

Document downloaded from:

<http://hdl.handle.net/10251/80571>

This paper must be cited as:

Bissoli, G.; Niños Rodenas, R.; Fresquet Corrales, S.; Palombieri, S.; Bueso Rodenas, E.; Rubio, L.; Garcia-Sanchez, MJ.... (2012). Peptidyl-prolyl cis-trans isomerase ROF2 modulates intracellular pH homeostasis in Arabidopsis. *Plant Journal*. 70(4):704-716. doi:10.1111/j.1365-313X.2012.04921.x



The final publication is available at

<http://dx.doi.org/10.1111/j.1365-313X.2012.04921.x>

Copyright Wiley

Additional Information

# Peptidyl-prolyl *cis-trans* isomerase ROF2 modulates intracellular pH homeostasis in *Arabidopsis*

Gaetano Bissoli<sup>1</sup>, Regina Niñoles<sup>1</sup>, Sandra Fresquet<sup>1</sup>, Samuela Palombieri<sup>1</sup>, Eduardo Bueso<sup>1</sup>, Lourdes Rubio<sup>2</sup>, María J. García-Sánchez<sup>2</sup>, José A. Fernández<sup>2</sup>, José M. Mulet<sup>1</sup>, and Ramón Serrano<sup>1,\*</sup>

<sup>1</sup>Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera, 46022 Valencia, Spain

<sup>2</sup>Departamento de Biología Vegetal, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos, 29071 Málaga, Spain

**Word count:** 7205

**Running title:** ROF2 modulates pH homeostasis

**Key words:**

H<sup>+</sup>-ATPase, K<sup>+</sup> transport, membrane potential, toxic cations, stress tolerance, *Arabidopsis thaliana*

\***Corresponding author.** Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-CSIC, Camino de Vera, 46022 Valencia, Spain. Tel.+ 34 96 387 7883; Fax: +34 96 387 7859; E-mail: [rserrano@ibmcp.upv.es](mailto:rserrano@ibmcp.upv.es). Co-corresponding author is José M. Mulet ([jmmulet@ibmcp.upv.es](mailto:jmmulet@ibmcp.upv.es))

## Abstract

Intracellular pH must be regulated close to neutrality to be compatible with cellular functions but the mechanisms of pH homeostasis and the responses to intracellular acidification are mostly unknown. We have found in the plant *Arabidopsis thaliana* that intracellular acid stress, generated by weak organic acids at normal external pH, induces several chaperone genes, including *ROF2* encoding a peptidyl-prolyl *cis-trans* isomerase of the FK506-binding protein class. Loss of function of *ROF2*, and specially the double mutation of *ROF2* and of its closely related gene *ROF1* results in acid sensitivity. On the other hand, over-expression of *ROF2* confers tolerance to intracellular acidification by increasing proton extrusion from cells. The activation of the plasma membrane proton pump ( $H^+$ -ATPase) is indirect: over-expression of *ROF2* activates  $K^+$  uptake, this causes depolarization of the plasma membrane that activates the electrogenic  $H^+$  pump. Depolarization of *ROF2*-overexpressing plants explains their tolerance to toxic cations such as lithium, norspermidine and hygromycin B, whose uptake is driven by the membrane potential. As *ROF2* induction and intracellular acidification are common consequences of many stresses, this mechanism of pH homeostasis may be of general importance for stress tolerance.

## Introduction

Living cells must regulate their intracellular pH within a narrow range of values close to neutrality that are compatible with cellular functions (Hochachka and Somero, 2002). In addition to this permissive role, intracellular pH may have regulatory functions (Felle, 2001), as observed during cell growth (Gillies, 1982; Perona and Serrano, 1988) and death (Gottlieb *et al.*, 1996; Ludovico *et al.*, 2001). Also, acid resistance of pathogenic bacteria is critical for survival in the stomach and inside phagosomes (Vandal *et al.*, 2009; Krulwich *et al.*, 2011). From an applied point of view, intracellular acidification is crucial for the action of weak acid food preservatives on spoilage microorganisms (Holyoak *et al.*, 1996; McPherson *et al.*, 2005).

In bacteria diverse mechanisms for pH sensing and cytoplasmic pH homeostasis have been identified (Krulwich *et al.*, 2011). However, in eukaryotic cells and in particular in plants the components of the pH homeostatic machinery, including intracellular pH sensors, signal transducing molecules, regulators of cation transport and cellular systems most sensitive to pH stress are mostly unknown (Felle, 2001). Only the carboxylation-decarboxylation reactions of the “biochemical pH stat” proposed by Davies (1986) are usually considered for pH homeostasis in plants.

One general source of intracellular pH stress is the metabolic production of carboxylic acids, a process that probably determined the early evolution of proton pumps and chemiosmotic energy coupling in primitive cells (Raven and Smith, 1976). Fungi and plants share a proton chemiosmotic circuit at their plasma membrane, with a proton-pumping P-ATPase generating the proton electrochemical gradient that drives secondary active transporters (Serrano, 1985). The activity of this H<sup>+</sup>-ATPase is crucial for growth and tolerance to intracellular acid stress in yeast (Vallejo and Serrano, 1989; Portillo and Serrano, 1989; Holyoak *et al.*, 1996; McPherson *et al.*, 2005) and plants (Young *et al.*, 1998; Haruta *et al.*, 2010). Known mechanisms of pH homeostasis include the slightly acidic pH optimum of the plasma membrane H<sup>+</sup>-ATPase (Serrano, 1985) and the activation of the enzyme by phosphorylation at its C-terminal regulatory domain in response to sugar metabolism (Lecchi *et al.*, 2007; Niittyla *et al.*, 2007) and acidification (Eraso and Gancedo, 1987; Bobik *et al.*, 2010). The protein kinases and signal transduction pathways involved in the regulation of H<sup>+</sup>-ATPases, however, remain unknown. Yeast (Schüller *et al.*, 1994; Moskvina *et al.*, 1999) and plant (Tena and Renaudin, 1998) MAP kinases are activated by multiple stresses, including acid stress, but their relationship to H<sup>+</sup>-ATPases has not been demonstrated.

As expected from the need for electrical balance during H<sup>+</sup> pumping, K<sup>+</sup> transport is also important for tolerance to acid stress (Mulet *et al.*, 1999; McPherson *et al.*, 2005) and it is also activated under conditions of intracellular acidification. During K<sup>+</sup> starvation this cation leaks out of the cells in exchange for H<sup>+</sup> and the cytosol is acidified, triggering activation of K<sup>+</sup> transport. 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 major high-affinity K<sup>+</sup> transporter inactivated by Ppz1 (Yenush *et al.*, 2002). In Arabidopsis the major root K<sup>+</sup> transporter AKT1 is phosphorylated and activated by the dimeric calcium-activated protein kinase CBL1/9-CIPK23 (Xu *et al.*, 2006). Mutants in these regulatory systems, however, are viable and therefore other cellular systems must participate in the regulation of H<sup>+</sup> and K<sup>+</sup> transport.

A genomic approach to investigate intracellular pH homeostasis has been to utilize the yeast null-mutant genomic collection to identify genes important for

tolerance to intracellular acid stress caused by weak organic acids (Mollapour *et al.*, 2004; Schüller *et al.*, 2004; Mira *et al.*, 2010a). Although many functions may have escaped the analysis because of gene redundancy and although there is some variation in the results from different laboratories, the cellular functions represented in all studies are (genes between brackets): tryptophan biosynthesis (*TRP1*, *TRP2*, *TRP5*), ergosterol biosynthesis (*ERG2*, *ERG6*), efflux of carboxylates (*PDR12*), potassium uptake (*TRK1*), vacuolar proton pumping (subunits of vacuolar H<sup>+</sup>-ATPase: *TFP1*, *VMA2*, *VMA4*), vesicle traffic (*VPS16*, *SSO2*, *SAC1*, *ARG82*), glycolysis (*PFK1*, *PFK2*) and transcription (*GAL11*, *RPB4*, *WAR1*). The absence of similar global studies in other organisms precludes conclusions about whether these may be considered general mechanisms.

In plants intracellular pH has been correlated with many regulatory phenomena, specially hypoxia (Drew, 1997; Felle, 2001) but most studies on acid stress refer to extracellular low pH and are concerned with acid rain and the salicylic acid pathway (Lee *et al.*, 2006) and with solubilization of soil aluminium, a very toxic cation counteracted by root secretion of chelating di- and tri-carboxylic acids (Iuchi *et al.*, 2007).

We have utilized *Arabidopsis thaliana* as a model organism to identify novel components of the intracellular pH homeostatic machinery. Our transcriptomic studies indicate that intracellular acidification increases expression of several chaperones also induced by heat stress and that include the peptidyl-prolyl *cis-trans* isomerase ROF2.

Chaperones or Heat-Shock Proteins (HSPs) of different families (HSP110, HSP90, HSP70-HSP40, Hsp60-Hsp10, small-HSPs and prolyl isomerases) participate in disaggregation and refolding of stress-denatured proteins (Nover, 1994). Prolyl isomerases not only facilitate protein folding but may also regulate the activity of other proteins (He *et al.*, 2004; Geisler and Bailly, 2007). ROF2 and ROF1 are highly related (85% identity) prolyl isomerases of *Arabidopsis* (Aviezer-Hagai *et al.*, 2007) that play different roles during heat stress. *ROF2* is not expressed under normal conditions but is highly induced by heat. On the other hand, *ROF1* is expressed under normal conditions and slightly induced by heat stress (Aviezer-Hagai *et al.*, 2007). ROF1 positively modulates thermotolerance by interacting with HSP90.1 and increasing the HSFA2-mediated accumulation of chaperones of the small-HSPs family (Meiri and Breiman, 2009). ROF2, on the other hand, does not interact with HSP90.1 and negatively modulates the HSFA2-mediated expression of small HSPs (Meiri *et al.*, 2010).

Our results indicate that *ROF2*, and probably also *ROF1*, plays a positive role in tolerance to intracellular acid stress. Over-expression of *ROF2* activates K<sup>+</sup> uptake, depolarizes the plasma membrane and indirectly activates the electrogenic H<sup>+</sup>-ATPase. Therefore, intracellular acid stress, probably through protein denaturation, induces chaperones such as ROF2 that not only help to refold proteins but also activate H<sup>+</sup> extrusion to restore intracellular pH.

## Results

### The transcriptomic response to intracellular acidification shares components with the “Misfolded Cytosolic Protein” response

We have determined the transcriptomic response of *Arabidopsis* to intracellular acidification caused by acetic acid, a weak organic acid that diffuses into cells and lowers internal pH (Krebs *et al.*, 1983; Holyoak *et al.*, 1996). In yeast there are several

reports on genes modulated by weak organic acids (Schüller *et al.*, 2004; Kawahata *et al.*, 2006; Mira *et al.*, 2010b) but in the case of Arabidopsis no previous studies existed. The transcriptional response to lowering of external pH has been described in Arabidopsis (Lager *et al.*, 2010) but these results are different to those of intracellular acidification at constant external pH (see below).

The total number of genes with significantly detected transcript levels was 15789, with 800 altered in expression more than 2-fold after 2 h incubation with 3.5 mM acetic acid (pH 5.5). Analysis of these genes indicated that most (502 genes; about 63 %) were repressed and much less (298 genes; about 37 %) induced. The array data were confirmed by RT-PCR for 5 genes (supplementary Figure S1).

The group of repressed genes was enriched (3-5 fold with respect to the genome) in genes related to Metabolism (GO Biological process). A more detailed analysis of the 107 genes repressed more than 4-fold (see supplementary Table S1B) indicates that 26 % of the genes correspond to the category of Metabolism (sugars, aromatic compounds and lipids) and 14 % to Transcription Regulators. This indicates remodeling of gene expression with reduction of metabolism.

We have compared the group of genes repressed by intracellular acid stress more than 2-fold (502 genes) with those repressed by other stresses (also more than 2-fold; Supplementary Table S2). There is significant overlap with genes repressed by heat (8.4 %; 42 genes) and osmotic stress (7.2 %; 36 genes), but the overlap was only 1 % (5 genes) in the case of genes repressed by low external pH and 0.6 % (3 genes) in the case of hypoxia. The microarrays of auxin treatment, oxidative stress and infection with *Pseudomonas syringae* had no repressed genes in common with the acetic acid microarray. Finally, 38 % of the genes repressed by acetic acid encode putative proteins of unknown function.

More information could be obtained from the analysis of genes induced by intracellular acid stress more than 2-fold. The functional categories (GO Biological process) enriched in the group of induced genes with respect to the genome (and with more than 5 genes in it) were: Response to heat (17-fold), Drug transport (15-fold), Response to high light intensity (15-fold) and Response to ROS (Reactive Oxygen Species; 6-fold). A more detailed analysis of the 65 genes induced more than 3-fold (see supplementary Table S1A) indicate that 17 % of the genes induced by acetic acid encode putative proteins of unknown function, a much lower value than in the case of repressed genes (see above). The dominant categories of induced genes of known function are: Metabolism (14 %), Transcription factors (12 %), Transporters (11 %), Protein folding (9 %) and Detoxification and antioxidant response (6 %). Some of these categories correspond to the enriched GO Biological processes: Protein folding (Response to heat), Transport (Drug transport) and Detoxification and antioxidant response (Response to ROS).

As detailed in supplementary Table S2, the overlap between acetic acid-induced genes and genes induced by heat (14.1 %; 42 genes), by osmotic stress (11.4 %; 34 genes), low external pH (10.1 %; 30 genes) and hypoxia (5.0 %; 15 genes) is small but significant. On the other hand, the overlaps with genes induced by auxin treatment (1.7 %; 5 genes) and by oxidative stress or biotic stress (less than 2 genes or 0.7 %) were insignificant.

The most conspicuous feature of the transcriptional response of Arabidopsis to intracellular acidification is that genes encoding heat-shock proteins (chaperones) of different types are induced. As indicated in Table 1, 8 out of 10 heat-shock genes induced by intracellular acid stress were also induced by misfolded proteins produced by the proline analog azetidine-2-carboxylic acid (Sugio *et al.*, 2009). This “Misfolded

Cytosolic Protein” response is a subset of the wider heat-shock response and represents the pathway triggered by misfolded proteins in the cytosol.

Other genes induced by intracellular acidification in Arabidopsis include nutrient-H<sup>+</sup> symporters for sulfate (*SEL1*) and phosphate (*PTH1* and *PTH2*), a channel for ammonium (*AMP1.2*) and two ABC-ATPases (*ABCG22* and *ABCB12*) (see supplementary Table S1A). Intracellular acidification inhibits H<sup>+</sup>-symporters (Hueso *et al.*, 2011) and this would explain the induction of the corresponding genes to improve nutrient uptake. ABC-ATPases may be involved in organic anion efflux as in the case of yeast Pdr12 (Piper *et al.*, 1998). A more detailed analysis of these genes is outside the focus of the present work.

### **Prolyl isomerase ROF2 is important for weak organic acid tolerance in Arabidopsis**

*ROF2* was the only prolyl isomerase gene (out of 55 in Arabidopsis; He *et al.*, 2004) induced by intracellular acid stress in our microarray (see Table 1). As mentioned in the introduction, *ROF2* has a closely related homolog called *ROF1* and both prolyl isomerases play different roles during heat stress (Aviezer-Hagai *et al.*, 2007). *ROF1* exhibits similar (but less intense) induction than *ROF2* by stresses such as heat, high light, high glucose, flagellin, abscisic acid, ultraviolet light and hydrogen peroxide (see <https://www.genevestigator.com/>) and *ROF1* was also induced (less intensively than *ROF2*) by intracellular acid stress (see supplementary figure S1).

In order to investigate if induction of *ROF1* and *ROF2* by intracellular acid stress correlates with a positive effect of these prolyl isomerases on tolerance to this stress, we have tested germination and growth in the presence of acetic, propionic and sorbic acids of Arabidopsis transgenic plants with over-expression and null mutations of these genes. The over-expressing lines exhibit a high constitutive expression of *ROF2* (supplementary Figure S2). The single mutants *rof1-1* and *rof2-1* (in the following *rof1* and *rof2*) are probably null mutants because the level of the mRNAs was undetectable (supplementary Figure S3). The results of Figure 1(a) and (b) indicate that in a germination assay *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 over-expression of *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*. This acetic and propionic acid-tolerance phenotype was observed with three independent transformed lines exhibiting high expression of *ROF2* (see supplementary figure S2). The acetic acid sensitivity of the *rof2* mutant could be complemented by transformation with the *ROF2* gene (supplementary figure S4), suggesting that ectopic expression of *ROF2* is biologically relevant.

As indicated in Figure 1(c) 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. The growth of plants (measured by fresh weight) was inhibited more by acetic acid in 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 more adult plants.

As indicated in supplementary figure S5, over-expression of *ROF2* improves germination in the presence of sorbic acid and the double mutant *rof1 rof2* germinates less well than wild type in the presence of this acid. Single *rof1* or *rof2* mutants exhibited little phenotype. The fact that similar phenotypes were observed with acetic, propionic and sorbic acids suggest that these phenotypes were due to intracellular acidification and not to specific metabolism of the acid.

### **Other stress and development phenotypes of *ROF* mutants**

In addition to tolerance to intracellular acid stress, *ROF* mutants exhibited phenotypes related to seed longevity and plant development. We show in Table 2 the results of a controlled deterioration test for seed longevity (accelerated aging) based on heat stress (23 h at 42 °C) of imbibed seeds (Prieto-Daena *et al.*, 2006). The double mutant *rof1 rof2* was more sensitive than wild type. In this assay only the double loss-of-function *rof1 rof2* gave phenotype: single *rof1* or *rof2* mutants and over-expression of *ROF2* exhibited similar germination than wild type after accelerated aging of seeds.

We have observed two developmental phenotypes in plants over-expressing *ROF2*: decreased branching is shown in Figure 2 (a) and delayed flowering in Figure 2 (b). In the first case single and double mutants of *rof1* and *rof2* have no effect on branching but the single *rof1* mutant or the double mutant *rof1 rof2* have a decrease in bolting time as measured by both “days after germination” and “number of leaves”.

### **Over-expression of *ROF2* increases acid extrusion, depolarizes the plasma membrane and stabilizes intracellular pH in the presence of acetic acid**

There are several possible mechanisms to improve tolerance to cytosolic acidification but increasing H<sup>+</sup> pumping out of the cells was the first one we tested. We have utilized a visual assay for H<sup>+</sup> efflux from root cells based on plates containing the pH indicator bromocresol purple. The yellow color of the dye in the surroundings of roots indicates external acidification and as indicated in Figure 3 (a) it was much greater in the case of *ROF2* over-expressing plants than in controls. The possible phenotype of the *rof1 rof2* double mutant could not be observed with this qualitative assay for H<sup>+</sup> efflux. We have also utilized a quantitative assay based on measuring the acidification of the external medium of the plants with a sensitive pH meter. The results of Figure 3 (b) indicate the 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 wild type. This defect could be 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* (Columbia-0) increased both the initial rate and the final extent of acidification.

The increase in external acidification upon over-expression of *ROF2* can be explained by activation of the plasma membrane H<sup>+</sup>-ATPase, of K<sup>+</sup> transport or both. K<sup>+</sup> transport is required for electrical balance during proton extrusion and although anion efflux may also contribute to electrical balance, K<sup>+</sup> uptake is usually more important. These mechanisms can be differentiated by the effect of over-expression of *ROF2* on the electrical membrane potential. As indicated in Figure 4, transgenic plants



over-expressing *ROF2* are depolarized, suggesting that *ROF2* activates  $K^+$  transport. Activation of the  $H^+$ -ATPase would have produced the opposite effect (hyperpolarization). In agreement with this conclusion we have measured the plasma membrane  $H^+$ -ATPase activity of control and *ROF2* transgenic plants and we did not observe significant differences ( $2.1 \pm 0.2$  and  $1.9 \pm 0.2$  nmoles Pi/min x mg protein, respectively; average of 4 determinations  $\pm$  standard error). The single *rof2* mutant has the same membrane potential than wild type but the double *rof1 rof2* mutant is hyperpolarized. The addition of acetic acid increases the potential in all genotypes, as expected from the known activation of the  $H^+$ -ATPase at low pH described in the Introduction. However, the relative values still indicate depolarization in *ROF2* over-expressing plants and hyperpolarization in the double *rof1 rof2* mutant.

We have measured the intracellular (cytosolic) pH of Arabidopsis root cells with double-barreled microelectrodes and as indicated in Figure 4 under normal conditions this parameter is similar in all genotypes (7.3-7.4). The cytosolic pH decreased by 0.7 pH units in wild type Arabidopsis after addition of 1 mM acetic acid (from 7.3 to 6.6). However, in transgenic plants over-expressing *ROF2* no pH decrease was observed under the same conditions. Apparently, the enhanced capability of transgenic plants for proton extrusion rapidly compensates the influx of protons mediated by diffusion and dissociation of acetic acid inside the cells. In the *rof2* single mutant acetic acid produced an acidification of 0.1 pH units and in the double *rof1 rof2* mutant of 0.3 units. The fact that *rof1 rof2* plants experience less cytosolic acidification than wild type is difficult to explain because these mutants have less capability for proton extrusion (Figure 3 (b)).

### ***ROF2* improves germination in the presence of toxic cations**

We have confirmed the depolarization of the plasma membrane in *ROF2*-overexpressing plants by testing the sensitivity of these plants to toxic cations. In yeast indirect estimates of plasma membrane electrical potential indicate that depolarization by increased  $K^+$  uptake improves the tolerance of cells to toxic cations such as sodium, lithium, norspermidine and hygromycin B (Mulet *et al*, 1999; Goossens *et al*, 2000). The interpretation is that the electrical potential (negative inside) drives the uptake of toxic cations by unknown channels and this model can now be tested in Arabidopsis plants depolarized by over-expression of *ROF2*. As indicated in Figure 5 over-expression of *ROF2* increases the tolerance of Arabidopsis to the toxic cations lithium, norspermidine and hygromycin B. The germination assay of stress tolerance determines the appearance of green, expanded cotyledons in the germinating seeds but the tolerance phenotype can also be appreciated by seedling size (Figure 5). These results are consistent with over-expression of *ROF2* depolarizing the plasma membrane and decreasing uptake of toxic cations. On the other hand, the double mutant *rof1 rof2* is more sensitive to toxic cations than wild type, a phenotype that can be explained by hyperpolarization of the plasma membrane and increased cation uptake.

### ***ROF2* activates $K^+$ transport**

Additional evidence for the hypothesis that over-expression of *ROF2* activates  $K^+$  transport was provided by the improved germination of *ROF2* transgenic plants in

media with low  $K^+$  levels (10  $\mu M$ ), while the *rof1 rof2* double mutant germinates less well than wild type in this medium (Figure 6 (a) and (b)).

Another confirmation of the connection between *ROF2* and  $K^+$  uptake was obtained by measurements of  $Rb^+$  uptake in different genotypes.  $Rb^+$  was utilized as  $K^+$  analog because measurements of  $K^+$  uptake are complicated by the high concentration of the cation inside cells. As indicated in Figure 7, in the transgenic plants over-expressing *ROF2*  $Rb^+$  uptake (at 30 min) is 2-fold greater than in control plants. On the other hand, in the *rof1 rof2* double mutant  $Rb^+$  uptake was 70% of control plants.

## Discussion

As a first step in our molecular analysis of intracellular pH homeostasis in Arabidopsis, we have investigated the transcriptional response to acetic acid, a weak organic acid that diffuses into cells and lowers cytosolic pH, as determined with pH-sensitive microelectrodes (Figure 4). Our results indicate that this response is different from responses to other stresses but include genes activated by other stresses, specially by heat stress.

The transcriptional response to heat stress has been divided into two major pathways: the “Misfolded Cytosolic Protein” response represents the pathway triggered by misfolded proteins in the cytosol and additional heat-shock responses are mediated by membrane sensors that activate MAP kinases and other pathways (Trotter *et al.*, 2002; Sugio *et al.*, 2009). The first one should not be confused with the “Unfolded Protein Response”, an incomplete name that refers to events in the lumen of the endoplasmic reticulum. The Misfolded Cytosolic Protein pathway is probably triggered by misfolded proteins that bind to heat-shock proteins (HSP70 and HSP90) and release heat-shock transcription factors (HSFs) from inhibition by these HSPs (Sorger, 1991). The transcriptional response to intracellular acid stress shares many genes with the response to Misfolded Cytosolic Proteins (Table 1) and this coincidence suggest that a low cytosolic pH probably generates misfolded proteins and that damage to protein structure may be one of the most important problems generated by intracellular acid stress. This conclusion is supported by results obtained in yeast, where the transcriptional response to intracellular acidification (Schüller *et al.*, 2004; Kawahata *et al.*, 2006; Mira *et al.*, 2010b) also includes many genes induced by azetidine-2-carboxylic acid (Trotter *et al.*, 2002).

It has been a surprise to find so little overlap between responses to intracellular acidification and to hypoxia because oxygen deficiency causes intracellular acidification due to the fermentative accumulation of lactic acid (Drew, 1997). The genes of two transcription factors induced by acetic acid, *HRE1* and *HRE2*, are also induced by hypoxia and are important determinants of the transcriptional response and survival of Arabidopsis under these conditions (Licausi *et al.*, 2010). The induction of *HRE1* and *HRE2* by acetic acid is accompanied by the induction of some fermentative genes modulated by these transcription factors under hypoxia: *ADH1* (alcohol dehydrogenase), *DL4665W* (lactate dehydrogenase), *T28P16.12* (ribose kinase), *ACO1* (ACC oxidase) and *HBI* (hemoglobin) (Licausi *et al.*, 2010). Most of the hypoxia responses, however, are probably triggered by low-oxygen levels and not by intracellular acidification, which is a late event in hypoxia (Drew, 1997). Actually, the mechanism of oxygen sensing in plants has recently been identified and it is based on an oxygen-dependent N-end rule degradation pathway acting on the ERF-transcription factor *RAP2.12* (Licause *et al.*, 2011). This factor has an N-terminal cysteine that

becomes oxidized in the presence of oxygen. This triggers ubiquitination and proteosomal degradation of RAP2.12. Under hypoxia RAP2.12 is stabilized, moves to the nucleus and activates anaerobic gene expression.

Dissaggregation and renaturation of misfolded proteins must be crucial for tolerance to stresses known to denature intracellular proteins such as heat-shock and, accordingly, over-expression of crucial chaperones (HSPs) has been shown to protect yeast (Sanchez *et al.*, 1992) and Arabidopsis (Nishizawa *et al.*, 2006) against heat. Protection was also observed against other stresses, suggesting that, in addition to heat, many abiotic stresses (osmotic, salt, starvation, cold) cause protein denaturation. In the case of intracellular acid stress our transcriptomic studies provide circumstantial evidence that protein denaturation also occurs.

We have identified a chaperone, the prolyl isomerase ROF2 induced by intracellular acid stress that by over-expression improves tolerance to this stress. This was demonstrated by a germination test and by the growth of plants at later stages. The *rof2* mutant had little phenotype but the double mutant *rof1 rof2* gave the expected acid sensitivity. As indicated at the Introduction, *ROF2* and *ROF1* play different roles during heat stress (Meiri and Breiman, 2009; Meiri *et al.*, 2010) but in our assays for seed longevity (Table 2) and germination in the presence of acetic (Figure 1) and sorbic (supplementary Figure 5S) acids the single mutants *rof1* and *rof2* behavior almost like wild type while the double mutant *rof1 rof2* is more sensitive to accelerated aging of seeds and to germination in the presence of weak organic acids. This suggests that for these phenotypes *ROF1* and *ROF2* are redundant genes.

Prolyl isomerases were originally identified as receptors for the immunosuppressive drugs cyclosporin A, FK506 and rapamycin (Harding *et al.*, 1986) and collectively referred as immunophilins. Later on these drug receptors were found to be peptidyl-prolyl *cis-trans* isomerases (Takahashi *et al.*, 1989; Fisher *et al.*, 1989; Harding *et al.*, 1989). At present they are classified into 3 families by sequence homology and drug binding: cyclophilins (binding cyclosporin A), FKBP (FK506-binding proteins, also binding rapamycin in yeast and animals) and parvulins (Arevalo-Rodriguez *et al.*, 2004; He *et al.*, 2004). The two prolyl isomerases induced by intracellular acid stress, *ROF2* and *ROF1*, belong to the FKBP family. Prolyl isomerases are important cellular chaperones involved in protein folding (He *et al.*, 2004; Arevalo-Rodriguez *et al.*, 2004). In addition, those containing tetratricopeptide domains (such as *ROF2* and *ROF1*) modulate the heat-shock response by binding to HSP90 in yeast (Duina *et al.*, 1998; Prodromou *et al.*, 1999) and in Arabidopsis (Aviezer-Hagai *et al.*, 2007). Finally, some prolyl isomerases 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 ABC-ATPases involved in auxin transport (Geisler *et al.*, 2004; Bailly *et al.*, 2008). 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.

Although *ROF2* probably participates in protein folding, our results indicate that it has the additional function of activating  $K^+$  uptake and indirectly  $H^+$  efflux to restore intracellular pH. The electrophysiological studies conducted in Arabidopsis root cells indicate that over-expression of *ROF2* depolarizes the plasma membrane (Figure 4), as expected from the observed activation of  $K^+$  transport (Figure 7). Further evidence for this mechanism is provided by the observation that over-expression of *ROF2* improves germination and early growth in medium with low  $K^+$  (Figure 6) and in the presence of toxic cations such as norspermidine, lithium and hygromycin B (Figure 5). Tolerance to

toxic cations has been interpreted by a model (Goossens *et al.*, 2000) where electrical membrane potential, set by the relative activities of the H<sup>+</sup>-ATPase (pump) and potassium uptake (leak), determine the uptake of toxic cations. Accordingly, depolarization produced by increased K<sup>+</sup> uptake should reduce uptake and toxicity of toxic cations and this is what we have observed. In all cases the *rof1 rof2* mutant exhibited opposite phenotypes than over-expression of *ROF2*, as expected from its decreased K<sup>+</sup> transport (Figure 7) and hyperpolarized membrane potential (Figure 4).

Our results add intracellular pH homeostasis and K<sup>+</sup> transport to the wide range of biological functions modulated by prolyl isomerases (in addition to protein folding, see above). The K<sup>+</sup> transporters activated by *ROF2* may correspond to the major K<sup>+</sup> uptake systems in Arabidopsis roots (AKT1 and HAK5; Gierth and Mäser, 2007). The effect of *ROF2* on transporters could be direct or through regulators of the systems and in this respect the CBL1/9-CIPK23 protein kinase (Xu *et al.*, 2006) that regulates AKT1 is a candidate to be regulated by *ROF2*. We are testing these hypothesis as well as the participation of auxin in *ROF2* action, as suggested by the developmental phenotypes of *ROF2* over-expressing plants (reduced branching and delayed bolting).

Proline isomerization may be a rate-limiting step in the conformational changes occurring during activation or transport function of K<sup>+</sup> channels/transporters. This “catalytic” mechanism has been suggested in the case of cyclophilin PIN1 from Arabidopsis (Wang *et al.*, 2010) and animal cells (Lee *et al.*, 2011). Alternatively, prolyl isomerases may bind to target proteins and change their conformation independently of the prolyl isomerase activity. This “stoichiometric” mechanism seems to apply in the interaction of animal cyclophilin A with rhodopsin (Baker *et al.*, 1994), HIV-1 Gag protein (Luban, 1996) and chemotaxis receptor CD147 (Song *et al.*, 2011) and in the case of mammalian FKBP52 and steroid hormone receptor (Riggs *et al.*, 2007). Mutants of Arabidopsis *ROF2* without prolyl isomerase activity would be needed to determine the mechanism (catalytic or stoichiometric) of regulation of K<sup>+</sup> transport. In this respect, some mutants of the mammalian homolog of *ROF2* (FKBP52) with reduced activity have been described (Riggs *et al.*, 2007).

As a conclusion, the finding that intracellular acidification induces a chaperone like *ROF2* that activates K<sup>+</sup> transport and H<sup>+</sup> extrusion establish a connection between protein folding and membrane transport that may be of general relevance to many abiotic stresses. Heat-shock has been shown to cause intracellular acidification (Weitzel *et al.*, 1987) and osmotic stress denatures cytosolic proteins (Burkewitz *et al.*, 2011). Therefore it is plausible that abiotic stresses cause both protein denaturation and intracellular acidification and that induction of chaperones like *ROF2* has the double purpose of protein folding and pH homeostasis.

## Materials and methods

### Arabidopsis growth and transformation

*Arabidopsis thaliana* (Columbia-0 ecotype) plants were grown under long-day greenhouse conditions (16 h light/8 h dark, at 23 °C, 130 μE x m<sup>-2</sup> x s<sup>-1</sup> 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 *et al.*, 1993; Clough and Bent, 1998).

For *in vitro* culture, seeds were surface sterilized with commercial bleach and rinsed with sterile water. The agar (0.8%) medium contained Murashige and Skoog (MS) basal salt mixture (Sigma; 0.4%), sucrose (1%) and 10 mM MES buffer acid taken to pH 5.5 with Tris base. Stratification was conducted during 3 days at 4°C and then plates were transferred to chambers under the conditions described above. When indicated, the medium was supplemented with 3.5 mM acetic or propionic acids or 0.55 mM sorbic acid (pH 5.5 with Tris base). Germination was scored after 4-5 days. For growth in liquid culture the MS medium was prepared without agar and using suspension-culture-6 well plates (Cellstar, Greiner). Each well contained 7 ml medium and 3 seedlings that have been cultivated during 6 days in solid medium. Plates were incubated with shaking (100 rev/min) under the conditions described above.

The potassium-free medium has already been described (Alejandro *et al.*, 2007) and for germination assays was supplemented with 10  $\mu$ M KCl.

### **Transcriptomic response of Arabidopsis to intracellular acid stress**

Seedlings grown during 6 days in solid medium were transferred to liquid medium and grown for 13 days. Four samples (3 plants each) were incubated with 3.5 mM acetic acid (pH 5.5) for 2 h and other 4 samples were incubated without acetic acid. RNA was extracted with the "RNeasy Mini Kit" (Quiagen). Thirty micrograms of RNA was retrotranscribed in the presence of modified nucleotides [5-(3-aminoallyl)-2-deoxy-UTP] by using an oligo(dT) primer. cDNA synthesis was performed at 50°C during at least 3 h, using the reverse transcriptase Superscript III (Invitrogen). The cDNA was then purified using Qiaquick (Qiagen) columns and labeled with Cy3 and Cy5 dyes as recommended by the manufacturer (Amersham Biosciences). cDNAs were then purified again with Qiaquick (Qiagen) columns and the labeling was confirmed by measuring the absorbance at the appropriate wavelength, using a Nanodrop ND 1000 (Thermo Scientific).

Arabidopsis oligonucleotide microarrays were obtained from Prof. David Galbraith (<http://www.ag.arizona.edu/microarray/>). The chosen design for the microarray experiments was a "balanced block" design with four biological replicates and two dye swaps. For analysis, they were submerged for 45 min in a prehybridization solution (0.1% SDS, 5x SSC [1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 1% bovine serum albumin) at 42°C. Microarrays were then hybridized with the labeled probes in hybridization solution (0.1% SDS, 5x SSC, 50% formamide, 0.1 mg/ml salmon sperm DNA) using hybridization chambers (Telechem) overnight at 42°C. After the hybridization, microarrays were washed in different dilutions of SSC and SDS and then scanned to obtain digital images using a GenePix 4000B scanner (Axon Instruments) with a 10- $\mu$ m resolution.

Digital images from microarrays were analyzed using the GenePix 4.0 (Axon Instruments) program. Only those genes whose intensity was 2-fold higher than local background intensity in at least one of the channels used were included in the analysis. The data from the four biological replicates were analyzed and normalized by the LOWESS method using the Acuity 4.0 program (Axon Instruments), and statistical selection of genes was performed with the significance analysis application (SAM) (Tusher *et al.*, 2001). Genes were selected as differentially expressed when the expression in acid treated cells was at least 2-fold higher or 2-fold lower than the

expression in the untreated and when applying the significance analysis (SAM), the false discovery rate (FDR) was lower than 5.5%.

Relevant functional categories were selected using the Gene Ontology Term Finder as described previously (Boyle *et al.*, 2004) and over-representation of categories in our data sets was analyzed by FATIGO (<http://babelomics.bioinfo.cipf.es/functional.html>). The Arabidopsis acid stress microarray data have been submitted to the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) and assigned the accession number GSE29259 (Arabidopsis: control-vs. acetic acid treatment). Confidential access by peer-reviewers can be achieved by username “renioro” and password “cerrallo”.

### **Comparison of genes modulated by intracellular acidification with transcriptional responses to other stresses**

With the exception of genes modulated by hypoxia (Liu *et al.*, 2005), the microarray data were obtained from the web page (<http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl>) and the information is summarized in supplementary Table S2. Data from other groups were not renormalized and genes induced and repressed were only considered when the data were statistically significant with p-value 0.05 and they were up and down regulated, respectively, more than 2-fold. Times of stress were selected to maximize overlap with our acetic acid microarray (2 h treatment) and corresponded to the shortest times employed (1 h in the case of osmotic, low external pH and auxin treatments; 3 h in the case of heat and hypoxia stresses; 4 h in the case of bacterial infection). Concerning heat stress, although the microarray of 1 h treatment had more overlap with our acetic acid microarray than the one of 3 h (83 versus 42 in the case of induced genes) the high number of regulated genes after 1 h heat stress (5513 induced and 4455 repressed) made more reasonable the comparison with the 3 h heat treatment (1173 induced and 1773 repressed).

Overlapping (Venn diagrams) of our acetic acid microarray with other data sets with transcriptional responses to other stresses was done at the web page (<http://www.bioinformatics.org/gvenn/index.htm>) and the results are summarized in supplementary Table S2.

### **Over-expression of *ROF2* in Arabidopsis**

The complete *ROF2* cDNA was obtained as plasmid RAFL 14-54-F14 from RIKEN (Tsukuba, Japan). The coding sequence was amplified from plasmid RAFL 14-54-F14 (RIKEN, Tsukuba, Japan) with primers upstream (5'-ATACTCGAGCAGAAAATGGAAGACGATTTTCGAC; *Xho* I site underlined) and downstream (5'-CGCGAATTCTCATGCCTTGGTGTCAATACTCAT; *EcoR* I site underlined), digested and ligated to binary plasmid pXCS-HAStrep (ampicillin resistance in bacteria, Basta resistance in plants and 2 X 35S promoter; Witte *et al.*, 2004). The 2 X 35S promoter contains a duplication of the upstream activating sequence of the cauliflower mosaic virus 35S promoter and provides very high expression levels of downstream genes (Kay *et al.*, 1987). The recombinant plasmid was introduced into *Agrobacterium tumefaciens* GV3101::pMP90RK and used to transform Arabidopsis, both the wild type Columbia-0 and the *rof2* mutant. Three transgenic lines with over-expression of *ROF2* were selected and in the case of wild

type transformation the northern analysis of the lines is shown in supplementary Figure S2.

### **Arabidopsis mutants with loss of function of *ROF* genes**

T-DNA insertion mutants with loss of function of *ROF1* (*rof1-1*; Salk\_024090) and *ROF2* (*rof2-1*; Sail\_35\_A02) were obtained from the European Arabidopsis Stock Centre at the University of Nottingham (NASC). The *rof1-1* T-DNA insertion is in the first exon (out of 12) and the *rof2-1* insertion in the sixth exon (out of 13). Both are null alleles because no expression could be detected by RT-PCR (supplementary Figure S3). Homozygous mutant plants (*rof1-1/rof1-1* and *rof2-1/rof2-1*) were selected and crossed to identify double homozygous *rof1-1/rof1-1 rof2-1/rof2-1* mutants. The double mutant was named in the following *rof1 rof2*.

### **Measurement of plasma membrane H<sup>+</sup>-ATPase activity in Arabidopsis roots: *in vitro* and *in vivo* assays**

For *in vitro* determination of activity of the plasma membrane H<sup>+</sup>-ATPase Arabidopsis plants were grown for 18 days in vertical plates with MS medium containing no sucrose under the conditions described above. About 0.5 g 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<sup>+</sup>-ATPase activity were as described (Serrano, 1988).

For *in vivo* assay of the plasma membrane proton pump we used qualitative and quantitative assays. In the first case plants were grown in vertical plates with normal solid MS medium for 16 days and then transferred to vertical plates of the same medium (pH 5.5) without MES buffer and with 0.003% bromocresol purple. This pH indicator turns yellow below pH 5.2 and after 8 h incubation the yellow color around roots indicates proton extrusion.

The quantitative assay is based on measuring the acidification of the external medium of plants with a sensitive pH meter (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 24 h in darkness. The starved plants (about 5 plants with 2 mg root weight) were then incubated in a vessel with 5 ml 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 15 min. 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 5 µl of 10 mM HCl (producing a pH decrease of about 0.05 units).

### **Electrophysiology of Arabidopsis root epidermal cells**

Seeds of *Arabidopsis thaliana* wild type (Col-0), *ROF2* over-expressing lines, *rof2* mutant and *rofl rof2* double mutant were surface esterilized, 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 °C, seeds were grown vertically at 25 °C for 10-15 days, at a light intensity of 150  $\mu\text{mol m}^{-2} \text{s}^{-1}$  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 (1.1 ml volume) with continuous perfusion of the assay medium (flux of 10 ml  $\text{min}^{-1}$ ), containing 0.1 mM  $\text{CaCl}_2$ , 0.1 mM KCl, 0.1 mM NaCl, 12 mM MES-Bis tris propane (pH 5.5). Epidermal root cells (located at  $\approx 0.5$  cm from root tip) of plant seedlings were impaled with single-barrelled borosilicate glass vertically pulled, filled with 500 mM KCl and connected to a high impedance differential amplifier (WPI FD223, World Precision Instruments, Sarasota, FL, USA).

Cytosolic pH and membrane potential was simultaneously measured using double-barrelled microelectrodes. Microelectrode pre-treatment and backfilling was similar to the  $\text{H}^+$ -selective microelectrodes described previously (Felle, 1987; Fernández *et al.*, 1999). After 30 min heating at 180 °C, the barrels containing the sensor were silanized 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 °C and backfilled with the  $\text{H}^+$ -sensor, once cold. The  $\text{H}^+$ -sensor was prepared by mixing the Hydrogen Ionophore II-cocktail A in a mixture of polyvinylchloride/tetrahydrofuran (40 mg  $\text{ml}^{-1}$ ) at a ratio 1/6 (v/v). The microelectrodes were filled with the  $\text{H}^+$ -sensor cocktail and stored in a dry chamber (silica gel) until the evaporation of tetrahydrofuran. The microelectrode barrel containing the  $\text{H}^+$  sensor was backfilled with a mixture of 500 mM KCl and 100 mM Mes-Tris adjusted to pH 4.3, whereas the voltage barrel and the reference electrode were filled with 500 mM KCl. The signals from the  $\text{H}^+$ -selective and voltage barrels were measured and simultaneously subtracted by the high impedance differential amplifier. The  $\text{H}^+$ -selective microelectrodes were calibrated before and after the measurements with buffers solution containing 96 mM KCl and 2.5 mM Mes – Bis tris propane (pH 5.3; 6.3 and 8.3). Calibrations curves showed slopes round 49 mV / pH unit.

### **Determination of rubidium uptake in Arabidopsis**

Potassium transport was measured by using rubidium as a tracer and the method has been described (Alejandro *et al.*, 2007). Basically, *Arabidopsis* seedlings were germinated and grown in potassium-free medium and after 15 days incubated in the same medium with 2 mM RbCl. At the indicated times samples were taken, washed, extracted with nitric acid, centrifuged and  $\text{Rb}^+$  determined in the supernatant by atomic absorption spectrophotometry.

### **Acknowledgements**

This work was supported by grants BFU2008-00604 of MICINN (Madrid) and PROMETEO/2010/038 of “Consellería de Educación” (Valencia). We thank Dr. Eugenio Grau (Sequencing Service, IBMCP, Valencia) for sequencing of the different



genes and Dr. Vicente Fornés (Instituto de Tecnología Química, Valencia) for assistance with the atomic absorption spectrophotometry. None of the authors has a conflict of interest to declare.

## Short legends for supplementary material

Table S1. Genes modulated by intracellular acidification. Table S1A. Genes induced by intracellular acidification (3-fold and more) in the plant *Arabidopsis thaliana*. Table S1B. Genes repressed (4-fold and more) by intracellular acidification in the plant *Arabidopsis thaliana*

Table S2. Information about the microarray experiments compared in the present work

Supplementary figures. Figure S1. Confirmation of 3 induced and 2 repressed genes of the acetic acid microarray. Figure S2. Northern analysis of *ROF2* expression in three transgenic lines that over-express *ROF2* (numbers 7, 16 and 17) and in non-transformed control (ctrl). Figure S3. The *rof1-1* and *rof2-1* alleles do not express the respective genes (less than 5 %).

## References

- Alejandro, S., Rodriguez, P.L., Bellés, J.M., Yenush, L., García-Sánchez, M.J., Fernández, J.A. and Serrano, R. (2007) An Arabidopsis quiescin-sulphydryl oxidase regulates cation homeostasis at the root symplast-xylem interface. *EMBO J.* **26**, 3203-3215.
- Arevalo-Rodriguez, M., Wu, X., Hanes, S.D. and Heitman, J. (2004) Prolyl isomerases in yeast. *Front. Biosci.* **9**, 2420-2446.
- Aviezer-Hagai, K., Skovorodnikova, J., Galigniana, M., Farchi-Pisanty, O., Maayan, E., Bocovza, S., Efrat, Y., von Koskull-Döring, P., Ohad, N., and Breiman, A. (2007) Arabidopsis immunophilins ROF1 (AtFKBP62) and ROF2 (AtFKBP65) exhibit tissue specificity, are heat-stress induced, and bind HSP90. *Plant Mol. Biol.* **63**, 237-255.
- Bailly, A., Sovero, V., Vincenzetti, V., Santelia, D., Bartnik, D., Koenig, B.W., Mancuso, S., Martinoia, E. and Geisler, M. (2008) Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. *J. Biol. Chem.* **283**, 21817-21826.
- Baker, E.K., Colley, N.J. and Zuker, C.S. (1994) The cyclophilin homolog NinaA function as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. *EMBO J.* **13**, 4886-4895.
- Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis* plants. *CR Acad Sci Paris/Life Sci* **316**, 1194-1199.
- Bobik, K., Boutry, M. and Duby, G. (2010) Activation of the plasma membrane H<sup>+</sup>-ATPase by acid stress. *Plant Signal Behav* **5**, 681-683.
- Boyle, E.I., Weng, S., Gollub, J., Jin, H., Botstein, D., Cherry, J.M. and Sherlock, G. (2004) GO::TermFinder—open source software for accessing gene ontology information and finding significantly enriched gene ontology terms associated with a list of genes. *Bioinformatics* **20**, 3710–3715.

- Burkewitz, K., Choe, K. and Strange, K.** (2011) Hypertonic stress induces rapid and widespread damage in *C. elegans*. *Am. J. Physiol. Cell Physiol.* **301**, C566-576.
- Clough, S.J. and Bent, A.F.** (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *A. thaliana*. *Plant J.* **16**, 735-743.
- Drew, M.C.** (1997) Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 223-250.
- Davies, D.D.** (1986) The fine control of cytosolic pH. *Physiol. Plant.* **67**, 702-716
- Duina, A.A., Kalton, H.M., Gaber, R.F.** (1998) Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. *J. Biol. Chem.* **273**, 18974-18978.
- Eraso, P. and Gancedo, C.** (1987) Activation of yeast plasma membrane ATPase by acid pH during growth. *FEBS Lett.* **224**, 187-192.
- Felle, H.H.** (1981) A study of the current-voltage relationships of electrogenic and passive membrane elements in *Riccia fluitans*. *Biochim. Biophys. Acta* **602**, 180-195.
- Felle, H.H.** (1987) Proton transport and pH control in *Sinapis alba* root hairs: a study carried out with double-barreled pH micro-electrodes. *J. Exp. Bot.* **38**, 340-354.
- Felle, H.H.** (2001) pH: signal and messenger in plant cells. *Plant. Biol.* **3**, 577-591.
- Fernández, J.A., García-Sánchez, M.J. and Felle, H.** (1999) Physiological evidence for a proton pump and sodium exclusion mechanisms at the plasma membrane of the marine angiosperm *Zostera marina* L. *J. Exp. Bot.* **50**, 1763-1768.
- Fisher, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T., Schmid, F.X.** (1989) Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* **337**, 476-478
- Geisler, M., Girin, M., Brandt, S., Vincenzetti, V., Plaza, S., Paris, N., Kobae, Y., Maeshima, M., Billion, K., Kolukisaoglu, U.H., Schulz, B. and Martinoia, E.** (2004) Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. *Mol. Biol. Cell* **15**: 3393-3405.
- Geisler, M. and Bailly, A.** (2007) Tête-à-tête: the function of FKBP in plant development. *Trends Plant Sci.* **12**, 465-473.
- Gierth, M. and Mäser, P.** (2007) Potassium transporters in plants-Involvement in K<sup>+</sup> acquisition, redistribution and homeostasis. *FEBS Lett.* **581**, 2348-2356.
- Gillies, R.J.** (1982) Intracellular pH and proliferation in yeast, Tetrahymena and sea urchin eggs. In *Intracellular pH: its measurement, regulation, and utilization in cellular functions* (Nuccitelli, R. and Deamer, D.W., eds), New York, NY: Alan R Liss, pp 341-359.
- Goossens, A., de la Fuente, N., Forment, J., Serrano, R. and Portillo, F.** (2000) Regulation of yeast H<sup>+</sup>-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. *Mol. Cell. Biol.* **20**, 7654-7661.
- Gottlieb, R.A., Nordberg, J., Skowronski, E. and Babor, B.M.** (1996) Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. *Proc Natl Acad Sci USA* **93**, 654-658.
- Harding, M.W., Hanschumacher, R.E. and Speicher, D.W.** (1986) Isolation and amino acid sequence of cyclophilin. *J. Biol. Chem.* **261**, 8547-8555.
- Harding, M.W., Galat, A., Uehling, D.E. and Schreiber, S.L.** (1989) A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature* **341**, 758-760.

- Haruta, M., Burch, H.L., Nelson, R.B., Barret-Wilt, G., Kline, K.G., Mohsin, S.B., Young, J.C., Otegui, M.S. and Sussman, M.R.** (2010) Molecular characterization of mutant Arabidopsis plants with reduced plasma membrane proton pump activity. *J. Biol. Chem.* **285**, 17918-17929.
- He, Z., Li, L. and Luan, S.** (2004) Immunophilins and parvulins. Superfamily of peptidyl prolyl isomerases in Arabidopsis. *Plant Physiol.* **134**, 1248-1267.
- Hochachka, P.W. and Somero, G.N.** (2002) *Biochemical Adaptation. Mechanism and Process in Physiological Evolution.* Oxford: Oxford University Press, pp 345-351.
- Holyoak, C.D., Stratford, M., McMullin, Z., Cole, M.B., Crimmins, K., Brown, A.J.P. and Coote, P.J.** (1996) Activity of the plasma membrane H<sup>+</sup>-ATPase and optimal glycolytic flux are required for rapid adaptation and growth of *Saccharomyces cerevisiae* in the presence of the weak-acid preservative sorbic acid. *App. Environ. Microbiol.* **62**, 3158-3164.
- Hueso, G., Aparicio-Sanchis, R., Montesinos, C., Lorenz, S., Murguía, J.R. and Serrano, R.** (2011) A novel role of protein kinase Gcn2 in yeast tolerance to intracellular acid stress. *Biochem. J.* in press, doi:10.1042/BJ20111264
- Iuchi, I., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Kobayashi, Y., Ikka, T., Hirayama, T., Shinozaki, K. and Kobayashi, M.** (2007) Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. *Proc. Natl. Acad. Sci. USA* **104**, 9900-9905.
- Kawahata, M., Masaki, K., Fujii, T. and Iefuji H** (2006) Yeast genes involved in response to lactic acid and acetic acid: acidic conditions caused by the organic acids in *Saccharomyces cerevisiae* cultures induce expression of intracellular metal metabolism genes regulated by Aft1p. *FEMS Yeast Res.* **6**, 924-936.
- Kay, R., Chan, A., Daly, M. and McPherson, J.** (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plan genes. *Science* **236**, 1299-1302.
- Kim, J.I., Sharkhuu, A., Jing, J.B., Li, P., Jeong, J.C., Baek, D., Lee, S.Y., Blakeslee, J.J., Murphy A.S., Bohnert, H.J., Hasegawa, P.M., Yun, D.-J. and Bressan, R. A.** (2007) *yucca6*, a dominant mutation in Arabidopsis, affects auxin accumulation and auxin-related phenotypes. *Plant Physiol.* **145**: 722-735.
- Krebs, H.A., Wiggins, D., Stubbbs, M., Sols, A. and Bedoya, F.** (1983) Studies on the mechanism of the antifungal action of benzoate. *Biochem. J.* **214**, 657-663.
- Krulwich, T.A., Sachs, G. and Padan, E.** (2011) Molecular aspects of bacterial pH sensing and homeostasis. *Nature Rev. Microbiol.* **9**, 330-343.
- Lager, I., Andreasson, O., Dunbar, T.L., Andreasson, E., Escobar, M.A. and Rasmusson, A.G.** (2010) Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. *Plant Cell Environ.* **33**, 1513-1528.
- Lecchi, S., Nelson, C.J., Allen, K.E., Swaney, D.L., Thompson, K.L., Coon, J.J., Sussman, M.R. and Slayman, C.W.** (2007) Tandem phosphorylation of Ser-911 and Thr-912 at the C terminus of yeast plasma membrane H<sup>+</sup>-ATPase leads to glucose-dependent activation. *J. Biol. Chem.* **282**, 35471-35481.
- Lee, Y., Park, J., Im, K., Kim, K., Lee, J., Lee, K., Park, J.-A., Lee, T.-K., Park, D.-S., Yang, J.-S., Kim, D. and Lee, S.** (2006) Arabidopsis leaf necrosis caused by simulated acid rain is related to the salicylic acid signaling pathway. *Plant Physiol. Biochem.* **44**, 38-42.

- Lee, T.H., Chen, C.H., Suizu, F., Huang, P., Schene-Fischer, C., Daum, S., Zhang, Y.J., Goate, A., Chen, R.H., Zhou, X.Z. and Lu, K.P. (2011) Death-associated protein kinase 1 phosphorylates Pin1 and inhibits its prolyl isomerase activity and cellular function. *Mol. Cell* **42**, 147-159.
- Licausi, F., van Dongen, J.T., Giuntoli, B., Novi, G., Santaniella, A., Geigenberger, P. and Perata, P. (2010) *HRE1* and *HRE2*, two hypoxia-inducible ethylene response factors, affect anaerobic responses in *Arabidopsis thaliana*. *Plant J.* **62**, 302-315.
- Licausi, F., Kosmacz, M., Weits, D.A., Giuntoli, B., Giorgi, F.M., Voesenek, L.A.J., Perata, P. and van Dongen, J.T. (2011) Oxygen sensing in plants is mediated by an N-end rule pathway for protein stabilization. *Nature* **479**, 419-422.
- Liu, F., VanToai, T., Moy, L.P., Bock, G., Linfood, L.D. and Quackenbush, J. (2005) Global transcription profiling reveals comprehensive insights into hypoxic response in *Arabidopsis*. *Plant Physiol.* **137**, 1115-1129.
- Luban, J. (1996) Absconding with the chaperone: essential cyclophilin-Gag interaction in HIV-1 virions. *Cell* **87**, 1157-1159.
- Ludovico, P., Sousa, M.J., Silva, M.T., Leao, C. and Corte-Real, M. (2001) *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. *Microbiology* **147**, 2409-2415.
- Mcpherson, N., Shabala, L., Rooney, H., Jarman, M.G. and Davies, J.M. (2005) Plasma membrane H<sup>+</sup> and K<sup>+</sup> transporters are involved in the weak-acid preservative response of disparate food spoilage yeast. *Microbiology* **151**, 1995-2003.
- Meiri, D. and Breiman, A. (2009) *Arabidopsis* ROF1 (FKBP62) modulates thermotolerance by interacting with HSP90.1 and affecting the accumulation of HsfA2-regulated sHSPs. *Plant J.* **59**, 387-399.
- Meiri, D., Tazat, K., Cohen-Peer, R., Farchi-Pisanty, O., Aviezer-Hagai, K., Avni, A. and Breiman A. (2010) Involvement of *Arabidopsis* ROF2 (FKBP65) in thermotolerance. *Plant Mol. Biol.* **72**, 191-203
- Mira, N.P., Palma, M., Guerreiro, J.F. and Sá-Correia, I. (2010a) Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. *Microbial Cell Factories* **9**, 79-85.
- Mira, N.P., Becker, J.D. and Sá-Correia, I. (2010b) Genome expression program involving the Haalp-regulon in *Saccharomyces cerevisiae* response to acetic acid. *Omic* **14**, 587-601.
- Mollapour, M., Fong, D., Balakrishnan, K., Harris, N., Thompson, S., Schüller, C., Kuchler, K. and Piper, P.W. (2004) Screening the yeast deletion mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative. *Yeast* **21**, 927-946.
- Moskvina, E., Imre, E.-M. and Ruis, H. (1999) Stress factors acting at the level of the plasma membrane induce transcription via the stress response element (STRE) of the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **32**, 1263-1272.
- Mulet, J.M., Leube, M.P., Kron, S.J., Ríos, G., Fink, G.R. and Serrano, R. (1999) A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. *Mol. Cell. Biol.* **19** 3328-3337.
- Niittyla, T., Fuglsang, A.T., Palmgren, M.G., Frommer, W.B. and Schulze, W.X. (2007) Temporal analysis of sucrose-induced phosphorylation

- changes in plasma membrane proteins of Arabidopsis. *Mol. Cell. Proteomics* **6**, 1711-1726.
- Nishizawa, A., Yabuta, Y., Yoshida, E., Maruta, T., Yoshimura, K. and Shigeoka, S.** (2006) Arabidopsis heat shock transcription factor A2 as a key regulator in response to several types of environmental stress. *Plant J.* **48**, 535-547.
- Nover, L.** (1994) The heat stress response as part of the plant stress network. In *Biochemical and Cellular Mechanisms of Stress Tolerance in Plants* (Cherry, J.H., ed.), Springer-Verlag, Berlin, pp. 3-45.
- Pérez-Pérez, J.M., Ponce, M.R. and Micol, J.L.** (2004) The ULTRACURVATA2 gene of Arabidopsis encodes an FK506-binding protein involved in auxin and brassinosteroid signaling. *Plant Physiol.* **134**, 101-117.
- Perona, R. and Serrano, R.** (1988) Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. *Nature* **334**, 438-440.
- Piper, P.W., Mahé, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R., Mülbauer, M., Coote, P.J. and Kuchler, K.** (1998) The Pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. *EMBO J.* **17**, 4257-4265.
- Portillo, F. and Serrano, R.** (1989) Growth control strength and active site of yeast plasma membrane ATPase studied by site-directed mutagenesis. *Eur J Biochem* **186**, 501-507.
- Prieto-Dapena, P., Castaño, R., Almoguera, C. and Jordano, J.** (2006) Improved resistance to controlled deterioration in transgenic seeds. *Plant Physiol.* **142**, 1102-1112.
- Prodromou, C., Siligardi, G., O'Brien, R., Woolfson, D.N., Regan, L., Panaretou, B., Ladbury, J.E., Piper, P.W. and Pearl, L.H.** (1999) Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones. *EMBO J.* **18**, 754-762.
- Raven, J.A. and Smith, F.A.** (1976) The evolution of chemiosmotic energy coupling. *J. Theor. Biol.* **57**, 301-312.
- Riggs, D.L., Cox, M.B., Tardif, H.L., Hessling, M., Buchner, J. and Smith, D.F.** (2007) Noncatalytic role of the FKBP52 peptidyl-prolyl isomerase domain in the regulation of steroid hormone signaling. *Mol. Cell. Biol.* **27**, 8658-8669.
- Sanchez, Y., Taulien, J., Borkovich, K.A. and Lindquist, S.** (1992) Hsp104 is required for tolerance to many forms of stress. *EMBO J.* **11**, 2357-2364.
- Schüller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C. and Ruis, H.** (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J.* **13**, 4382-4389.
- Schüller, C., Mamnun, Y.M., Mollapour, M., Krapf, G., Schuster, M., Bauer, B.E., Piper, P.W. and Kuchler, K.** (2004) Global phenotypic analysis and transcriptional profiling defines the weak acid stress response regulon in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **15**, 706-720
- Serrano, R.** (1985) *Plasma Membrane ATPase of Plants and Fungi*, Boca Raton, FL: CRC Press, Chapter 3, pp 48-52.
- Serrano, R.** (1988) H<sup>+</sup>-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. *Meth Enzymol* **157**, 533-544.
- Song, F., Zhang, X., Ren, X.B., Zhu, P., Xu, J., Wang, L., Li, Y.F., Zhong, N., Ru, Q., Zhang, D.W., Jiang, J.L., Xia, B. and Chen, Z.N.** (2011) Cyclophilin A

- (CyPA) induces chemotaxis independent of its peptidylprolyl cis-trans isomerase activity: direct binding between CyPA and the ectodomain CD147. *J. Biol. Chem.* **286**, 8197-8203.
- Sorger, P.K.** (1991) Heat shock factor and the heat shock response. *Cell* **65**, 363-366
- Sugio, A., Dreos, R., Aparicio, F. and Maule, A.J.** (2009) The cytosolic protein response as a subcomponent of the wider heat shock response in *Arabidopsis*. *Plant Cell* **21**, 642-654.
- Takhashi, N., Hayano, T. and Suzuki, M.** (1989) Peptidyl-prolyl *cis-trans* isomerase is the cyclosporin A-binding protein cyclophilin. *Nature* **337**, 473-475.
- Tena, G. and Renaudin, J.-P.** (1998) Cytosolic acidification but not auxin at physiological concentration is an activator of MAP kinases in tobacco. *Plant J.* **16**, 173-182.
- Trotter, E.W., Kao, C.M.-F., Berenfeld, L., Botstein, D., Petsko, G.A. and Gray, J.V.** (2002) Misfolded proteins are competent to mediate a subset of the responses to heat shock in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277**, 44817-44825
- Tusher, V.G., Tibshirani, R. and Chu, G.** (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**, 5116–5121.
- Vallejo, C.G. and Serrano, R.** (1989) Physiology of mutant with reduced expression of plasma membrane H<sup>+</sup>-ATPase. *Yeast* **5**, 307-319.
- Vandal, O.H., Nathan, C.F. and Ehrt, S.** (2009) Acid resistance in *Mycobacterium tuberculosis*. *J. Bacteriol.* **191**, 4714-4721.
- Wang, Y., Liu, C., Yang, D., Yu, H. and Liou, Y.-C.** (2010) *Pin1At* encoding a peptidyl-prolyl *cis/trans* isomerase regulates flowering time in *Arabidopsis*. *Mol. Cell* **37**, 112-122
- Weitzel, G., Pilatus, U. and Rensing, L.** (1987) The cytoplasmic pH, ATP content and total protein synthesis rate during heat-shock protein inducing treatments in yeast. *Exp. Cell Res.* **170**, 64-79.
- Witte, C.-P., Noël, L.D., Gielbert, J., Parker, J.E. and Romeis, T.** (2004) Rapid one-step protein purification from plant material using eight-amino acid StrepII epitope. *Plant Mol. Biol.* **55**, 135-147.
- Xu, J., Li, H.-D., Chen, L.-Q., Wang, Y., Liu, L.-L., He, L. and Wu, W.-H.** (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K<sup>+</sup> transporter AKT1 in *Arabidopsis*. *Cell* **125**, 1347-1360.
- Yenush, L., Mulet, J.M., Ariño, J. and Serrano, R.** (2002) The Ppz protein phosphatases are key regulators of K<sup>+</sup> and pH homeostasis: implications for salt tolerance, cell wall integrity and cell cycle progression. *EMBO J.* **21**, 920-929.
- Yenush, L., Merchan, S., Holmes, J. and Serrano, R.** (2005) pH-responsive, posttranslational regulation of the Trk1 potassium transporter by the type1-related Ppz1 phosphatase. *Mol. Cell. Biol.* **25**, 8683-8692.
- Young, J.C., DeWitt, N.D. and Sussman, M.R.** (1998) A transgene encoding a plasma membrane H<sup>+</sup>-ATPase that confers acid resistance in *Arabidopsis thaliana* seedlings. *Genetics* **149**, 501-507.

**Table 1.** Heat-shock response genes induced 2-fold or more by intracellular acid stress (Acid; this work) and by stress caused by misfolded cytosolic proteins (MCP) in *Arabidopsis* (Sugio *et al.*, 2009). A dash indicates the gene is not induced. Genes in bold are induced by both stresses.

Protein subfamily	Acid	MCP
HSP100	<b>HSP101</b>	<b>HSP101</b>
HSP90	<b>HSP90.1</b>	<b>HSP90.1</b>
HSP70/K	-	<i>HSP70</i>
	-	<i>HSP70-2</i>
	-	<i>HSP70T-2</i>
	<i>FES1A</i>	-
HSP40/J	-	<i>At1g71000</i>
	-	<i>At2g20560</i>
	-	<i>At4g28480</i>
small HSPs	<b>HSP23.5-M</b>	<b>HSP23.5-M</b>
	-	<i>HSP23.6-M</i>
	-	<i>HSP17.6A</i>
	-	<i>HSP17.6B</i>
	<b>HSP17.6C</b>	<b>HSP17.6C</b>
	-	<i>HSP17.4A</i>
	-	<i>HSP17.4B</i>
<b>HSP15.7-CI</b>	<b>HSP15.7-CI</b>	
Prolyl isomerases	<b>ROF2</b>	<b>ROF2</b>
	-	<i>At1g01940</i>
HSF	<b>HSFA2</b>	<b>HSFA2</b>
	<i>HSFA5</i>	-
	-	<i>HSFB2A</i>
	-	<i>HSFB2B</i>
	<b>HSFB1</b>	<b>HSFB1</b>

**Table 2.** Germination of seeds of different genotypes after treatment in water during 23 h at 42 °C. Non-treated seeds of all genotypes had 99-100 % germination (seedlings with green cotyledons) after 4 days in MS medium. Data as mean  $\pm$  standard error (4 determinations). *OERFO2* corresponds to line 17 over-expressing *ROF2*.

Genotypes	% germination
Wild type	33 $\pm$ 5
<i>rof1</i>	27 $\pm$ 3
<i>rof2</i>	38 $\pm$ 2
<i>rof1 rof2</i>	7 $\pm$ 1
<i>OEROF2</i>	32 $\pm$ 4



## LEGENDS TO THE FIGURES

**Figure 1.** Arabidopsis ROF1 and ROF2 prolyl isomerases modulate germination in media with weak organic acids.

(a) Representative seedlings of Arabidopsis wild type, *rof1* mutant, *rof2* mutant, double mutant *rof1rof2*, and line 17 over-expressing *ROF2* (OE *ROF2*) 4 days after plating in normal pH 5.5 MS medium and in this medium supplemented with 3.5 mM acetic acid or 3.5 mM propionic acid as indicated. (b) Statistical data from 4 experiments like the one in part A. The mean per cent of seedlings with green, expanded cotyledons and the standard error are given. The size of the seedlings is also a measure of acid tolerance. (c) Weight of plants after germination in medium without acetic acid for 7 days and then growing in liquid medium for 12 days without (normal medium) or with 2 mM acetic acid as indicated. The percent inhibition by the acid is calculated.

**Figure 2.** Developmental phenotypes of *ROF* mutants.

(a) Branching phenotype. Bud number refers to the number of secondary inflorescence shoots from axils of rosette leaves and were counted after 45 days of growth under long-day illumination. Data are the mean of 30 plants and bars correspond to the standard error. Two *ROF2* over-expressing lines (numbers 7 and 17) were employed. The *yuc6-ID* mutants is presented as an example of high-auxin mutant with strong apical dominance (Kim *et al.*, 2007). (b) Bolting phenotype. Bolting time (days after germination) and number of rosette leaves at time of bolting for the different genotypes grown under long-day illumination.

**Figure 3.** Over-expression of *ROF2* increases proton extrusion from Arabidopsis roots.

(a) Arabidopsis plants over-expressing *ROF2* (line 17; OE *ROF2*) and control plants were incubated in solid MS medium at pH 5.5 without MES buffer and with 0.003% bromocresol purple. The yellow color of the pH indicator around OE *ROF2* roots but not around control roots indicates increased proton extrusion in transgenic plants. (b) Quantification of proton efflux from roots. Each point is the average of three repetitions and the standard errors (not shown for clarity) were 0.005-0.010  $\mu\text{moles H}^+$ .

**Figure 4.** Electrical membrane potential and cytosolic pH of *ROF2* mutants. (a) Over-expression of *ROF2* (line 10) depolarizes the plasma membrane and stabilizes cytosolic pH in the presence of acetic acid. Records of plasma membrane electrical potential ( $E_m$ ) and cytosolic pH (pH) of a root epidermal cell in a typical experiment. The addition (1 mM Ac) and removal (Wash) of acetic acid is indicated. (b): mean values ( $\pm$  standard error, number of experiments between brackets) of membrane potential and cytosolic pH of control, (wild type Arabidopsis), OE *ROF2* (line 10 over-expressing *ROF2*), *rof2* (single mutant) and *rof1 rof2* (double null mutant in *ROF1* and *ROF2*).

**Figure 5.** Gain and loss of function of *ROF2* modulate tolerance to toxic cations. (a) Representative seedlings of Arabidopsis wild type, double mutant *rof1rof2*, and line 17 over-expressing *ROF2* (OE *ROF2*) after plating in normal MS medium and in medium supplemented with 25 mM LiCl (5 days), 2.8 mM norspermidine (4 days) or 20  $\mu\text{g/ml}$  hygromycin B (14 days) as indicated. Other conditions as in figure 1. (b) Statistical data from 4 experiments like the one in part (a). The mean per cent of seedlings with green, expanded cotyledons and the standard error are given. The size of the seedlings is also a

measure of acid tolerance. Similar results were obtained with two additional transgenic lines (numbers 7 and 16, see supplementary figure S2).

**Figure 6.** Gain and loss of function of *ROF2* modulate germination in low  $K^+$  medium. (a) Representative seedlings of Arabidopsis wild type, double mutant *rof1rof2*, and line 17 over-expressing *ROF2* (OE*ROF2*) after plating in normal MS medium (control) and in medium with only  $10 \mu M K^+$ . Other conditions as in figure 1. (b) Statistical data from 4 experiments like the one in part (a).

**Figure 7.** Gain and loss of function of *ROF2* modulate rubidium ( $Rb^+$ ) uptake.  $Rb^+$  is utilized as an analog of  $K^+$ . The uptake of  $Rb^+$  at 15 and 30 min by Arabidopsis plants wild type, double mutant *rof1rof2*, and line 17 over-expressing *ROF2* (OE *ROF2*) is shown. The experiment was repeated twice with similar results.