
pH: $-\log_{10}$ H^+ concentration
pHc: cytosolic pH
Pi: inorganic phospate
pKa: $-\log_{10}$ of constant of H^+ dissociation in weak acids
PM: plasma membrane
PPI: peptidyl prolyl *cis-trans* isomerase
PPi: Pyrophosphate
PSE: Na_2HPO_4 SDS EDTA buffer
RB: Right Border
ROF2: Rotamase FKBP 2 (gene)
ROS: Reactive Oxygen Species
r.p.m.: revolutions per minute
SAIL: Syngenta *Arabidopsis* Isertion Library
SDS: Sodium Dodecyl Sulfate (detergent)
SKOR: Stelar K^+ Outward Rectifier
SOC: Super Optimal broth with Catabolite repression
SOC1: Suppressor of Over-expression of CO 1 (gene)
SPIK: Shaker Pollen Inward K^+ Channel
SSC: Saline Sodium Citrate
TCES: Tris, NaCl, EDTA, SDS
TE: Tris EDTA buffer
TPK: Tandem Pore K^+ Channel
TRH1: Tiny Root Hair 1 (gene)
v/v: volume/volume
w/v: weight/ volume

INTRODUCTION

It is necessary a short preface to explain the relevance of the issues treated below. The regulation of intracellular pH is important for both applied and basic science. The applied (agronomical) aspect refers to two physiological phenomena: a) intracellular acidification is a common consequence of many abiotic stresses such as heat and water stress and it contributes to cellular damage; b) pH homeostasis is connected to transport of toxic cations such as sodium because the H^+ pump and K^+ channels determine the membrane potential that drives uptake of toxic cations (Mulet *et al*., 1999). Plant physiology has developed different systems to regulate pH in different plant compartments. There is a lot of information on pH homeostasis in mitochondria and chloroplast, but the regulation at the level of cytoplasm and plasma membrane (PM) is poorly understood (Taiz and Zeiger, 2006). Cytoplasmic pH homeostasis deserve intensive investigation because in addition to its role in stress, it controls cell expansion, nutrient transport and hormones signaling, like auxin and abscisic acid (Mahfouz *et al.*, 2005; Lager *et al.*, 2010; Grebe, 2005, Kang *et al.*, 2010).

pH homeostasis

In a constantly changing environment, organisms have developed different strategies to maintain their internal media relatively constant. These mechanisms are known generically as homeostatic processes and allow the organism to maintain internal conditions within a tolerable range (Gao *et al.*, 2004). An essential aspect of this regulation of plant cell homeostasis is ion transport, which is maintained through the regular and continuous input and output of ions between the different cellular compartments and the extracellular environment. Thus at the end the balance of each ion is optimized with the requirements of the cell in every compartment. This process is determined by membrane transporters and the proteins that regulate them. Many transporter proteins in plant cells use the electrochemical gradient across the membrane for its activity. This gradient is generated by proton pumps with the energy obtained from the hydrolysis of adenosine triphosphate (ATP) (Palmgren, 2001). The control of intracellular pH is essential for life, because it influences numerous physiological processes such as protein synthesis, DNA and RNA synthesis, the cell cycle and changes in the ionic conductance of the membranes. In addition proteins are sensitive to proton concentration changes because they could suffer

irreversible denaturation or changes of ionizable groups. The pH also regulates the activity of key enzymes and metabolic steps (Kurkdjian and Guern, 1989). Plants are able to generate huge differences in pH between the two sides of the cell membranes to optimize cellular requirements and allow co-transport using this gradient. Thus the cytoplasmic pH remains close to neutrality (pH 7.2-7.5), while in the external environment and in the vacuolar lumen the pH is 5-5.5 (in the case of the vacuole this pH is essential for the activity of acid hydrolases and the vesicle fusion processes). With this low pH in the vacuole the cells have the possibility to store a massive amount of carboxylic acids, the main ones are: citric acid, oxalic acid and malic acid. On the other hand the action of vacuolar proton pumping ATPase and pyrophosphatase can increase the cytosolic pH (pH_C). An extreme example in the plant kingdom of these mechanisms are chloroplasts: the pH inside a thylakoid can reach pH 4, but in the stroma compartment can be 8. This is because the pumping of protons from the stroma into the thylakoid lumen by the photosystems and cytochromes bf. The result is a pH gradient through the thylakoid membrane with an excess of protons inside (Britto and Kronzuaker, 2005).

pH regulation mechanism

Although the cell attempts to maintain a constant pH in each compartment, factors associated with metabolism, solute transport or environmental factors may disturb this balance. In plant cells metabolic production of organic acids is the major source of disturbance of pH. Other factors that disrupt the intracellular pH are anaerobic metabolism, light-dark transitions, changes in temperature and variations in external pH. In order to cope with these disturbances, plant cell has different mechanisms to maintain pH homeostasis such as:

Intracellular buffering

A molecule of water lacks buffering capacity, so living organisms have developed their own buffering molecules. The metabolism of living beings constantly produce organic acids (for example lactic and other carboxylic acids) which change the pH so the role of physiological buffers became essential. The buffers consist of non-ionized and ionized salts whose formation involves production or consumption of protons (Smith and Raven, 1979). In the cell the main physiological buffer is the bicarbonate buffer (first pKa= 6.4)

and also the phosphate buffer (second pKa= 6.9) and proteins through its ionizable groups (mostly imidazole, pKa around 7). The buffering capacity of these compounds is relatively low compared with the intensity of changes in proton concentration, thus cytoplasmic buffers are not sufficient to compensate these imbalances, so there are other systems able to mantain intracellular homeostasis

Biochemical pH-stat

This is a more effective regulatory system than the intracellular buffers. The model was proposed by Davies (Davies, 1986). It is based on the synthesis and degradation of malate by the coordinated action of phosphoenolpyruvate (PEP) carboxylase and the malic enzyme (Davies, 1973) (Figure 1a). Since the pH optimum of PEP carboxylase is greater than the neutral pH, when the pH of the cytosol increases the enzyme catalyses the formation of more oxaloacetate (OAA) from PEP (Figure 1a and 1b) releasing H^+ and lowering the pH. The OAA is subsequently transformed into malic acid by the enzyme malate dehydrogenase. By contrast, when there is acidification of the cytosol malic enzyme, which has an optimum pH below 7, decarboxylates more malate, leading to pyruvate (Figure1a). As shown in Figure 1c its mechanism requires the consumption of H^+ (Smith and Raven, 1979). Therefore, this model gives to the malate metabolism a key role in pH homeostasis. Whereas most of the malate present in the cell is stored in the vacuole, channels and transporters participate in the flow of malate between the cytosol and vacuolar lumen and *vice versa*, so they play an important role in maintaining pH homeostasis (Martinoia *et al.*, 2007).

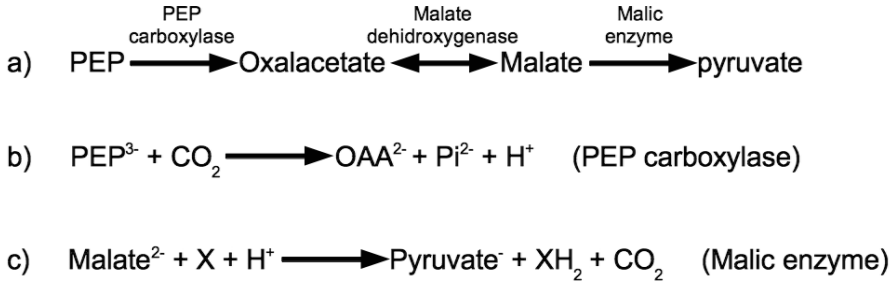


Figure 1: biochemical mechanism of the process of pH-stat proposed by Davies in 1986: **a**) **o**verall process scheme; **b**) reaction of carboxylation of PEP by the action of PEP carboxylase with the consequent release of H^+; **c**) malate decarboxylation reaction to give rise to pyruvate consuming H^+. (PEP: phosphoenolpyruvate; OAA: oxaloacetate; Pi: inorganic phosphate; X / XH_2: final substrate reduced after the red-ox coenzymes NAD+ or NADP+).

Biophysical pH-stat

Eukaryotic cells can control the pH of the different compartments regulating cellular action of several transporters of protons located in the cell membranes. The main proteins involved in pH homeostasis are (Figures 2 and 3):

H^+-ATPases

In *Arabidopsis thaliana*, there are three kinds of H^+-ATPases: the F-type in mitochondrial membrane and chloroplast, the V-type localized in the tonoplast and P-type in the plasma membrane. Thus the H^+-ATPases of the PM expel H^+ to the apoplast, generating a membrane potential between -100 and -200mV, negative for the cytosolic compartment and positive for apoplast. On the other hand, vacuoles, mitochondrial matrix and stroma of the chloroplasts are more acidic than the cytoplasm, so there is also an active transport protons from the cytoplasm through the vacuolar membrane and inner membranes of mitochondria and chloroplasts (the outer membrane of these organelles are permeable to ions) (Palmgren, 2001). The gene family of P-type H^+-ATPase consists of eleven members (AHA1-AHA11) in *Arabidopsis thaliana*. All of them except AHA8 encode proteins and a single cell can have more than one isoform, with AHA1 and AHA2 being the most abundant followed by AHA3. The activity of these proteins is regulated at different levels. It is known that salt stress and/or osmotic stress induce its expression (mainly AHA2) and this increases the amount of AHA protein but this regulation is not very important. The pH optimum of this enzyme is around 6.6, much lower than the cytosolic pH itself (pH_C= 7.5). A drop of pH_C increases activity of PM H^+-ATPase, and therefore, the expulsion of protons (Michelet and Boutry, 1995). The H^+-ATPases have a C-terminal domain of self-inhibition, so to activate the pump this domain should be phosphorylated at the penultimate threonine and then it can bind to a 14-3-3 protein to stabilize a non-inhibiting conformation. There are certain kinases and phosphatases that determine the activity of the pump at this level. V-type H^+-ATPase is located, not only in the vacuole, but also in different vesicles of the endomembrane system. This enzyme uses the energy of ATP hydrolysis to pump H^+ into vesicles, enabling compartment acidification capable of powering secondary active transport through these membranes (Krebs *et al.*, 2010).

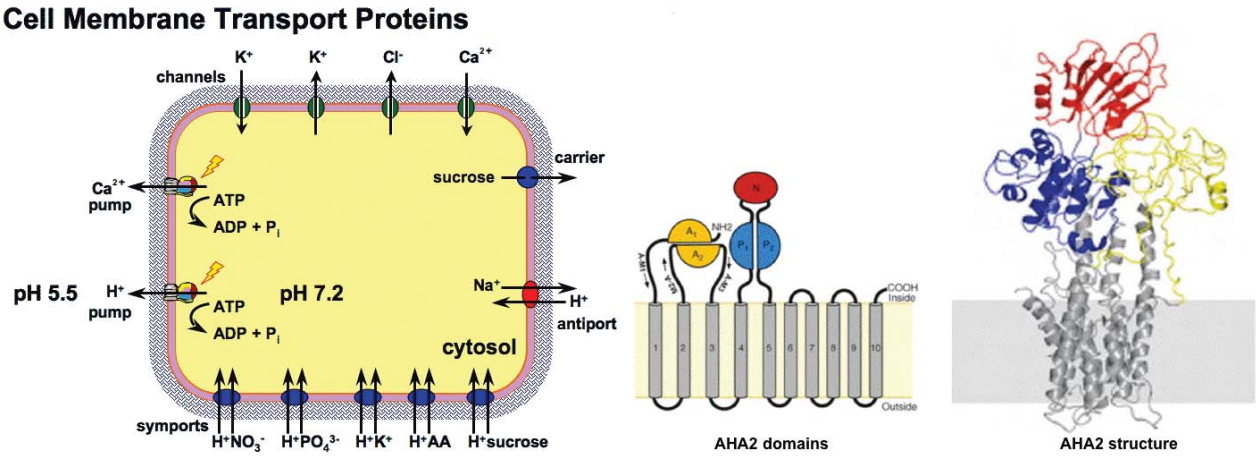


Figure 2: **a**) cell scheme of PM transporters; **b**) and **c**) PM H^+-ATPase (AHA2) that hydrolyzes ATP to pump protons from inside the cell into the apoplast (http://plantphys.info/index.html, Bublitz *et al.*, 2010).

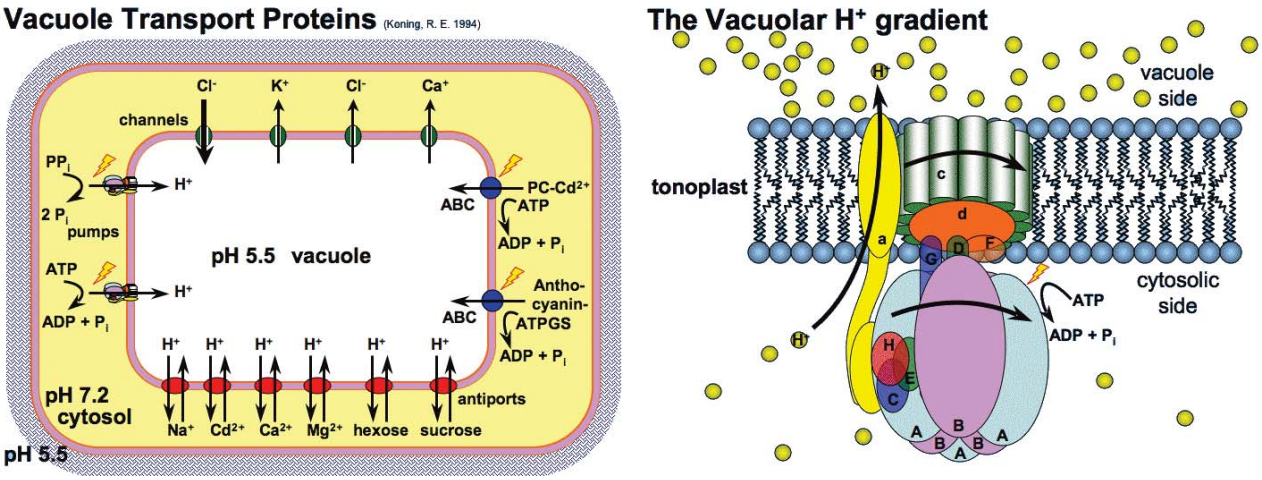


Figure 3: **a**) scheme of tonoplast transporters; **b**) a schematic V type H^+-ATPase that consumes ATP to move protons inside the vacuole (http://plantphys.info/index.html).

On the vacuole membrane there is another kind of proton pump: the pyrophosphatase, a protein that transports H^+ into the vacuole using pyrophosphate as substrate. Both enzymes contribute to generate the electrochemical potential of the tonoplast, and regulate the pH of the different cellular compartments (Zhen *et al.*, 1997).

ATPases and secondary active transport

Toxic cations enter passively through nonspecific channels. However, anions should enter the cell by carrier proteins that are energized by symport with protons. The symport can be defined as the transport of two substrates simultaneously in the same direction, by the same protein. In the case of proton symport, a proton gradient has previously being created and it is used by the other substrate to enter with it. These transporters are essential for plant nutrition (taking care of the uptake of nutrients: phosphorus, nitrogen, sulphur, etc.). And, indeed, are highly expressed in cell types specialized in nutrient uptake from the soil, such as the epidermal cells of the root hairs (Gaxiola *et al.*, 2007). Thus, the ATPases have an essential role in maintaining the pH homeostasis of these ions as they allow the action of other proteins (symporters or antiporters). As indicated in Figure 4.

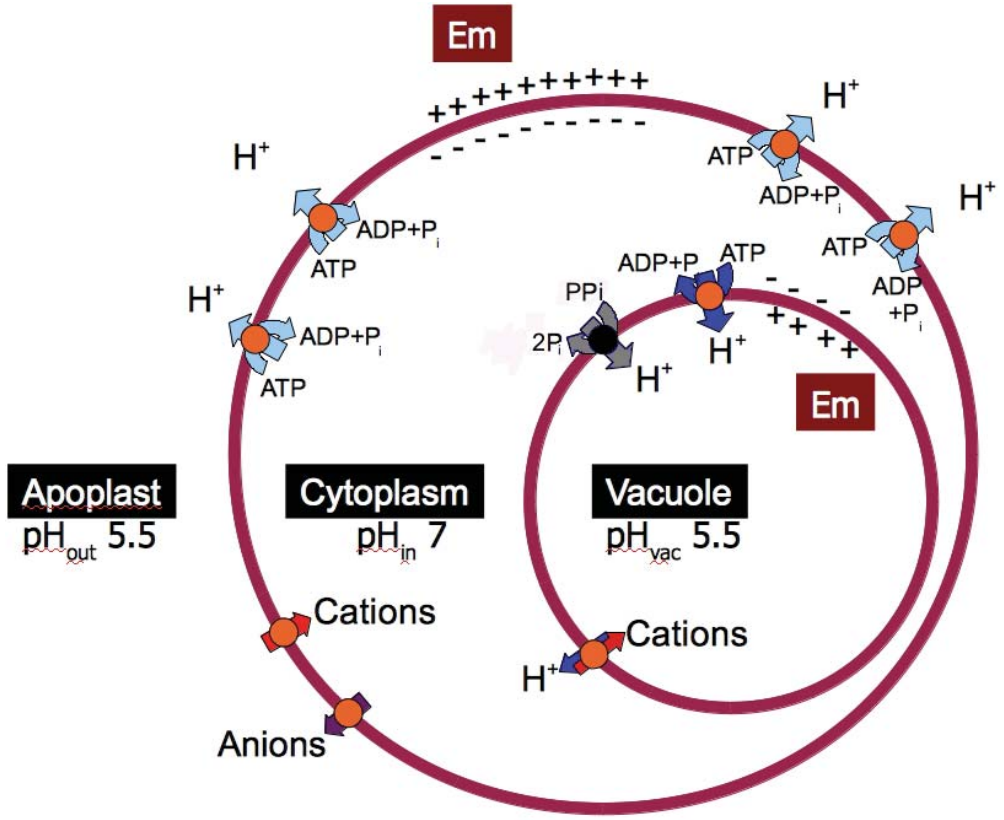


Figure 4: H^+-ATPase extrudes protons and the consequent membrane potential promote ion transport by channels and transporters.

Regulation of pH homeostasis in *Saccharomyces cerevisiae*

Also in yeast there are cytoplasmic buffers and biophysical mechanisms, mediated by the action of different types of transporters for H^+, which are involved in maintaining pH homeostasis in this organism as in plant cells. Regarding H^+ transport, it is similar to plants, with a PM H^+-ATPase and a vacuolar V-type ATPase as the main proteins involved in pH regulation in different intracellular compartments (Ariño, 2010). The H^+-ATPase is responsible of the creation of the H^+ electrochemical gradient across the PM in yeast (Serrano *et al.*, 1991). On the other hand, several antiporters, symportes and cation/proton are also involved to a lesser extent, the maintenance of intracellular pH homeostasis (Ariño, 2010).

Weak organic acids and pH homeostasis

Weak acids are those that in aqueous solution, maintain a balance between its protonated form (HA) and the unprotonated form (A^-). This balance depends on the pH of the medium and the pKa of the acid. pKa is the pH at which [HA] = [A^-]. An increase in pH will increase the deprotonated/protonated ratio, while a decrease of pH will decrease it. The uncharged form of weak acid is relatively permeable and can diffuse freely through the plasma membrane. Once in the cytosol, the acid dissociates into H^+ and A^-. This increase in proton concentration in the cytoplasm leads to a decrease in intracellular pH and altered homeostasis. For weak acids the concentration of protonated and unprotonated forms follows strictly the Henderson-Hasselbach equation (Figure 5).

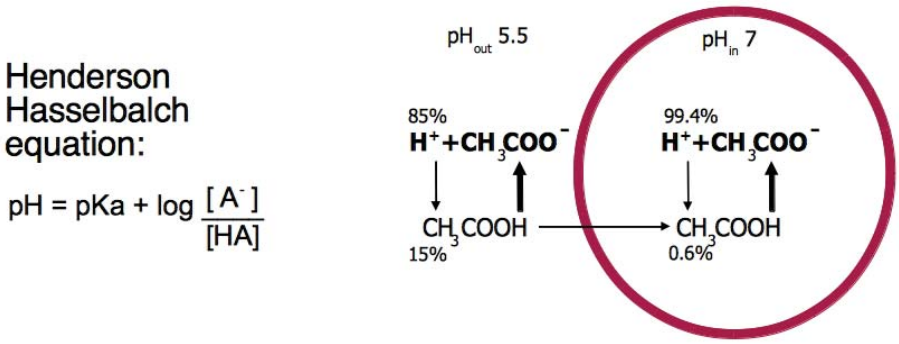


Figure 5: Henderson-Hasselbach equation with schematic entrance of acetic acid into a plant cell.

Mechanisms of weak organic acid toxicity in micro-organisms

The mechanisms of organic acid toxicity and tolerance in *E. coli* are depicted in Figure 6. It's important to say that weak organic acids have two different effects. First, there is an antimicrobial effect due to the acidity itself, that is, the drop of extracellular pH. The second, more important, is the specific antimicrobial effect due to the undissociated form. The antimicrobial effect of many organic acids is exerted through the undissociated form, and this factor is more important than the decrease in external pH, that at low concentrations of the acid may be negligible. The dissociated form of the acid, being an anion, is highly polar and therefore does not cross the PM of microorganisms. The undissociated form, by contrast, does cross the membrane. Once inside, the acid can dissociate and lower intracellular pH (Östling and Lindgren, 1993). This can seriously affect cell metabolism by affecting the pH gradient and interferes with transport of amino acids, phosphate and other nutrients. In addition, many enzymes essential for microbial metabolism are inactivated at acidic pH (Bearson *et al.,* 1997). Another negative consequence of this process is the increase in cell turgor. Upon dissociation of the acid inside the cell, internal anion concentration increases. This triggers a compensation mechanism of the electrical charge that forces to increase the levels of Na^+, K^+ and/or glutamate, leading to an increase intracellular ionic contenent. This process causes a large increase in mechanical pressure on the wall of the microorganism, with eventually break out (Foster, 1999). Food industry has used these phenomena to its advantage. Weak organic acids (as acetic, propionic, lactic, sorbic and benzoic acid) are used as food preservatives (Hirshfield *et al.*, 2003). Most studies on weak organic acids have been performed in bacteria and yeast and little is known in plants, despite their great importance in conditions such as germination in soil rich in organic acids such as humus. The toxicity of acids is given by the accumulation of anions and the decrease of pH in the cell cytoplasm. Increasing the concentration of anions has shown to produce an increase in the transport of potassium ions in the cell, which increases the turgor pressure, and the flow of water into the cell. To maintain turgor pressure and cell volume, the glutamate is transported outside the cell (Foster, 1999). This activity changes the osmolarity of the cytoplasm, which in turn reduces the potential for cell growth and viability. On the other hand, high levels of anions of weak acids in the cytoplasm may have effects on

metabolism, because they can influence the activity of enzymatic reactions. In addition, in yeast subjected to acetic acid stress, this can induce programmed cell death, depending on the dose of acid (Ludovico *et al.*, 2003). This induction of cell death was related to intracellular acidification and production of reactive oxygen species (ROS). It is also important to note that the non-dissociated weak organic acids can be inserted into the lipid bilayer at concentrations that depend on their hydrophobic index. These molecules can interfere with the activity of membrane proteins and thus may cause an inhibition of growth when they reach a critical level in the lipid bilayer.

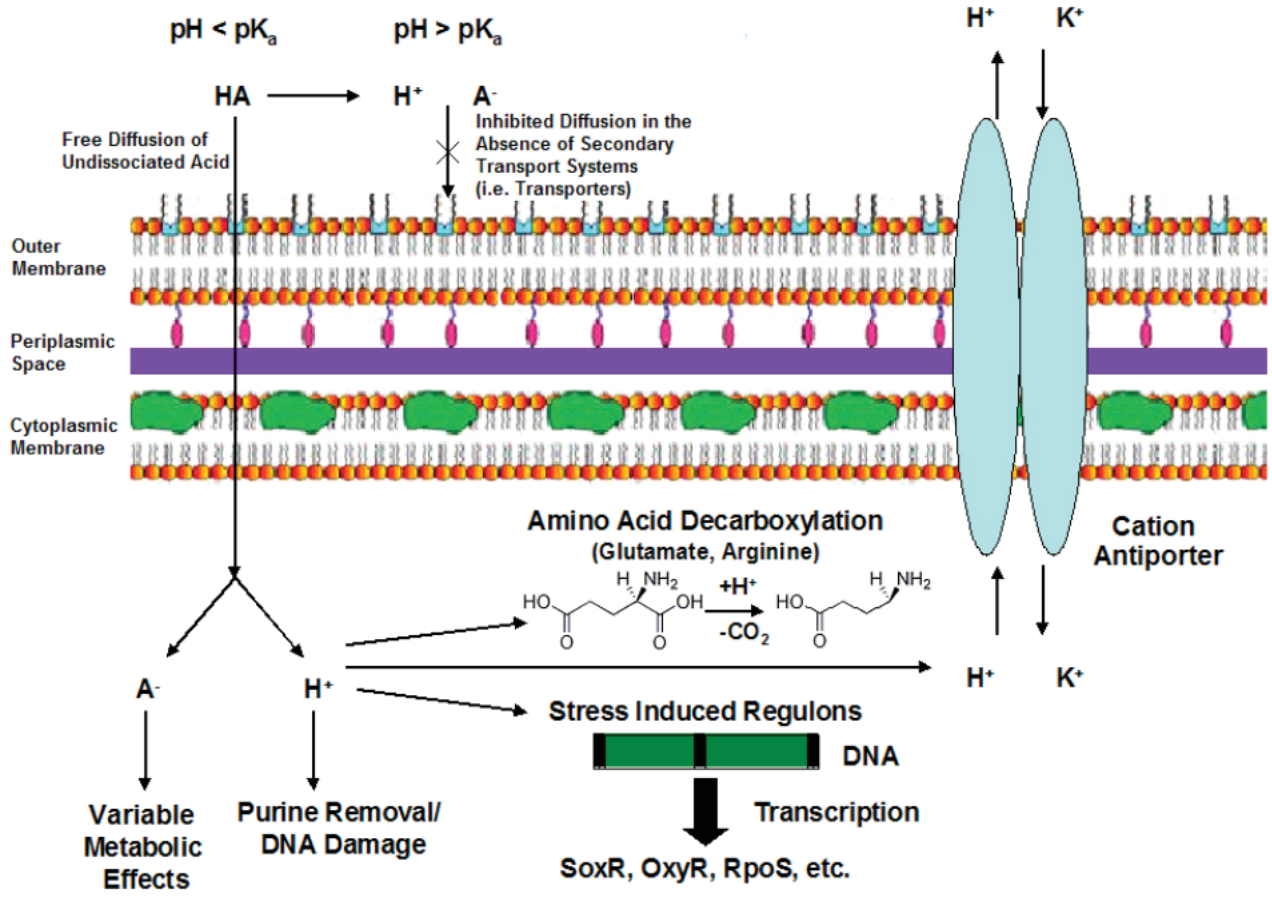


Figure 6: an overview of organic acid toxicity and tolerance mechanisms in *E. coli*. Diffusion of undissociated acid molecules can occur freely in acidic medium but is limited to transport systems at neutral or basic pH. The toxic effects associated with organic acids are the result of both anion specific affects on metabolism as well as increased internal proton concentrations. Effects on internal pH are mitigated by transport of protons out of the membrane, consumption of protons by decarboxylation reactions, and, more generally, induction of stress regulons. Anion specific tolerance mechanisms are not well characterized (Warnecke and Gill, 2005). Proton extrusion by bacterial F-ATPase is not shown, as well as the induction of reactive oxygen species by intracellular acidification.

Acid stress and pH signaling in yeast

The spontaneous growth of yeast in the must was used in the past for wine production. In this process the presence of weak organic acids is relevant because it helps to reduce bacterial growth. Yeast grows so well because has developed mechanisms to protects himself against low external and intracellular pH. As we discussed before, low intracellular pH affects enzymatic reactions and protein stability and induces the perturbation of many cellular activities including growth and can lead to cell death. A change of pH can trigger specifically different physiological processes, including the activation of the proteins responsible for maintaining ion homeostasis. In yeast there is a pH-sensitive interaction between the phosphatase Ppz1 and its inhibitory subunit Hal3 (Yenush *et al.*, 2005). At low intracellular pH Hal3 binds and inhibits Ppz1 and this results in increased phosphorylation and activation of Trk1, a high-affinity K^+ transporter inactivated by Ppz1 (Yenush *et al.*, 2002). Therefore, the known regulation of K^+ transport in yeast occurs by direct perception of intracellular acidification by a multi-subunit protein phosphatase (Hal3-Ppz1) acting on the transporters. Mutants in these regulatory systems, however, are viable and therefore other cellular systems must participate in the regulation of H^+ and K^+ transport. The acidification of the vacuolar compartment by the vacuolar H^+-ATPase is important for protein sorting and zymogen activation (Nelson and Nelson, 1990; Yamashiro *et al.*, 1990) and this enzyme also contributes to cytosolic pH (pH_C) homeostasis during acid stress (Nelson and Nelson, 1990). The K^+ transporter Trk1 (Mulet *et al.*, 1999) and the ABC-ATPase Pdr12 that extrudes the anions of organic acids (Piper *et al.*, 1998) are also important for acid tolerance. *WAR1* encodes a transcription factor that mediates expression of *PDR12* (Causton *et al.*, 2001; Schüller *et al.*, 2004). Other genes identified in these studies are only indirectly related to pH homeostasis. Tryptophan biosynthesis is important for weak organic acid tolerance because tryptophan transport in auxotrophic strains is inhibited by weak organic acid stress (Bauer *et al.*, 2003). The important role of phosphofructokinase (*PFK1* and *PFK2*) has been anticipated by biochemical studies that demonstrated inhibition *in vitro* and *in vivo* of this pace-maker glycolytic enzyme at acidic pH (Krebs *et al.*, 1983). Other targets of acid toxicity seem to be general transcription (*GAL11, RPB4*) and vesicle traffic (Brett *et al.*, 2005).

The soil acidity

In plants the situation is different and more complex, there are different kinds of soil acidity. In acidic soils, aluminium and manganese can become very soluble and therefore toxic. In addition, soil acidity reduces the capacity of the plant to absorb phosphorus, calcium, magnesium and molybdenum. Boron, copper and zinc are also toxic in the soil at low pH. Soil pH has little effect on nitrogen uptake (McNulty and Boggs, 2010).

Soil acidity can be originated by:

• Aluminosilicate clays.

• Hydrated aluminium (Al) and iron (Fe) oxides can be found amorphous or colloidal crystalline state, encapsulating other particles between the layers of the crystal lattice, at low pH these oxides solubilize and release H^+.

• Soluble salts: these salt are exchanged with adsorbed aluminium and this produces an increase in soil acidity.

• The humus or organic matter is rich in weak organic acid such as acetic or propionic.

pH signaling in *Arabidopsis thaliana*

Some studies have used various weak organic acids (acetic, propionic and benzoic acid) to induce acidification in the cell cytoplasm of several plant systems (Kurkdjian and Guern, 1989). In plants there are two mechanisms widely studied that cause intracellular pH acidification: one is important in pathogen response and triggers the cell death program: the plant does scorched earth around the pathogen to save the whole plant. And the other one is the plant cell growth, still partially understood at the moment.

Plant cell growth

There are many open questions on how plants grow. The acid growth theory is the most accepted model and is based on the fact that the plant growth hormone Indol Acetic Acid

(IAA) activates the PM H^+-ATPases. This acidifies the apoplast by expulsion of protons, triggering a process that weakens the cell wall, hyperpolarizates the PM and increases the uptake of K^+. The entry of K^+ into the cell causes osmotic changes that allow water to enter through the aquaporins in the cytosol, therefore, the cell turgor favours cell expansion (Maurel, 2007, Hager, 2003). The K^+ transport takes place through low affinity systems or channels (Lebaudy *et al.*, 2007) and through high affinity transporters such as HKT and HAK (Rodríguez-Navarro and Rubio, 2006). The IAA can activate the PM H^+-ATPase through specific kinases or induce the synthesis of new molecules of H^+-ATPase. Moreover, potassium uptake is activated not only by the hyperpolarization of the membrane, but also by the action of IAA on K^+ transporters. IAA can activate directly the K^+ transporters or promote their expression (Philippar, 1999). The cell wall acidification by the H^+-ATPase reaches pH 5.0, which activates certain enzymes, including expansins. This weakens non-covalent bonds between cell wall polysaccharides. Furthermore, other enzymes like glucanases, the xyloglucan endotransglycosylases (XET) and xyloglucan hydrolases (XGH) have been associated with the weakening of the cell wall. It is believed that these enzymes rearrange and decompose, respectively, the hemicellulose (mainly xyloglucans) and promote the action of expansins (Cosgrove and Durachko, 1994; Fry *et al.,* 1992; Taiz and Zeiger, 1996).

K^+ transport in *Arabidopsis*

At cellular level K^+ is the most abundant cation in the plant cytosol, while other ions such as Na^+, Cl^-, Ca^{2+} and H^+ are ejected into the apoplast or compartmentalized into the vacuole. At physiological level, plant growth requires large amounts of K^+, so it is necessary to transport it from the soil to the growing organs (Lebaudy A. *et al., 2007*). K^+ uptake and transport within the plant are mediated by two types of integral membrane proteins: K^+ channels and K^+ transporters. Plants do not have K^+-pumping ATPases as in animal cells and some bacteria. The channels are characterized by forming aqueous pores where the ligand (K^+) is free to bind the protein active site simultaneously from the two sides of the membrane and no conformational change occurs during passage. Channels, however, are often controlled by opening/closing of a “gate”, but this conformational change does not occur in every transport cycle. Turnover numbers of channels are very high, greater than $10^7 s^{-1}$. The transporters, on the other hand, have a rate-limiting conformational change every catalytic cycle to allow binding of the transported

ion (K^+) first from one side and then release it from at theother side of the membrane (Ames and Lecar, 1992). Turnover numbers are much smaller than in the case of channels (10-1000 s^{-1}). Another important parameter in transport is the affinity. High affinity mechanism (mainly transporters, but not only) means K_m in the μM range and lower affinity mechanism (frequently channels) means a K_m in the mM range (Chiu *et al.* 2002).

K^+ channels

As reported in the scheme in Figure 7 there are three main families of K^+ channels: Shaker, TPK and Kir. All of them are voltage-gated K^+ channels, that is, gating occurs according to the membrane potential.

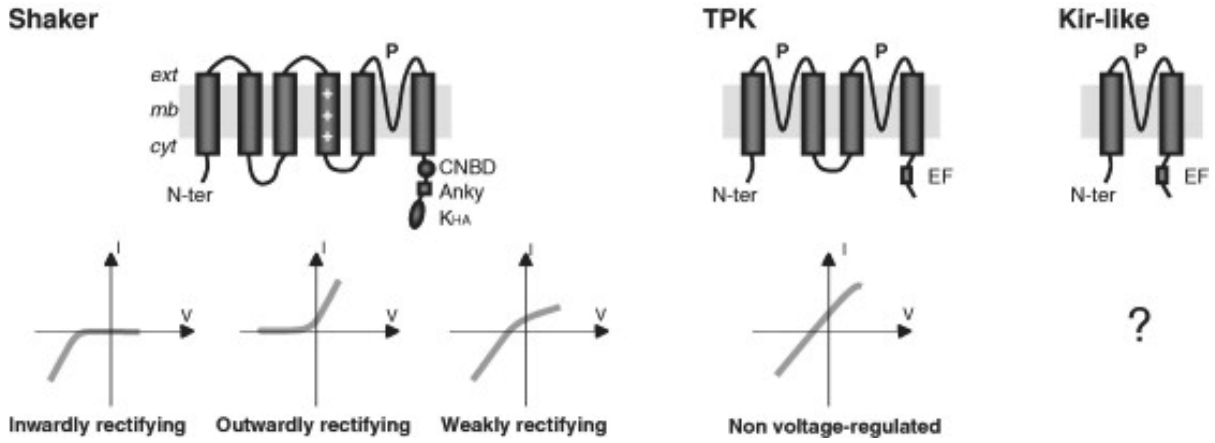


Figure 7: scheme of domains in three types of K^+ Channels. P is the pore forming domain (four such domains form one pore). CNBD is a domain with homology to cyclic nucleotide binding domains. Anky is an ankyrin-repeat domain. EF is a calcium binding domain.

Shaker channels

Shaker channels are strongly regulated by membrane potential. They can be divided into three subfamilies (called IRC, ORC and WIRC) depending on the voltage range within which they are active and their specific permeability. Otherwise they can be divided by sequence homology into five groups and they can form heterotetramers like in Figure 8b

(Lebaudy *et al*., 2007). Nine genes belong to the Shaker family and it is necessary to assembly four gene products to get a functional channel (Figure 8c). They can form homomeric or heteromeric channels, it should be noted that the function of heteromerization is to change their functional properties. Patch-clamp studies showed that the expression of AtKC1/KAT3, in absence of AtAKT1, did not form inward channels. On the other hand AtAKT1 without AtKC1/KAT3, increases the influx rate. Homomeric channels like AKT1 or KAT1 can work alone with a defined activation threshold of membrane potential. With the co-expression of AKT2 and KAT1, KAT1 and KAT2, AKT2 and KAT2, AKT1 the activation threshold changes membrane potential and also their macroscopic conductance (Jeanguenin *et al.,* 2011). In guard cells the most abundant channel is the heteromeric 2KAT1 and 2KAT2 (Lebaudy *et al.*, 2008).

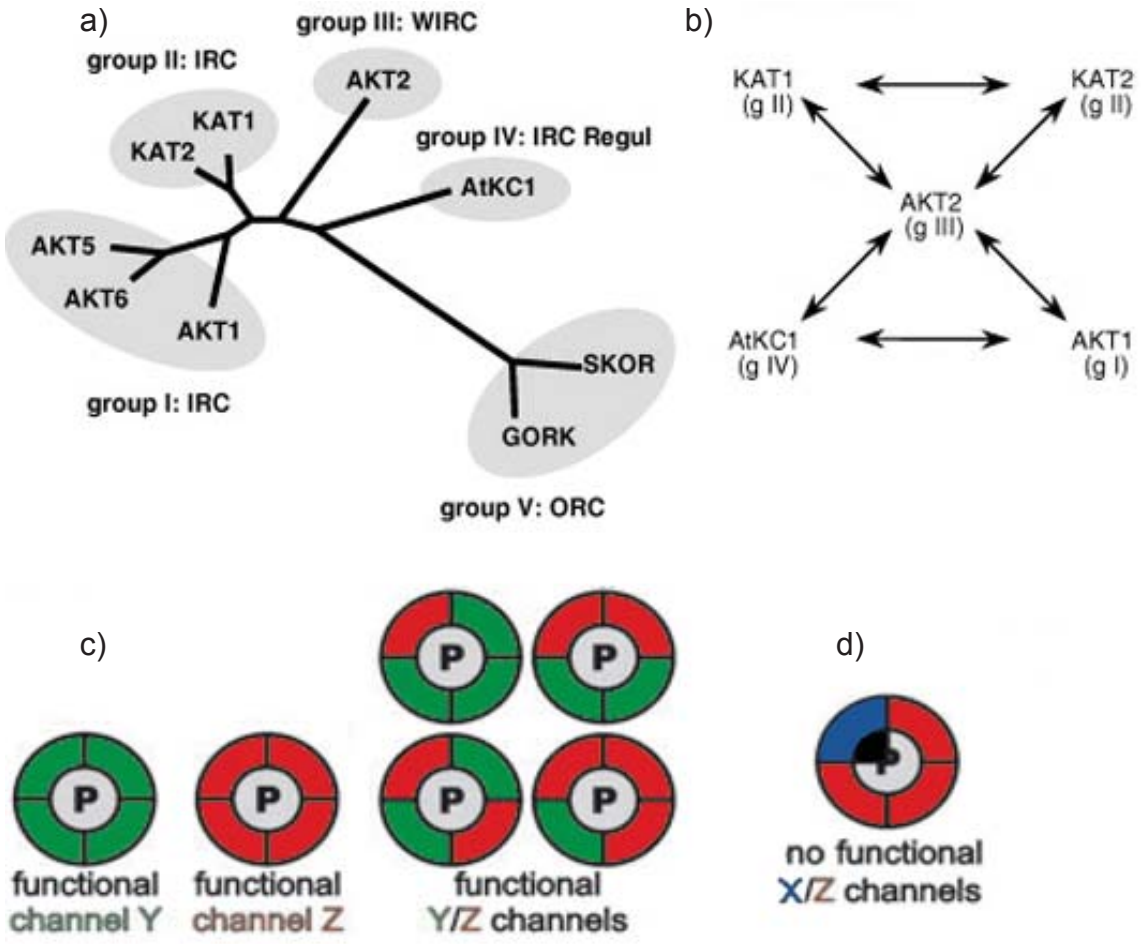


Figure 8: **a**) phylogenetic tree of shaker channels; **b**) scheme of functional combinations (heteromers) of different Shaker gene products (Lebaudy *et al.*, 2007); **c**) the possibles functional combinations for a single functional Shaker homomer Y or Z, functional heteromers Y/Z. A mutant gene product by T-DNA can produce a homomer X with a blocked pore. **d**) Theorically dominant negatives are possible for all combinations of heteromers X/Z (Lebaudy *et al.*, 2008).

Inwardly Rectifying Conductance (IRC)

They are activated by hyper-polarization of the membrane and are involved in the entry of K^+ into the cell. These are channels whose activation is independent of the extracellular K^+ concentration and possess a high K^+/ Na^+ selectivity. In *Arabidopsis* the major K^+ uptake system in roots is the AKT1 channel (Hirsch *et al.*, 1998). It is documented (Figure 9) that the *akt1* knock-out mutant grows poorly in media with low levels of K^+, (≤100μM KCl; www.arabidopsis.org). AKT1 is phosphorylated and activated by the calcium-activated protein kinase CBL1/9-CIPK23 (Xu *et al.*, 2006). K^+ uptake should be activated by intracellular acidification. This acid signal could be converted into an increase of free calcium by H^+/Ca^{2+} exchange at the plasma membrane or vesicle levels (Felle, 2001). The K^+ channels KAT2 and KAT1 are located mainly in guard cells of leaves and mediate K^+ influx during the opening of stomata (Pilot *et al.*, 2001). SPIK (Shaker Pollen Inward K^+ channel) is involved in pollen K^+ uptake it is required for proper development of the pollen tube (Mouline *et al.*, 2002).

a)

b)

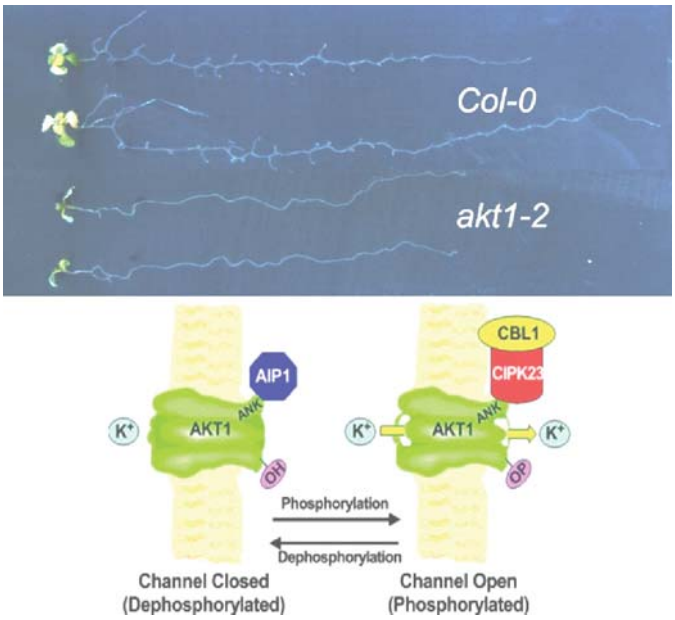


Figure 9: **a**) Col-0 and *akt1-12* knock-out mutant seedlings grown in MS with 10μM KCl after 10 days; **b**) AKT1 regulation by phosphorylation (Lee *et al*., 2007)

Weakly Inwardly Rectifying Conductance (WIRC)

They are activated by hyperpolarization of the membrane, but they can work also in normal conditions (normal membrane potential). These channels show a high ratio for K^+/Na^+ selectivity (Véry and Sentenac, 2002) and may mediate both the entry and the K^+ efflux from the cell (Véry and Sentenac, 2003). Currently there are two known members of this subfamily, AKT2/AKT3, two transcripts of the same gene that are located in the parenchyma zone, close to phloem and xylem and are believed to be involved in input and output K^+ from phloem (Lacombe, 2000). Similarly to the situation of AKT1, CBL4/CIPK6 Ca^{+2} sensor/protein kinase complex modulates AKT2 activity. Loss of either CBL4 or CIPK6 leads to the same developmental phenotypes as complete loss of AKT2 function. AKT2 modulation by CBL4/CIPK6 complexes could be explained by protein phosphorylation. But a recent paper does not support the regulation of AKT2 by CIPK6 dependent phosphorylation. The activity of this K^+ channel is promoted by a physical interaction with a kinase but it is phosphorylation-independent. The key here is translocation of the kinase from endoplasmic reticulum membrane to PM where it activates AKT2. Loss-of-function mutants of either CBL4, CIPK6 or AKT2 exhibit a developmental phenotype, specifically delayed development and flowering in these mutants as compared to wild type (Held *et al.*, 2011).

Outwardly Rectifying Conductance (ORC)

They are activated by depolarization of the membrane potential and are involved in K^+ output efflux. These are channels whose activation is dependent on the concentration of K^+ and with great K^+ / Na^+ selectivity (Véry and Sentenac, 2002). Like the IRK-type channels, ORC are located in the PM of the cells. The two most prominent members of this subfamily are SKOR (Shaker K^+ Outward Rectifying) and GORK (Gated Outwardly Rectifying K^+ Channel). SKOR is located mainly in the root and mediates the loading of K^+ in the xylem vessels (Gaymard *et al*., 1998). GORK is expressed in roots and in guard cells of stomata, where it mediates the efflux of K^+ during stomatal closure (Pilot *et al.*, 2001).

KCO-2P channels (K^+ Channel Outward rectifying 2 Pore)

Also known as TPK (Tandem Pore K^+ Channel), as it was observed that not all of them are outward rectifying type. In *Arabidopsis*, there are five members of the family KCO-2P, KCO1, KCO2, KCO4, KCO6 and KCO5. It has been shown that KCO1, KCO5 and KCO2 are located in the tonoplast membrane (Lebaudy *et al.*, 2007). The most studied has been KCO1, located mainly in root and stem, which is a channel selective for K^+ that allows both entry and exit of this cation in the vacuole (Gobert *et al.*, 2007) and is regulated by Ca^{+2} and cytosolic pH (Czempinski *et al.*, 1997). KCO4 is predominantly expressed in pollen and is located in the PM (Becker *et al.*, 2004), while KCO6 shows very high expression levels in roots and leaves (Schönknecht *et al.*, 2002).

Kir-like channels (K^+ inward rectifying like)

Also known as KCO-1P channels (K^+ Outward Ca^{+2} Channel rectifying Pore 1). In animals the channels of this family act as IRK-type channels and given their structural similarity it is very likely that they do the same in plants (Véry and Sentenac, 2002). In *Arabidopsis* only one member, KCO3 has been identified (Czempinski *et al.*, 1999).

Non-voltage-gated Channels

With respect to partially selective ion channels that are permeable to K^+, we can highlight two families:

GLR (Glutamate Receptors)

It is a family of channels that in *Arabidopsis* has 20 members and all are expressed in roots (Chiu *et al.*, 2002). Like their counterparts in animals, they are permeable to K^+, Na^+ and / or Ca^{+2} (Nakanishi *et al.*, 1990).

CNGC Channels (Cyclic Nucleotide-Gated Channel)

In *Arabidopsis* they are 20 members of this family characterized and its structure is highly homologous to Shaker-type channels, but they are not regulated by voltage. The data available suggests that these channels are permeable to monovalent and divalent cations

(K^+, Na^+ and Ca^{+2}), regulated by cyclic nucleotides and calmodulin (Véry and Sentenac, 2002).

K^+ Transporters

These transporters are involved in K^+ transport through the PM and tonoplast and are associated with high affinity transport, although this association is not always true (Dalton, 1984), and it had been observed that they are upregulated in conditions of deficiency of K^+ in the extracellular medium (Rodriguez-Navarro and Rubio, 2006). Potassium transporters can be grouped into three families.

HAK (High Affinity K^+ Uptake)/ KUP (K^+ Uptake Permease)

This family was first identified in *Escherichia coli* and named KUP, 13 members are present in the *Arabidopsis* genome (Mäser, 2001) divided into four phylogenetic groups. There are four groups identified phylogenetically but only two are well characterized, the I and II groups. In *Arabidopsis*, the group I transporters have high affinity for K^+ and low affinity for Na^+ and NH_4^+ (Bañuelos *et al.*, 2002). It is unknown whether these transporters are energized by co-transport with H^+ (Véry and Sentenac, 2003). The main members of this group are: HAK5, KT5, KUP12 and HAK7. Group II is characterized by mediating low-affinity K^+ transport (K_m ~ 5mM), although it appears that some transporters of this family may also have high affinity for K^+ as is the case KUP1 (Rubio *et al.*, 2000). They have high affinity for K^+ and Rb^+, also low selectivity for Na^+, so it can be blocked by competitive inhibition by this cation (Véry and Sentenac, 2003). Other members of this group are: KT2/KUP2 (KT: K^+ Transporter) KT3/KUP4, KT4/KUP3, HAK8 and HAK6. Some members of this family seem to be expressed in roots and are involved in the response to potassium deficiency in soil (Ashley et al., 2006). KUP4/TRH1 function was associated with several development defects in the loss-of-function lines. It was observed that root growth deviated from vertical, a severe defect in root hair development and curiously the addition of higher levels of K^+ does not rescue phenotypes. Only the addition of exogenous IAA (60nM) counteracts these defects. Finally, the GUS assay with the auxin-responding gene DR5 (Figure 10), reveals an accumulation of endogenous auxin in the *trh1* mutant (Vicente-Agullo *et al.*, 2004). These observations suggest a link of KUP4/TRH1 activity with auxin transport.

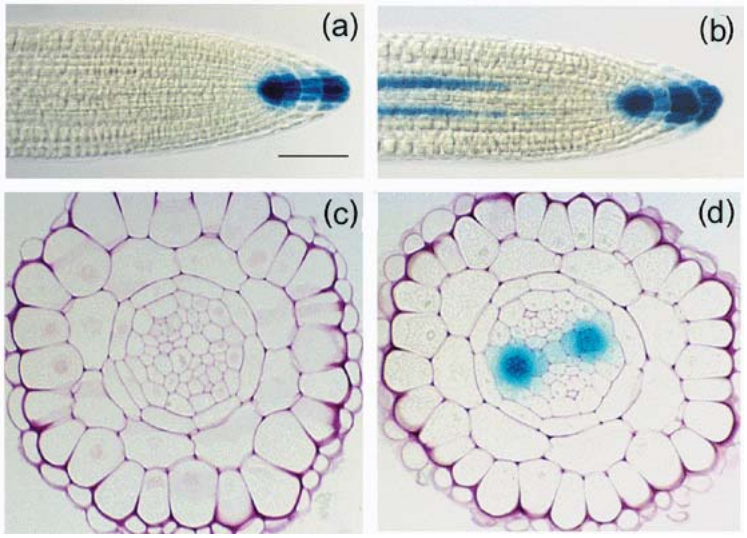


Figure 10: expression of DR5:GUS in wild type (wt) roots grown on Murashige-Skoog solidified media. Highest GUS activity was detected in the quiescent centre, in young columella and mature columella root cap but not in the stele. (**a**) DR5:GUS expression in the tip of the 4 days after germination wt root; (**b**) and (**c**) kup4/*trh1* plants grown on Murashige–Skoog solidified media display additional site of DR5:GUS expression in the protoxylem cell files; (**d**) DR5:GUS expression in the tip of the 4 days after germination trh1 roots (Vicente-Agullo *et al.*, 2004)

Antiporter family

This family includes proteins with cation / H^+ antiporter activity which are involved in the transport of K^+, Na^+ and Ca^{+2} and other monovalent / divalent cations (Mäser *et al.*, 2001). This family is subdivided into two subfamilies, according to the classification system of conveyors TC (Saier, 2000): CPA2 CPA1. CPA1 subfamily in *Arabidopsis* includes 8 types of antiporter Na^+/H^+ (NHX1-8), of which only one is involved in the transport of K^+, AtNHX1. It is a H^+ antiporter that shows affinity for both Na^+ and by K^+ (Venema *et al.*, 2002). It is located in the tonoplast and is implicated in the accumulation of K^+ in the vacuole, Na^+ detoxification of the cytoplasm and probably in cytosolic pH homeostasis (Apse, 1999). The subfamily CPA2 in *Arabidopsis* is composed of 34 members. Of these 6 were identified by their homology to bacterial output antiporter K^+ (Kef, K^+ Efflux) and postulated that might be involved in the entry of K^+ in the vacuole (Mäser, 2001). The other members of this family are called CHX (Cation H^+ Exchanger) and are involved in antiport with H^+ of various monovalent and divalent cations, including K^+. Notably CHX17 expressed in the PM of the epidermis and the cortex of mature roots and seems to be involved in root K^+ uptake (Cellier *et al.*, 2004).

Family of high affinity K^+ transporters

In the case of *Arabidopsis*, the only member of this family HKT1 does not transport K^+ but Na^+. However, in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) some of the members of this family have been identified as proton symporters of Na^+ / K^+ (Véry and Sentenac, 2002).

H^+-ATPase and K^+ transport can play more roles

To put in order the precedent aspects about the proteins implicated in the pH homeostasis, the most important for the cell ion homeostasis is the activity of H^+-ATPase (PM and vacuolar) to extrude the protons in safe compartments. First it maintains constant the internal pH (Young *et al*., 1998) and second it accumulates toxic cations in the vacuole by secondary transport. In the PM the situation is the opposite: high membrane potential also drives a major uptake of toxic cations. The number of these proteins per weight unit and their activation by phosphorylation give the plant strength against these adverse conditions. Also the K^+ channel AKT1 is important for homeostasis because is the main K^+ transporter in roots (for example in root epidermal cells), and a high rate of K^+ entrance depolarizes the membrane potential and reduces uptake of toxic cations. All the others K^+ channels and transporters probably are useful for the K^+ fine-tuning in all plant cells or compartments. At this point it is necessary also to mention another important class of carriers, the ATP-Binding Cassette (ABC) transporters. They form a very large superfamily and are ubiquitous with an ATP-dependent pumping activity. They transporters for a very wide range of substrates, including lipids, drugs, heavy metals, and can confer multidrug resistances to the cells. The yeast ABC transporters confer drug resistance and extrude antifungal compounds (Kim *et al.*, 2010). Also, some anions of weak organic acid like sorbate and benzoate are extruded by these transporters (Hatzixanthis *et al.*, 2003). In plants there are ABC ATPases described as importers of malate in guard cells and also flavonoids transporters (Kang *et al*., 2010). Lately, their implication in phytohormone fluxes was observed. For example ABCB1/PGP1 and ABCB19/PGP19 are auxin efflux carriers (Figure 11; Mravec *et al*., 2008). In the cell wall (pH 5.5), IAA molecules are present in both ionized and protonated forms. As described before for acetic acid, the hydrophobic, protonated IAAH (pKa= 4.8) enters into the cell passively through the plasma membrane.

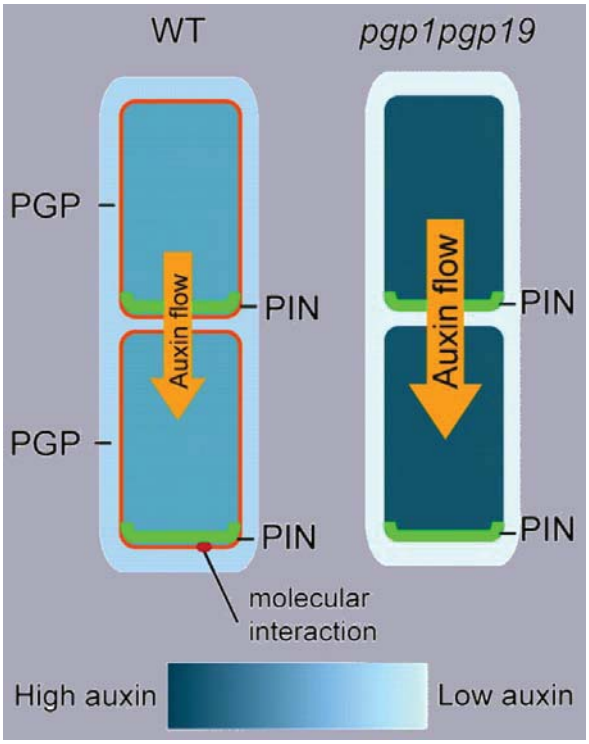


Figure 11: schematic representation of the auxin levels in wild-type and ABCB1, ABCB19 knock-out.

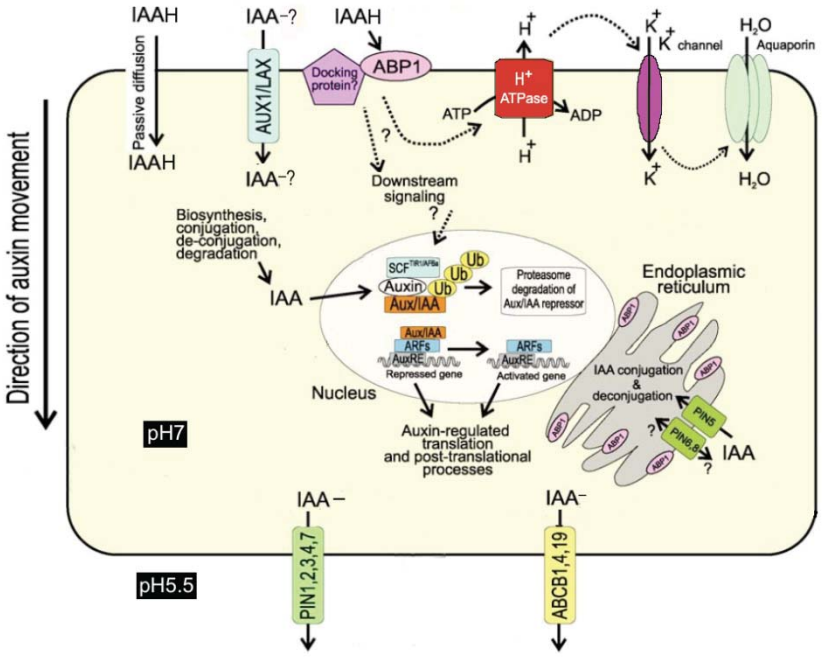


Figure 12: IAA entrance, exit, store and perception in a cell of *Arabidopsis* (adapted from Simon and Petrášek, 2011).

Experiments monitoring ^{3}H-IAA uptake observed in oocytes expressing AUX1 did not find any evidence of a carrier-mediated uptake of the deprotonated form IAA, although such a mechanism might be expected for IAA uptake at higher pH (Carrier *et al.*, 2008). Once inside the cytoplasm (pH 7), the IAAH dissociates and the IAA^- ion cannot passively move

out of the cell and therefore becomes trapped inside (IAAH->IAA- $+H^+$). In this case, ABCB1/PGP1 and ABCB19/PGP19 are involved to transport IAA^- ions out of the cell into apoplast compartment (Figure 11). On the other hand PINs can release the IAA^- molecule indirectly to the next cell by way of the ABP1 IAA-receptor. This second exit pathway has a higher rate, and it determines the auxin polarity transport and affects the formation of secondary roots (Figure 12). pH is a critical parameter for ABP1-auxin affinity. The pH in the ER lumen is estimated to be close to 7.0, and ABP1 affinity to auxin is nearly zero at this pH (Tian *et al*., 1995). The highest affinity was determined at approximately pH 5.5 (Löbler and Klämbt, 1985; Shimomura *et al*., 1986; Tian *et al*., 1995), which is a typical pH of the extracellular matrix. Stress conditions induce the ABA response and such conditions are known to elevate extracellular pH. This increases the level of ionized ABA level into the apoplast (ABAH->ABA^- $+H^+$). In this situation ABA^- cannot passively diffuse across the PM, in contradiction to the need to rapidly deliver the stress hormone into the cell to elicit a timely response. In this case ABA uptake may be mediated by another ABC transporter, ABCG40/PDR12. Many aspects about the mechanisms of auxin and ABA signaling remain to be elucidated. It is not demonstrated, but it is common the idea that there must be coordination between pH and growth-development-stress because both these two phytohormones, IAA and ABA, are weak organic acid species (Kang *et al*., 2010).

Mislocalization of ABCB1, ABCB4, ABCB19

The *Arabidopsis* mutant *Twisted Dwarf 1* (*twd1*) knock out displays a severe reduced development and a reduced cell elongation, a disoriented growth of all organs in both the epidermal and whole plant level. These phenotypes can also be observed in knock-out mutants of the ABC transporters *abcb*1, *abcb*4, *abcb*19 and it is more evident in double mutants. There is evidence that TWD1/FKBP42 interacts with the cytoplasmically localized C terminus of ABCB19. The role for TWD1/FKBP42 in creating functional ABCB19 is consistent with its reported interaction with the chaperone Hsp90 (Wu *et al*., 2010). So loss of TWD1 causes the mislocalization of ABCB1, ABCB4, and ABCB19 to the ER instead of to the PM. This behavior was confirmed by two independent ways: by transmission electron microscopy of immunogold-labeled samples in the case of ABCB19 and by confocal microscopy of fluorescent tagged fusion proteins (Wu *et al.*, 2010). The ER localization reported was obtained by *in vivo* confocal microscopy of a functional TWD1-CFP driven by the native promoter by the overlap of *twd1* phenotype with the *abcb1, abcb19* double mutant. It was also studied the localization of other unrelated class

of IAA efflux carriers with a completely different result. For example the PIN-FORMED2 auxin transporter or PM marker proteins was not affected by loss of TWD1/FKBP42 gene. The three ABCB components are synthesized at the ER compartment and TWD1/FKBP42 assist the passage to the PM, in this way the correct auxin movement allows the normal development of the plant. TWD1/FKBP42 belong at the immunophilin (PPI) superfamily whose biological functions are still largely uncharacterized in plant.

Auxins

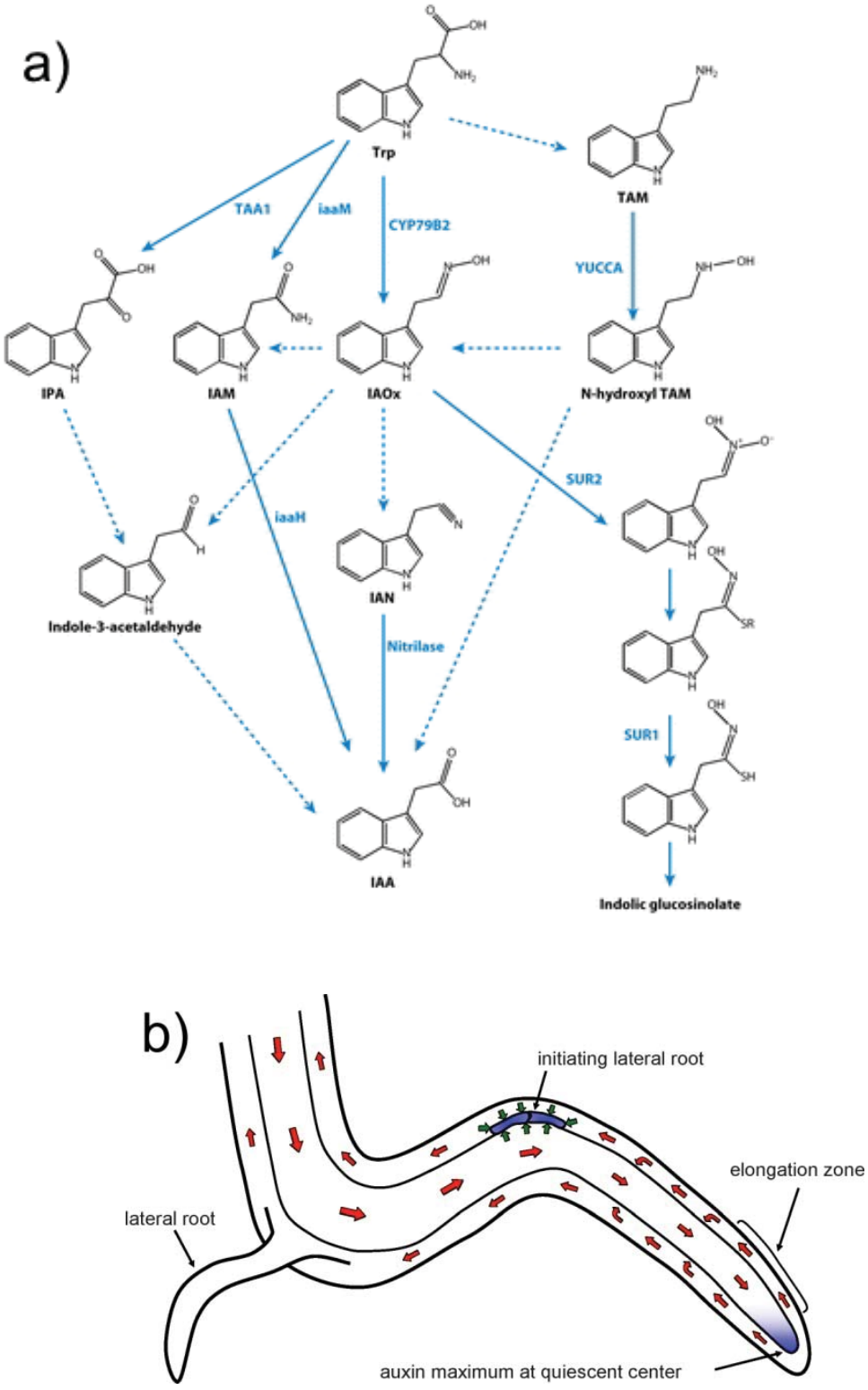
IAA synthesis

IAA comes from the meristematic zone, it is right there where it is synthesized. Actually not all IAA synthetic pathways are elucidated: in many IAA pathways, some pathogens interfere in the IAA plant pathways or it is possible to find pathogens with only some intermediate species isolated. At the moment there are only two clear things: the amino acid tryptophan plays a central role in IAA biosynthesis but also exists a pathway independent of this amino acid (Zhao, 2010).

Long distance IAA movements

After its synthesis, from the apical meristem, IAA goes toward the root tip, creating concentration gradients across various plant tissues (acropetal flow; Smith, 2008). When the auxin reaches the root follows its movement by the phloem (root central section). Once in the quiescent centre, changes the direction: close to the epithelial cell comes back (basipetal flow) and this flow and leads the secondary root primordia initiation. A plant connection between hormones and chaperones has been identified. Curiously AtTWD1 / FKBP42 shares some similarity with FKBP506 a chaperone that participates in insulin intracellular transport. Sequence homologies and analogous functions of many of the protein components of the two systems further suggest parallel mechanisms (see Figure 13c). There are data about the first stages of this process, with vesicle formation on the top side of the cell (closest to the synthesis site). This ends with exocytosis to the bottom side of the cell and the recycling of some vesicles. With endocytosis and exocytosis the cell takes IAA and gives IAA. As reported before for the IAA entrance, there are several possibilities, but auxin can only exit the cells via vesicle with basal-localized efflux carriers (Taiz and Zeiger, 2006, www.plantphys.net). This asymmetric transport mechanism

maintains cellular polarity. The genes that encode the auxin polar efflux are the PINs and ABCB/PGPs genes that can interact together cooperatively augment the IAA^- efflux.



c)

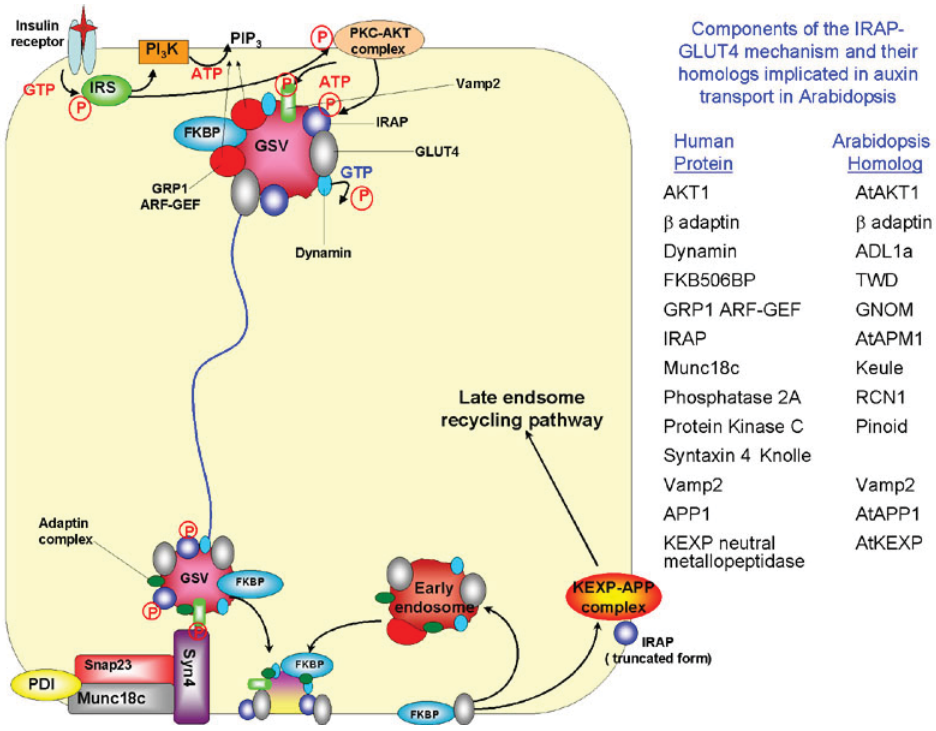


Figure 13: **a**) scheme of several IAA synthesis pathways, dashed arrow means hypothetical pathway; **b**) IAA movement into the root; **c**) an external signal (hormone binding) triggers a phosphatidilylinositol / PINOID phosphorylation cascade that activates asymmetric vesicular trafficking by: 1) causing dissociation of either an FKB506 binding protein TWD1 or Cyclophilin 5; 2) relocation of an inhibitory GNOM from an endomembrane compartment to PIP3–enriched plasma membranes; 3) recruitment of a dynamin.

Cell to cell movement

IAA is expelled out of cells by PIN1 into the apoplast, where is caught by ABP1, then delivered to the second cell as in Figure 14a. It seems that ABP1 alone promotes the PIN1 recycling and the consequent reduced exit of IAA. ABP1-IAA stabilizes PIN1 in the PM increasing the extrusion from first cell (Sauer and Kleine-Vehn, 2011). For the second cell the ABP1-IAA and ABP1 effects are opposite: ABP1-IAA complex promotes the clathrin endocytosis so the IAA entrance rate in the second cell increase. Unliganted ABP1 inhibits the clathrin-mediated endocytosis (Robert *et al*., 2010). All these regulations involve genes like ROP6 and ROP2.

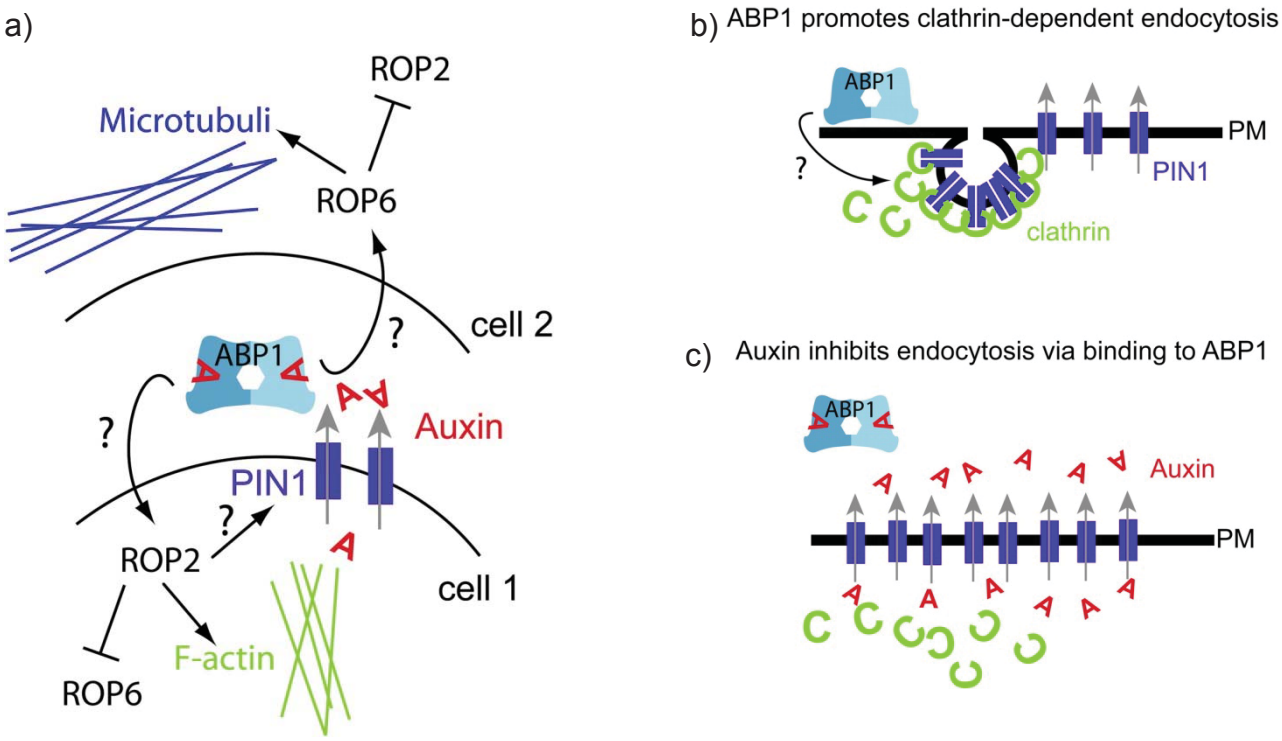


Figure 14:
a) general view of ABP1-IAA complex between onsecutive cells;
b) ABP1 action on PIN1;
c) ABP1-IAA action on PIN1 (http://www.sciencedirect.com/science/article/pii/S0092867410010755).

IAA genetic response

Downstream of all these processes IAA reaches the nucleus and induces the auxin dependent transcription. This hormone is pivotal for plant development: cell growth, root branches, meristem activity, flowering time, phototropism and gravitropism depend on modulated auxin signals. Like in jasmonate and giberellin signaling, the auxin signal is strictly tuned, uses the same strategy and even shares some proteins and the 26S proteasome. In the absence of auxin the target promoter zones are occupied by repressor proteins called Aux/IAAs. This situation prevents the RNA polymerase II and action on the auxin response genes. In the presence of auxin, the IAA-receptor TIR1 triggers the ubiquitination and degradation of these repressors by an ubiquitin ligase containing TIR1 as the subunit recognizing target proteins. Now the RNA polymerase II can induce the auxin genes response. The IAA-receptor is degraded by the 26S proteasome. New receptor components are *de novo* synthesized by the same auxin response genes to give a precise feedback regulation (Santner and Estelle, 2009).

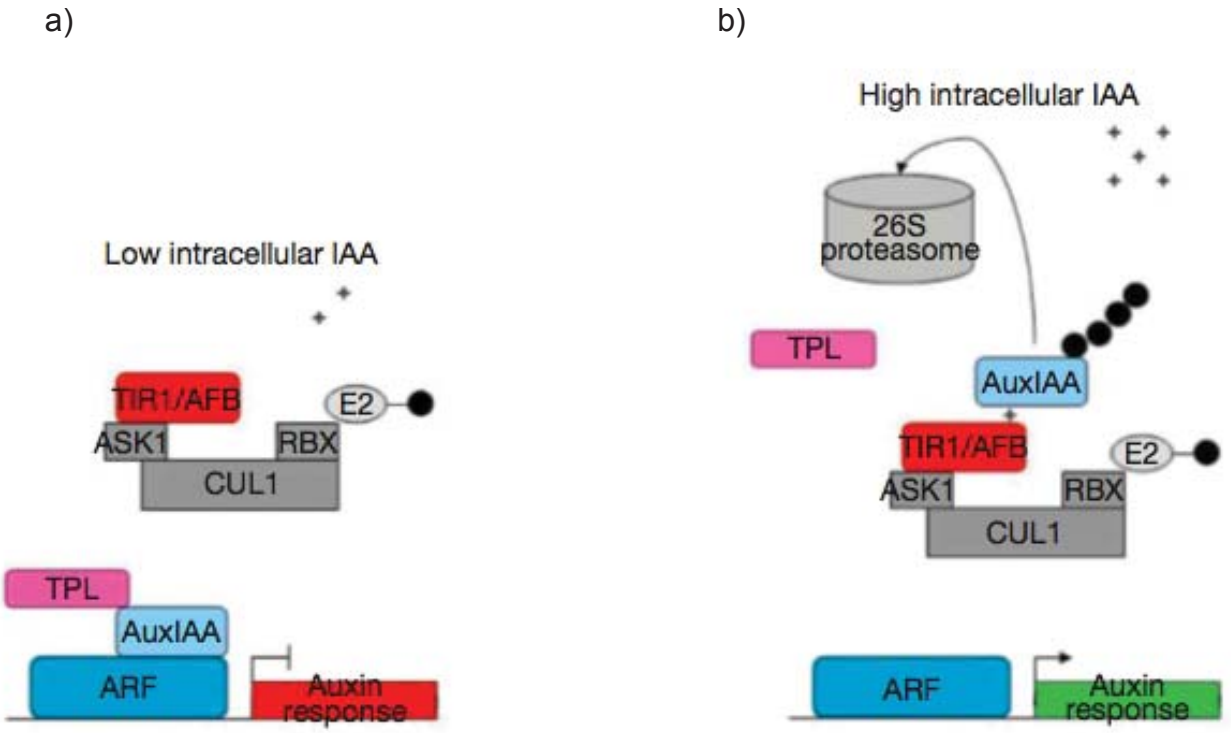
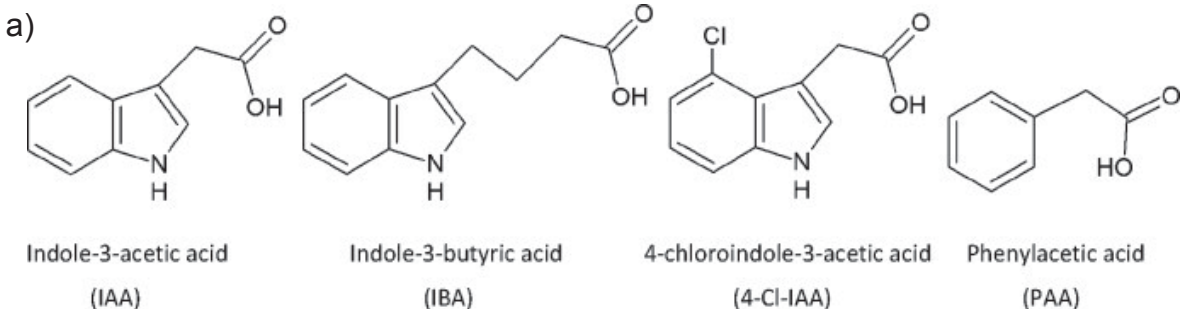
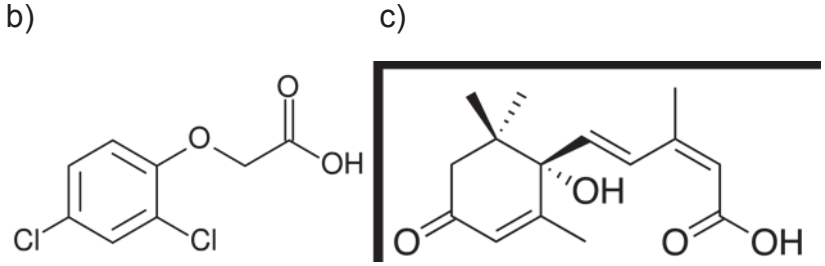


Figure 15: **a**) inhibition; **b**) and activation of auxin response genes (Santner and Estelle, 2009)

Different auxins, different roles





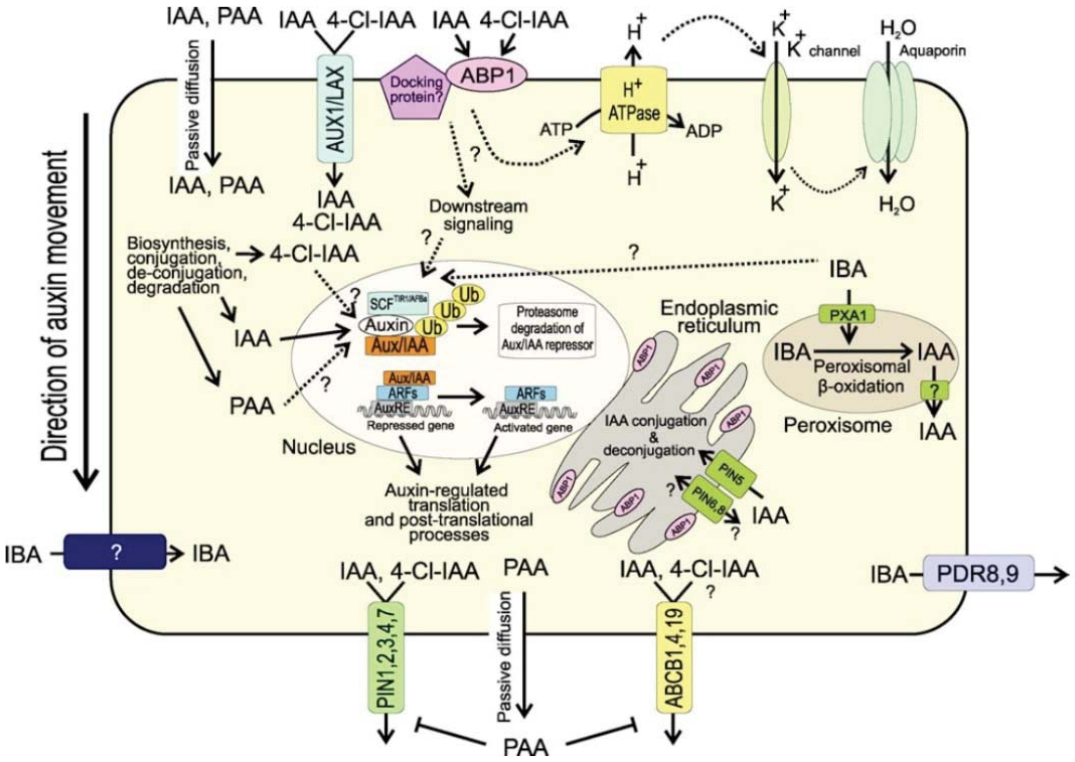


Figure 16: molecules with carboxylic group: **a**) endogenous auxin; **b**) 2,4D a synthetic auxin and **c**) Abscisic acid; **d**) plant cell with different auxins (Simon and Petrášek, 2011).

The auxins into the nucleus serve as a kind of molecular glue that stabilizes the interaction between the TIR1 receptor and the repressors or Auxin Response Factors (ARFs, see Figure 15b). Though the word auxin includes the endogenous and the synthetic compounds, the effects for each molecule can be different. For example, the commonly used synthetic molecule 2,4D has a pKa= 2.64, is poorly transported by auxin efflux carrier and does not bind to the ABP1. This means that it misses one of the best described auxin effect: the auxin-stimulated activity of PM H^+-ATPases and the subsequent cellular expansion. The elevated H^+ release is compensated by an inward release of K^+ ions, which provides a positive turgor pressure for cell elongation and expansion. At non-genomic levels the other auxins also increase cytosolic Ca^{2+} level and activate reactive oxygen species through the activation of phosphatidylinositol 3-kinase activity. Furthermore, as already described, with 2,4D missing the auxin promoting cell-to-cell transport, the exocytosis and the endocytosis rates do not change. All these processes are considered fast auxin responses because they start in few seconds/minutes after the exogenous auxin sub-ministration. As all the other auxins, 2,4D induces the same genetic response: the expression of new proteins, their synthesis and their effect, such as cell duplications and it takes several hours (a slow response). As for PAA, the term

endogenous auxin is approximative, because root symbiotic bacteria can release this hormone with anti-microbial activity and the plant can uptake it. PAA has a pKa= 4.31 and induce weakly IAA response. Unfortunately, the specifity of TIR1 for binding of PAA has not been tested yet. Data suggest that ABP1 has a high affinity to this molecule (Simon and Petrášek, 2011). 4-Cl-IAA is not present in *Arabidopsis*, but in other plants is present and it is more active than IAA, maybe for its long term stability. It has a pKa= 4.75, several assays suggest a fast response in cell expansion by H^+-ATPase. IBA is the storage form, 25-30% of total and it is even higher, under drought and salinity stress conditions. IBA has a pKa=4.82 level, IAA is its precursor and can converted back. It is exported out of cell by ABCB/PDR transporters (See the Figure 16d). IAA is the most abundant and studied, can be conjugated with amino acid, sugar and peptides. The release of active IAA from the conjugated form is under control of hydrolytic enzymes. It has a pKa= 4.75. An "antagonist" of auxin (stress signaling), abscisic acid, shows chemical property similar to the "endogenous" auxins (growth signaling) with a pKa= 4.75 (Figure 16c).

Immunophilins

Historically Peptidyl Prolyl *cis-trans* Isomerization proteins (PPIs) were the subject of intense research, because two subfamilies were originally identified as the cellular targets of immunosuppressant drugs. There are three subfamilies of immunophilins or peptidyl prolyl *cis-trans* isomerases (Figure 17; Lu *et al*.,2007)

a) FK-506 Binding Proteins (FKBP) are targets for FK-506 (an immunosuppressant) and for rapamycin (an immunosuppressive drug).

b) Cyclophilins are cellular target of cyclosporin A, an immunosuppressive drug actually clinically used.

c) Parvulins the third subfamily, like Pin1 protein, bind to and isomerizes specific phosphorylated Ser/Thr-Pro motifs in certain proteins.

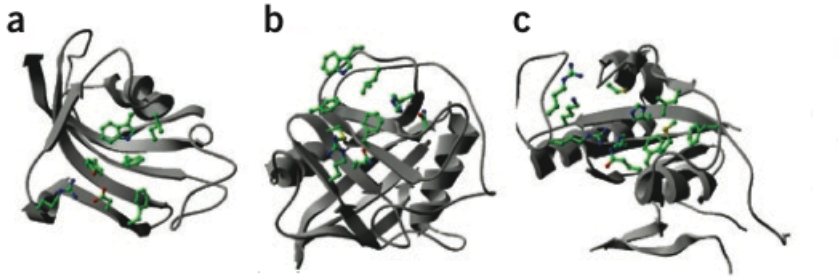


Figure 17: immunophilins **a**); **b**); **c**) structures of three sub-families Cyclophilins, FKBPs, Parvulins (Lu *et al.,* 2007).

FKBPs in yeast and *Arabidopsis*

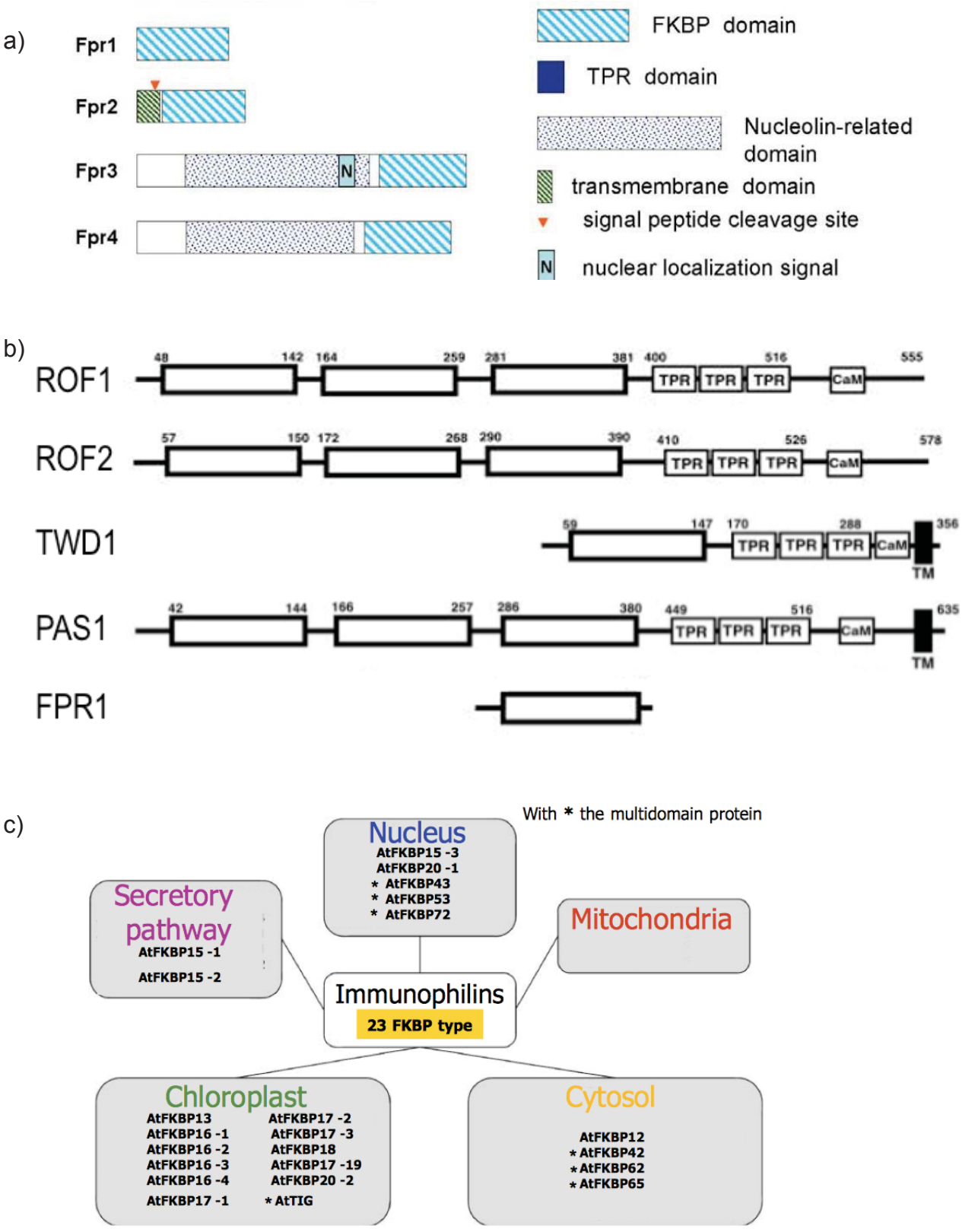


Figure18:
a) an overview in yeast of FKBPs (Arevalo-Rodriguez *et al.,* 2004);
b) examples of multidomain FKBPs, and the simplest FKBP in yeast, FPR1 (Romano *et al.,* 2005).
c) a schematic distribution of FKBPs in *Arabidopsis* by bioinformatic prevision (He *et al.,* 2004);

The immunophilins such as cyclophilins (CYP) and FKBPs can be classified as single domain or multiple domains (Figure 18). Simple domain immunophilins consist of a FKBP or CYP catalytic domain and the signal peptide. And there are others, which have multiple functional domain. Of the 23 *Arabidopsis* FKBPs, 16 are characterized as simple domains. The rest of AtFKBPs contain multiple FKBP domains. Four of them, including TWD1 (FKBP42), ROF1 (AtFKBP62), ROF2 (AtFKBP65) and PAS1 (AtFKBP72) have three tetratricopeptide repeat domains (TPR: binding sites for other proteins) and a calmodulin domain (which acts as a receptor for Ca^{+2}) (He *et al*., 2004). 50% of the *Arabidopsis* FKBPs are found in the chloroplast, other FKBPs are located in the endoplasmic reticulum, nucleus and in the cytosol. None of the FKBPs is considered to be located in the mitochondria, although previous studies suggest that these proteins could also be present there (Breiman *et al.*, 1992, Luan *et al.*, 1994). In yeast there are 4 FKBPs (Figure 18a) and 8 Cys A. A mutant of all these genes is viable and indicating that their function is not essential.

Enzymatic activity and its substrates

The three subfamilies, Cyclophilins, FKBPs and Parvulins, are genetically unrelated but share similar active site (Lu *et al.,* 2007, green residues in Figure 19) and promote the same folding reaction: the *cis trans* isomerization of peptide bonds preceding a proline residue. As reported before immunophilins were identified by their capability to inhibit the immune system in presence of some drugs. For this reason the number of papers is higher for mammalian cells than for model organism like yeast and Arabidopsis.

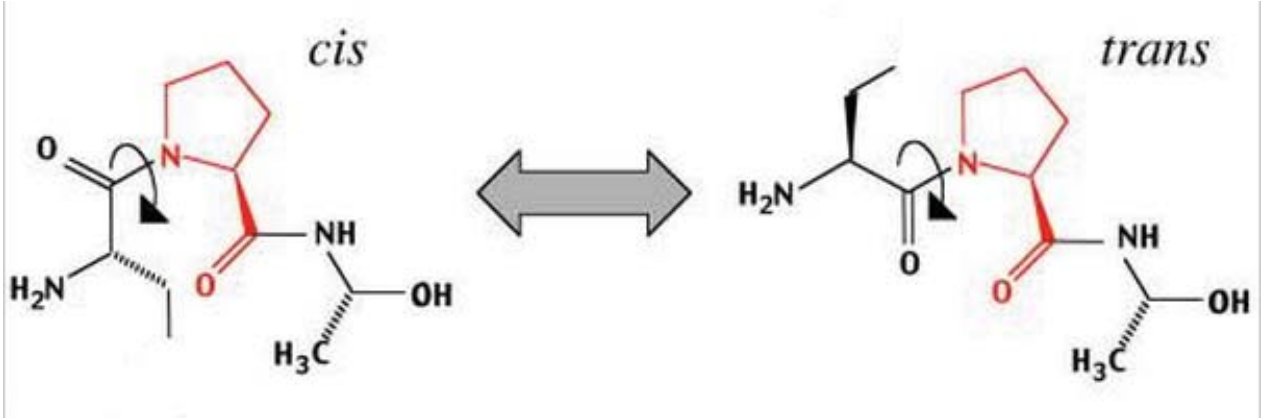


Figure 19: *cis trans* isomerization of the proline residue, in red (Shaw, 2002)

Interleukin-2 tyrosine kinase (Itk) is involved in the intracellular signaling events leading to T cell activation. It contains SH3, SH2, and catalytic domains common to many other kinases. The peculiarity is that Itk catalytic activity is inhibited by the peptidyl prolyl isomerase activity of CypA. NMR structural studies combined with mutational analysis show that a proline-dependent conformational switch within the Itk SH2 domain regulates substrate recognition and mediates regulatory interactions with the active site of CypA. CypA and Itk form a stable complex in Jurkat T cells that is disrupted by treatment with cyclosporin A. Activation may be accompanied by phosphorylation of Tyr-180 in the SH3 domain, expelling a bound proline-rich domain. c*is trans* isomerization around Asn-286–Pro-287 causes pronounced conformational changes in the C terminus of the Itk SH2 domain, leading to Itk kinase activation (Figure 20; Brazin *et al.*, 2002).

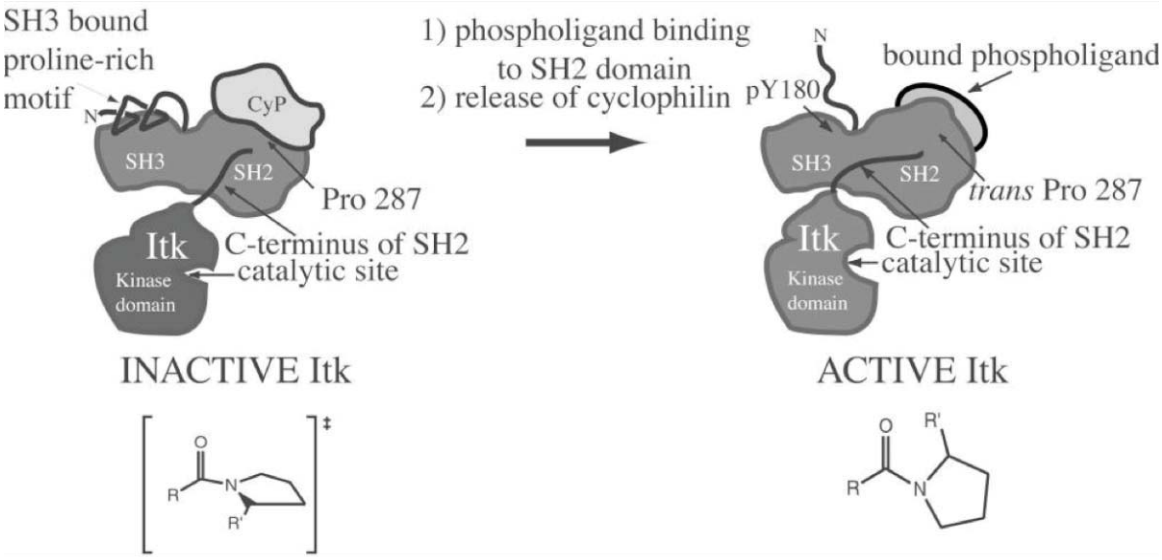


Figure 20: model for Itk regulation. Two “states” of Itk are depicted inactive, CypA-bound Itk; and active, phospho-ligand-bound Itk. The proposed configuration of the Asn-286–Pro-287 imide bond is illustrated for the inactive (Brazin *et al.*, 2002).

5-Hydroxytryptamine type 3 (5-HT_3) receptors bind neurotransmitter and can be targets of PPIases. In this proteins a specific proline (Pro8*) triggers the opening (gating) of anion channel (Lummis *et al.,* 2005). Located at the apex of the loop between the second and third transmembrane helices (M2–M3, see Figure 21), this proline can link binding of neurotransmitter to gating through a *cis–trans* isomerization of the protein backbone. Using unnatural amino acid mutagenesis, a series of proline analogues with varying preference for the *cis* conformer were incorporated at the 8* position. Proline analogues that strongly favor the *trans* conformer produced no functional channels. Among the functional mutants there was a strong correlation between the intrinsic *cis–trans* energy gap of the proline analogue and the activation of the channel, suggesting that *cis–trans* isomerization of this single proline provides the switch that interconverts the open and closed states of the channel. Consistent with this proposal, nuclear magnetic resonance suggest that a molecular rearrangement at Pro8* is the structural mechanism that opens the receptor pore.

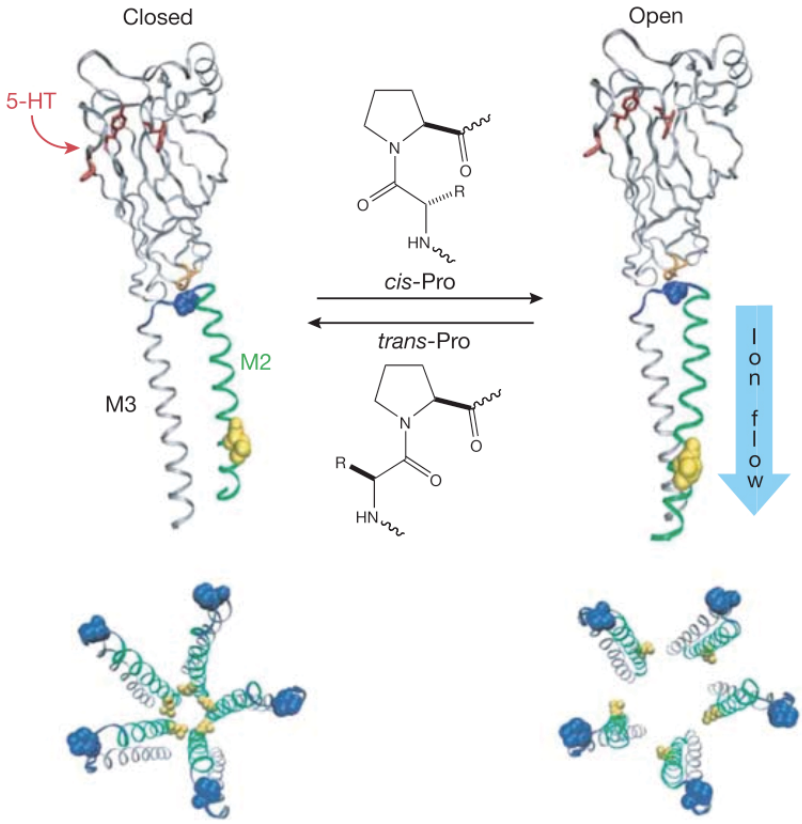
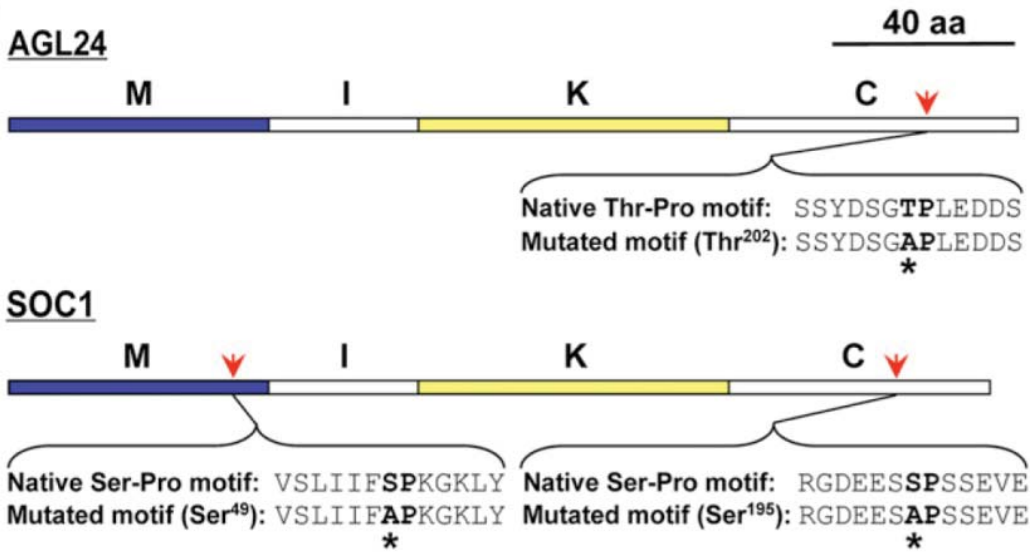


Figure 21: a single 5-HT_3 illustrating how *cis trans* isomerization at Pro8* could function as a hinge for movement of M2 during gating (Lummis *et al.,* 2005).

In *Arabidopsis thaliana* the PIN1-type parvulin 1 (not to be confused with the PIN1 auxin efflux tansporter) controls the floral transition. Its substrates are two MADS protein: AGAMOUS-LIKE 24 (AGL24) and SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1). As usual in this subfamily, PIN1 (Pin1At) recognizes the phosphorylated forms and changes the *cis-trans* conformation. When PIN1A is deleted, the isomerization rate goes down dramatically. This also occurs if AGL24 and SOC1 are mutated in the phosphorylation sites (Thr^{202} for AGL24 and Ser^{49} and Ser^{195} for SOC1), next to the *cis-trans* isomerization site (see Figure 22; Wang et al., 2010).

a)



b)

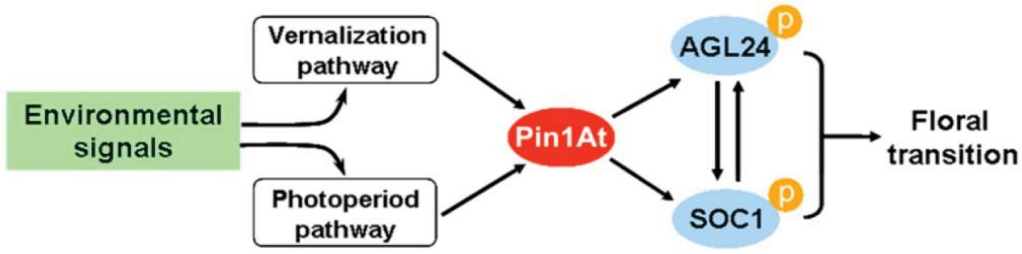


Figure 22: **a**) the two Pin1At substrates, AGL24 and SOC1 with their domains and the specific sites of phosphorylation that are next to the *cis-trans* isomerization site; **b**) model response to environmental signals, the vernalization and photoperiod pathways regulate the transcriptional levels of Pin1At responsible of the floral transition mechanism of AGL24 and SOC1.

ROF1 (FKBP62) and ROF2 (FKBP65)

Within the larger family of FKBPs from *Arabidopsis*, we find FKBP62 (ROF1) and FKBP65 (ROF2). These proteins are homologous, they share the same domains and a high identity of sequences (85%) (Aviezer-Hagai *et al.*, 2007). Both proteins contain a domain that has peptidyl prolyl *cis trans* isomerase activity and binding site for FK506, a TPR region responsible for binding to HSP90, and a calmodulin domain (Blecher *et al.*, 1996, Reddy *et al.*, 1998, Kurek *et al.*, 1999). Enzyme activity (peptidyl prolyl *cis-trans* isomerase) of the FKBP domain confers chaperone function, helping to the correct folding of other proteins. This catalytic activity is inhibited by binding to the immunosuppressant drug FK506. (Pirkl et al., 2001, Kurek et al., 2002). Previous analysis demonstrated that expression of ROF1 and ROF2 is regulated by ageing, abiotic and biotic stresses (Figure 23). ROF1 and ROF2 are expressed in vascular tissues, in seedlings and flowers. Expression increases upon heat stress. It was demonstrated that ROF1 binds to heat shock protein HSP90, and their interaction occurs via the TPR domain (Aviezer-Hagai *et al.*, 2007). In addition, the expression of ROF1 increases when the plant is subjected to high levels of NaCl and wounding (Vucich and Gasser, 1996). It was shown that HSP90 interacts with ROF1 in the cytoplasm and, when exposed to heat stress, both proteins bind to form a complex in the nucleus (Meiri and Breiman, 2009). Its location is due to the interaction of HsfA2 with HSP90.

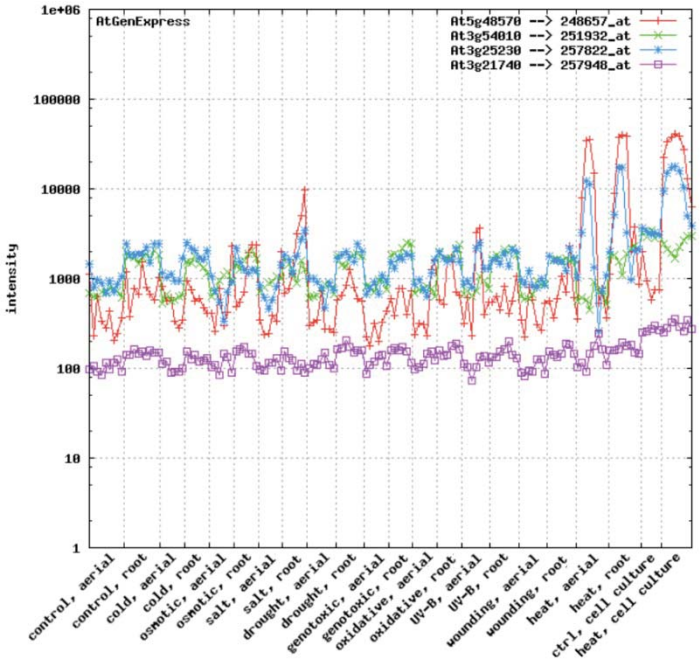


Figure 23: expression levels of ROF1 (At3g25230), ROF2 (At5g48570), PAS1 (At3g54010) and TWD1 (At3g21640) after different treatment (http:// www.weigelworld.org)

Heat Shock Proteins and co-chaperons

There are six major families within Heat Shock Proteins (HSPs): Hsp70 family (DnaK/Ssa); J-protein/Hsp40 family (DnaJ/Ydj1); Hsp60 family (GroEL); Hsp90 family; Hsp100 (Clp) family and small Hsp family (Rajan *et al*., 2009). In normal conditions Heat Shock Proteins (HSPs) are responsible for the refolding of denatured protein and the folding of new synthesized proteins. They have a key role also in protein complex maturation, the translocation of proteins through membranes (Noël *et al*., 2007) and the degradation of misfolded proteins (Rajan *et al*., 2009). During abiotic and biotic stresses there is an increase of their action. Two of the main heat shock types, HSP90 and HSP70, share these roles and are highly conserved in all organisms. In *Arabidopsis*, four HSP90 homologues localize to the cytosol (HSP90.1, HSP90.2, HSP90.3 and HSP90.4), and three HSP90 localize in the mitochondria (At3g07770), chloroplast (CR88), and endoplasmic reticulum (SHD/GRP94). The four cytosolic HSP90s are highly homologous to each other (86%-99% identity), which suggests that their activity is identical in *Arabidopsis*. HSP90.2, HSP90.3 and HSP90.4 mRNAs accumulate constitutively, while HSP90.1 mRNA increases extensively after heat shock in *Arabidopsis* seedlings (Yamada *et al*., 2008). HSP70 type is ubiquitous and only HSP101 (a cytosolic protein) seems to solubilize protein aggregates under heat stress condition. Normally a HSP does not work alone, for example a complex containing Hsp90 and its client protein often includes other proteins called ‘co-chaperones’, which work cooperatively together with Hsp90 to ‘chaperone’ the client protein.

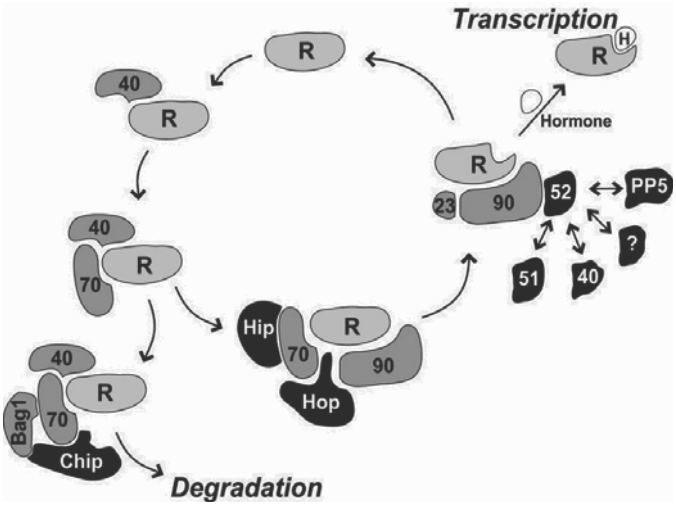


Figure 24: assembly of steroid receptor chaperone complex and its recycling for next cycle or complex degradation (Smith, 2004).

Hsp90 requires many specific co-chaperones for its function corresponding to a specific group of client proteins. Several Hsp90 and Hsp70 co-chaperones contain the tetratricopeptide repeat (TPR) domain, which interacts with the highly conserved EEVD motif at the C-terminal ends of Hsp90 and Hsp70. The acidic side chains in EEVD interact with a subset of basic residues in the TPR binding pocket called a 'carboxylate clamp'. The carboxylate clamp residues are conserved in the TPR domains of known Hsp90/Hsp70 co-chaperones. In *Arabidopsis* there are 36 proteins with this carboxylate clamp domain, including ROF1 and ROF2 (Prasad *et al.*, 2010). In mammals the mature steroid hormone receptor complexes contain any one of several co-chaperones that mutually compete for binding to Hsp90. These co-chaperones share a tetratricopeptide repeat (TPR) domain that mediates binding to the highly conserved C-terminal MEEVD sequence in Hsp90. Among the TPR co-chaperones observed in steroid receptor complexes are immunophilins such as two members of the FK506-Binding Protein (FKBP) family FKBP51 and FKBP52 (hsp56, p59, HBI) and cyclophilin 40 (CyP40). FKBP51 and FKBP52 are characterized by a conserved binding domain for the immunosuppressive drug FK506, and they show peptidyl prolyl isomerase activity as well as a chaperone-like activity *in vitro*. FKBP52 has a stimulating effect on many steroid hormone receptors (Smith, 2004). In plants, ROF1 and ROF2 are the most similar proteins to the mammalian FKBP51 and FKBP52. This suggests that ROF1 and ROF2, like the mammalian homologous FKBPs, could have a role in hormonal responses too. This is something promising, discovering the roles of ROF1 or ROF2 can be useful at the academic level but also in some agricultural applications. It could be easier to work directly on a crop plant but crop plants normally miss a lot of genetic tools and its life cycle is longer. It is better to choose a model system to reach the specific knowledge for a second application step in the target specie.

Arabidopsis thaliana, Saccharomyces cerevisiae: two model systems

Arabidopsis thaliana is a self-pollinating plant, dicot, family *Brassicaceae*. It is related to many crop plants such as mustard, cabbage or turnip, but has no agronomic value. The plant is distributed naturally in many regions of Europe, Asia and North America. Many ecotypes have been collected from the wild, being *Columbia* and *Landsberg erecta* the most used in research (Meinke, 1998). The plant is small, about 30cm tall, its compact size allows a higher dose of planting (up to 10,000 plants/m^2), has a reduced genome (125 Mb). The complete genome sequence now defines 25500 genes, distributed among 5 chromosomes. Their life cycle is short, about eight weeks under optimal conditions, it is a very prolific plant with seeds that in optimum storage conditions are viable for several years. It is used as a model organism in studies of cell and molecular plant biology, allowing quick and effective testing hypotheses that then can be extrapolated to plants of agronomic interest. It is possible to apply to *Arabidopsis thaliana* most molecular biology tools, such as mutagenesis, mutant screening, positional cloning etc. High efficiency genetic transformation of *Arabidopsis* plants is achieved by flower infiltration with an *Agrobacterium tumefaciens* suspension (Bechtold and Pelletier, 1998), ad this simple method has generated numerous collections of mutant lines (Azpiroz-Leehan and Feldman 1997, Bent 2000). In some of these collections generated by insertion of T-DNA or transposons, the regions flanking the T-DNA or transposon have been sequenced in many lines. This allows reverse genetics, that is, to investigate the phenotypic effect of altering a particular gene. These collections include more than 200,000 lines (Kuromori *et al*., 2004, Rosso *et al*., 2003, Pan *et al*., 2003). Finally, indicate that the major advantage of *Arabidopsis thaliana* over bacteria and yeast is to be a multicellular organism, which can address studies of integrated response to stress. *Arabidopsis thaliana* genes can be functionally expressed when fused to GFP, allowing the localization of proteins in the living plant organs by fluorescence microscopy. Already in the 1930's *Saccharomyces cereviseae* was introduced in the laboratories, its ease of manipulation transformation, and the homologous recombination for reverse genetics have contributed to advances in molecular and cellular biology. It was a reference organism for long time, moreover in 1996 it was published (Goffeau *et al.*, 1996) the complete sequence of the first eukaryotic organism: *Saccharomyces cerevisiae*. The ease of genetic manipulation has enabled the

dissection of the function of gene products from other eukaryotes in the yeast system. Among all eukaryotic model organism, *Saccharomyces cerevisiae* combine several advantages. It is unicellular and can be grown on defined media providing complete control over environmental parameters (Strathern *et al.*, 1981, Guthrie, 1991). A large number of examples provide evidence that a substantial percentage of cellular functions are highly conserved from yeast to mammals and that corresponding genes can often complement each other. *Saccharomyces cerevisiae* genome (12.8Mb) is about 200 times smaller than human genome. Its genome contains approximately 6,000 Open Reading Frames (ORFs), most of which are likely to encode specific proteins. The genome of *Saccharomyces cerevisiae* has 16 chromosomes ranging in size between 250Kb and 2500Kb (Goffeau *et al.*, 1996). Yeast has a generation time of approximately 90 minutes so mass production of cells is straightforward. Simple procedures for isolation of high molecular weight DNA and mRNA are routinely used in many laboratories. High efficiency transformation of yeast cells is achieved using $CH_3COO^-Li^+$ procedure (Ito *et al.*, 1983) or by electroporation. A large variety of vectors have been designed to express genes of interest in yeast cells. Moreover, a large number of yeast strains carrying auxotrophic markers, drug resistance markers or defined mutations are available. The ease of gene disruption using simple step gene replacement is unique in yeast. The yeast system has also proven an invaluable tool to clone and to maintain large segments of foreign DNA in YACS, which have been extremely useful for many genome projects of higher eukaryotes. *Saccharomyces cerevisiae* has been used to create a comprehensive genome-wide data sets for a eukaryotic organism covering areas such as, post-translational protein modification, gene expression patterns in response to many different stress conditions, and phenotypic analysis of loss-of-function mutations corresponding to all non-essential genes (Merchan, 2007). A special mention is necessary for the protein-protein interaction networks: actually the two-hybrid system and the pull down of proteins by affinity purification are largely used to identify protein-protein interactions. Thus, this model organism represents an important point of reference in the study of gene function in higher eukaryotes, such as plants and humans. In fact, the basis of much of what we know about basic cellular processes, such as protein regulation, cell cycle progression and cellular metabolism was first discovered in yeast.

OBJECTIVES

The present study has the following objectives:

1. To investigate if gain and loss of function of ROF2, a peptidyl prolyl *cis trans* isomerase of *Arabidopsis thaliana* induced by intracellular acid stress, has an impact on tolerance to this stress

2. To elucidate the mechanism of tolerance to intracellular acidification (caused by weak organic acids) conferred by over-expression of ROF2

3. To characterize additional phenotypes of the ROF2 over-expressing plants

MATERIALS AND METHODS

Plasmids maps

In the present study we used the following two plasmids (pictures from 25 to 27):

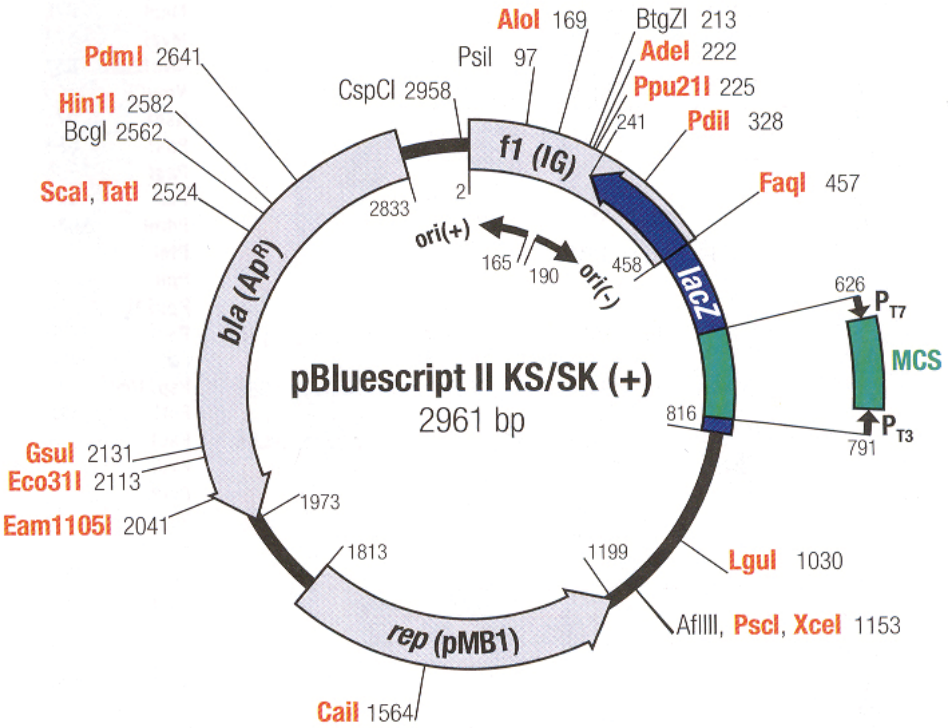


Figure 25: pBluescriptSK(+) a multicopy plasmid with ampicillin resistance. It has a good multi cloning site (MCS). This is inside to the lacZ and allows colour selection. In presence of X-Gal and IPTG only the colonies with a insert that broke the LAcZ have a white colour, the other ones are blue. (Stratagene)

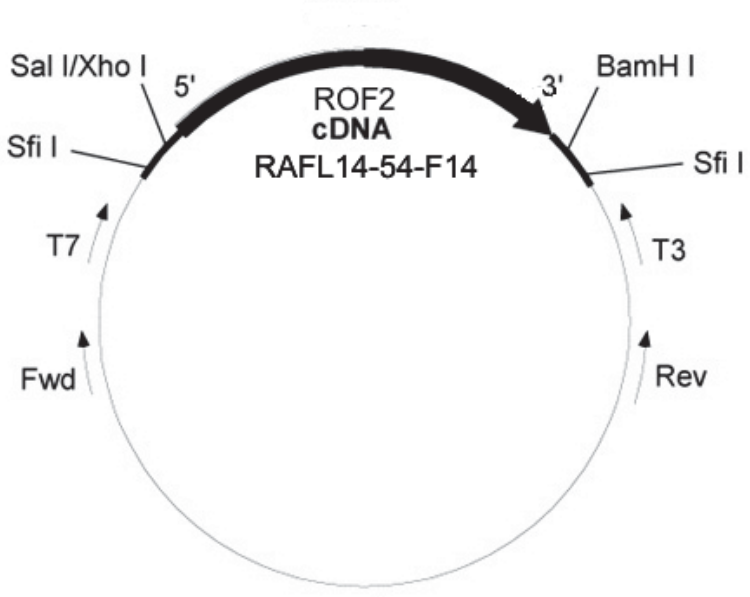


Figure 26: plasmid with full lengh cDNA of ROF2 gene. It was obtained by RIKEN BRC Experimental plant division (http://www.brc.riken.jp/lab/epd/Eng/catalog/RAFL.shtml).

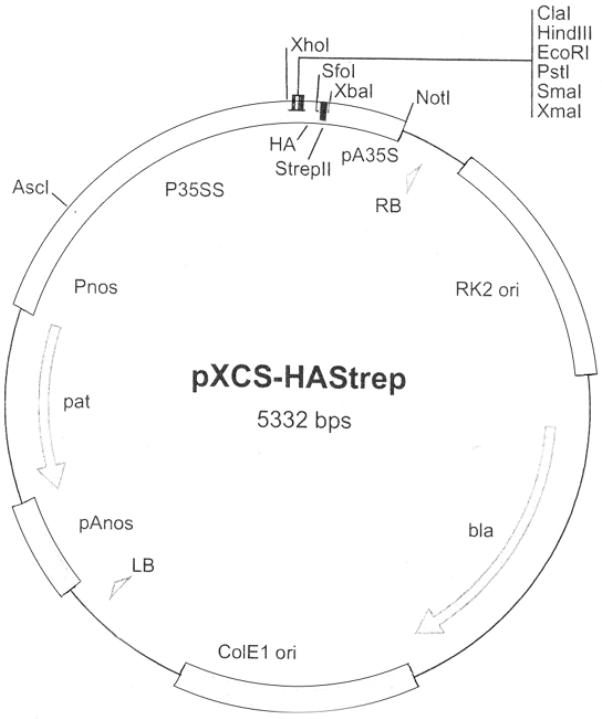


Figure 27: pXCS-HAStrep a plasmid for over- expression of recombinant proteins in plants with the viral 35S promoter. It contains a cloning site which allows the cloning of a gene in frame with a HA tag and strepII tag (for protein purification by pull down). Also has a bacterial gene for ampicillin resistance and BASTA® selection in plants (Witte *et al.* 2004).

Arabidopsis solutions

Murashige-Skooge medium (MS)

The agar medium contained 0.4% Murashige and Skoog (1962) salts (MS) with 1% (w/v) sucrose, 10mM Morpholino Ethane-Sulfonic acid (MES) and 0.8% (w/v) agar, pH adjusted to pH 6 with Tris base. In acid stress conditions the pH was adjusted to 5.5.

We added acetic acid (after autoclaving) from 1M stock solution buffered at pH 5.5 with Tris-base to a final concentration to the medium in the Petri dish of 3.5mM.

Different concentrations of Norspermidine, Hygromycin B and LiCl were prepared by adding appropriate amounts of reagents to the basal medium as before after autoclaving. For liquid culture, the MS medium was prepared without agar, and using 250ml flasks or suspension-culture-6-well plates (Greiner).

K^+ -free medium

2.5mM $Ca(NO_3)_2$, 2mM $MgSO_4$, 0.1mM NaFeEDTA, 80mM $Ca(H_2PO_4)_2$, 25mM $CaCl_2$, 25mM H_3BO_3, 2mM $ZnSO_4$, 2mM $MnSO_4$, 0.5mM $CuSO_4$, 0.5mM Na_2MoO_4, 0.01mM $CoCl_2$, 1% sucrose and 2.5mM MES. NH_4^+ was added as $NH_4H_2PO_4$ at final concentration of 2mM. The pH was adjusted to 5.7 with $Ca(OH)_2$.

Arabidopsis methods

Plant material, growth and transformation

Arabidopsis thaliana (Columbia-0 ecotype) plants were grown under greenhouse conditions (16h light / 8h dark, at 23ºC, 130μE · m^{-2} · s^{-1} and 70% relative humidity) in pots with soil-vermiculite mixture as described (Alejandro *et al.*, 2007). Plants grown in pots were transformed by flower infiltration (Bechtold and Pelletier, 1998; Clough and Bent, 1998). For *in vitro* culture, seeds were surface sterilized with commercial bleach and rinsed with sterile water. Stratification was conducted during 3 days at 4ºC and then plates were transferred to chambers under the conditions described above. When indicated, the medium was supplemented with 3.5mM acetic or propionic acids (pH 5.5 with Tris-base). For growth in liquid culture the MS medium was prepared without agar and using suspension-culture-6 well plates (Cellstar, Greiner). Each well contained 7ml medium and 3 seedlings that have been cultivated during 6 days in solid medium. Plates were incubated with shaking (100r.p.m.) under the conditions described above.

Selection of At5g48570 and At3g25230 knock-out *Arabidopsis* lines

One At5g48570 (*ROF2*) Arabidopsis knockout mutant line (Sail_355_A02), and one At3g25230 (*ROF1*) Arabidopsis knock-out mutant line (SALK_024090) were obtained from the Salk Institute (Genomic Analysis Laboratory). The seeds of ROF2 were selected by 50μM BASTA®, for 4 days, the seeds of the second one were sown and grown on MS medium with 50 mg/ml of kanamycin for 6 days. Resistant seedlings were transplanted to soil for 3 weeks, after which genomic DNA was extracted from the individual plants (more details about genomic DNA extraction method in appendix, page 111).

PCR reaction (genotyping)

Homozygous knockout mutant plants were selected by PCR using three primers.

ROF2 reactions:

ROF2 LP 5´-CTT GCG TAT TTT CCA GCT TTG-3´,
ROF2 RP 5´-CAG TCA AGT AAG CTC GGC-3´,
SAIL-LB3 5´-TAG CAT CTG AAT TTC AT AAC CAA TCT CGA TAC AC-3´.

ROF1 reactions:

ROF1 LP 5´-ATG ATT GAG GAT GAC AAA TGG-3´,
ROF 1 RP 5´-GAT TCC CCA TAT GCT AGC TCC-3´,
SALK-LBb1.3 5´-ATT TTG CCG ATT TCG GAA-3´

The same primers were used to select the double mutant after the breeding between *rof2* and *rof1* homozygotes.

Over-expression of *ROF2* in transgenic plants

ROF2 cDNA containing the complete open reading frame was obtained from Genomics Science Center, RIKEN, Japan.

The cDNA was cloned directionally into the *Xho* I and *Eco R*I sites of pXCS-HAStrep by PCR with

ROF2XhoI 5'-ATA CTC GAG CAG AAA ATG GAA GAC GAT TTC GAC-3'
ROF2EcoRI 5'-CGC GAA TTC TCA TGC CTT GGT GTC AAT ACT CAT-3'

To generate *35S::FPR1* lines we amplified the gene from *FPR1*-YCp50 (kindly doned by M. Hall lab.) using the primers

FPR1XhoI 5'-GGG CTC GAG ATG TCT GAA GTA ATT-3'
FPR1PstI 5'-GGA AAA GAC GTC GTT GAC CTT CAA CAA-3'

and cloned directionally into *Xho* I and *Pst* I sites of pXCS-HAstrep. Both plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101::pMP90RK by electroporation. *A. thaliana* (Columbia ecotype) wild-type plants were transformed by flower infiltration (Bechtold and Pelletier, 1998).

Transgenic plants with *35S::ROF2* and *35S::FPR1* constructions were screened on MS agar medium containing 50µM BASTA®, and the expression of the transgene was further confirmed by northern analysis as described in appendix pages 110 (plant RNA extraction), 113 (gel separation), and 114 (transfering and radiolabeled revelation), or by RT-PCR as described in Bissoli *et al.,* 2012.

K^+ Determination

Five-day-old seedlings were grown in liquid culture for 12 days and then transferred onto a second liquid culture. For sampling at different times, seedlings were collected, rinsed twice briefly with cold 30mM $MgCl_2$ and once with cold distilled water. For K^+ determination, samples were dried at 50ºC for 3–4 days. After dry weight measurement, samples were extracted with 0.1M HNO_3 for 30min. at room temperature, cell debris were removed by filtration and mono-valent cations were determined by atomic absorption spectrophotometry (Naranjo *et al*., 2003).

Measurement of Rb^+ uptake

Potassium-free medium was prepared for measurement of rubidium uptake. 5-day-old seedlings from MS agar plates were transferred to suspension-culture well containing 7ml of liquid MS medium. After 10 days, the seedlings were collected, rinsed briefly with water and added to a 7ml uptake solution containing potassium-free medium supplemented with 2mM RbCl. The uptake was performed at 23ºC under white fluorescent light. At the completion of uptake, the seedlings were rinsed twice with 7ml of cold 20mM $MgCl_2$ and once with water. Finally, the seedlings were treated, Rb^+ content was determined by atomic absorption spectrophotometry.

Measurement of PM H^+-ATPase activity in *Arabidopsis* roots

Plants were grown for 18 days in vertical plates with medium containing no sucrose under the conditions described above. About 0.5g fresh weight of roots were frozen with liquid nitrogen and homogenized with mortar and pestle. The homogenization buffer, differential centrifugation, resuspension medium and specific assay for plasma membrane H^+-ATPase activity were as described (Serrano, 1988).

Root acidification assay

Plant were grown for 18 days on MS and spread on MS plates (without MES buffer) containing 0.005% (w/v) of bromocresol purple; pH 6 to adjusted with KOH. Plates were visualized after 6 hours in a lighted growth chamber and the yellow colour provided a qualitative estimation of external acidification (more details in appendix, page 112).

The quantitative assay is based on measuring the acidification of the external medium of plants with a pH electrode (Crison GLP22 with pH electrode 52.08).

Plants were grown for 12 days in vertical plates with normal MS medium and then transferred to a plate with sterile water and incubated 24h in darkness. The starved plants (about 5 plants with 2mg root weight) were then incubated in a vessel with 5ml of MS medium without sucrose and without buffer and the pH was adjusted to 5.7 with KOH. Only the roots were submerged and shaking was effected with a magnetic bar.

Proton pumping was started by addition of sucrose (1% final) and the pH recorded during the first 15min. Data were analyzed with a PC computer using the GLP-PC software from Crison. Total pH change was 0.03-0.10 pH units for the different samples and buffering

power was calibrated by addition of 5µl of 10mM HCl (producing a pH decrease of about 0.05 units).

Electrophysiology of *Arabidopsis* root epidermal cells

Membrane potential (Em) and intracellular pH were determinated by María Jesús García Sánchez and Jose A. Fernandez in the "Departamento de Biología Vegetal" from "Universidad de Málaga", following the protocol reported below.

Seeds of *Arabidopsis thaliana* wild type (*Col-0*) and ROF2 over-expressing lines were surface sterilized, washed with sterile distilled water and grown aseptically in Petri dishes with agar and MS medium as described above. After 48 h in the dark at 4 ºC, seeds were grown vertically at 25ºC for 10-15 days, at a light intensity of 150µmol·m^{-2}·$sec.^{-1}$ with a photoperiod of 16/8 h light/dark.

Membrane potentials were measured using the standard glass microelectrode technique (Felle, 1981). Seedlings were mounted in a Plexiglas chamber (1.1ml volume) with continuous perfusion of the assay medium (flux of 10ml·$min.^{-1}$), containing 0.1mM $CaCl_2$, 0.1mM KCl, 0.1mM NaCl, 12mM MES-Bis tris propane (pH 5.5). Epidermal root cells (located at ≈ 0.5cm from root tip) of plant seedlings were impaled with single-barrelled borosilicate glass vertically pulled, filled with 500mM KCl and connected to a high impedance differential amplifier (WPI FD223, World Precision Instruments, Saratosa, FL, USA).

Cytosolic pH and membrane potential was simultaneously measured using double-barrelled microelectrodes. Microelectrode pre-treatment and backfilling was similar to the H^+-selective microelectrodes described previously (Felle, 1987; Fernández *et al.*, 1999). After 30 min. heating at 180ºC, the barrels containing the sensor were sylanized by the addition of one drop of dimethyldichlorosilane (0.1% in benzene) in the blunt end of the pipette. The microelectrodes then were heated again for 60 min at 180 ºC and backfilled with the H^+-sensor, once cold. The H^+-sensor was prepared by mixing the Hydrogen Ionophore II-cocktail A in a mixture of polyvinylchloride/tetrahydrofuran (40mg/ml) at a ratio 1/6 (v/v). The microelectrodes were filled with the H^+-sensor cocktail and stored in a dry chamber (silica gel) until the evaporation of tetrahydrofuran. The microelectrode barrel containing the H^+ sensor was backfilled with a mixture of 500mM KCl and 100mM Mes-Tris adjusted to pH 4.3, whereas the voltage barrel and the reference electrode were filled with 500mM KCl.

The signals from the H^+-selective and voltage barrels were measured and simultaneously subtracted by the high impedance differential amplifier. The H^+-selective microelectrodes were calibrated before and after the measurements with buffers solution containing 96mM KCl and 2.5mM MES – Bis tris propane (pH 5.3; 6.3 and 8.3). Calibrations curves showed slopes round 49mV / pH unit.

Fluorescence microscopy of GFP fusion proteins

The binary plasmid to express a ROF2-GFP fusion was constructed as follows.
The coding sequence of ROF2 was amplified from plasmid RAFL 14-54-F14 (RIKEN, Tsukuba, Japan) as described above. The PCR product was phosphorylated and inserted in the *Eco R*V site of pBluescript SK-. A *Bam H* I - *Xho* I digest of this plasmid was inserted in the *Xho* I and *Bam H* I sites of plasmid JM743. This plasmid was made by inserting a fragment containing the GFP sequence (Chiu *et al.*, 1996) in the *Bam H* I and *Eco R* I sites of plasmid pBluescript SK-. An *Eco R* V - *Pvu* II fragment from this plasmid, containing the GFP and polilinker region of pBluescript, was inserted in the *Sma* I site of plasmid pXCS-HAStrep (Witte *et al*., 2004) with the right orientation. The recombinant ROF2-GFP plasmid was introduced into *Agrobacterium tumefaciens* GV3101::pMP90RK and used to transform *Arabidopsis*. The fluorescence of GFP fusion proteins was observed in roots of the transgenic plants by a Leica TCS-SL confocal microscope and a laser scanning confocal imaging system. A 488nm excitation wavelength and a 510nm emission wavelength were used.

Hormonal determination

We used only the root part of seedlings grown in long day condition for 18 days. For IAA determination we prepurified by solid phase extraction and after we asked the help of laboratories with the liquid chromatography apparatus coupled to mass spectrometer. For IAA a private specialized laboratory permormed the measures following the method descripted in Tromas *et al.*, 2009. Indeed Aurelio Gomez laboratory crew (in Castellon) performed the ABA extraction in aqueous solution and the concentrations were determinated with the method described in Arbona *et al*., 2010.

RESULTS

Initial data

Our starting point was a microarray analysis of genes induced by acid stress in *Arabidopsis* (Bissoli *et al., 2012*).

Arabidopsis genome encodes 23 immunophilins but only two were over-expressed upon weak organic acids condition: *ROF1* and *ROF2*, with the second one being expressed at higher levels. These data were confirmed with semi-quantitative PCR (supplemental data of Bissoli *et al.*, 2012).

in silico analysis

At the beginning of our work we could not find any bibliographic reference about *ROF2*, whereby we submitted queries at the Genevestigator about several FKBP genes (subfamily) and later at the genes that share higher homology with *ROF2*. Thus, we compared the expression levels of *ROF2* with *TWD1* (At3g21640), *PAS1* (At3g54010) and *ROF1* (At3g25230) in different abiotic stress conditions. All of them have FKBP, TPR and CaM domains as ROF2 and we have seen that the expression level of ROF2 changes frequently, suggesting us that it was implicated in many different kinds of stresses (Figure 28).

TWD1 and *PAS1* mRNA levels are poorly similar to ROF2, while ROF2 reaches the top expression levels with heat treatments, like ROF1. Again with Genevestigator, it was possible to see the expression profile during the different steps of development and we saw that ROF2 and ROF1 have similar trends in many development steps (Figure 29a). Finally these data indicated that ROF2 expression level, in normal conditions, was always lower than ROF1.

These genes show an homology of 85% and with the help of another bioinformatic tool, the *Arabidopsis* eFP Browser (in The Bio-Array Resource for Plant Biology site) we tested the ROF1 and ROF2 co-expression in roots of young rosette plant, just the organ that we expected a response to stress generated by weak organic acids, Figure 29b. And in the same web site was observed a detailed root map of topological expression

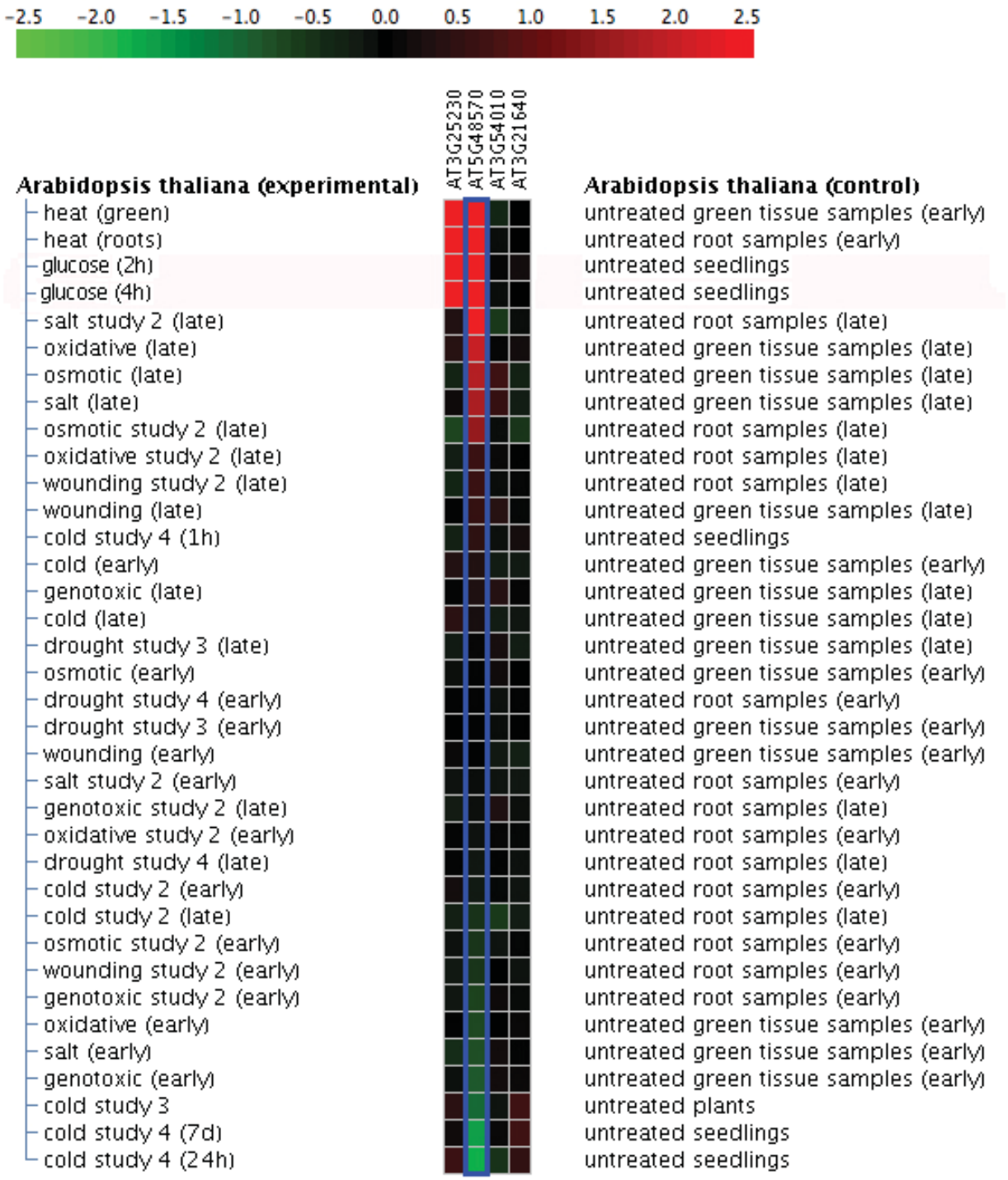


Figure 28: Genevestigator plot with expression levels of AtROF1 (At3g25230), AtROF2 (At5g48570), AtTWD1 (At3g21640) and AtPAS1 (At3g54010) after several abiotic stresses (http://www.genevestigator.com/gv/).

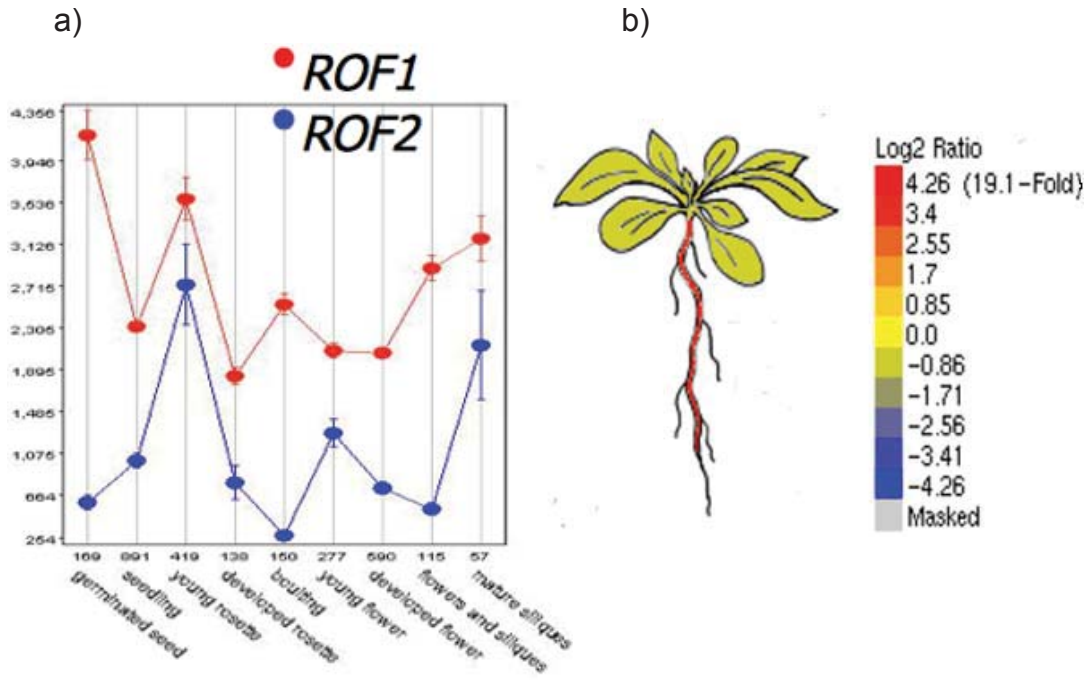


Figure 29:
a) expression levels of ROF1 and ROF2 during the different plant development steges (http://www.genevestigator.com/gv/); **b**) higher level of expression for both ROF1 and ROF2: in vegetative root (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

We found another microarray data base indication that suggested a genetical relation between ROF1 and ROF2.

The results were analized by Pearson Correlation Coefficients (r-values) for all genes against two queries that were displayed on a scatter plot. In this plot all the blue point belong to a probe of 121 AtGenome1 or 322 ATH1 arrays stored in with *Arabidopsis* data mining tools (http://www.arabidopsis.leeds.ac.uk/act/, Leeds University, UK, Manfield *et al.*, 2006).

In our case we have seen the correlation between the different immunophilins and the best correlation was between ROF1 and ROF2 with a result of +0.76 a high correlation value, that suggest that the two have a similar behavior, in all different conditions of the probe. In the correlation the top scale value is 1.

With all these bioinformatic data we thought that it is possible that these two genes share a similar function in plant cells. We planed to generate ROF2 *Arabidopsis* over-expression lines and T-DNA knock-out mutant lines, the singles *rof2* and *rof1* and precisely for the possible overlap roles, also the double mutant *rof1,rof2.*

a) b)

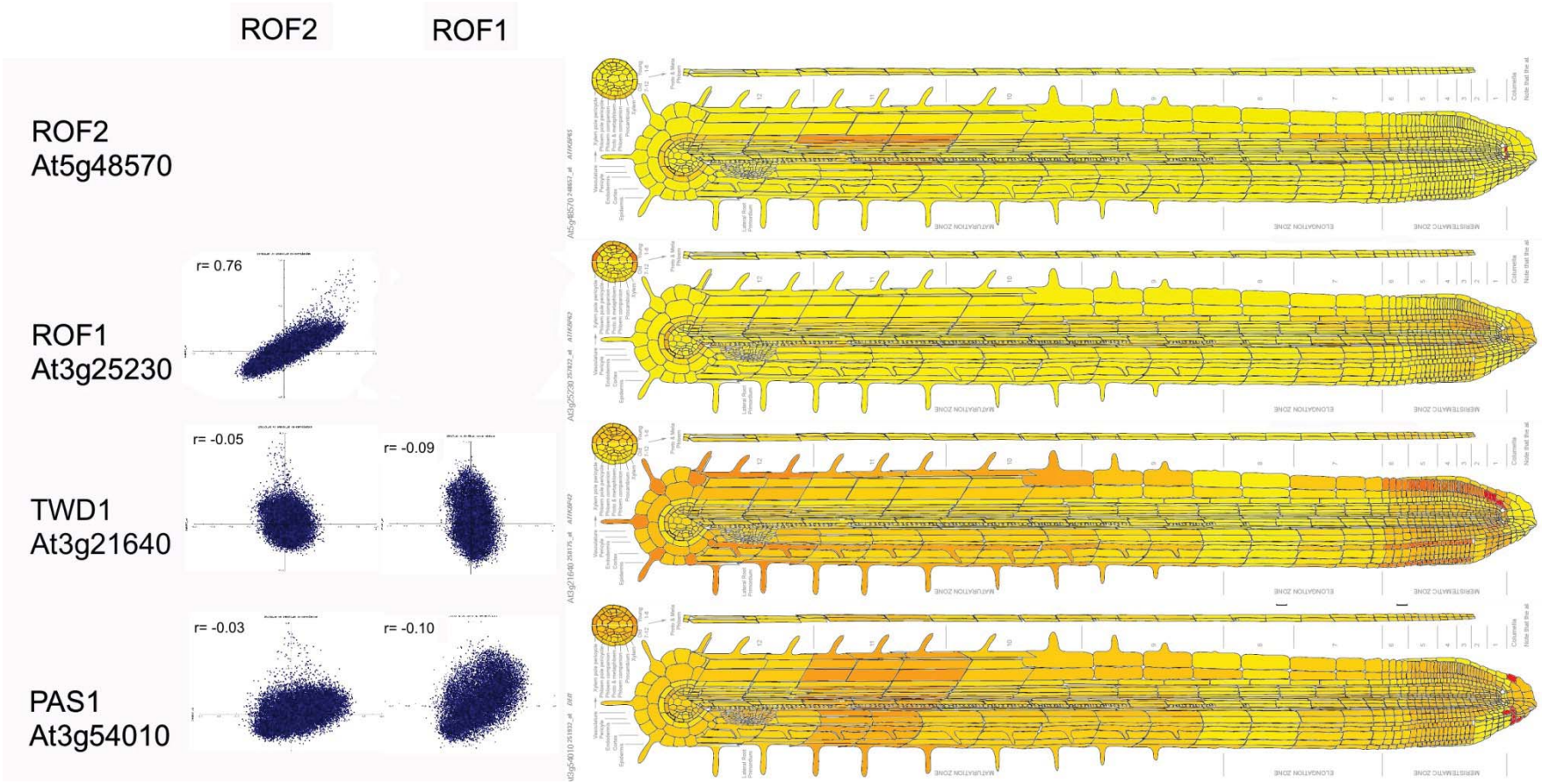


Figure 30:
a) coexpression scatter plot with r-values (www.arabidopsis.leeds.ac.uk/act/coexpanalyser.php)
b) Topological expression scheme in root

Plant immunophilin mutants

Loss-of-function

We have isolated T-DNA mutant lines that interrupt the At5g48570 gene (*ROF2*). We choose several lines with the interruption into the coding region sequence and as close as possible to the start codon. We selected the T-DNA homozygote lines with Basta® and PCR genotyping to make sure they were homozygous. For At3g25230 gene (*ROF1*), we followed the same strategy and selection, except that we used the specific antibiotic for selection of the Salk T-DNA lines: kanamycin.

In addition to the selection, we performed expression analysis by RT-PCR for the two alleles *rof1-1* and *rof2-1* (in the following *rof1* and *rof2*) and the results of Figure 31 indicated that they are null mutants because the level of the mRNAs was undetectable. We crossed *rof1* and *rof2* homozygote mutants and after a couple of generations, selecting by Basta® and kanamycin, as described before, to get the double mutant knock-out. At the end, to be absolutely sure, we tested the double mutant line selected with PCR to confirm the ROF1 and ROF2 T-DNA interruption in the same plant.

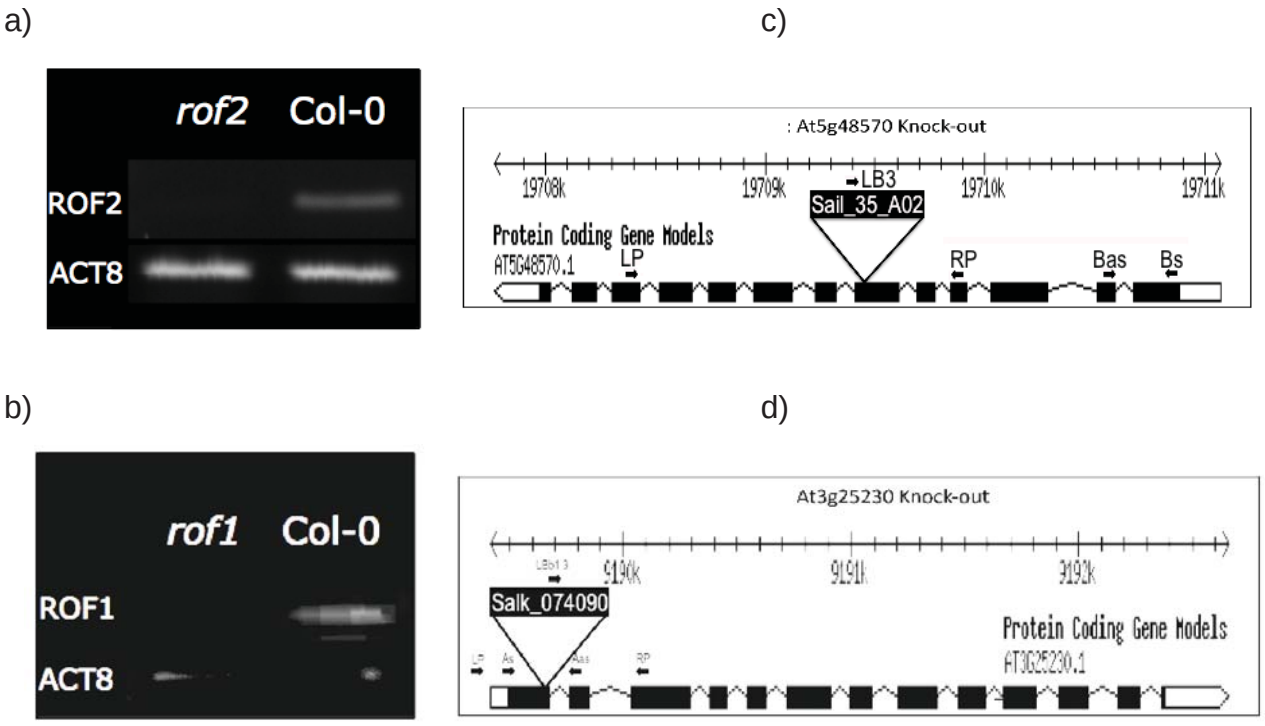


Figure 31: RT-PCR semi-quantitative analysis of ROF2 **a**) and ROF1 **b**) and relative map of knock-out T-DNA mutants: Sail_35_A02 **c**) and Salk_074090 **d**). The small black arrows on the map indicate the primers for the semi-quantitative RT-PCR (Bas and Bs, As and Aas) and the primers for the genotyping analysis (RP and LP).

Gain of Function

At the beginning we used the simplest yeast immunophilin FPR1. It contains only the *cis-trans* domain and in our germination assay in presence of acetic acid, we observed a tolerance of FPR1 over-expression lines (northern analysis in Figure 32a) and a sensitivity of *rof2* line. *rof1* behavior was quite similar to the Col-0.

After the preliminary *35S::FPR1* positive results, we were convinced to make the *35S::ROF2* lines (northern analysis in Figure 32b), and the *rof1,rof2* double mutant. After this promising experiment it seemed to us more significant that the first hits in FPR1 BLAST analysis were ROFs genes.

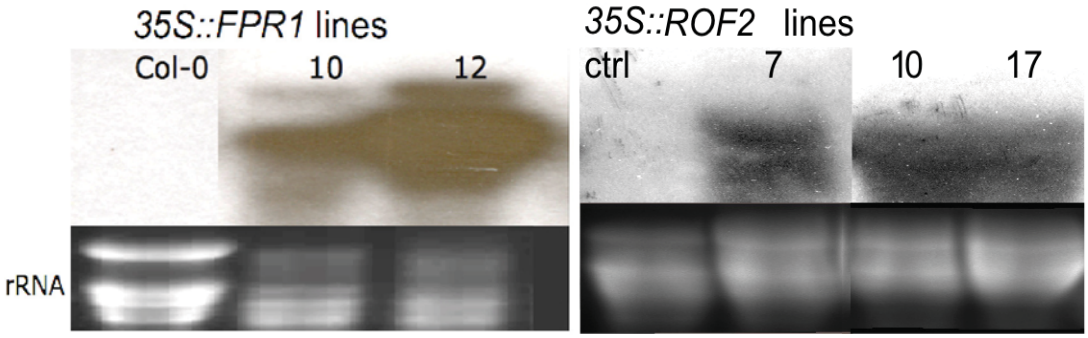


Figure 32: **a**) FPR1 over-expression *Arabidopsis* lines by northern blot analysis; **b**) northern blot revelation with ROF2 radiolabeled probe in three independent *Arabidopsis* over-expression lines, exactly the lines that we used in our experiments. In the bottom side the RNA quantification.

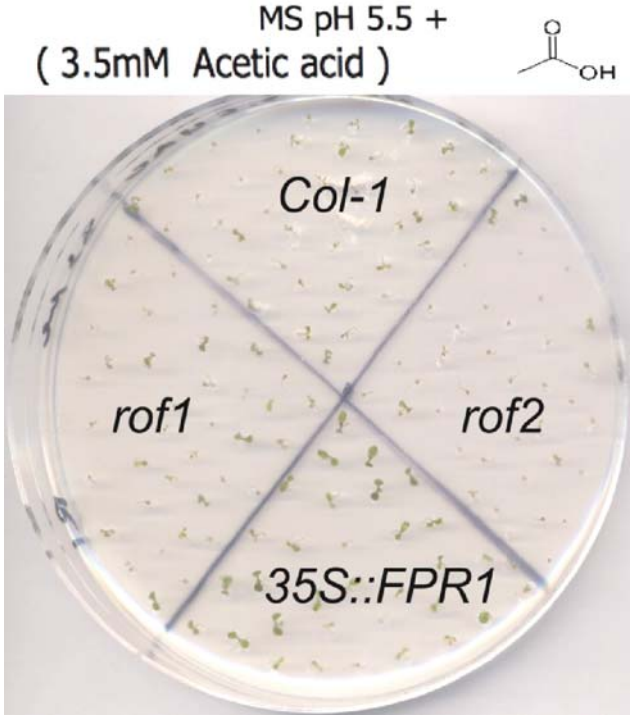
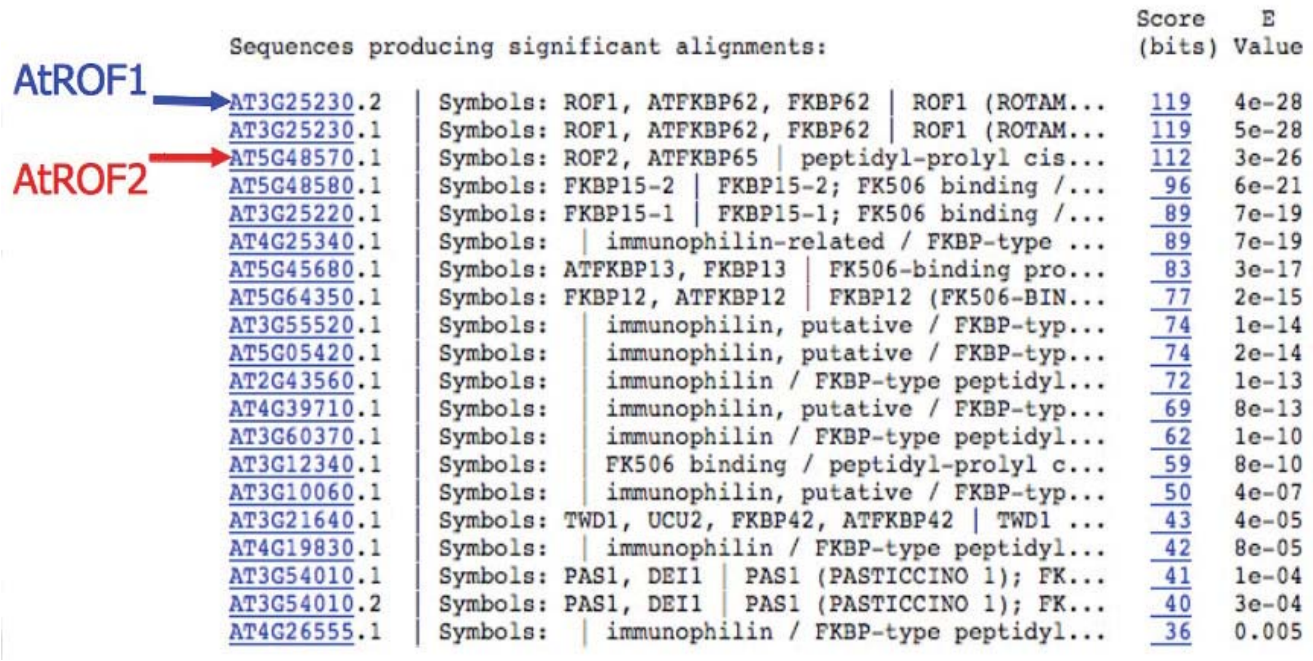


Figure 33: *35S:FPR1 Arabidopsis* line with *rof1* and *rof2* germination in CH_3COOH after 4 days.

Table 1: ScFPR1 BLASTp versus *Arabidopsis* protein database in NCBI (http://blast.ncbi.nlm. nih.gov/)



Sequences producing significant alignments:		Score (bits)	E Value
AT3G25230.2	Symbols: ROF1, ATFKBP62, FKBP62 | ROF1 (ROTAM...	119	4e-28
AT3G25230.1	Symbols: ROF1, ATFKBP62, FKBP62 | ROF1 (ROTAM...	119	5e-28
AT5G48570.1	Symbols: ROF2, ATFKBP65 | peptidyl-prolyl cis...	112	3e-26
AT5G48580.1	Symbols: FKBP15-2 | FKBP15-2; FK506 binding /...	96	6e-21
AT3G25220.1	Symbols: FKBP15-1 | FKBP15-1; FK506 binding /...	89	7e-19
AT4G25340.1	Symbols: | immunophilin-related / FKBP-type ...	89	7e-19
AT5G45680.1	Symbols: ATFKBP13, FKBP13 | FK506-binding pro...	83	3e-17
AT5G64350.1	Symbols: FKBP12, ATFKBP12 | FKBP12 (FK506-BIN...	77	2e-15
AT3G55520.1	Symbols: | immunophilin, putative / FKBP-typ...	74	1e-14
AT5G05420.1	Symbols: | immunophilin, putative / FKBP-typ...	74	2e-14
AT2G43560.1	Symbols: | immunophilin / FKBP-type peptidyl...	72	1e-13
AT4G39710.1	Symbols: | immunophilin, putative / FKBP-typ...	69	8e-13
AT3G60370.1	Symbols: | immunophilin / FKBP-type peptidyl...	62	1e-10
AT3G12340.1	Symbols: | FK506 binding / peptidyl-prolyl c...	59	8e-10
AT3G10060.1	Symbols: | immunophilin, putative / FKBP-typ...	50	4e-07
AT3G21640.1	Symbols: TWD1, UCU2, FKBP42, ATFKBP42 | TWD1 ...	43	4e-05
AT4G19830.1	Symbols: | immunophilin / FKBP-type peptidyl...	42	8e-05
AT3G54010.1	Symbols: PAS1, DEI1 | PAS1 (PASTICCINO 1); FK...	41	1e-04
AT3G54010.2	Symbols: PAS1, DEI1 | PAS1 (PASTICCINO 1); FK...	40	3e-04
AT4G26555.1	Symbols: | immunophilin / FKBP-type peptidyl...	36	0.005

Positive control in germination assays

We improved the germination results with statistical data. And all the experiments were corroborated with the positive control. The positive control as in the Figure 34 is the germination of different lines in MS medium.

The germination in normal condition, without stress, for each mutant line, it must be close to 100%. If the germination control is partial, the assays in stress conditions are more difficult to interpret. For this reason we used only fresh seed material (15-45 days old). Furthermore, not only the starting material is important, also seed sterilization can affect the germination. Indeed seeds that stayed in contact with hypochlorite (in the previous sterilization step) for long time had a lower germination rate, so this control was useful to check the sterilization step too.

As usual, in our experiments we leave the sterilized seeds for a couple of days to do the stratification treatment, our goal was to facilitate seed germination.

In summary, with all these simple precautions it was possible to reach a minimal rate of 98%, for all the transgenic lines at the same time. This gives us data experiment sufficiently precise to differentiate the mutant line.

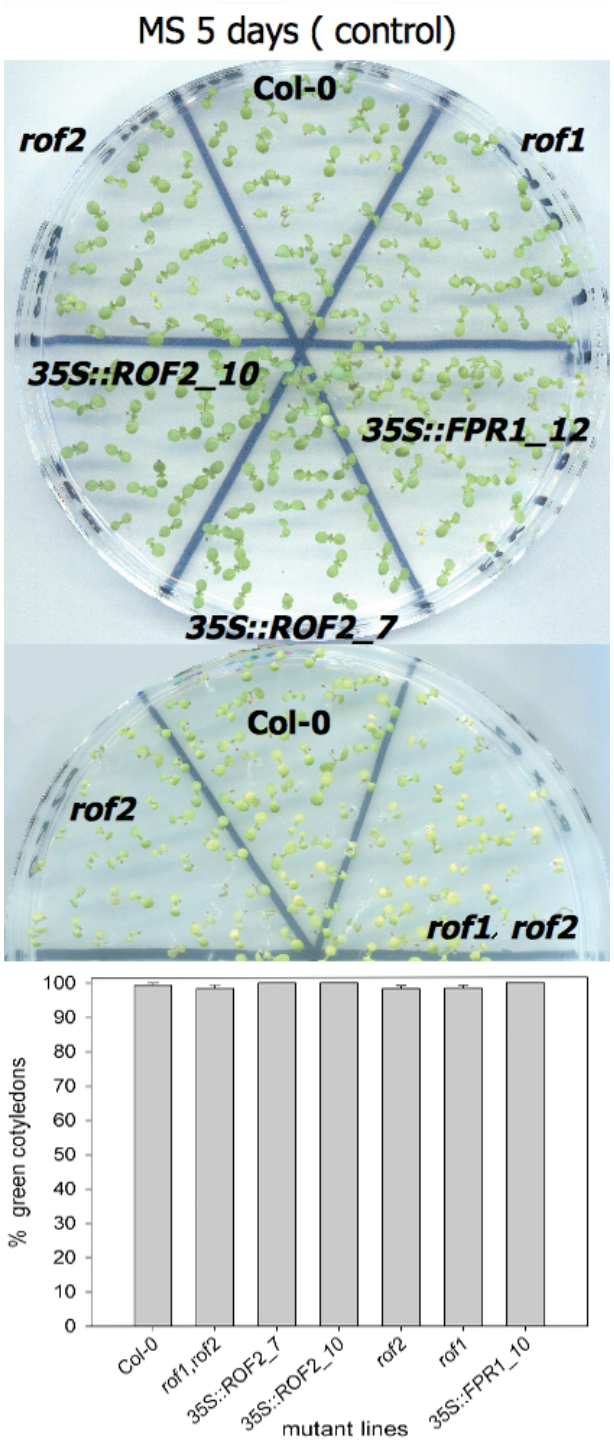


Figure 34:
(top) representative seedlings of *Arabidopsis* wild-type (Col-0), line 7 and 10 over-expressing ROF2 (35S::*ROF2*) *rof2* single mutant, *rof1* single mutant, double mutant *rof1,rof2* line 10 overexposing FPR1, 5 days after plating in normal pH 5.5 MS. (bottom) its statistical analysis.

Germination in weak acid stress conditions

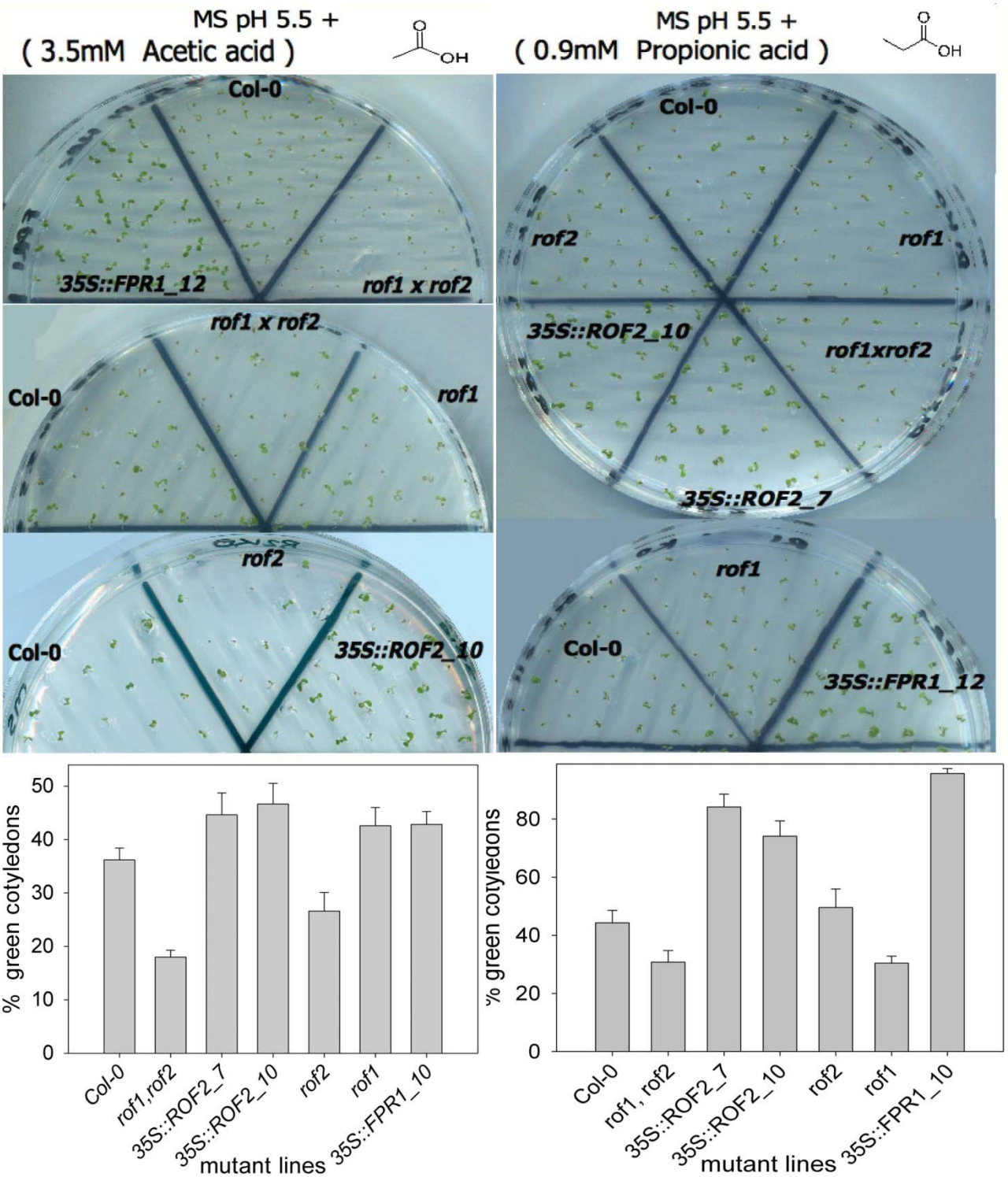


Figure 35. Top panel representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant, double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1,rof2*) and line 10 over-expressing FPR1 (*35S::FPR1*), 4 days after plating in pH 5.5 MS supplemented with 3.5mM acetic acid (CH_3COOH). Bottom panel: statistical analysis.

Figure 36: Top: representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant, double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1,rof2*) and line 10 over-expressing FPR1 (*35S::FPR1*), 4 days after plating in pH 5.5 MS supplemented with 0.9mM propionic acid (CH_3CH_2COOH). Bottom: statistical analysis.

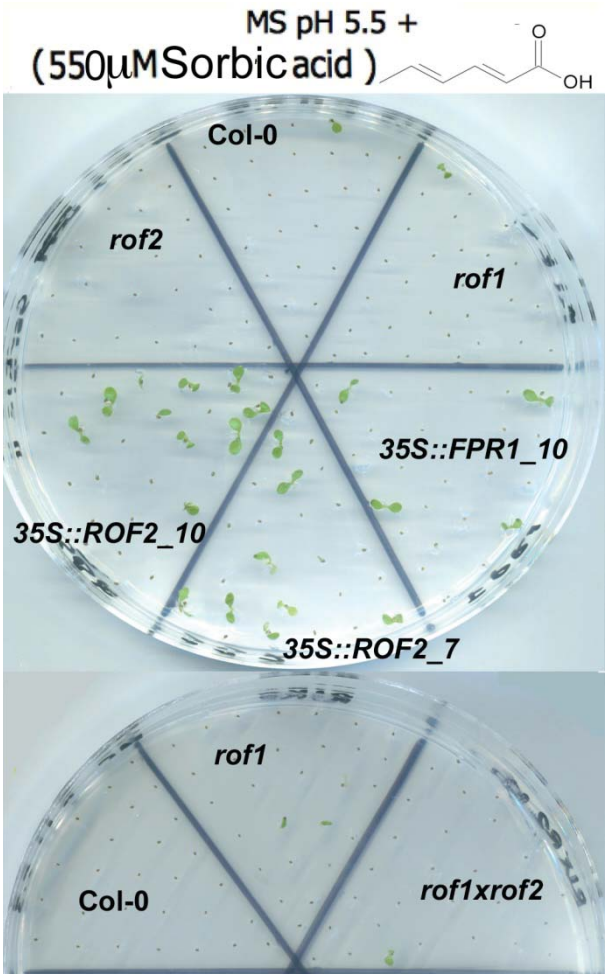


Figure 37: representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant, double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1*,*rof2*) and line 10 over-expressing FPR1 (*35S::FPR1*), 4 days after plating in pH 5.5 MS supplemented with 550µM sorbic acid.

The representative germination assays in Figures 35-36 indicates that *rof1* and *rof2* single mutants are slightly more sensitive to acetic acid than wild-type and this phenotype was exacerbated in the double mutant *rof1 rof2*. In the case of propionic acid the *rof1* mutant was more sensitive than the *rof2* mutant. On the other hand *35S::ROF2* improves tolerance to both acetic and propionic acids as determined by the appearance of green, expanded cotyledons in the germinating seeds. In addition, seedling size in the presence of the acids was greater in the plants over-expressing *ROF2*. The phenotypes in acetic and propionic acid were observed with three independent transformed lines exhibiting high expression of *ROF2*. We recognize a similar trend also in presence of a third weak organic acid (Figure 37), the sorbic one. We observed consistent behaviors in acetic, propionic and sorbic acids, suggesting that these phenotypes were due to intracellular acidification rather than specific metabolism of a single weak acid.

Growth in presence of acetic acid in liquid medium

We recorded the growth (fresh weight) of 18 days old seedlings in liquid medium (MS pH 5.5) and in the presence of acetic acid to prove several things: first the behavior in adult plant. Indeed germination is only a short step in the plant development. Young seedlings are missing a lot of organs or they are not completely developed. During the germination step the energy and the nutrient come from the seed, and the relation with the envirorment can be partial.

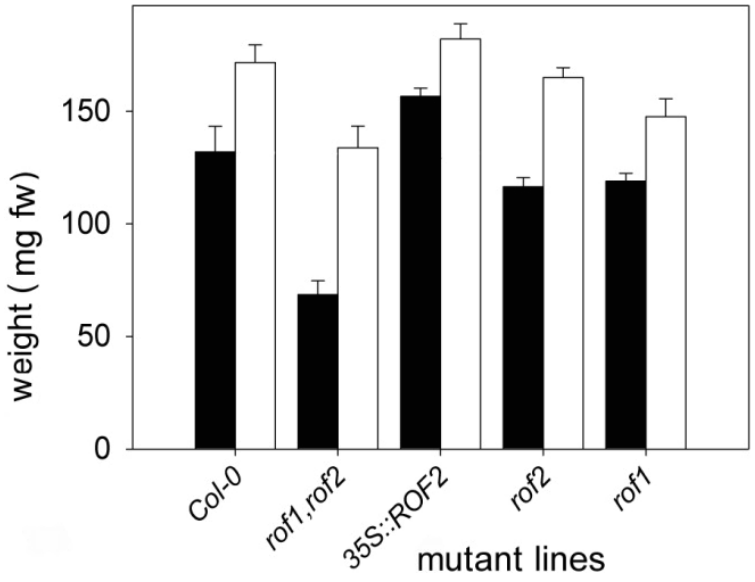


Figure 38: weights of seedlings after germination for 7 days in medium without acid and growing 12 days in liquid medium in presence of CH_3COOH 2.5mM (black bars) or in normal condition (white bars).

Also adult leaves with their vascular vessels and the plant age can change the homeostasis aptitude of the whole plant. Our results extend the role of ROFs genes in later steps because also in older *35S::ROF2* seedlings, ROF2 confers weak organic acid tolerance. In Figure 38 we can see the sensitivity and tolerance to acetic acid were also observed after germination for 7 days in medium without acid and growing the seedlings for 12 days in the absence or presence of acetic acid. Second, germination is only a parameter that gives the survival rate. The growth of plants measured by fresh weight, give us independent data where the value describes better the health of a single plant. So in this experiment the growth inhibition by acetic acid affects more the double mutant *rof1 rof2* (49%) than in wild-type (22%) while single mutants *rof1* or *rof2* exhibited similar inhibition than wild-type. Plants over-expressing ROF2 were less inhibited by acetic acid than wild-type. This indicates that the acetic acid phenotypes can be observed not only on germination but also in older plants.

ATPase activity

At that moment all our data suggested that some PPIs confer weak acid stress tolerance. ROF2 should be involved in pH homeostasis in yeast and *Arabidopsis* because in PPI over-expression lines the growth inhibition due to low cytosolic pH is smaller. Consequently, PM H^+-ATPase was the first suspect. We did specific enzymatic assay for each mutant lines. We isolated the mitochondrial fraction of the root tissue, rich in PM H^+-ATPase and with several controls we quantified the ATPase activity to hydrolyze its substrate ATP (figure 39). An unexpected result revealed us that there are no marked differences between the mutants, actually the PM H+-ATPase activity of ROF2 over-expressing plants was slightly less than in wild type. Therefore it was necessary to explain the weak organic acid resistance phenotype of ROF2 over-expressing plants by another mechanism. For example, we thought that the proton extrusion could be activated only at low pH_c or we searched some other hypothetical environmental interference. At this point we started to check proton extrusion and membrane potential values. At the same time we began to use toxic cations.

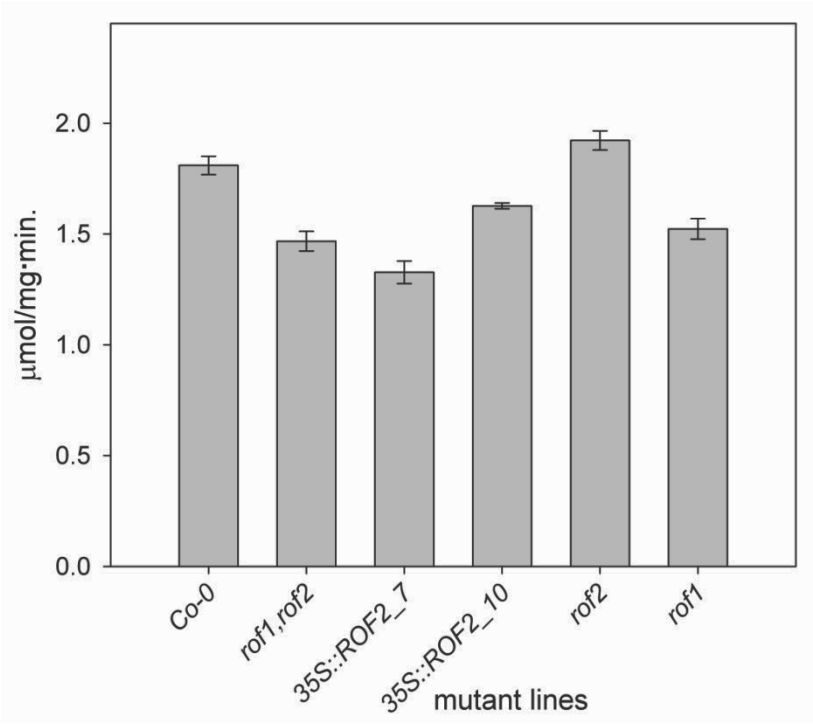


Figure 39: ATPase activity on mithocondrial fraction (rich in PM ATPase) of *Arabidopsis* root tissue

Tolerance to toxic cations

We tested again the germination in the Figures 40-41-42: a high electric membrane potential (negative inside) drives the uptake of toxic cations by unknown channels and this model can now be tested in *Arabidopsis.* We used Li^+, Hygromicyn B, and Norspermidine at toxic levels for the *Arabidopsis* seedlings. Interestingly, as indicated in Figure 40, 41 and 42 gain-of-function of *ROF2* increases the tolerance of *Arabidopsis* plants to toxic cations. While loss of function of *rof1* and *rof2* (double mutant) and single mutants decrease tolerance in a similar way as measured by the germination assay (per cent appearance of green, expanded cotyledons in the germinating seeds and seedling size).

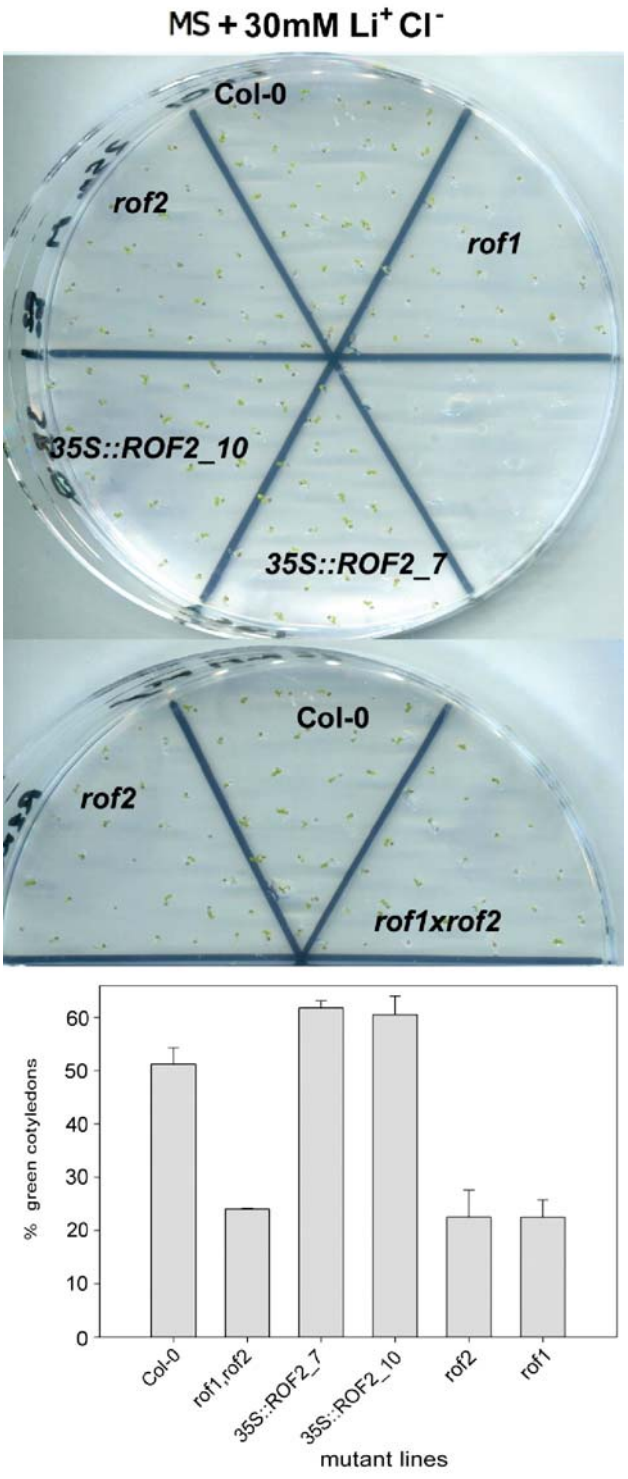


Figure 40: representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant and double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1,rof2*), 4 days after plating in MS supplemented with 300mM of LiCl.

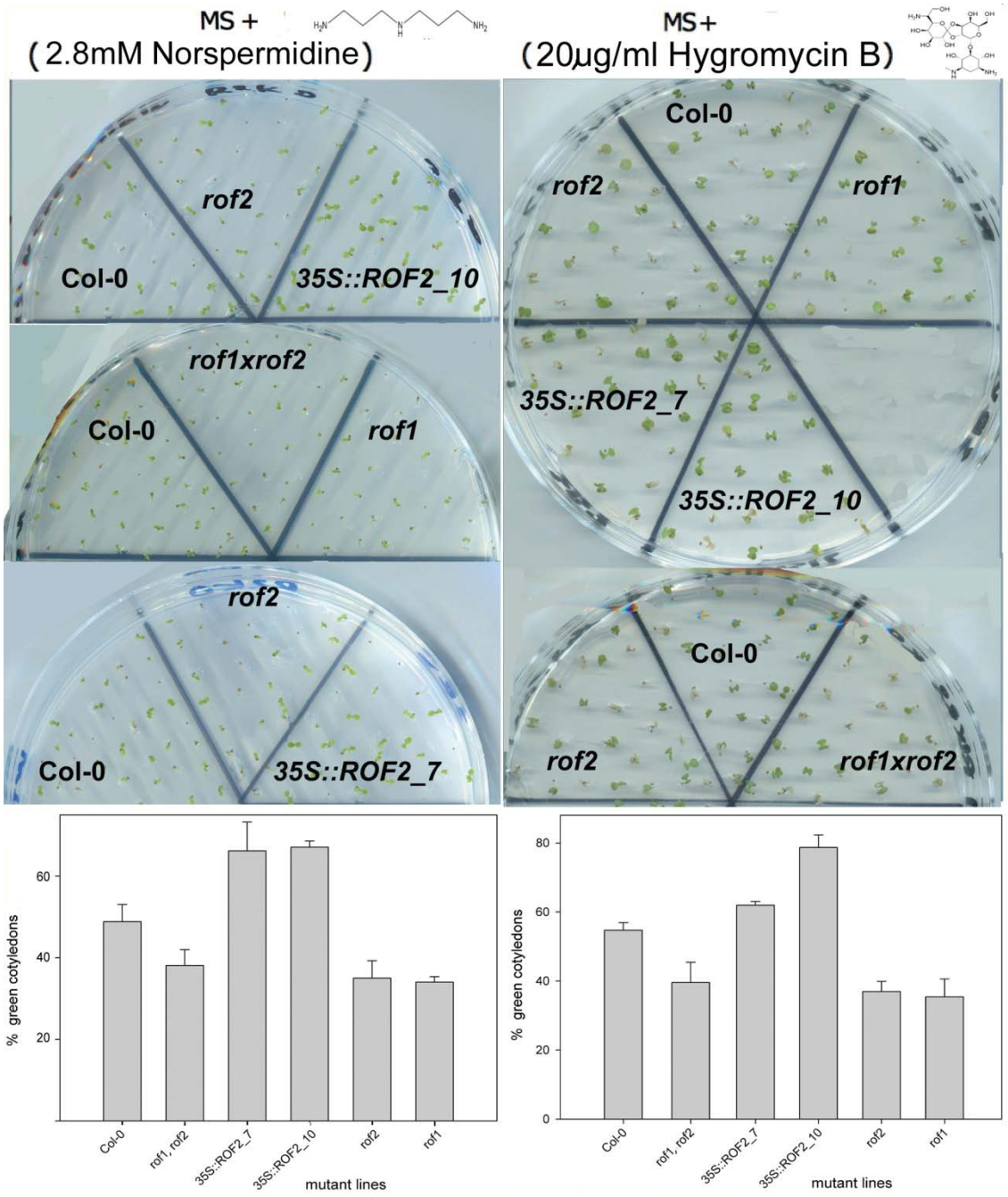


Figure 41: representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant and double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1,rof2*), 4 days after plating in MS supplemented with 2.8mM Norspermidine.

Figure 42: representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant and double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1,rof2*), 4 days after plating in MS supplemented with 20µg/ml of Hygromicyne B.

Germination in low K^+ medium

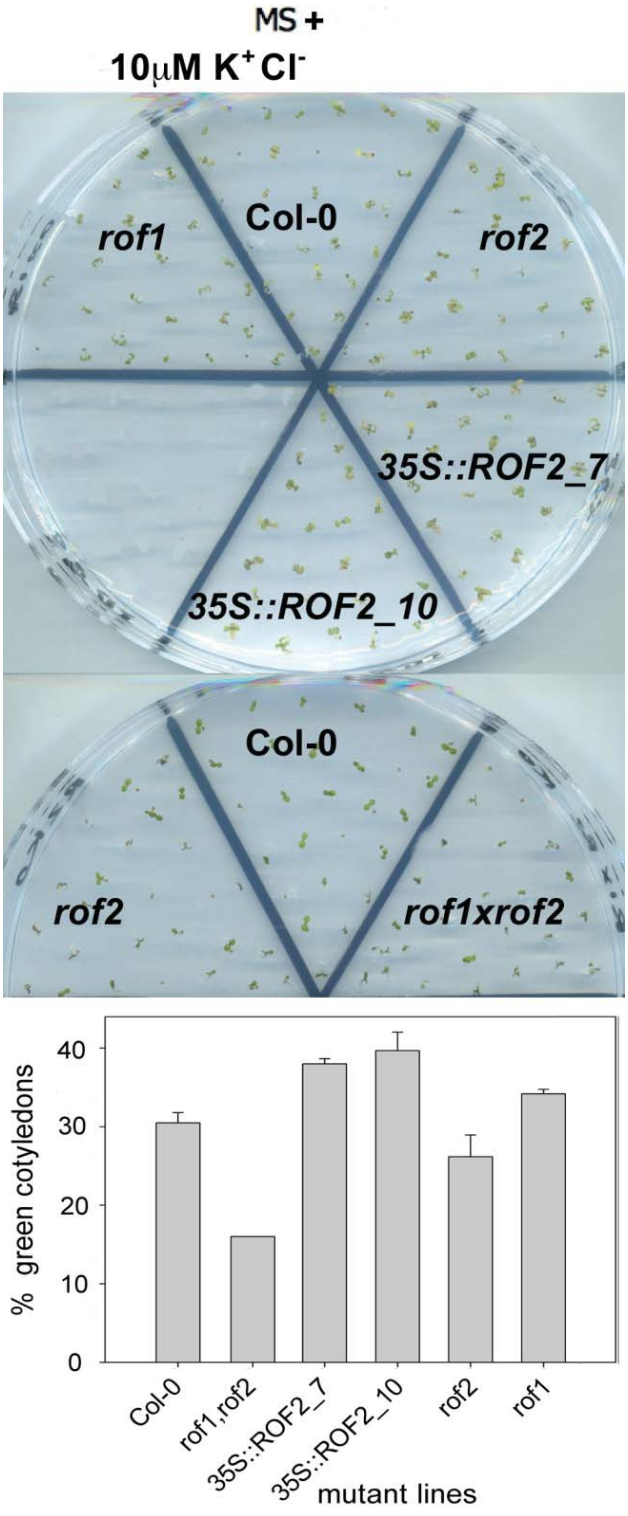


Figure 43: representative seedlings of *Arabidopsis* wild type (Col-0), line 7 and 10 over-expressing ROF2 (*35S::ROF2*), *rof2* single mutant, *rof1* single mutant, double mutant *rof1-1/rof1-1 rof2-1/rof2-1* (*rof1,rof2*), 7 days after plating in MS with (low K^+ level) 10μM KCl.

The results with toxic cations suggested that the PM electrical potential could be affected by the gene dose of ROFs: depolarized by over-expression and hyperpolarized by loss-of-funtion. One possible mechanism for this regulation is that ROF activates K^+ transport in roots. One confirmation of this hypothesis was provided by the improved germination of *ROF2* over-expressing plants in medium with low K^+ levels, 10µM, while the *rof1 rof2* double mutant germinated less well than wild-type in this medium (Figure 43). In normal MS medium, K^+ concentration is 20mM and for young seedlings the K^+ massively come from the medium and only in very small quantities from the seed. Since K^+ is everywhere: in the glass of bottles and electrode of pH meter, in the non-purified water, in the agar and in the salts of nutrients, to reach a low K^+ level in growth media we prepared the medium without K^+ salts, using agarose and milliQ water and we used only plastic containers avoiding external surplus K^+. At 10µM, K^+ is the growth bottleneck and both the two *35S::ROF2* lines seem to help the K^+ uptake because their germinations are better than in wild-type and in the *rof1 rof2* double mutant. Germination of *rof2* single mutant in low K^+ medium is slightly less than wild-type, while for *rof1* mutant is similar to wild type.

K^+ uptake

Germination is an indirect assay for K^+ transport and we did also direct uptake measurement of Rb^+, a non-physiological cation that serves as tracer of K^+. We did a kinetic assay to get a detailed (and direct) data to compare the Rb^+ accumulation in the different mutant lines (Figure 44). Rb^+ uptake was increased in *35S::ROF2* over-expressing lines with respect to wild-type and it was decreased in the double mutant. This was a key assay together with the germination at low K^+ because it gave us the model to explain all the phenotypes in weak organic acid medium and in presence of toxic cations.

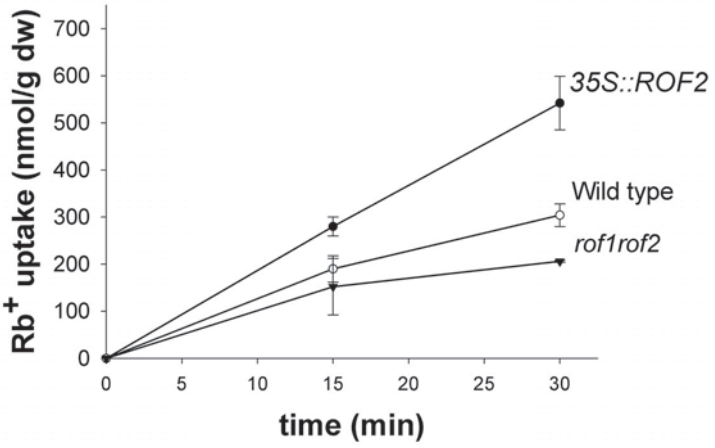


Figure 44: time course of Rb^+ uptake in wild-type, *35S::ROF2* and *rof1, rof2*. It is utilized as an analog of K^+. Uptake missing in the cell metabolism, for this reason it is easy its measurement (missing the basal level). Gain and loss of function of *ROF2* modulate Rb^+ uptake.

H^+ extrusion

A qualitative, visual assay for acid extrusion from *Arabidopsis* roots indicates that over-expression of *ROF2* increases proton efflux (Figure 45). Yellow colour of the pH indicator bromocresol purple in the surroundings of roots indicates external acidification and it was much greater in the case of *ROF2* over-expressing plants than in controls. We have also utilized a quantitative assay based on measuring acidification of the external medium of the plants using a sensitive pH meter. Seedlings preincubated in water were supplemented with sucrose at time zero. Despite we were close to the limit of detection of the equipment, we could take significant curves, choosing plants with similar size, reduced liquid volumes and a efficient liquid solution mixing. The results in Figure 46 indicate that both the initial rate of proton efflux and the final extent of external acidification are much lower in the *rof1,rof2* double mutant than in the wild-type. This defect was corrected by transformation of this mutant with the ROF2 gene, again showing that ectopic expression of ROF2 is biologically relevant. Finally, over-expression of ROF2 in wild-type *Arabidopsis* increased both the initial rate and final extent of acidification.

Figure 45: *Arabidopsis* plants over-expressing ROF2 (line 17; 35S::ROF2) and control plants were incubated in solid MS medium at pH 5.5 without MES buffer and containing 0.005% Bromocresol purple. The yellow colour of the pH indicator around the *35S::ROF2* roots but not around the control roots indicates increased H^+ in transgenic plants.

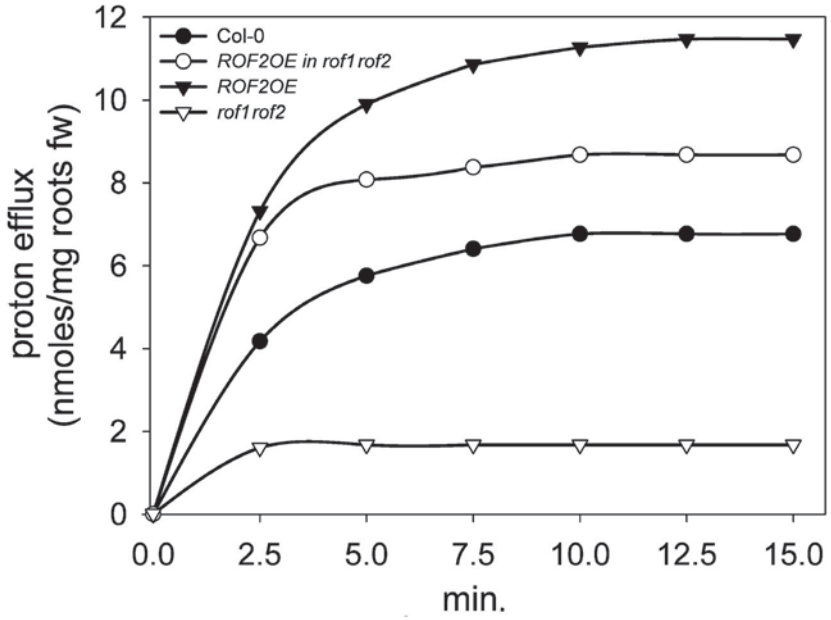


Figure 46: quantification of proton efflux from roots. Values are the means of three repetitions, and the standard errors (not shown for clarity) were 0.5 – 1.0 nmol H^+/mg roots; Sucrose was added at time 0 to start proton extrusion. Calibration pulses were made with 5 nmoles HCl at the end of measurements.

Electrophysiological measurements

In a collaboration with the laboratory of Prof. José A. Fernández of the "Universidad de Málaga", they performed electrophysiological measurements of membrane potential and cytosolic pH with our mutants. The recordings of figure 47 and the corresponding data of table 2 tell us that *35S::ROF2* over-expressing root epidermal cells have a depolarized PM with respect to wild type. Also, at the bottom of Figure 47, it is shown that after acetic acid addition to the ROF2 gain-of-function line, we do not appreciate a change in the pH_c, while the wild type cells are acidified. These results are in agreement with a model where increased K+ transport depolarizes the PM and, by electrical balance, stimulates H^+ efflux. For unknow reasons, the double mutant *rof1 rof2* does not exhibit a lower pH than wild type upon acetic acid treatment, although as expected the membrane potential was hyperpolarized.

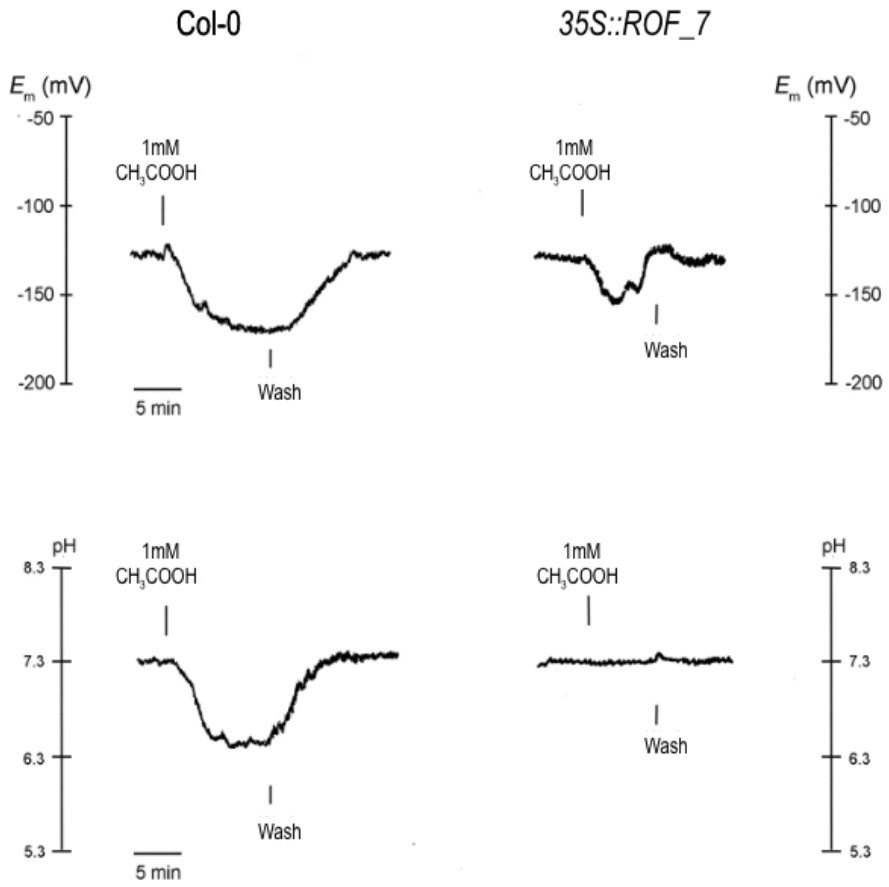


Figure 47: representative electrical membrane potential (Em) and cytosolic pH of ROF2 mutants: over-expression of ROF2 (line 10) depolarizes the plasma membrane and stabilizes cytosolic pH in the presence of acetic acid. Curves show the plasma membrane Em and cytosolic pH (pH) of a root epidermal cell in a typical experiment. The time of addition of acetic acid (1 mM Ac) and its removal (Wash) is indicated.

Table 2: electrical membrane potential and cytosolic pH of ROF2 mutants; Em and cytosolic pH of control (wild-type Arabidopsis), *35S::ROF2* (line 10 over-expressing ROF2), *rof2* (single mutant) and *rof1,rof2* (double null mutant in ROF1 and ROF2) plants. Values are means ± standard error (number of experiments in parentheses).

	Membrane potential (mV)		Cytosolic pH	
Acetic acid:	-	+	-	+
Control	-139 ±4 (13)	-164 ±3 (3)	7.3 ±0.1 (4)	6.6 ±0.1 (4)
35S::ROF2	-116 ±2 (11)	-130 ±2 (3)	7.4 ±0.1 (4)	7.4 ±0.05 (4)
rof2	-141 ±3 (4)	-155 ±3 (3)	7.3 ±0.1 (3)	7.2 ±0.05 (3)
rof1 rof2	-148 ±4 (10)	-173 ±3 (10)	7.3 ±0.1 (5)	7.0 ±0.05 (5)

Sub-cellular localization of ROF2 changes during intracellular acid stress

The activation of K^+ transport in *Arabidopsis* by *ROF2* could occur at different levels, from gene expression to transporter activity. We have started the characterization mechanism by analyzing the sub-cellular localization of ROF2 in *Arabidopsis* root cells by using transgenic plants expressing a *ROF2::GFP* fusion (GFP: Green Fluorescent Protein). Under normal conditions the ROF2-GFP chimeric protein is concentrated in the nucleus and it is also present with less intensity in the cytosol (Figure 46, first row). This situation changed upon addition of acetic acid to lower intracellular pH, where ROF2-GFP was excluded from the nucleus and appeared exclusively at the cytosol (Figure 46, second row). Maybe the green halo around the nucleus indicates localization at the endoplasmic reticulum. But to be sure it would be necessary to use a fluorescent marker of this organelle, such as a GFP-fused protein specific of the endoplasmic reticulum. One plausible explanation for this change in sub-cellular localization is that the effect of PPIs on K^+ transport may be at the protein activity level operating from the cytosol. Acid stress may favors exit of ROF2 from the nucleus to facilitate activation of K^+ transport and H^+ extrusion.

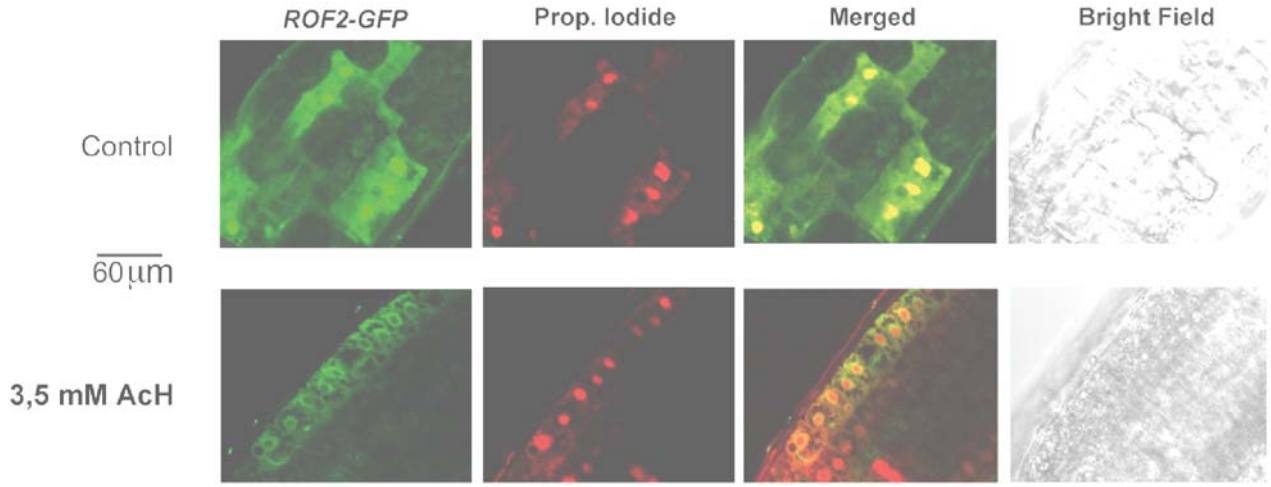


Figure 48: localization with confocal fluorescence microscopy of root cells from transgenic plants expressing a ROF2-GFP fusion. In the upper panels plants were incubated with normal medium while in the lower panels they were incubated 3 hours in the presence of 3.5mM acetic acid before observation.

Flowering delay

In the absence of stress, the different mutant lines have a different behavior in their development. When we collected the seeds to make the germination tests, we observed that ripened siliques appeared in some mutants earlier than in others. In particular the *rof1* and the *rof1 rof2* double mutants anticipated the seed maturation approximately 10-20 days before wild-type. On the other hand the gain-of-function of *ROF2* (*35S::ROF2*) delayed its maturation and the senescence in general. In Figure 49 we appreciate the differences in flowering time. The delay of gain-of-function lines is in agreement with a higher rosette leaf number and these results suggest that over-expression of ROF2 may increase auxin levels or sensitivity.

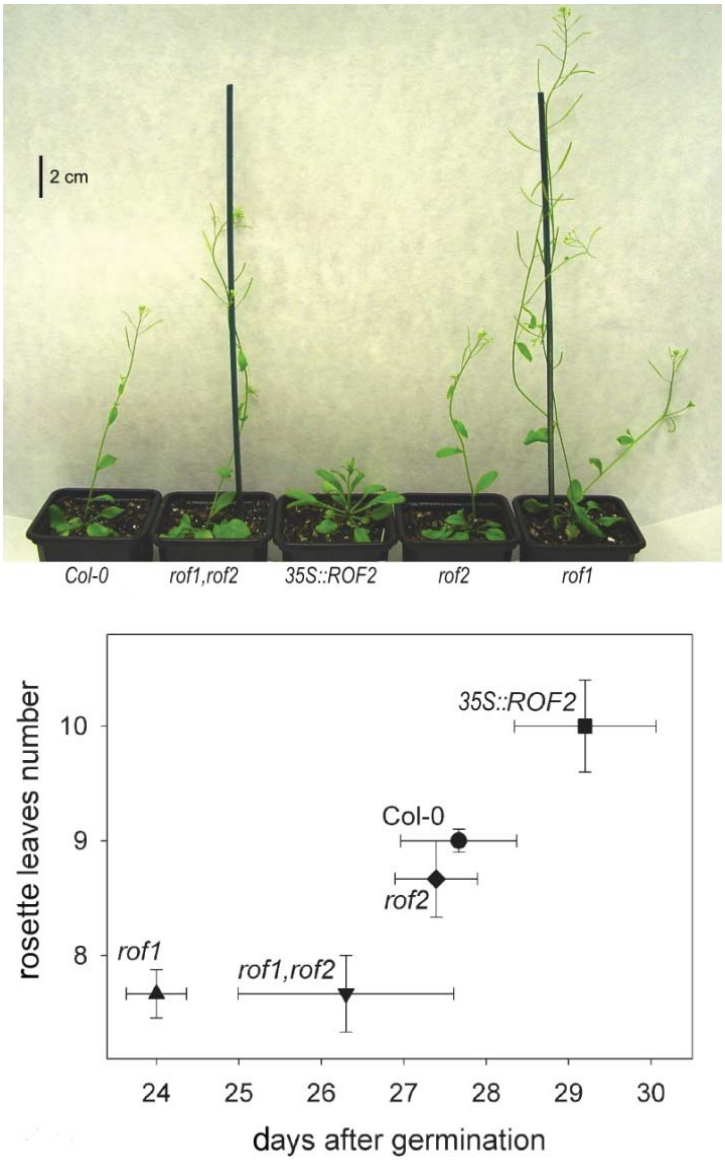


Figure 49:
a) 29th day of Col-0 and ROF mutant lines grown under long days;
b) bolting time and number of rosette leaves at time of bolting for *rof1* and *rof2* knock-out single mutants, *rof1,rof2* double mutant, wild-type (Col-0), and ROF2 over-expression. Plants were growth under long days (16 hour light and 8 h darkness).

Apical dominance and seeds

Another developmental phenotype was apical dominance. In this physiological phenomenon the main central bud of the plant is dominant over leave axillary buds. Normally it is indicative of high levels of auxin signaling, such as in the *yuc6-1D* mutant (Kim *et al.*, 2007). We have observed that *35S::ROF2* lines have a reduced number of buds with respect to wild type, the same phenotype than the *yuc6-1D* mutant. The knock-out mutant lines exhibited no phenotype by this assay (Figure 50a). In addition *35S::ROF2* lines share with *yuc6-1D* another phenotype: increased seed size (Figure 50b). The *rof1 rof2* double mutant has the opposite phenotype: a smaller seed size. The *rof1* and *rof2* single mutants are similar to wild-type.

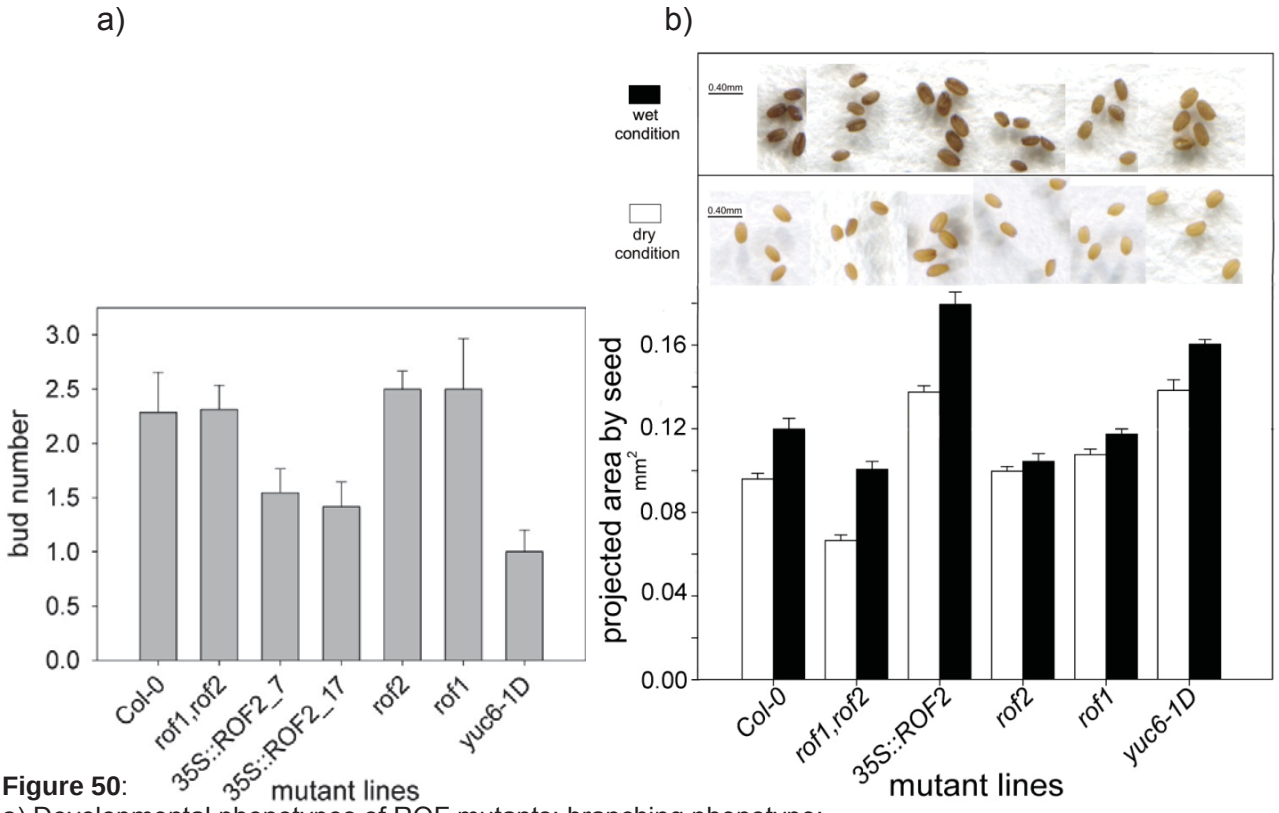


Figure 50:
a) Developmental phenotypes of ROF mutants: branching phenotype;
bud number refers to the number of secondary inflorescence shoots growing from the axils of rosette leaves, counted after 45 days of growth under long-day illumination. Values are means standard error (n = 30 plants). Two ROF2 over-expressing lines (numbers 7 and 17) were used. Data for the *yuc6-1D* mutants are presented as an example of a high-auxin mutant with strong apical dominance (Kim *et al*., 2007).
b) Seed proiected areas of wild-type (left), *rof*s mutants (middle) and yucca6-1D (right) before (bottom) and after imbibing water (top) for 4 d (seed skin is present). To calculate the means of projected areas, we took at least 40 seeds proceding from different parents of the same *Arabidopsis* line.

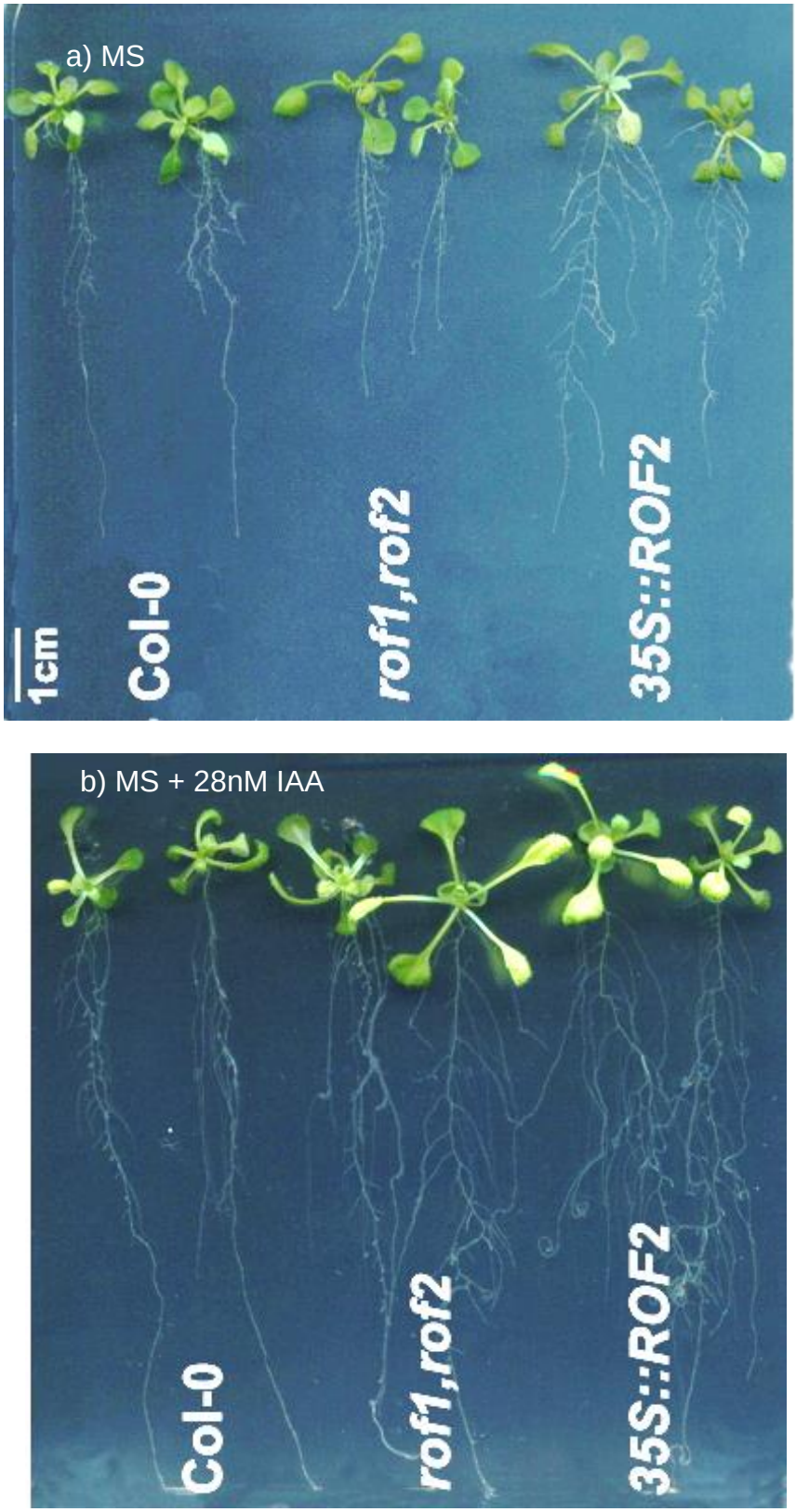


Figure 51:
a) Seedling grown in MS vertical plates for 18 days with different developed root. **b**) Seedling grown in vertical plates of MS and 28nM IAA (18 days). *rof1 rof2* line with exogenous IAA became similar to the wild-type seedlings.

After the characterization of all these possible shoot auxin phenotypes, we focused our attention on the root part of the plant. Here we noticed a higher number of secondary roots for the *35S::ROF2* plants. On the other hand *rof1 rof2* mutant roots were devoid of secondary roots and, as usual, wild-type seedlings were in the middle of the two extremes (Figure 51a). This is also additional evidence that higher expression of ROF2 correlates with phenotypes typical of mutants with high auxin signaling. Then to be sure about this positive relation between ROF2 and IAA, we tried to revert the *rof1 rof2* root phenotype using exogenous IAA. We observed that by adding 28nM of IAA we got *rof1 rof2* seedlings with a higher developed root organ, rather more secondary roots and longer. Interestingly, adding exogenous IAA at the *35S::ROF2* seedlings we could appreciate the spiral wrap tip of the roots, a loss of gravitopism. The results for the endogenous IAA content were as expected for the loss-of function mutant lines, that is, less auxin (Figure 51a) whereas the *35S::ROF2* line contained more auxin than the *rof1 rof2* mutant but less than wild type. Apparently, loss-of-function of ROF decreases auxin levels while gain-of-function of ROF2 increases auxin sensitivity and slightly decreases auxin levels. We have also determined the levels of the growth inhibitor hormone abscisic acid (ABA) and observed that the ROF2 over-expressing line has less of this hormone that wild type, while loss-of-function mutants were like wild type (Figure 52b). All these hormone measurements are preliminary and will be confirmed in the new future within the Plant Hormone Analysis Service of our institute (IBMCP), that is installing a core-service for the determination of all plant hormones based on an ultrasensitive LC-MS/MS system.

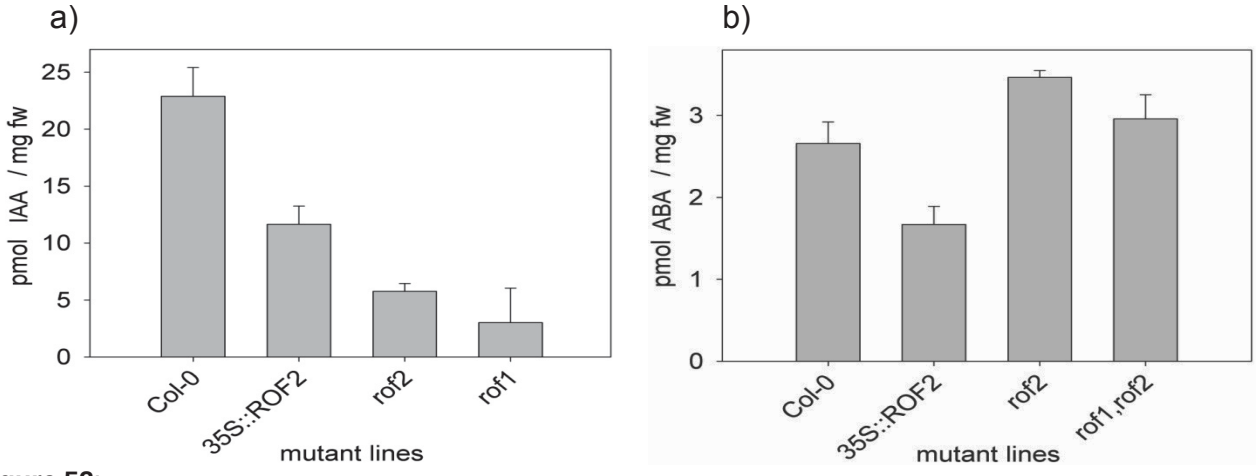


Figure 52:
a) root IAA levels of different lines; **b**) root ABA concentrations. In both hormone determinations we took three samples for each line.

Cold sensitivity

The published data of Aviezer-Hagai *et al.*, 2007 and Meiri *et al.*, 2009, relate ROF genes to HSP proteins and heat stress. But our experience suggested a phenotype at low temperatures. Upon small changes of temperature, we observed differences in seedling size between the mutant lines. Also the *in silico* data in Figure 28 indicated a lower mRNA level of ROF2 in this cold condition. In Figure 53 we found a similar phenotype of cold sensitivity for shoot growth of *rof1 rof2* double mutant and for *35S::ROF2.* The 35S promoter does not changes its expression in cold condition, but the *35S::ROF2* lines show the same behavior in the cold than the knock-out mutant.

Shibasaki *et al.* (2009) have shown that inhibition of root growth and gravity response by cold is due to inhibition of intracellular trafficking of auxin efflux carriers. Indeed, at low temperatures vesicle trafficking generally is slower than normal, and mutants with alterated function in vescicles can affect severely the growth of whole plant. The traffic of vesicles coincides with the form of transport that the plant uses for transporting auxin carriers and therefore the polar gradient that is established between the source and the sink of this molecule is affected by cold. It is not clear if this mechanism can be applied to the inhibition of leaf growth by cold and how ROF proteins could participate.

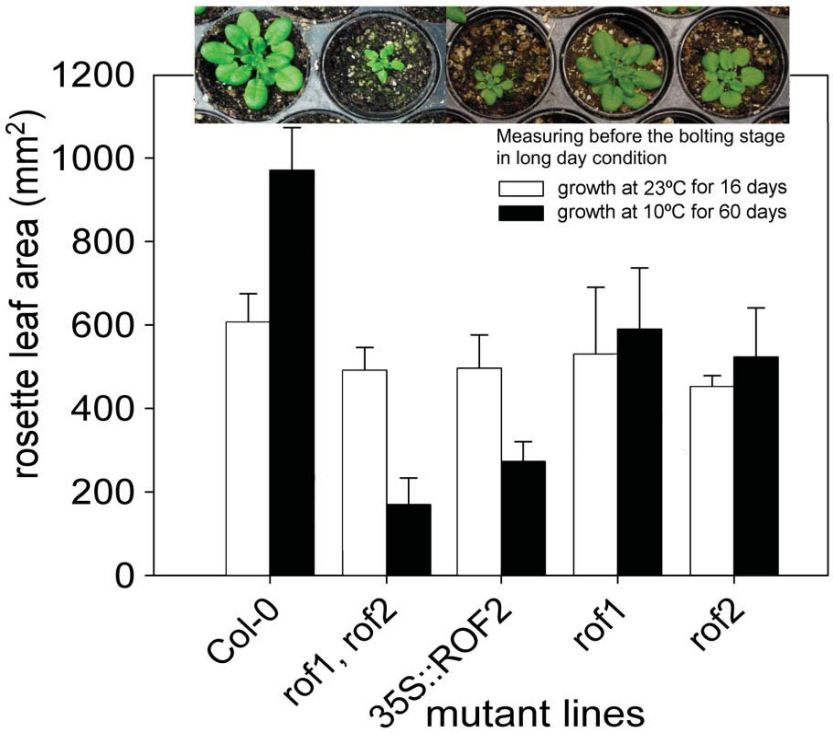


Figure 53: In black bars it can be seen the developmental inhibition caused by low temperature (4ºC long day) calculated by rosette leaves areas (upper side) after 60 days. White bars are the rosette areas of seedlings grown at 23ºC under long day condition.

Post-transcriptional regulation

We used again the chimeric construction over-expressing the ROF2-GFP protein to observe its behavior in presence of different levels of IAA and if it is implicated in vescicle trafficking. We already knew that under normal conditions the chimeric ROF2-GFP protein is concentrated in the nucleus and it is also present with less intensity in the cytosol (Figure 54a). The situation changed upon addition of IAA. The strong promoter 35S does not change its expression level in presence of IAA but we observed in Figure 54b that the IAA treatment affects the GFP fluorescent signal and it dissapears with time. This could be explained if the ROF2-GFP protein were degraded by the proteasome after IAA supplementation. In samples treated with MG132 the fusion protein remained stable, indicating that MG132 protects ROF2-GFP against proteasome degradation (Figure 54f,g).

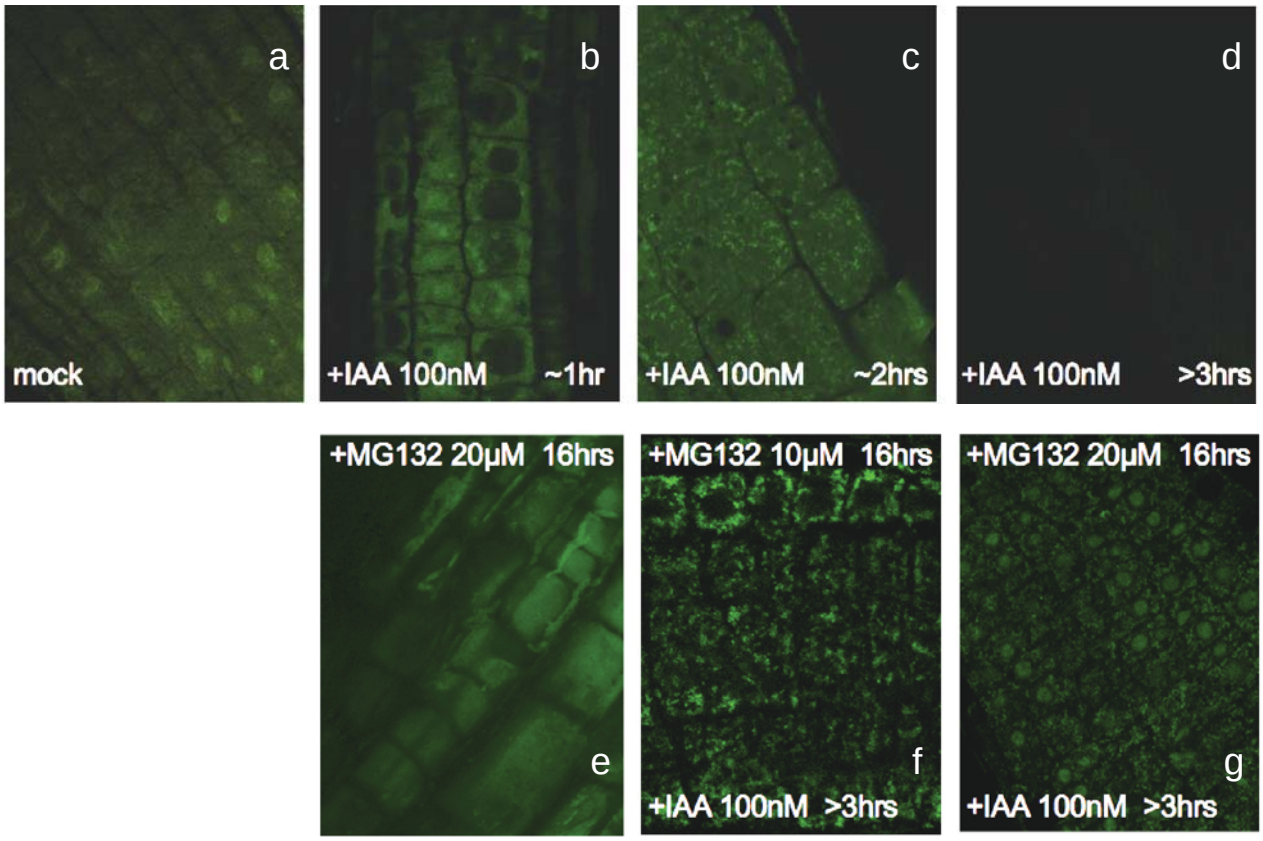


Figure 54: **a**) control ROF2-GFP is located in cytosol and nucleos; **b**) first hour started the spot formation (these are in low number) by IAA; **c**) in the second hour appared marked spot and the spot number increased; **d**) after the third hour ROF2-GFP disapear; **e**) ROF2-GFP control with only MG132; **f**) and **g**) ROF2-GFP with IAA and MG132 treatment at low and high concentration (for MG132 treatments we followed Son *et al*.,2010 and Jang *et al*., 2010).

The result was clear (Figure 54e as control) because we saw a gradually increasing accumulation of green protein in spots (Figure 54f and 54g) upon proteosomal inhibition by MG132.

In order to investigate the physiological relevance of this IAA-induced degradation of ROF2 protein, we tested the effect of auxin in our pH-sensitive colorimetric assay (with bromocresol purple). The presence of exogenous IAA (75nM IAA) changes the results: *35S::ROF2* plants are the first to change the pH indicator in the absence of IAA (Figure 55a), but in the presence of IAA these plants are the last ones to effect this change. Figure 55b is an example: after an exposure of 16 hours, all the other lines have over-come the *35S::ROF2* one. As auxin responses follow a bell-shaped curve, with an optimum preceding inhibition by excess hormone, these results could indicate a higher perception of IAA hormone in *35S::ROF2* plants, changing the 75 nM IAA concentration from stimulatory in wild type and loss of function lines to inhibitory in the gain of function line.

a) Mock b) IAA 75nM

Figure 55:
Visualization of medium acidification around the root system using a colorimetric assay based on the pH-sensitive dye bromocresol purple at pH 6. **a**) Normally the *35S::ROF2* lines show the higer acidification. **b**) On the other hand in presence of exogenous IAA (75nM) there is an inhibition of H^+ extrusion for the *35S::ROF2* lines (on the right) by suggests an specific hypersentivity of this line to IAA.

DISCUSSION

In the present work we have found that the prolyl isomerase ROF2 participates in two independent plant functions: ion homeostasis and auxin signaling. Ion homeostasis in plants is determined mainly by H^+ transport.

The plasma membrane and the vacuolar H^+-ATPase generate an electrochemical potential through the membrane that is used for other ions to move until the optimal concentration in each compartment and the homeostais is attained. In weak organic acid stress conditions, the cytosolic pH drops and several metabolic and cellular reactions are affected. The plant response to weak organic acid is poorly characterized. We used acetic acid to characterize this response. Acetic Acid is a weak organic acid that diffuses into cells and lowers cytosolic pH, as determined using pH-sensitive microelectrodes. In a transcriptional analysis of acetic acid treated plants we identified several groups of induced genes, and found that the response partially overlaps with other kind of stresses, specially heat stress. Among the genes upregulated we identified the co-chaperone ROF2 (a peptydil-prolyl *cis-trans* isomerase or PPI), and also a related one named ROF1. In the literature PPI were originally identified as receptors for the immunosuppressive drugs cyclosporin A and FK506 (Harding *et al.*, 1986), that is the reason why were originally called immunophilins. Later, these drug receptors were found to be peptidyl prolyl *cis-trans* isomerases (Fisher *et al.,* 1989; Harding *et al.*, 1989; Takahashi *et al.,* 1989). Immunophilins are classified into three families by sequence homology and specific drug binding.

The two prolyl isomerases induced by intracellular acid stress, ROF2 and ROF1, belong to the FKBP family. PPI are important cellular chaperones that are involved in protein folding (Arevalo-Rodriguez *et al*., 2004; He *et al.,* 2004). In addition, those containing tetratricopeptide domains with carboxylate clamps, such as ROF2 and ROF1, modulate the heat-shock response by binding to HSP90 in *Arabidopsis* (Aviezer-Hagai *et al.,* 2007; Prassad *et al.,* 2010) and in yeast (Duina *et al.,* 1998; Prodromou *et al*., 1999). We also found that over-expression of *ROF2* in plants improves tolerance to intracellular acid stress. We were able to confirm this by germination test and by assays with plants during vegetative or reproductive growth. Though our microarray and RT-PCR, show that *ROF2* expression is dramatically increased by acetic acid (Bissoli *et al.,* 2012), under normal growth conditions *ROF2* is poorly expressed. So we assume that the role of *ROF2*

as a general chaperone is marginal. Our results also indicate that *ROF1* and *ROF2* function is only partially redundant. Figure 49, indicates that phenotypes related to auxin distinguish between *ROF2* and *ROF1* and we are thinking that *ROF2* is also involved in a conformational regulation such as AtPin1 (another PPI), via the AGL24 and SOC1 proteins (Wang *et al.,* 2010). Also in the literature we have found that ROF1 plays different roles than ROF2 during heat stress (Meiri and Breiman, 2009; Meiri *et al.,* 2010), but in respect to weak organic acids, their function seems to be redundant. Single mutants *rof1* and *rof2* are quite similar, in germination and rate of growth in presence of weak organic acids, compared to wild-type (Figure 35, 36, 37, 38). *ROF1* expression is always at higher levels (in absolute values) than *ROF2* and shares with it a high sequence identity percentage, suggesting in many cases they play overlapping roles. The double mutant *rof1 rof2* showed a higher acid sensitivity than wild-type and single mutants, supporting the redundance hypotesis of *ROF1* and *ROF2* with regard to stress induced by weak organic acids.

Despite the acid tolerance of *ROF2* overexpression and its in vivo fast H^+ extrusion (Figure 45, 46), we excluded a direct activation of PM ATPase by ROF2. Electrophysiological measurements indicate a more depolarized PM upon ROF2 overexpression while in the double mutante *rof1 rof2* there is hyperpolarization. We could observe in *rof1 rof2* germination assays a lower percentage of germination than wild type in presence of three different toxic cations. According to the model described by Mulet *et al.* (1999), membrane depolarization correlates with low uptake of toxic cations (lithium, norspermidine, hygromycin B) and less toxicity. If ROF2 overexpression caused activation of PM ATPase, the cells would reach an elevated membrane potential and a higher absorption of toxic cations, just the opposite of what we noticed. In accordance with this model, direct meausurements of PM ATPase activity (Figure 39) indicate that in *35S::ROF2* line it is similar to that of mutant lines. Rubidium uptake measurements together with the data of germination at low K^+ medium helped us to interpret that our phenotypes were due to higher absorption of K^+. To summarize all these data, we asume that PM ATPase activity has a limited range of operation, because cells can not maintain a high membrane potential and K^+ (a depolarizing cation) improves the ATPase action.

As mentioned before, ROF1 and ROF2 are PPI proteins. Some members of this family such as TWD1/FKBP42 and PAS1/FKBP72 modulate plant growth and development (Pérez-Pérez *et al.,* 2004; Geisler and Bailly, 2007), flowering time (Wang *et*

al., 2010) and activity of ABC ATPases involved in auxin transport (Geisler *et al.,* 2004; Bailly *et al.,* 2008). We have observed that the 35S::ROF2 over-expressing lines have increased apical dominance and seed size, induced root branching and delayed time of flowering. Furthermore we measured in roots the levels of IAA and ABA and both hormones are present in the 35S::ROF2 mutant at a lower level than wild-type. The *rof1 rof2* double mutant seems similar to wild-type. With these results we cannot explain easily the ROF2 influence on development. Further measurements of IAA levels in shoots are needed.

The phenotypes of the plant lines overexpressing ROF2 and the double knock-out mutant in cold conditions, suggest a link between ROF2 and auxin transport because Shibasaki *et al.,* (2009) have showed that cold inhibits growth and gravitropism by reducing vesicle trafficking of auxin efflux carriers. We further investigated this relationship and we could see that in the presence of IAA, a ROF2-GFP fusion is degraded, a quite frequent phenomenon in proteins involved in hormone regulation (Kelley and Estelle, 2012). In presence of exogenous IAA, we observed an inhibition of root H^+ extrusion in ROF2 over-expressing plants, probably because *35S::ROF2* plants are hypersensitive to IAA. To confirm the relation between auxins and ROF2 we observed a *35S::ROF2-GFP* after treatment with exogenous IAA and the 26S proteosome inhibitor MG132 (Figure 54 and Figure 55) and confirm that ROF2 stability depends on Auxins. Moreover it is already known that disaggregation and renaturation of misfolded proteins is crucial for stress tolerance. The denaturation of intracellular proteins, such as heat shock and the over-expression of crucial chaperones (HSPs) was observed to protect yeast (Sanchez *et al.,* 1992) and Arabidopsis (Nishizawa *et al.,* 2006) against heat and osmotic, salt, starvation, cold stresses.

These novel roles of prolyl isomerases are unrelated to the heat-shock response, and may involve interaction of these chaperones with transcription factors, membrane transporters and other cellular proteins. For example there could be a higher sensitivity at the receptor level when auxin binds to the same TIR1 pocket that docks the Aux/IAA substrate. In our case the root branching phenotypes suggest a hypotetical interaction (direct or indirect) with a couple of specific Aux/IAA such as IAA19 or IAA17 (Tatematsu *et al.*, 2004 and Fukaki *et al*., 2002). This protein family is characterized by a domain designed to the degradation by proteasome 26S upon higher levels of IAA. Actually the

domain II sequence is used as a reporter to map the auxin response (Brunoud *et al.,* 2012). Two contiguous prolines in GWPPV are the core of this domain II and it was speculated (Dharmasiri *et al.,* 2003) that a prolyl isomerase regulation is required within domain II of Aux/IAAs for the recognition by SCF (Skp, Cullin, F-box containing complex).

Concerning intracellular pH homeostasis, the best model that explains our results is that ROF2 regulates K^+ transporters. ROF2 probably participates in protein folding, as mentioned before, and our results indicate that it has the additional function of activating K^+ uptake and indirectly modulating H^+ efflux to restore intracellular pH. The effect of ROF2 on these transporters may be direct or it may act through regulators of the systems, and in this respect, the CBL1/9–CIPK23 protein kinase (Xu et al., 2006) that regulates *AKT1* is relevant. *AKT1* is a candidate for regulation by ROF2 and currently we are testing this hypothesis. Also we are looking for kinases that can be regulated by ROF2 through prolyl isomerization and that are active in either cytosol and nucleous compartments, where ROF2-GFP is localized. If the regulation through ROF2 dependes on a kinase, this hypothetical protein kinase must be also involved in osmotic response, flowering time, seed storage and lateral root development. And what is more important, this kinase should interact with ROF2. This protein should be activated if it is a positive determinant by ROF2 in stress conditions and normal growth conditions. Otherwise ROF2 could work like a deactivator of a negative kinase for the same mentionated phenomena.

We are thinking that proline isomerization may be a rate-limiting step in the conformational changes occurring during the activation or transport function of K^+ channels/transporters. Alternatively, prolyl isomerases may bind to target proteins and change their conformation independently of their prolyl isomerase activity (Wu *et al.,* 2010). Therefore, it is plausible that abiotic stresses cause both protein denaturation and intracellular acidification, and that induction of chaperones such as ROF2 has the double purpose of protein folding and pH homeostasis. Acidification of cell compartments is important also for auxin transport and signal transduction and therefore determines the development of the whole plant. Now we are interested to discover if the K^+ uptake induced by ROF2 is related to the auxin signaling. For example we are testing the Rb^+ uptake in presence of IAA for each lines to distinguish the role between *rof1* and *rof2* mutant lines. Now we are performing these assays including also the ROF1 overexpression line (Meiri et al. 2009) to see differences with our ROF2 overexpression lines.

CONCLUSIONS

1) A transcriptomic study has shown that genes encoding HSP chaperones are the most induced by intracellular acid stress generated by weak organic acids. Between these HSPs, we identified the ROF2 prolyl isomerase gene as highly induced by acid stress.

2) ROF2 overexpression confers resistance to intracellular acid stress generated by weak organic acids (acetic, propionic and sorbic). On the other hand, the double mutant *rof1 rof2* is sentitive to this stress. ROF1 is a prolyl isomerase partially redundant with ROF2 and very similar to it.

3) ROF2 overexpression results in a higher H^+ extrusion from roots, while the *rof1 rof2* double mutant has a reduced H^+ extrussion. However, neither gain nor loss of function of ROF have affect the in vitro activity of the PM H^+-ATPase.

4) ROF2 overexpression confers tolerance to toxic cations like lithium, norspermidine and hygromicyn B. The *rof1 rof2* double mutant is more sensitive to these cations than wild type. This can be explained by the observation that over-expression of ROF2 depolarizes the PM and therefore the uptake of toxic cations will be decreased. In the *rof1 rof2* double mutant the plasma membrane is hyperpolarized and the uptake of toxic cations increased.

5) Germination in low K^+ medium and measurements of Rb^+ uptake indicate that ROF2 activates K^+ transport and this explains the depolarization of the plasma membrane upon over-expression of ROF2.

6) The activation of the proton pump by ROF is indirect, based on activation of secondary K^+ uptake. The ensuing decrease in membrane potential, a physical parameter that inhibits the PM H^+-ATPase, explains the increased rate of proton efflux from roots.

7) *35S::ROF2* lines present several alterations in flowering time, apical dominance, rosette leaves number, root branching and increased seed sizes. All these results suggest that ROF2 is a positive regulator of auxin (IAA) signaling. Gain and loss of function of ROF reduced development at low temperature by unknown mechanisms.

8) IAA treatments on *35S::ROF2* lines destabillizes the ROF2 protein, inducing its degradation and decreasing the rate of H^+ extrussion.

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APPENDIX

Rutinary protocols

E.coli solutions

Antibiotics

For stock solution dissolve with milliQ water, sterilize by filtration through a 0.45µm disposable filter.
Final concentration of Ampicilin in Petri dishes or liquid media 100µg/ml.
Final concentration of Kanamicin in Petri dishes or liquid media 50µg/ml.
Final concentration of Carbenicilin in Petri dishes or liquid media 100µg/ml.

LB (Luria-Bertani) medium

We dissolved in 950ml deionized water:
NaCl 10g
Bacto Yeast Extract (Difco #0127-17-9) 5g
Bacto Tryptone (Difco #0123-17-3)10g
Adjust the pH to 7.0 with NaOH, volume to 1 litre. If necessary we added 15g/l of Bacto Agar.
We autoclaved 20 minutes at 2atm.

X-gal: 5-Bromo-4-chloro-3-indolyl-β-D-galactoside

Make a stock solution by dissolving X-gal in dimethylformamide to make a 20mg/ml solution. Use a glass or polypropylene tube. The tube containing the solution should be wrapped in aluminum foil to prevent damage by light and should be stored at -20°C.
It is not necessary to sterilize X-gal solutions by filtration.
In the Petri dish final concentration 80µg/ml.

IPTG: Isopropylthio-β-D-galactoside (m.w.=238.3g/mol)

Make a solution of IPTG by dissolving 2g of IPTG in 8ml of distilled H_2O.
Adjust the volume of the solution to 10ml with distilled H_2O and sterilize by filtration through a 0.22micron disposable filter.
Dispense the solution into 1ml aliquots and store them at -20°C.
In the Petri dish final concentration 0.5mM.

E.coli methods

Plasmid Purification (miniprep)

This protocol was originally obtained from Diane England and modified by Andy Blasband, Applied Biosystem June 15, 1991. “DNA Sequencing Protocols” edited by Collin A. Graham and Alison J. M. Hill
1) Pellet 1.5ml of colture grown for 1 minute in a centrifuge
2) Resuspend pellet in 200µl of GTE buffer (50mM Glucose, 25mM Tris pH8, 10mM EDTA pH 8) by pipeting up and down.
3) Add 300µl of 0.2N NaOH and 1% SDS buffer (freshly made), mix by tube inversion. Do not vortex. Incubate on ice for 5min.
4) Add 300µl of 3.0M CH_3COOK, pH4.8. Mix tube by inversion. Do not vortex. Incubate on ice 5min.
5) Spin in microcentrifuge for 10 min. at R.T. and transfer the supernatant (aproximately 700-750µl) to a new tube.

6) Add RNAse A to a final concentration of 20µg/ml (aproximately 2µl of 10mg/ml stock). Incubate at 37ºC for 20min.
7) Do a chloroform extraction in a 2ml tube using two chloroform volumes. Do not use phenol, do use straight chloroform. Mix layers by shacking not vortexing. Spin extraction for 1min. to separate phases and remove the aqueous phase to a clean tube.
8) Precipitate DNA y adding an equal volume of isopropanol (700 to 750µl). Place the tube on ice for 10 min.
9) Pellet DNA for 15 min. at R.T. Rinse pellet with 500µl of 70% EtOH. Pour off EtOH and dry the pellet in a speed vacum.
10) Dissolve the pellet in 32µl of milliQ autoclaved water. Add 6.4µl of NaCl 5M and 40µl of 13% PEG8000. Mix well and on ice for 20min. longer incubations will improve yelds.
11) Spin for 15min. at 4ºC. Remove the supernatant and rinse pellet with 500µl of 70% EtOH.
12) Pour off Ethanol, dry pellet in a speed vacum and resuspend in 20 to 30µl.
For normal DNA grade I did not the Points 10 and 11.

Second DNA purification (electrophoration grade)

Add at 50µl DNAstarting solution (with a lot of salts)
1) 500µl n-Butanol, vortex for 1min.
2) Centrifuge at maximum speed and pour off the supernatant.
3) Wash the pellet with 70% Ethanol, centrifuge 5min. at maximum speed pour off the supernatant
4) Speed vacum for a couple of minute add 10µl milliQ water autoclaved

Competent cells (Heat-Shock)

Inoculate a single colony in 3-5 ml LB and let it grow overnight (O.N.) at 37°C under vigorous shaking.
Diluite O.N. culture 1:100 in fresh LB.
Grow to O.D.=0.3-0.4.
Pellet the cells (10', 4500 r.p.m., 4°C in falcon tubes).
Carefully remove all the LB medium.
Resuspend in 1/10 of the original volume of TSS.
Freeze in liquid nitrogen 100 µl aliquots.
Store at –70°C.

Transformation (Heat-Shock)

Thaw the competent cells in ice (DH5α strain).
1) Add the DNA (max 40-50 ng in 1-5 µl) and mix.
2) Put 30min. at 4ºC
3) Put for 45sec. at 42°C
4) Put 2min. at 4ºC
5) Add 0.9 ml SOC medium (or, in case of necessity, also LB)
6) Incubated 1h at 37°C under vigorous shaking
7) Plate on selective LB plates.

Competent *E. coli* (Electrophoration)

1) Inoculate a single colony in 3-5 ml LB and let it grow overnight (O.N.) at 37°C under vigorous shaking.
2) Dilute O.N. culture 1:100 in fresh LB (1 ml in 100 ml).
3) Grow at 37°C to $O.D._{600}$=0.5-0.6.
4) Pellet the cells (10', 4500r.p.m., 4°C in falcon tubes).
5) Carefully remove all the LB medium.
6) Resuspended the pellet in 100ml of ice-cold water.
7) Pellet the cells (15', 4500r.p.m., 4°C in falcon tubes).
8) Resuspended the pellet in 25ml of ice-cold water.
9) Pellet the cells (15', 4500r.p.m., 4°C in falcon tubes).
10) Resuspended the pellet in 10ml of ice-cold 10% glycerol.
11) Pellet the cells (20-30', 4500r.p.m., 4°C in falcon tubes).
12) Resuspended the pellet in 1.2ml of ice-cold 10% glycerol.
13) Dispensed into 50-80µl aliquots.
14) Froze in liquid nitrogen and store at –70°C.

Transformation (Electrophoration)

Settings for electroporation (0.1 cm cuvettes):
•25 µF •200 Ohm •1700-1800 Volts

1) Thawed 50 µl of electrocompetent cells at 4ºC. (Pre-cooled the cuvettes).
2) Add 1ng of supercoiled vector or 3-5 ng of ligated vector to the cells. Mix carefully.
3) Put the cells and the DNA into the cuvette and beat it a couple of times on the table to get rid of air bubbles.
4) Dry quickly the cuvette and put it into the holder (better pre-cooled) and give the pulse.
5) Add as soon as possible 1 ml SOC and transfer the culture in a bacteriology tube or a 1.5ml eppendorf tube.
6) Let grow 1 h at 37°C, under stirring
7) Plate on selective LB agar plates
8)Let grow O.N. at 37°C.

Agrobacterium tumefaciens methods

Competent cells *(Electrophoration)*

It was employed *A. tumefaciens* GV3101::pMP90RK strain ampicilin/carbenicilin resistance.
1) It was prepared 10ml of satured *Agrobacterium* culture in LB liquid with carbenicilin (60µg/ml) at 28ºC for 2-3 days.
2) For each 1ml of saturated culture it was inoculated 100ml of LB for an O.N. and incubated at 28 ºC.
3) Centrifugate these cells at 5000 r.p.m. for 15-20min. and wash 2 times with cold H_20 MilliQ at 4ºC.
4) The cells were resuspended in 4ml of glycerol at 10% at 4ºC split in 0.2ml aliquot and stored at –80ºC.

Transformation (Electroporation)

1) We used electroporation cells size of 0.2 cm and the condition electroporation were 2KV, 1KW, 25mF.
2) We added at 0.2ml of *A. tumefaciens* competent cells 0.1-0.5mg of plasmid.
3) After the electroporation step we added 1ml SOC medium at the cells and we incubated at 28ºC for 2-3 hours.
4) The transformants cells were selected on LB medium plates with carbenicillin (45-70µg/ml).

Arabidopsis protocols

Seed sterilization

For *in vitro* culture, seeds were surface sterilized by soaking in 70% ethanol containing 0.1% Triton X-100 for 15 min, followed by commercial bleach (2.5%) containing 0.05% Triton X-100 for 10 min and rinsing three times with sterile water. Stratification of the seeds was conducted during 3 days at 4ºC.

Plant RNA extraction

Before than an northern analysis or a RT-PCR reaction it is necessary do a RNA purification from a plant tissue in our case roots of plants of 16-17 days old. For a good RNA quality is important to preserve the samples in cold conditions and using the gloves to avoid the RNAse action.
1) Put in a 15ml tube 1ml Phenol/Choloroform/Isoamilic alcohol (PCI) 25:24:2 with a 1ml of TCES (0.2M Tris-HCl pH8, 0.2M NaCl, 50mM EDTA, 2%w/v SDS) and pre-heated in a thermoblock at 50ºC
2) Grind with mortar and pestle 1g of fresh material stored in liquid nitrogen
3) Pour into the pre-hated tube and incubate for 15min.
4) Centrifuge at 10000r.p.m. (revolutions per minute) for 10min.
5) Put in a new 2ml tube the aqueous phase with a volume of chloroform: isoamilic alcohol and mix.
6)Centrifuge 12000r.p.m. for 10min. and put the aqueous phase in a new 1.5ml tube
7) Add a volume of LiCl 6M mix gently and store at -20ºC for 3-4 hours
8) Centrifuge at maximum speed for 10min.
9) Wash the precipitate with 70% ethanol, dry with speedvacum, add water and store at -80ºC.

Arabidopsis transformation by floral dip

A colony of *A. tumefaciens* transformed with the plasmid of interest, was utilized to inoculate 200ml LB medium with carbenicilin 60µg/ml, it was incubated at 28ºC until saturation.
The cells were collected by centrifugation (5000r.p.m. for 15min. a RT) and they were resuspended in 400ml of infiltration solution (5% saccharose, 0,02%v/v Silwet L-77 detergent).

Genomic DNA Extraction with CTAB (Soltis' Lab Protocol):

http://www.flmnh.ufl.edu/soltislab/Soltis_site/Protocols.html

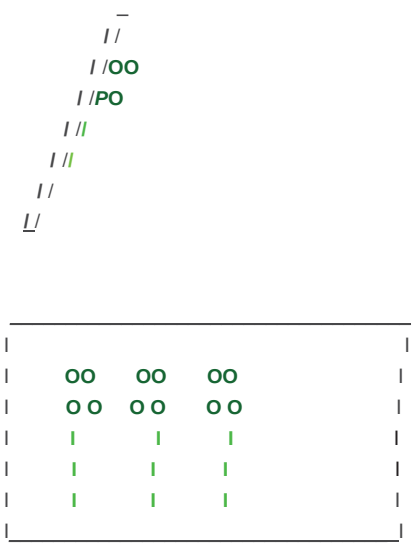
1) Prepare CTAB buffer, use within 2-3 days, store capped:
Add β-mercaptoethanol (Fisher Cat#: BP176-100) and only if it is not *Arabidopsis* add polyvinylpyrrolidone (soluble) (Fisher Cat#: BP431-500) and stir to dissolve right before starting the extractions: CTAB PVP β-merc
0.5 ml 0.02g 2.5µl
5 ml 0.2g 25µl
20ml 0.8g 100µl
2) Weigh out 10-20mg of silica-dried plant tissue better from original young leaves.
3) Grind tissue with blue pestles
a. Pestles can be reused, store in 10% bleach solution and rinse well with water before using them.
b. Grinding can be done with or without the aide of liquid nitrogen and/or washed and autoclaved sand.
4) Add 500µl of CTAB buffer and grind samples a bit more.
5) Incubate samples at 55°C for 1hr to overnight.
6) Add 500µl of 24:1 Chloroform:IsoAmylAlcohol and mix well by shaking tubes.
7) Centrifuge for 5-10 minutes at maximum speed.
a. Following centrifugation, you should have three layers: top: aqueous phase, middle: debris and proteins, bottom: chloroform.
b. Go on to the next step quickly so the phases do not remix
8) Pipette off the aqueous phase taking care not to suck up any of the middle or chloroform phases. Pipetting slowly helps with this.
9) Place the aqueous phase into a new labeled eppendorf tube.
a. Add RNAse let stand RT or 37°C for 10-20'.
10) Estimate the volume of the aqueous phase.
11) Add 0.08 volumes of cold 7.5M CH_3COONH_4.
12) Add 0.54 volumes (using the combined volume of aqueous phase and added CH_3COONH_4) of cold isopropanol (=2-propanol).
13) Mix well.
14) Let sit in freezer for 15 min to O.N.
a. Longer times means more DNA, but also more contaminants.
15) Centrifuge for 3 min at maximum speed.
16) Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.
17) Add 700µl of cold 70% Ethanol and mix
18) Centrifuge for 1 min at maximum speed.
19) Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.
20) Add 700µl of cold 95% Ethanol and mix
21) Centrifuge for 1 min at maximum speed.
22) Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.
23) Dry the pellet:
a. Place samples in the speed vac for 20 min.
or
b. Invert samples on a Kim-wipe and let stand for 1 hr or until dry.
24. Resuspend samples with 100µl of TE buffer or water. Allow to resuspend for 1hr at 55°C or overnight in refrigerator before running a test gel using 5µl of the DNA.

in vivo ATPase activity assay

Initial MS (Morashige and Skooge, 1962) 6 cm diameter plates: put stratificated and sterilized seeds to growth in a adapted Arabidopsis chamber for 5-6 days.

Prepare 3 different liquid stocks:
-MES-Tris 0.5M pH5.5 buffer (0.5M MES, use Tris 1M to reach the pH5.5)
-Sucrose-50% weight / volume
-MS-salts 4% solution (Murashige & Skoog medium basal salt mixture; cod. M0221, Duchefa)
Autoclave all stock solutions (quickly remove the sucrose otherwise gives a dark color). After the autoclave, store at room temperature the MS salts and Sucrose stocks; best store the MES-Tris stock at 4ºC.

Fill 2 square plates (12x12cm) with 100ml of
0.8g Agar
2ml Sucrose
2ml MES-Tris pH5.5
10ml MS salts 4%
up to 100ml with water and autoclave.
At the same time autoclave also several cheesecloth sheets shaped to fit into the 12x12cm square plates.
After the medium solidification, firstly spread the cheesecloth on the 12x12 agar plates and spread in a parallel way the young seedlings
The cheesecloth helps to separate the roots from the pH5.5 medium (which disturbs the next step)
Put the plates oblique: the aerial part is moved over the void with respect to the roots.



When grown, the roots accumulate in the vacuole or apoplast the medium pH (5.5), just before the end of vegetative step it is possible to do the final assay.

Fill 2 square plates (12x12cm) with 120ml of
2.4ml 50% w/volume
Sucrose 12ml 4% MS salts
0.005% Bromocresol purple (12mg) (Bromocresol Purple B5880, Sigma Aldrich)
adjust to pH 5.7 with KOH 1M
Then we put 0.96g Agar and up to 120ml with water

Autoclaved Bromocresol purple plates can store at 4ºC.

Spread the seedling in the Bromocresol purple plates, put in adapted Arabidopsis chamber with the light on and wait the indicator changes.
Col-0 it takes 12-16 hours depending of the light source and the plant health.

Reference:
Merlot S, Leonhardt N, Fenzi F, Valon C, Costa M, Piette L, Vavasseur A., Bernard G., Boivin K., Müller A., Giraudat J. Leung J. (2007). "Constitutive activation of a plasma membrane H^+-ATPase prevents abscisic acid-mediated stomatal closure". *The EMBO journal* 26 (13): 3216-3226.

RNA and DNA Methods

DNA Electrophoresis

Gels of 0.7% was used to check the plasmid or genomic DNA quality, 1% was used to separate DNA fragments.
Gel: Agarose melted in TBE 0.5X with 0.05% Ethidium bromide
Buffer: TBE 0.5X with 0.05% Ethidium bromide
TBE 10x (Tris-Borate EDTA buffer): 108g Tris-base, 55g H_3BO_3, 40ml of 0.5M EDTA (pH8), until 1l with milliQ water.
Loading buffer 6X(agarose gel): 0.25%w/v bromophenol blue, 0.25%w/v xylene cyanol FF,15%w/v Ficoll (Type 400; Pharmacia) in milliQ water.
Bands detection was realized by UV tube of 254nm.

RNA Electrophoresis

Denaturant gel

To do the northern blot analysis it is necessary to do slice gels for the RNA separation with a denaturant gel.
1% Agarose
MAE 1X: 10%v/v MAE10X (200mM MOPS, 50mM CH_3COONa, 10mM EDTA adjust at pH7 with NaOH) and 2.2% formaldheyde (stock 37%).
Electrophoresis buffer: MAE1X and 1.1% formaldheyde
Samples loading buffer: 1.25X (20% v/v formaldheyde, 55%v/v formamide, 12.5%v/v MAE10X, 8%v/v glycerol, 0.05%w/v bromophenol blue, 0.001% w/v Ethidium bromide)
Before loading heat the samples 56ºC for 10min. The same treatment for the invitrogen RNA ladder. For each lane we loaded at least 30ng of RNA, previously we determinate the concentration with a NanoDrop equipment.

No denaturiant gel

To check the RNA quality is useful the preparation of no denaturing gel: it is faster than denaturing one.
1% agarose gel melted with TAE1X (TAE50X, 1l: Tris-base 242g, 0.5M EDTA pH 8.0 100ml, glacial acetic acid 57.1ml, dH2O to nearly 1l, adjust the pH to 8.5).
The samples were loaded with the same 1.25X loading buffer and thermal treatment as in denaturing gels, 10min at 95ºC.

Transfering RNA to the membrane

After the separation with the denaturant gel we took a picture to get the intensity of original bands.
We eliminated the formaldehyde from the gel with 50ml of SSC10X for 30 min. for 2 times.
SSC20X: 3M NaCl, 0.3M Tri-Sodium Citrate adjusted at pH 7 with HCl and autoclaved.
On wet paper (SSC10X) we put the gel and on top the nylon membrane (wet the Hybond N® with SSC10X) and other 3 wet paper.
After we added dry paper that took the humidity of the bottom layers.
At the end we left to dried the membrane and fixed the RNA with UV light(120 mJ for 2 times).

Radiolabelling probe

We prepared radioactively-labeled probes with the random priming method. Its main characteristic is the presence of exanucleotides of all possible sequences that are contained in Oligo Labeling Buffer (OLB). The complementary DNA strand is synthesized by “Klenow” fragment of DNA polymerase I, using the random exanucleotides as primers. In the polymerase reaction the dCTP (deoxy-Cytidine Tri-Phosphate) is substituted by the radiolabelled nucleotide form (^{32}P-dCTP).
During the synthesis of the new strand, this special nucleotide is incorporated conferring the radioactivity property at the probe.
In order to obtain the probe we chose the gene fragment of interest (normally 300bp long) we amplified it by PCR.

1) We denatured it, 15µl (40-80ng) of the PCR product 10 min. at 95ºC and
2) We mixed with 5µl of OLB5X. OLB5X: 25mM $MgCl_2$, 5mM Dithiothreitol, 0.1mM MES pH 6.8 adjusted with Tris, 0.1mM dATP, 0.1mM dTTP, 0.1mM dGTP.
3) We added 2.5µl of BSA (Bovine Serum Albumin, 1mg/ml, 10X concentrated)
4) We put the enzyme 1µl of “Klenow” fragment
5) At the end 2µl of the ^{32}P-dCTP (20µCi in total).
6) Incubation O.N. at R.T.
7) We stopped the reaction with 200µl of TE solution.
8) Finally we incubated at 95ºC for 10min., just before the membrane hybridization step.

Northern blot pre-hybridization and hybridization

1) We did the membrane pre-hybridization in a hybridization tube with 25ml of Phosphate SDS EDTA buffer (PSE: 300mM Na_3PO_4 pH 7.2, 7% SDS, 1mM EDTA) to clean the membrane at 65ºC for 30 min.
2) We did the membrane hybridization poured off the old PSE solution, after we added other 25ml PSE and all the (just before denatured) labelled probe incubating at 65ºC O.N.
3) We recycled the PSE solution with the probe in a Falcon tube and storing at -20ºC.
4) We started washing steps with a buffer with high salt levels Saline Sodium Citrate (SSC)
In the hybridization tube 2 times 50ml of SSC4X with 0.1% of SDS for 15min.
In a plastic container at least 1 time 50ml of SSC4X with 0.1%SDS for few minutes, checking with the Geiger counter the level of radioactivity.
5) In a cassette we revealed the Northern blot with photographic film