Title: Salt Stress Induces Internalization of Plasma Membrane Aquaporin into the Vacuole in *Arabidopsis thaliana*.

Authors: Masamichi Ueda^a, Nobuhiro Tsutsumi^{a, b}, Masaru Fujimoto^{a, b, 1}

Author affiliations: ^aLaboratory of Plant Molecular Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

^bJapan Science and Technology Agency (JST), CREST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

¹**Corresponding author:** Masaru Fujimoto

Address: Laboratory of Plant Molecular Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Tel: +81-3-5841-5076

Fax: +81-3-5841-5183

E-mail address: amfuji@mail.ecc.u-tokyo.ac.jp

Abstract

Salt stress is a major environmental stress for plants, causing hyperosmotic, ionic and drought-like stresses. Plasma membrane intrinsic protein 2;1 (PIP2;1), which forms a water channel that regulates water flux thorough the plasma membrane (PM), is constitutively trafficked between the PM and the *trans*-Golgi network (TGN) in *Arabidopsis thaliana*. Salt stress is known to relocate PIP2;1 to intracellular compartments, probably to decrease the water permeability of the root. However, the destination of internalized PIP2;1 and the mechanism by which PIP2;1 is internalized remain unclear. Here, we examined the effects of salt stress and inhibitors of endocytosis on the intracellular localization of green fluorescent protein–fused PIP2;1 (GFP-PIP2;1) in *A. thaliana* root epidermal cells. Salt stress decreased the fluorescence of GFP-PIP2;1 at the PM and increased it in the vacuolar lumen as shown by staining of the vacuolar membrane. The internalization of PIP2;1 was suppressed by an inhibitor of clathrin-mediated endocytosis and by inhibitors of two kinases that appear to have roles in salt stress, phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol 4-kinase (PI4K). Inhibiting PI4K suppressed the salt-induced endocytosis of GFP-PIP2;1 at the PM, whereas inhibiting PI3K suppressed the trafficking of GFP-PIP2;1 after its internalization. These results suggest that salt stress induces the internalization of PIP2;1 from the PM to the vacuolar lumen, and that these processes are dependent on clathrin, PI3K and PI4K.

Keywords: Arabidopsis; Salt stress; Aquaporin; Endocytosis; Clathrin

Abbreviations: BFA, Brefeldin A; CIE, clathrin independent endocytosis; CLSM, confocal laser scanning microscope; CME, clathrin-mediated endocytosis; DMSO, Dimethyl Sulfoxide; FIR, fluorescent intensity ratio; GFP, green fluorescent protein; MS, Murashige and Skoog; PAO, phenylarsine oxide; PI, phosphoinositide; PIP, plasma membrane intrinsic protein; PI3K, phosphatidylinositol 3-kinase; PI4K, phosphatidylinositol 4-kinase; PM, plasma membrane; PtdIns, phosphatidylinositol; PVC, prevacuolar compartment; TGN, *trans*-Golgi network; TyrA23, tyrphostin A23

Introduction

Plants, being immobile, must adapt to a variable environment. Accumulation of salt in the soil reduces crop yield in many parts of the world [1,2]. Salt stress on plants has two forms, a rapid hyperosmotic stress and a slow ionic stress [3]. Hyperosmotic stress is caused by the presence of soluble salts outside the root and imposes a water deficit on plants [3,4]. Ionic stress, which is caused by the invasion of sodium ions (Na^+) and chloride ions (Cl^-) into plant cells, produces ion imbalances [4,5]. These effects can reduce growth, cause premature senescence, or, in severe cases, kill the plants [3,4].

Aquaporins are membrane proteins that allow water molecules and small neutral solutes to diffuse across the membrane [6,7]. Plants aquaporins consist of four subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins, nodulin-26-like intrinsic proteins, and small basic intrinsic proteins [8]. Salt stress downregulates the mRNA levels of PIPs and tonoplast intrinsic proteins, and these changes are simultaneously accompanied by a decrease in the water permeability of the root [9]. PIP2;1 is mainly expressed in the root of *Arabidopsis thaliana*. Salt stress is thought to block the transport of newly synthesized PIP2;1 from the endoplasmic reticulum to the PM and to redistribute PM-localized PIP2;1 to intracellular compartments such as spherical bodies and intracellular fuzzy compartments [9,10]. Salt stress also accelerates the recycling of PIP2;1 between the PM and the *trans*-Golgi network

(TGN) [11,12]. In the absence of salt stress, the recycling pathway of PIP₂;1 was found to involve clathrin-mediated endocytosis (CME), while in the presence of salt stress it was found to involve clathrin independent endocytosis (CIE) [11,13]. Lipid rafts, which are microdomains in the PM, may have a role in salt-induced CIE [13]. However, the destination of salt-induced internalized PIP₂;1, whether PIP₂;1 redistributes from the PM or not, and the requirements of salt stress-responsive internalization of PIP₂;1 are unclear [14].

Phosphoinositides (PIs) are membrane lipids that include phosphatidylinositol (PtdIns) and its phosphorylated derivatives, and function in a variety of cell activities, including membrane trafficking [15]. Two kinases that phosphorylate PtdIns, PtdIns 4-kinase (PI4K) and PtdIns 3-kinase (PI3K), seem to have roles under salt stress. Salt stress increases the abundance of the product of PI4K [16], as well as its association with clathrin-coated vesicles [17] and PI3K is required for salt stress-induced endocytosis [18]. These findings raise the question of whether PI4K and PI3K have roles in the trafficking of PIP₂;1 under salt stress.

To investigate the effect of salt stress on the trafficking of PIP₂;1, we analyzed the localization of PIP₂;1 in the roots of *A. thaliana* plants constitutively expressing green fluorescent protein (GFP)–fused PIP₂;1. To clarify the salt-induced redistribution of PIP₂;1, we analyzed the effects of tyrphostin A23 (TyrA23), which inhibits CME, LY294002, which inhibits PI3K, and phenylarsine oxide (PAO), which inhibits PI4K. Brefeldin A (BFA), an

inhibitor of endocytic and secretory pathways [19], was also used to clarify the roles of PI3K and PI4K on PIP2;1 trafficking. Our results show that PIP2;1 is internalized from the PM to the vacuolar lumen in response to salt stress. The inhibitor assays show that the internalization of PIP2;1 is regulated by CME, PI3K and PI4K. Although previous studies found that CIE was involved in the salt-induced internalization of PIP2;1, the present results show that CME is also involved. These results provide clues for identifying the factors regulating membrane trafficking of PIP2;1 under salt stress.

Materials and Methods

Plant materials and growth conditions

A. thaliana (ecotype Columbia-0) plants expressing GFP-PIP2;1 and GFP-LTI6B under the Cauliflower mosaic virus 35S promoter [20] were kindly provided by Dr. J. Takano (Osaka Prefecture University). *SYPI32pro::GFP-SYP132* in pENTR, which was kindly provided by Dr. M.H. Sato (Kyoto Prefectural University) [21], was subcloned into pHGW by using Gateway cloning technology (Invitrogen). The construct was transformed into *Agrobacterium tumefaciens* strain C58C1 and transgenic plants harboring *SYPI32pro::GFP-SYP132* were generated by floral dipping [22]. Plants were grown on 1 × Murashige and Skoog (MS) agar plates at 22 °C under continuous light.

Salt stress and pharmacological assays

Eight-day-old *A. thaliana* seedlings were transplanted to liquid MS medium containing ~0 mM NaCl (no added NaCl; control condition) or 100 mM added NaCl (salt stress condition). The transplanted seedlings were incubated in the dark at 22 °C for 0, 3 or 6 hours before microscopic observations. 2 µM FM4-64 (Invitrogen, 4 mM stock solution in Dimethyl Sulfoxide (DMSO)) was added to the control and salt stress media at 0 hour. For

pharmacological assays using each endocytic inhibitor, DMSO (0.1 % v/v, control), 50 μ M TyrA23 (Calbiochem, 50 mM stock in DMSO), 50 μ M LY294002 (Sigma-Aldrich, 50 mM stock in DMSO) or 50 μ M PAO (Sigma-Aldrich, 50 mM stock in DMSO) was added to the liquid MS media with 2 μ M FM4-64 at 0 hour. For pharmacological assays using BFA, 50 μ M BFA (Sigma-Aldrich, 50 mM stock in DMSO) was added to each medium 3 hours after the beginning of endocytic inhibitor treatments.

Fluorescence microscopy

A. thaliana roots were placed on a glass slide (76 $\times$ 26 mm, Matsunami) and covered with a 0.12–0.17 mm thick cover glass (22 $\times$ 40 mm, Matsunami). Root epidermal cells in the elongation zone were observed by using confocal laser scanning microscope (CLSM) (C1si, Nikon) with an oil immersion objective lens (numerical aperture = 100 $\times$ 1.40). GFP and FM4-64 was excited at 488-nm Ar/Kr laser. Emission signals of GFP and FM4-64 were detected through 515/30-nm filter and 590/70-nm filter, respectively. Acquired images were processed with Photoshop CS6 (Adobe Systems).

Quantitative analysis

Microscope images were analyzed with Fiji [23] and ImageJ [24] for calculating the averages of fluorescence intensity of the intracellular- and the PM- regions. More than 4 cells were analyzed per root.

Statistical analysis

The results were expressed as means $\pm$ standard divisions of biological replicates. Student's *t*-test and Tukey's honestly significant difference were employed to analyze statistical significance.

Results

Salt stress induces the internalization of PIP2;1 from the PM to the vacuolar lumen.

To investigate the effects of salt stress on the intracellular trafficking of PIP2;1, we compared the temporal changes of subcellular localizations of GFP-PIP2;1 in the root epidermal cells of *A. thaliana* seedlings that were transplanted into the control and salt stress media in the dark. Just after transplantation (0 h), almost all of the GFP-PIP2;1 was localized to the PM in both conditions (Fig 1A, left panel). At 6 h, most of GFP-PIP2;1 fluorescence remained localized to the PM in the control cells (Fig 1A), while in the salt stressed cells, GFP-PIP2;1 fluorescence decreased in the PM and accumulated in vacuole-shaped structures (Fig 1A, arrowheads). These results are shown graphically in Fig 1B (left panel), which shows the intracellular-to-PM GFP fluorescent intensity ratio (FIR) increasing more rapidly in the salt stressed cells than in the control. To identify the vacuole-shaped structures in which PIP2;1 accumulated under the salt stress, we used FM4-64, a fluorescent marker of vacuolar membranes [25]. FM4-64 staining showed that at 6 h, the fluorescence of GFP-PIP2;1 was mainly enclosed in vacuolar membranes (Fig 1C), showing that PIP2;1 is internalized from the PM to the vacuolar lumen in response to salt stress.

In contrast to PIP2;1, two other GFP-labeled PM proteins (SYP132 and LTI6B

[20,21]) largely remained localized to the PM under both control and salt stress conditions (Fig 1A and B, middle and right panels). These results strengthen the idea that salt stress specifically affects PIP2;1.

Salt-induced internalization of PIP2;1 is suppressed by inhibition of CME.

To examine whether clathrin-mediated endocytosis (CME) is involved in salt-induced internalization of PIP2;1, we analyzed the effects of TyrA23, an inhibitor of CME. TyrA23 (50 μ M) strongly suppressed salt-induced internalization of GFP-PIP2;1 from the PM to the vacuolar lumen in root epidermal cells, but had no detectable effect under the control condition (Fig 2A). The suppression of internalization is shown graphically in Fig 2B. These results indicate that salt-induced vacuolar internalization of PIP2;1 requires clathrin-mediated endocytosis.

Salt-induced internalization of PIP2;1 is suppressed by inhibitors of phosphatidylinositol kinases

To examine whether phosphoinositides (PIs) are involved in salt-induced internalization of PIP2;1, we analyzed the effects of LY294002, an inhibitor of phosphatidylinositol 3-kinase

(PI3K), and PAO, an inhibitor of phosphatidylinositol 4-kinase (PI4K). Both LY294002 and PAO significantly suppressed the salt-induced internalization of GFP-PIP2;1 from the PM to the vacuolar lumen in root epidermal cells (Fig 2A and B). These results indicate that salt-induced vacuolar internalization of PIP2;1 depends on the activities of phosphatidylinositol kinases, especially PI4K.

To investigate whether LY294002 and PAO affect distinct processes of the salt-induced internalization of PIP2;1, we examined the effects of Brefeldin A (BFA), an inhibitor of endocytic and secretory pathways. BFA inhibits the recycling of proteins from endosomes to the PM, which causes the post-Golgi organelles including Golgi apparatus, the TGN and the prevacuolar compartment (PVC) to form aggregated structures called BFA compartments [19]. In the presence of BFA, GFP-PIP2;1 fluorescence was detected at the PM, in the vacuolar lumen and in BFA compartments (Fig 3, upper panels; arrows, arrowheads, and asterisks, respectively). However, in the presence of both BFA and LY294002, fluorescence was detected at the PM and in the BFA compartments but not in the vacuolar lumen (Fig 3, middle panels). And in the presence of both BFA and PAO, the fluorescence of both GFP-PIP2;1 and FM4-64 was detected only at the PM (Fig 3, lower panels). These results suggest that, under salt stress, PI4K, which is inhibited by PAO, is important for the endocytosis of PIP2;1 at the PM, while PI3K, which is inhibited by LY294002,

is important for the trafficking of PIP₂;1 to the vacuolar lumen after endocytosis at the PM.

Discussion

PIP2;1 is internalized to the vacuolar lumen responding to the salt stress

Under non-salt stress condition, PIP2;1 localizes to the PM (Fig 4A). The present results demonstrate that PIP2;1 is internalized from the PM to the vacuolar lumen in response to salt stress (Fig 4B). Under salt stress, root hydraulic conductivity is reduced to prevent water loss from the cells [9]. The reduction of PM-localized PIP2;1 under salt stress may partially account for the decrease in root hydraulic conductivity since it may suppress symplastic water transport across the PM.

The behavior of PIP2;1 appears to depend on its phosphorylation state [10]. For example, phosphorylation of Ser²⁸³ appears to be necessary for the PM localization of PIP2;1 and that the appearance of intracellular PIP2;1-containing compartments in response to NaCl treatment requires a non-phosphorylated form of Ser²⁸³ [10]. Further studies are needed to determine the involvement of phosphorylation of PIP2;1 in its transport to the vacuoles.

Salt-induced vacuolar internalization of PIP2;1 is dependent on CME, PI3K and PI4K.

The recycling of PIP2;1 between the plasma membrane (PM) and the *trans*-Golgi network (TGN) has been thought to involve clathrin-mediated endocytosis under non-salt stress

condition (Fig 4A), while, under salt stress condition, it has been thought to involve clathrin independent endocytosis (CIE) [11] (Fig 4B). However, our results suggest that PIP2;1 is endocytosed at the PM to the TGN by the activity of CME and PI4K, and then, transported to the vacuole via the PVC by the activity of PI3K (Fig 4B, red arrows).

TyrA23, an inhibitor of CME, suppressed the salt-induced internalization of PIP2;1 from the PM to the vacuolar lumen (Fig 2A and 2B). Salt stress was found to modulate the recycling mechanism of PIP2;1 from CME to CIE [11], though CME of PIP2;1 was reported to be still active under salt stress [13]. Our results suggest that CME is important for the salt-induced vacuolar internalization of PIP2;1. Under salt stress, PIP2;1 may be modified at the PM, such as by phosphorylation [10] depending on the type of trafficking (CME dependent vacuolar internalization or CIE dependent recycling). PAO, an inhibitor of PI4K, severely suppressed the salt-induced vacuolar internalization of GFP-PIP2;1 (Fig 2) and kept GFP-PIP2;1 at the PM (Fig 3, lower panels). PtdIns4P and PtdIns(4,5)P₂, PI products of PI4K, are reported to regulate CME in a coordinated manner [26]. Taken together, our results show that the salt-induced vacuolar internalization of PIP2;1 is started by the PI4K-dependent CME (Fig 4B).

LY294002, an inhibitor of PI3K, significantly suppressed the salt-induced vacuolar accumulation of GFP-PIP2;1 (Fig 2), but, in the presence of BFA, did not inhibit the

internalization of GFP-PIP2;1 from the PM to the BFA compartments, which are aggregations of post-Golgi organelles including Golgi apparatus, the TGN and the PVC [19] (Fig 3, middle panels). These results suggest that, under salt stress, PI3K does not regulate endocytosis at the PM but is important for the trafficking of PIP2;1 after endocytosis from the PM. PtdIns3P, which is a PI product of PI3K, is mainly localized on the vacuolar and PVC membranes [27], where it is involved in trafficking various proteins from the TGN to the vacuoles [28]. In view of these reports, our results suggest that PI3K is required for the transport of PIP2;1 from the TGN to the vacuolar lumen (Fig 4B). Further studies of PIP2;1 dynamics with various fluorescent markers of post-Golgi organelles and with a loss-of-function mutant of PI3K should help to clarify the role of PI3K in the salt-induced internalization of PIP2;1.

Acknowledgments

We thank Dr. Junpei Takano (Osaka Prefecture University) for kindly providing GFP-PIP2;1 and GFP-LTI6B Arabidopsis seeds, and Dr. Masahiko Sato (Kyoto Prefectural University) for kindly providing the plasmid of pENTR. This research was supported by a grant to N. T. from Japan Science and Technology Agency (CREST).

References

- [1] S. Owens, Salt of the earth. Genetic engineering may help to reclaim agricultural land lost due to salinisation, EMBO Rep. 2 (2001) 877-879.
- [2] A. Fita, A. Rodríguez-Burruezo, M. Boscaiu, J. Prohens, O. Vicente, Breeding and Domesticating Crops Adapted to Drought and Salinity: A New Paradigm for Increasing Food Production, Front Plant Sci. 6 (2015) 978.
- [3] R. Munns, M. Tester, Mechanisms of salinity tolerance, Annu Rev Plant Biol. 59 (2008) 651-681.
- [4] P.M. Hasegawa, R.A. Bressan, J.K. Zhu, H.J. Bohnert, PLANT CELLULAR AND MOLECULAR RESPONSES TO HIGH SALINITY, Annu Rev Plant Physiol Plant Mol Biol. 51 (2000) 463-499.
- [5] X. Niu, R.A. Bressan, P.M. Hasegawa, J.M. Pardo, Ion Homeostasis in NaCl Stress Environments, Plant Physiol. 109 (1995) 735-742.
- [6] C. Maurel, M.J. Chrispeels, Aquaporins. A molecular entry into plant water relations, Plant Physiol. 125 (2001) 135-138.
- [7] O. Da Ines, W. Graf, K.I. Franck, A. Albert, J.B. Winkler, H. Scherb, et al, Kinetic analyses of plant water relocation using deuterium as tracer - reduced water flux of *Arabidopsis pip2*

aquaporin knockout mutants, *Plant Biol.* 12 (Suppl. 1) (2010) 129-139.

[8] U. Johanson, M. Karlsson, I. Johansson, S. Gustavsson, S. Sjövall, L. Fraysse, et al, The complete set of genes encoding major intrinsic proteins in *Arabidopsis* provides a framework for a new nomenclature for major intrinsic proteins in plants, *Plant Physiol.* 126 (2001) 1358-1369.

[9] Y. Boursiac, S. Chen, D.T. Luu, M. Sorieul, N. van den Dries, C. Maurel, Early effects of salinity on water transport in *Arabidopsis* roots. Molecular and cellular features of aquaporin expression, *Plant Physiol.* 139 (2005) 790-805.

[10] S. Prak, S. Hem, J. Boudet, G. Viennois, N. Sommerer, M. Rossignol, et al, Multiple phosphorylations in the C-terminal tail of plant plasma membrane aquaporins: role in subcellular trafficking of AtPIP2;1 in response to salt stress, *Mol Cell Proteomics.* 7 (2008) 1019-1030.

[11] D.T. Luu, A. Martinière, M. Sorieul, J. Runions, C. Maurel, Fluorescence recovery after photobleaching reveals high cycling dynamics of plasma membrane aquaporins in *Arabidopsis* roots under salt stress. *Plant J.* 69 (2012) 894-905.

[12] A. Martinière, X. Li, J. Runions, J. Lin, C. Maurel, D.T. Luu, Salt stress triggers enhanced cycling of *Arabidopsis* root plasma-membrane aquaporins, *Plant Signal Behav.* 7 (2012)

529-532.

[13] X. Li, X. Wang, Y. Yang, R. Li, Q. He, X. Fang, et al, Single-molecule analysis of PIP₂;1 dynamics and partitioning reveals multiple modes of *Arabidopsis* plasma membrane aquaporin regulation, *Plant Cell*. 23 (2011) 3780-3797.

[14] C. Maurel, Y. Boursiac, D.T. Luu, V. Santoni, Z. Shahzad, L. Verdoucq, Aquaporins in Plants, *Physiol Rev*. 95 (2015) 1321-1358.

[15] T. Munnik, E. Nielsen, Green light for polyphosphoinositide signals in plants, *Curr Opin Plant Biol*. 14 (2011) 489-497.

[16] C. Pical, T. Westergren, S.K. Dove, C. Larsson, M. Sommarin, Salinity and hyperosmotic stress induce rapid increases in phosphatidylinositol 4,5-bisphosphate, diacylglycerol pyrophosphate, and phosphatidylcholine in *Arabidopsis thaliana* cells, *J Biol Chem*. 274 (1999) 38232-38240.

[17] S. König, T. Ischebeck, J. Lerche, I. Stenzel, I. Heilmann, Salt-stress-induced association of phosphatidylinositol 4,5-bisphosphate with clathrin-coated vesicles in plants, *Biochem J*. 415 (2008) 387-399.

[18] Y. Leshem, L. Seri, A. Levine, Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt

tolerance, *Plant J.* 51 (2007) 185-197.

[19] Y.C. Tse, S.W. Lo, S. Hillmer, P. Dupree, L. Jiang, Dynamic response of prevacuolar compartments to Brefeldin A in plant cells, *Plant Physiol.* 142 (2006) 1442-1459.

[20] S.R. Cutler, D.W. Ehrhardt, J.S. Griffiths, C.R. Somerville, Random GFP::cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency, *Proc Natl Acad Sci U S A.* 97 (2000) 3718-3723.

[21] K. Enami, M. Ichikawa, T. Uemura, N. Kutsuna, S. Hasezawa, T. Nakagawa, et al, Differential expression control and polarized distribution of plasma membrane-resident SYP1 SNAREs in *Arabidopsis thaliana*, *Plant Cell Physiol.* 50 (2009) 280-289.

[22] S.J. Clough, A.F. Bent, Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*, *Plant J.* 16 (1998) 735-743.

[23] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, et al, Fiji: an open-source platform for biological-image analysis, *Nat Methods.* 9 (2012) 676-682.

[24] C.A. Schneider, W.S. Rasband, K.W. Eliceiri, NIH Image to ImageJ: 25 years of image analysis, *Nat Methods.* 9 (2012) 671-675.

[25] S. Bolte, C. Talbot, Y. Boutte, O. Catrice, N.D. Read, B. Satiat-Jeunemaitre, FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells, *J Microsc.* 214 (Pt 2)

(2004) 159-173.

[26] Y. Zhao, A. Yan, J.A. Feijó, M. Furutani, T. Takenawa, I. Hwang, et al, Phosphoinositides regulate clathrin-dependent endocytosis at the tip of pollen tubes in *Arabidopsis* and tobacco, *Plant Cell*. 22 (2010) 4031-4044.

[27] M.L. Simon, M.P. Platre, S. Assil, R. van Wijk, W.Y. Chen, J. Chory, et al. A multi-colour/multi-affinity marker set to visualize phosphoinositide dynamics in *Arabidopsis*, *Plant J*. 77 (2014) 322-337.

[28] D.H. Kim, Y.J. Eu, C.M. Yoo, Y.W. Kim, K.T. Pih, J.B. Jin, et al, Trafficking of phosphatidylinositol 3-phosphate from the trans-Golgi network to the lumen of the central vacuole in plant cells, *Plant Cell*. 13 (2001) 287-301.

Figure legends

Fig 1. Salt-induced internalization of PIP2;1 into the vacuolar lumen.

(A) CLSM images of epidermal cells in the elongation zone of *A. thaliana* roots expressing GFP-PIP2;1, GFP-SYP132, and GFP-LTI6B, respectively. For each protein, the left and right panels show images of root epidermal cells under control (non-salt stress) and salt stress conditions at 0 hours (upper) and 6 hours (lower). Arrowheads indicate the accumulation of GFP-PIP2;1 in the vacuole-shaped structures. Scale bars = 10 μ m.

(B) Bar graphs representing the intracellular-to-PM fluorescent intensity ratio (FIR) of GFP-PIP2;1, GFP-SYP132 and GFP-LTI6B. White and gray columns indicate the FIR after 0, 3 and 6 hours of treatment with the control and salt stress condition, respectively. Data represent means $\pm$ standard divisions with the number of roots analyzed (Student's *t*-test; ***P* < 0.01).

(C) CLSM images of GFP-PIP2;1 (green) and FM4-64 (red) of an epidermal cell in the elongation zone of *A. thaliana* root after 6 hours of salt stress treatment. The merge image shows that the internalized GFP-PIP2;1 perfectly matches the locations of the vacuoles. Arrowheads indicate vacuole-shaped structures. Scale bars = 10 μ m.

Fig 2. Effects of inhibitors of CME, PI3K and PI4K on the internalization of PIP2;1.

(A) CLSM images of GFP-PIP2;1 (green) and FM4-64 (red) of *A. thaliana* root epidermal cells, which were treated with 0.1% Dimethyl Sulfoxide (DMSO) (upper panels), 50 μ M tyrphostin A23 (TyrA23) (upper middle panels), 50 μ M LY294002 (lower middle panels) and 50 μ M phenylarsine oxide (PAO) (lower panels) under control (non-salt stress) and salt stress conditions for 6 h in the dark. Scale bars = 10 μ m.

(B) Bar graphs representing the intracellular-to-PM fluorescent intensity ratio (FIR) of GFP-PIP2;1. Hatched and closed columns indicate the FIR after 6 hours of treatment under the control and salt stress condition, with DMSO (green), TyrA23 (orange), LY294002 (blue), and PAO (magenta), respectively. Data represent means $\pm$ standard divisions. Different letters indicate statistically significant differences (Tukey's honestly significant difference; $\alpha = 0.05$, $n = 3$).

Fig 3. Distinct effects of LY294002 and PAO on the internalization of PIP2;1 under the presence of BFA.

CLSM images of GFP-PIP2;1 (green) and FM4-64 (red) of *A. thaliana* root epidermal cells that were treated with 0.1% Dimethyl Sulfoxide (DMSO) (upper panels), 50 μ M LY294002 (middle panels), and 50 μ M phenylarsine oxide (PAO) (lower panels) under salt stress condition in the

dark for 3 h, followed by another 3 h incubation with media containing each inhibitor and 50 μ M Brefeldin A (BFA). Arrows, arrowheads, and asterisks indicate the localization of GFP-PIP2;1 at the plasma membrane (PM), in the vacuolar lumen, and in the BFA compartments, respectively. Scale bars = 10 μ m.

Fig 4. Schematic illustrations of the trafficking pathways of PIP2;1 under non-salt stress and salt stress conditions.

(A) Under non-salt stress condition, PIP2;1 mainly localizes to the plasma membrane (PM), and recycles between the PM and the *trans*-Golgi network (TGN) regulated by clathrin-mediated endocytosis (CME) (green arrows). Hardly any PIP2;1 accumulates in the vacuole. PAO, phenylarsine oxide; PVC, prevacuolar compartment; TyrA23, tyrphostin A23.

(B) Under salt stress condition, PIP2;1 recycles between the PM and the TGN through clathrin independent endocytosis (CIE) (green arrows) and is internalized from the PM to the vacuolar lumen (red arrows). PIP2;1 is also endocytosed at the PM through the phosphatidylinositol 4-kinase (PI4K) –dependent CME, and transported to the vacuolar lumen, which requires the activity of phosphatidylinositol 3-kinase (PI3K).

Figure 1

Figure 2

Figure 3

Figure 4