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Salt stress triggers enhanced cycling of Arabidopsis root plasma-membrane aquaporins

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Aquaporins of the plasma membrane intrinsic protein (PIP) subfamily are channels which facilitate the diffusion of water across the plant plasma membrane (PM). Although PIPs have been considered as canonical protein markers of this compartment, their endomembrane trafficking is still not well documented. We recently obtained insights into the constitutive cycling of PIPs in Arabidopsis root cells by means of fluorescence recovery after photobleaching (FRAP). This work also uncovered the behavior of the model isoform *At*PIP2;1 in response to NaCl. The present addendum connects these findings to another recent work which describes the dynamic properties of *At*PIP2;1 in the PM in normal and salt stress conditions by means of single particle tracking (SPT) and fluorescence correlation spectroscopy (FCS). The results suggest that membrane rafts play an important role in the partitioning of *At*PIP2;1 in normal conditions and that clathrin-mediated endocytosis is predominant. In salt stress conditions, the rate of *At*PIP2;1 cycling was enhanced and endocytosis was cooperated by a membrane raft-associated salt-induced pathway and a clathrin-dependent pathway.

Keywords: aquaporin, constitutive cycling, salt stress, fluorescence recovery after photobleaching, single particle tracking, fluorescence correlation spectroscopy

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Plant plasma membrane (PM) proteins are synthesized in the endoplasmic reticulum (ER) and are targeted to the surface of the cell via the secretory pathway. Once in the PM, they undergo constitutive cycling, i.e., repeated cycles of endocytosis and exocytosis, before being eventually directed to the vacuole for degradation.[1] In plant cells, aquaporins of the Plasma membrane Intrinsic Protein (PIP) subgroup, channels which facilitate the diffusion of water across the PM, have emerged as molecular models for studying membrane protein trafficking. Maize *Zm*PIP2;4 and *Zm*PIP2;5 and *At*PIP2;1 of *Arabidopsis thaliana* have, for example, been found to carry a functional diacidic motif in their N-terminal tail, to facilitate their export out of the ER.[2,3] Similar export signal motifs also exist in other membrane proteins.[4-6] *At*PIP2;1 has been used by many laboratories as a marker of the PM,[7-12] and indirect evidence for its clathrin-dependent endocytosis and constitutive cycling between the PM and endosomal compartments have been obtained. Yet, knowledge of *At*PIP2;1 endomembrane trafficking is still fragmentary.

Our two recent studies by Luu et al.[13] and Li et al.[14] aimed at filling these gaps, by analyzing in detail the mobility of PIPs at the PM and key steps controlling their constitutive cycling. Until now, this process had been explored using a combination of pharmacologies to inhibit endo-, and exocytosis and classical confocal laser scanning microscopy. Treatment of Arabidopsis root cells by the fungal toxin brefeldin A (BFA), which inhibits the function of adenosine ribosylation factor GTPases by interacting with their associated guanine nucleotide exchange factors (see refs. 15 and 16), provokes the aggregation of vesicles originating from both the secretory and the endocytic pathways in so-called "BFA compartments." Consequently, BFA has

tentatively been considered as a blocker of PM protein exocytosis. Although the molecular targets of tyrphostin A23 (A23), a tyrosine analog, have not been unequivocally demonstrated in plant cells, this compound has been used to tentatively inhibit clathrin-mediated endocytosis.[17-21] Finally, the lipophilic styryl dyes FM1-43 and FM4-64 have also been used as endocytic tracers[22-26] and although they selectively label the lipid membrane, theses molecules have been accepted as markers of bulk endocytosis. The drawbacks of these approaches were, besides the undetermined specificity of some pharmacological compounds, a relatively low resolution of standard confocal imaging. In the Luu et al. paper, we have applied fluorescence recovery after photobleaching (FRAP) techniques to root epidermal cells of Arabidopsis plants ectopically expressing fusions between *At*PIPs and green fluorescent protein (GFP), to specifically address the cycling dynamics of these PM proteins with no need of drug application. In agreement with previous observations on other membrane proteins,[27] the lateral diffusion of the PIP constructs was found to be extremely low, and therefore did not contribute significantly to the observed fluorescence recovery. In that work, we proposed that the signal monitored after photobleaching was mainly emitted from the fusion proteins in the endosomes and those yet to reach the PM by exocytosis, i.e., proteins in cytoplasmic compartments which were moving relatively quickly compared with those anchored in the PM. This finding therefore allowed us to explore the mode of PIP cycling. We validated our claim that we were observing differential compartmentation of *At*PIPs using a combination of FRAP with a consistent set of pharmacological treatments. Drugs used included BFA, A23 or the synthetic auxin analog, naphthalene-1-acetic acid, which all reduced significantly the recovery of fluorescence after photobleaching, indicating an impairment in the constitutive cycling of the constructs.

To gain more insight into the dynamic properties of *At*PIPs in the PM, Li et al. used single particle tracking (SPT) in continuous images of a GFP-*At*PIP2;1 construct recorded by variable-angle evanescent wave microscopy, together with fluorescence correlation spectroscopy (FCS). In this study, we again observed that the lateral diffusion of the *At*PIP construct is extremely low compared with that of GFP-LTi6a, another marker protein of the PM.[28,29] SPT in root cells treated with methyl-β-cyclodextrin (MβCD), a sterol disrupting reagent, or using colocalization with mCherry-Flot1, a marker protein of membrane rafts, indicated that partitioning of the PIP construct in the PM depends on PM sterol content and is related to membrane rafts. Treatment with A23 provoked a marked increase in the density of the aquaporin construct in the PM, as monitored by FCS, suggesting that more constructs dwell on the membrane after the inhibition of clathrin-mediated endocytosis.

In previous studies, we have monitored the effect of salt (NaCl) stress on the subcellular localization of Arabidopsis root aquaporins by expression of fusions of these aquaporins with GFP.[30-32] A treatment with 100 mM NaCl induced, after 2–4 h, an increase in intracellular labeling by the *At*PIP fusions, which was interpreted as an intracellular relocalization of these proteins. However, root hydraulic conductivity, which has been shown to be determined in part by PIP activity,[33] was inhibited at a much faster rate (reduced by half after 45 min). In addition, FM1-43 uptake in root cells allowed us to conclude that salt stress enhances bulk-flow membrane endocytosis as soon as 45 min after application.[24] The whole set of data prompted us to explore the trafficking of *At*PIPs in the early phase (< 30 min) of the response of root cells to salt stress.

Several lines of evidence show that the cellular dynamics of *At*PIP2;1 is indeed markedly altered during the early phase of the salt stress response. First, FCS showed that the density of GFP-*At*PIP2;1 at the PM dropped to 46% of the control value as soon as 10 min after salt exposure, thereby confirming that salt promotes PIP internalization. Second, SPT revealed that the diffusion coefficient and the restricted diffusion of GFP-*At*PIP2;1 in the PM of salt-treated root epidermal cells were increased by 100% and 60%, respectively. FRAP analysis in the same stress conditions also showed that the amplitude of long-term recovery of fluorescence was increased by 2-fold. Here, we showed that the contribution of lateral diffusion of the constructs within the PM to this process was negligible and therefore hypothesized that the faster kinetics of recovery may reflect an enhanced cycling of *At*PIP-GFP. Complementary analyses suggested that overall endosomal labeling was unchanged while the rates of construct endocytosis and exocytosis (cycling) were simultaneously increased. Together these results suggested an enhanced cycling of *At*PIPs, with a higher surface exchange and membrane domain restricted diffusion, which seem to be two typical markers of enhanced endocytosis.

A critical issue is now to understand which pathway(s) is involved in PIP endocytosis under salt stress conditions. The importance of clathrin-dependent endocytosis was addressed by FCS in A23 treated-cells. In these experiments, A23 treatment increased the density of PIPs in the PM by ~50% relative to cells treated in only 100 mM NaCl, suggesting that A23 was somewhat able to counteract the effect of salt, and that clathrin-mediated endocytosis also intervenes under salt stress conditions. In FRAP experiments, the amplitude of the short-term response was slightly reduced by A23. The long-term response was by contrast similar between salt-treated roots with and without A23. This suggests that, in the latter experiments, an enhanced clathrin-mediated endocytosis could overrun the effect of this drug. Higher drug concentrations might be tested, but would probably impair the vitality of the cells (see ref. 15). The involvement of other endocytosis-mediating components can also be invoked. Adaptors (AP) are protein complexes which interact with the cytoplasmic tail of the cargoes and allow their recruitment into budding vesicles in specific intracellular compartments (see refs. 34 and 35 for review). Micro-adaptins form one of the four subunits of the AP complexes and were identified as interacting with tyrosine motifs in cargo cytoplasmic tails. The AP-2 complex is known to mediate endocytosis from the PM under standard conditions but the micro-adaptin involved (5 have been described in Arabidopsis) is still unknown. Here we

hypothesize that, under salt stress, AP-2 complexes (which are presumably sensitive to A23) and also other AP complexes mediate the endocytosis of *At*PIPs. Alternative hypotheses have been explored by Li et al. using MβCD to disturb membrane rafts. Treatment of root epidermal cells under salt stress by MβCD increased by > 30% the density of *At*PIP2;1-GFP in the PM when compared with cells exposed to salt alone, whereas this treatment had no effect on their density in non-stressed conditions. These data indicate that a raft-associated endocytic pathway influences endocytosis, specifically in salt stress conditions.

In conclusion, our two recent studies delineate a working model for PIPs, whereby, in normal conditions, membrane rafts play an important role in aquaporin partitioning and diffusion at the cell surface. Clathrin-mediated endocytosis is significantly involved in this process. In salt stress conditions, converging evidence from the two studies indicate an enhanced cycling of PIPs between the PM and endosomes located in the membrane vicinity. Whereas clathrin-mediated endocytosis is likely to operate in these conditions, a membrane raft-associated endocytic pathway seems to be activated, thereby contributing to enhanced PIP endocytosis. These findings confirm that the regulation of endocytosis represents an early and general response of plant cells to challenging biotic or abiotic conditions (see ref. 36). Besides the identification of the molecular components involved in salt-induced cycling of *At*PIP, one key question is to understand the rationale for this enhanced cycling and its relation to the intrinsic activity of *At*PIPs.

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