The *Arabidopsis thaliana* Immunophilin ROF1 Directly Interacts with PI(3)P and PI(3,5)P_2 and Affects Germination under Osmotic Stress

Debora Karali [1], David Oxley [2], John Runions [3], Nicholas Ktistakis [4], Theodora Farmaki [1]*

1 Institute of Applied Biosciences, Centre for Research and Technology – Hellas, Thermi, Thessaloniki, Greece, 2 The Mass Spectrometry Group, Babraham Institute, Cambridge, United Kingdom, 3 School of Life Sciences, Oxford Brookes University, Oxford, United Kingdom, 4 Signalling Programme, Babraham Institute, Cambridge, United Kingdom

Abstract

A direct interaction of the Arabidopsis thaliana immunophilin ROF1 with phosphatidylinositol-3-phosphate and phosphatidylinositol-3,5-bisphosphate was identified using a phosphatidylinositol-phosphate affinity chromatography of cell suspension extracts, combined with a mass spectrometry (nano LC ESI-MS/MS) analysis. The first FK506 binding domain was shown sufficient to bind to both phosphatidylinositol-phosphate stereoisomers. GFP-tagged ROF1 under the control of a 35S promoter was localised in the cytoplasm and the cell periphery of Nicotiana tabacum leaf explants. Immunofluorescence microscopy of Arabidopsis thaliana root tips verified its cytoplasmic localization and membrane association and showed ROF1 localization in the elongation zone which was expanded to the meristematic zone in plants grown on high salt media. Endogenous ROF1 was shown to accumulate in response to high salt treatment in Arabidopsis thaliana young leaves as well as in seedlings germinated on high salt media (0.15 and 0.2 M NaCl) at both an mRNA and protein level. Plants over-expressing ROF1, (WSROF1OE), exhibited enhanced germination under salinity stress which was significantly reduced in the rof1^2 knock out mutants and abolished in the double mutants of ROF1 and of its interacting homologue ROF2 (WSrof1^2/2^2). Our results show that ROF1 plays an important role in the osmotic/salt stress responses of germinating Arabidopsis thaliana seedlings and suggest its involvement in salinity stress responses through a phosphatidylinositol-phosphate related protein quality control pathway.

Citation: Karali D, Oxley D, Runions J, Ktistakis N, Farmaki T (2012) The Arabidopsis thaliana Immunophilin ROF1 Directly Interacts with PI(3)P and PI(3,5)P_2 and Affects Germination under Osmotic Stress. PLoS ONE 7(11): e48241. doi:10.1371/journal.pone.0048241

Editor: Haibing Yang, Purdue University, United States of America

Received February 17, 2012; Accepted September 21, 2012; Published November 2, 2012

Copyright: 2012 Karali et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research project is co-financed by the E.U.-European Social Fund (75%) and the Greek Ministry of Development-GSRT (25%) (PENED) as well as a European Molecular Biology Organization (EMBO) short term fellowship ASTF 395.00-2007 and the Biotechnology and Biological Sciences Research Council (BBSRC). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mfarmaki@certh.gr

Introduction

Phosphatidylinositol phosphates (PIPs) are phospholipids with important regulatory properties in signaling and trafficking processes [1]. PIPs possess a characteristic subcellular localiza-timses ucselor52inllular thelonth7egucognion

propeler structure may be utilized for binding [20]. Because of

Bacterially Expressed ROF1 Interacts with PI(3)P and PI(3,5)P_2 through its FKBD Domains

ROF1 and its truncated mutants (Figure 2B; Table S1; Table S2) were over-expressed with an N-terminal GST tag and C terminal-His tag following transformation of a BL21 bacterial cell line with the pALEX vector used for their cloning. Following double purification using Glutathione and Nickel columns ROF1 and its truncated mutants were eluted and used in a lipid overlay assay (Figure 3). ROF1 specifically associated with both the PI(3)P as well as with the PI(3,5)P_2 (Figure 3A). The FKBD1 domain was shown sufficient for PIP binding and appeared to possess a higher affinity for the PI(3,5)P_2 stereoisomer and reduced affinity for the PI(3)P (Figure 3A, B). A competition assay, using the two stereoisomers as competing lipids, produced a specific reduction in the case of the PI(3,5)P_2, however, an addition of PI(3)P as a competing lipid reduced signal for both the PI(3)P and the PI(3,5)P_2 in the ROF1 and the FKBD1 assay (Figure 3A, B). Although the FKBD1 was shown to possess a high affinity for the PI(3,5)P_2 its absence did not abolish binding to the PI(3,5)P_2 stereoisomer as demonstrated by the ability of the 2FKTPR construct to interact with both lipids (Figure 3C). On the other hand, FK3TPR, only interacted with the PI(3)P (Figure 3C). Although the TPR-calmodulin binding domain alone was also over-expressed, we were unable to solubilise it and the entire amount of protein remained inside the inclusion bodies. The results suggest a differential capacity of the FKBD domains for lipid binding as well as the ability of ROF1 to bind to PI(3,5)P_2 in the absence of the FKBD1 inspite the fact that FKBD2 and FKBD3 do not possess a structural identity to β spectrin and predictable PIP binding sites, at least not to the same extent as the FKBD1 does (Figure S2).

Use of the Αντι-ROF1 for the Characterization of the PI(3,5)P_2 Binding Site

A polyclonal antibody was raised against the bacterially expressed ROF1 as described in the Materials and Methods. Taking into account that αντι-ROF1 was raised against the denatured protein, ROF1 truncated mutants (Figure 2B) expressed in BL21 cells were purified and run on a polyacrylamide gel. Western blotting using

Figure 3. Lipid overlay assays in order to characterize ROF1 binding to different PIP stereoisomers. Dot blotting of different PIP stereoisomers probed with 1 μg/ml of the ROF1 protein (A) and its truncated mutant 1FK (FKBD1) (B) (Figure 2B; Figure S1) and detected with α-GST. (−): No competing lipid has been added in the incubation, [PI(3,5)P$_2$]: 50 μM of the lipid has been added during the incubation; [PI(3)P]: 50 μM of the lipid has been added during the incubation. (C) Dot blotting of different PIP stereoisomers probed with 2 μg/ml of the ROF1 truncated mutants 2FKTPR and FK3TPR (Figure 2B; Figure S1) and detected with α-GST. (D) Characterization of the binding site of ROF1 to PI(3)P and PI(3,5)P$_2$ using the N-terminus specific antibody anti-ROF1. Overexpressed ROF1 was pre-incubated with different concentrations of the antibody, as indicated, prior to its use in a lipid overlay assay and detected with anti-GST.
doi:10.1371/journal.pone.0048241.g003

showed a complete recognizing ability for the ROF1 providing that the polylysine rich patch (KKKLLK) was present (NROF1 construct) (Figure 2B; Figure S1; Figure S3C). Taking into account the specific site of antibody recognition, we used the antibody in order to block ROF1 prior to the incubation in the lipid overlay assay. Different concentrations of the ROF1 antibody were used in a mixture with the protein (0 to 2 μl/ml) and following incubation in a lipid overlay assay as previously described, α-GST was used for the detection of protein-lipid interaction (Figure 3D). The antibody pre-incubation reduced binding affinity for the 3,5 phosphorylated lipid but not for the lipid phosphorylated at the third position of the inositol ring (Figure 3D).

Antibody specificity was further verified using both *A. thaliana* wild type and mutant lines (WDsLOX, WS *rof1*$^{-}$, WS *rof1*$^{-}$/2^{-}) stressed at 37°C for up to 4 hrs. Results showed (Figure 4; Figure S4) that the anti-ROF1 specifically recognizes endogenous ROF1 and to a very low extent its homologous partner ROF2 providing the latter has been accumulated following heat stress [24] (Figure 4).

ROF1 is Induced Under Salinity Stress

3 week old *A. thaliana* plants were incubated with 0, 0.2 or 1 M NaCl. In young leaves collected at different time points, ROF1 and ROF2 accumulation was observed at an mRNA level following salt treatment (Figure 5A). A fast induction within 3 mins appeared following incubation with 1 M NaCl for both ROF homologues. mRNA accumulation was also observed in the case of ROF1 in seedlings germinated on high salt/osmotic media compared to the control (MS media). However, this accumulation was not observed for ROF2 (Figure 5B). ROF1 accumulation was also observed at a protein level (Figure 5C). No apparent differences were, however, observed in ROF1 protein accumulation at high salt concentrations (1 M) compared to lower salt concentrations (0.2 M) but an earlier reduction was observed for the 1 hr 1 M NaCl treatment compared to the 0.2 M incubation. Protein accumulation was also observed in the case of whole seedlings, germinated in high salt MS media (0.15 and 0.2 M NaCl) compared to the control seedlings (Figure 5D). The presence of ROF1 was also confirmed in non-germinated seeds only 3 hrs after water imbibition (Figure 5E).

Over-expressed and Endogenous ROF1 Localization Under Control and Osmotic/salinity Stress Conditions

Using an *A. tumefaciens* plant expression vector we transiently transformed tobacco leaves with constructs containing a full length ROF1. A cytoplasmic localization of the protein was observed using a 3D reconstruction (Figure 6A) as demonstrated by the traces (empty spaces) that the embedded organelles mark in the cytoplasm and the cytoplasmic strands formed. A peripheral localization may also be observed, more clearly seen in a single confocal section (Figure 6B). ROF1 localization was distinct to GFP localization since no nuclear localization of the GFP tagged-ROF1 was observed (Figure 6C). In order to confirm the nature of the peripheral localization of ROF1, a plasmolysis effect caused by 0.25 M NaCl treatment was used. As it is shown (movie S1), the peripheral staining of ROF1 is not due to a protein association with the cell wall.

In order to further verify ROF1 subcellular localization immunofluorescence microscopy with *A. thaliana* root tips was used. *A. thaliana* seedlings were germinated on 0.8% agar media containing 1× MS and immunofluorescence microscopy was performed as described in the Materials and Methods. Incubation

Figure 4. Recognition of ROF1 and ROF2 using anti-ROF1. 5-day old *A. thaliana* wild type, ROF1 knock-out ($rof1^{2}$) of both Columbia and Wassilevskija background and ROF1/2 double mutants (WS $rof1^{2}/2^{2}$) were incubated at 37C for up to 4 hrs. anti-ROF1 specifically detects the ROF1 protein in plant extracts and to a less extent a second upper band (ROF2) following heat treatment only. Columbia ROF1 knock-out belongs to the WDsLox line.
doi:10.1371/journal.pone.0048241.g004

with pre-immune serum or αντι-ROF1 staining of $rof1^{2}$ plants did not produce any fluorescence (results not shown) confirming antibody specificity as described above. In untreated plants and plants treated for a short period of time with high NaCl (0.4 M for 2 mins) ROF1 was mainly localized in the elongation zone of the root tip (seedlings were immersed in the solution washed briefly and then fixed) (Figure 7A, B; Movie S2). However, in plants grown on high salt media (0.2 M NaCl), the localization pattern changed, expanding throughout the meristematic zone (Figure 7C; Movie S3). The cytoplasmic localization of ROF1 was confirmed with immunofluorescence microscopy (Figure 7A; Movie S2) whereas a stronger peripheral staining was observed in plants grown on high salt media (Figure 7C; Movie S3) probably indicating membrane association. In any case, no nuclear localization was observed.

Figure 5. Gene expression and protein accumulation study for ROF1. (A) Real time PCR of wild type *A. thaliana* plants treated for different time points and under different salt concentrations (0 M, 0.2M and 1M NaCl). Results are expressed as fold increase relative to the control set at 1. Control represents mock treatment with H_2O for the respective time points to the NaCl treatment. (B) Real time PCR of control (C) wild type 4-day old *A. thaliana* seedlings germinated on MS media and of seedlings germinated and grown on media containing NaCl or mannitol. Results are expressed as fold increase relative to the control set at 1. Control represents plants grown on MS media. Results are average of three independent experiments performed in triplicates. (C) Western blotting of wild type *A. thaliana* young leaves obtained from plants which have been treated for different time points and at different salt concentrations (0 M, 0.2M and 1M NaCl). Control represents mock treatment with H_2O for the respective time points to the NaCl treatment. (D) Western blotting of control (C) wild type 4 day old *A. thaliana* seedlings germinated on MS media and seedlings germinated and grown on MS media containing different NaCl concentrations. (E) Western blotting of WS and WSROF1OE seeds 3 hrs after water imbibition.
doi:10.1371/journal.pone.0048241.g005

Figure 6. ROF1 localisation using tobacco plants transiently transformed and expressing GFP tagged ROF1 and GFP only. (A) Control

Effect of Osmotic/ionic Stress on the Germination of Wild Type and ROF Mutant *A. thaliana* Seeds

Several lines of Columbia wild type (Col-WT), Columbia *rof1*^{2} (Col-*rof1*^{2}), Wassilevskija wild type (WS), WS *rof2*^{2} and WS *rof1*^{2}/*rof2*^{2}, WS complemented mutants (WSROF1CM) and WS ROF1 over-expressions (WSROF1OE) were confirmed by western blotting using the αντι-ROF1 for ROF1 detection (Figure 4; Figure S4). Columbia WT as well as *rof1*^{2} seeds on a Columbia background were germinated on media containing different NaCl concentrations. Although no difference was recorded on the germination rate of plants grown on media without NaCl in relation to the WT (Figure S5A, D) a significant delay was

recorded on the germination of *rof1*^{2} plants germinated on media containing salt concentrations of 0.2 M NaCl (Figure S5B). The NaCl effect on plant germination was also observed with the Wassilevskija wild type and *rof1*^{2} (Figure 8A). Germination was nearly eliminated at 0.2 M NaCl when the WS*rof1*^{2}/2^{2} double mutants were used (Figure 8A, C). In order to show that the osmotic effect of ROF1 is exclusively due to the loss of the ROF1 gene complemented WS*rof1*^{2} as well as WSROF1OE lines were used. It was shown that gene complementation completely recovered the wild type phenotype whereas gene over-expression resulted in a highly enhanced germination under salinity stress (Figure 8A). In order to differentiate between the ionic and the osmotic effect, WS, WSROF1OE, WS*rof1*^{2}, WSROF1CM, WS*rof2*^{2} and WS*rof1*^{2}/2^{2} were geminated on media containing 0.4 M mannitol and the osmotic effect on plant germination was confirmed (Figure 8B, C; Figure S5C).

ROF1 Affects *A. thaliana* Seed Germination through a Phosphatidyl-Inositol-3 Kinase (PI3K) Related Pathway

A. thaliana WSROF1OE and WS*rof1*^{2}/2^{2} seeds were germinated in the presence or absence of wortmannin or LY294002 under both control and salinity stress conditions. Germination

Figure 8. Germination efficiency of ROF1 and ROF2 mutants under osmotic/salinity stress. (A) Germination rate of the Wassilevskija background plants WS, WS rof1^{2}, WS rof2^{2}, WS rof1^{2}/2^{2}, WSROF1CM and WSROF1OE on MS media containing 0.2 M NaCl. (B) Germination rate of the wild type Wassilevskija background plants WS, WS rof1^{2}, WS rof2^{2}, WS rof1^{2}/2^{2}, and WSROF1OE on MS media containing 0.4 M mannitol. Three independent characterized lines (in each experiment) were used for each genotype. The results show the average of three independent experiments

Figure 9. Effect of PI3K inhibitors on seed germination. Seed germination efficiency of WSROF1OE and rof1 WS ^{2}/2^{2} at different time points under control (A) or salinity stress (0.1 M NaCl) (B) in the presence or absence of 33 μM wortmannin or 60 μM LY294002. The results show the average of four independent experiments performed for each treatment. Values are means and bars are SDs.

doi:10.1371/journal.pone.0048241.g009

our work suggests its involvement in salt stress tolerance since its absence significantly reduces and further abrogates germination efficiency under salinity stress in the $rof1^{2}/2^{2}$ double mutant (Figure 8). Unlike ROF1, ROF2 mRNA and protein do not accumulate-at least not to a detectable by our antibody level-neither under control (also shown by previous studies) nor under salinity stress (as it occurs during heat stress) (Fig 5). ROF2 became detectable only following its enrichment specifically on the $PI(3,5)P_2$ column as demonstrated by our mass spectrometry data, pointing to its specific involvement in a PIP associated osmotic stress related mechanism. The involvement of such a mechanism in osmotic/salinity stress responses was supported by experiments with the PI3K inhibitors wortmannin and LY294002 (Figure 9).
Our results point to a PI3K related mechanism involved in seed germination under salinity stress through a ROF1 function. Seeds over-expressing ROF1 showed better germination efficiency in the presence of the inhibitors under normal conditions compared to the knock out plants. In contrast and under salt stress conditions and inhibitor addition, ROF1 overexpression did not enhance seed germination (as it happens in the absence of the inhibitor) and

Fluorescence and Confocal Microscopy

Localization studies of GFP-tagged ROF1 as well as immunofluorescence microscopy were performed using fluorescence and Confocal microscopy. Fluorescence microscopy was performed using an Axioskop 40 fluorescence microscope with a A-Plan Varel contrast 40x/0.65 (Zeiss, Germany). Images were captured using a ProgRes digital camera system from Jenoptic (Germany). For confocal microscopy two confocal microscopes were used; Live cell imaging was performed using a laser Scanning confocal Microscope TCS SP5 SN: 5100000134 with an HCX PL APO CS 63.0×1.20 water UV lens. A pinhole 1 and 1.6 zoom were used. Scan speed was 400 Hz. Resolution was 1024×1024 with 8 bits/pixel. Excitation was achieved with a 488 Argon laser for the GFP and a DPSS 561 for the RFP. Emission bandwidth for GFP was 504.0 nm–521.2 nm and the emission bandwidth for RFP 574.2 nm - 620.1 nm. Fixed tissue imaging was performed using an HC X PLAPO CS 40.0×0.85 dry lens. Pinhole was 2, zoom 1.7 and resolution 1024×1024. Excitation was achieved using a 405 nm Diode for DAPI and a 561 DPSS for Cy3 red fluorescence. Emission bandwidth for DAPI was 423 nm – 475.6 nm and the emission bandwidth for Cy3 567 nm – 619.4 nm. Image analysis was performed using the LAS AF software version 2.1.0 as well as the ImageJ (Java) software. Also, a Zeiss LSM 510 confocal microscope with a EC Plan-Neofluar 40x/1.30 oil DIC M27 objective was used. was 26
fluorescence4

1 ... 1 ... 0.2 ...
(AVI)

2 ... 1 ... 1 ...
(AVI)

3 ... 1 ... 1, 0.2 ...
(AVI)

... 1
(XLSX)

Acknowledgments

We would like to thank Prof. Adina Breiman for the Wassilevskija seeds, WS, WSrof1[2], WSrof2[2], WS ,W/2S

46. Farmaki T, Sanmartin M, Jimenez P, Paneque M, Sanz C, et al. (2007) Differential distribution of the lipoxygenase pathway enzymes within potato chloroplasts. J Exp Bot 58: 555–568.
47. Zou X, Osborn MJ, Bolland DJ, Smith JA, Corcos D, et al. (2007) Heavy chain only antibodies are spontaneously produced in light chain-deficient mice. J Exp Med 204: 3271–3283.
48. Panagiotidis CA, Silverstein SJ (1995) pALEX, a dual-tag prokaryotic expression vector for the purification of full-length proteins. Gene 164: 45–47.
49. Kargiotidou A, Deli D, Galanopoulou D, Tsaftaris A, Farmaki T (2008) Low temperature and light regulate delta 12 fatty acid desaturases (FAD2) at transcriptional level in cotton (Gossypium hirsutum Td [((potat2i1287(7Pal.(13rTJ -1o(Me9:t7io09(in5(5)406cFAD294.892 11.3976 Td [509.)-717(Kargiotido)-14(u)2692(A,)276

ipraseao(fom(s)-247(are)-249dDifferi)-13n(tiasly)-459(regun)-12(tMed)-249bys temper(ture)3253(ndo)]TJ 0 -1.716 TD [(ligh.f)-336(J)-337(Exp)-335(Bot)-335618: