Critical Review

Vesicular Trafficking and Salinity Responses in Plants

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Abstract

Research spanning three decades has demonstrated that vesicles pinch off from the plasma membrane and traffic through the cytoplasm of plant cells, much as previously reported in animal cells. Although the well-conserved clathrin-mediated mechanism of endocytosis has been well characterized, relatively little is known about clathrin-independent pathways in plants. Modulation of endocytosis by both physical stimuli and chemical ligands has been reported in plants. Here, we review the effect of salinity—one of the most deleterious environmental assaults—on endocytosis and intracellular trafficking. © 2015 IUBMB Life, 67(9):677–686, 2015

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Introduction

Vesicular trafficking is the concerted process of intracellular movement of membrane-enclosed material, which is indispensable for the functioning of a living cell. A major thoroughfare delivers vesicle-enclosed biosynthetic cargo from the endoplasmic reticulum (ER), via the Golgi (and also from the Golgi itself), to the plasma membrane (PM) or to the vacuoles (anterograde biosynthetic trafficking). Retrograde trafficking of cargo back to the ER from the Golgi is also documented. The generation of vesicles by invagination of the PM is termed endocytosis. Endocytosed cargo is routed through various intermediary stages, ultimately destined either for the vacuole or for recycling to the PM. Both the endocytic uptake and the subsequent intracellular trafficking are subject to precise regulation, mediated by molecules that are now being identified.

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In plant cells, endocytosis was considered to be unlikely due to the high turgor pressure against which the endocytic vesicles have to form. The barrier function of the cell wall made uptake of large molecules from the extracellular milieu also unlikely. However, extensive research has now unequivocally demonstrated the existence of endocytosis in plant cells. In addition to the presence of homologs of endocytic proteins present in animals, structures characteristic of endocytosis such as clathrin-coated pits and vesicles (see below) were observed by electron microscopy (1). A major breakthrough in the study of endocytosis in plants was the introduction of the lipophilic probe Fei Mao (FM4)-64 in the late 1990s (reviewed in ref. (2)). FM dyes are cell impermeable and fluoresce brightly only when embedded in a lipid environment, properties which made these dyes ideal candidates for labeling the PM and tracking endocytic internalization as well as the movement of endocytosed vesicles. Fluorescent tagging of PM proteins enabled the visualization of their uptake together with FM4-64, which helped to establish that the source of the vesicles was the PM. The use of photoconvertible fluorescent tags enabled monitoring of the endocytosis of PM proteins in real time (3). Once the phenomenon of endocytosis in plants was established, mechanistic details began to emerge. These investigations were borrowed from and depended heavily on the more extensively studied mechanisms of endocytosis in animal cells.

Clathrin-Mediated Endocytosis

This endocytic pathway is characterized by the assembly of the coat protein clathrin. The clathrin coat provides structural rigidity to the endocytosed vesicle and defines its shape. Scission of the vesicles from the PM is affected by the large GTPase, dynamin. The Arabidopsis genome has two genes that code for clathrin heavy chains (CHC1 and CHC2), and three that code for clathrin light chain (CLC1, CLC2, and CLC3). There are 16 proteins that comprise the Arabidopsis dynamin-related protein (DRP) family. These are subdivided into six groups (DRP1–6) based on sequence similarities and localization patterns (4). Using variable-angle total internal reflection fluorescence (VA-TIRF) microscopy, DRP2B, DRP1A, and DRP1C have been shown to be involved in endocytic vesicle formation together with clathrin on the PM (5,6). DRP2A has also been shown to form distinct foci on the PM together with DRP2B (7). Intriguingly, electron microscopy revealed the outer diameter of clathrin-coated vesicles in Arabidopsis (~60 nm; ref. (3)) to be much smaller than those found in animal systems (100–150 nm), possibly an adaptation to function against the high turgor of plant cells (8). The importance of clathrin-mediated endocytosis (CME) in plants has been convincingly demonstrated by the finding that plants that are homozygous null for both CHC isoforms are nonviable and that expression of a dominant-negative truncated version of CHC1 (HUB domain) causes severe endocytic as well as developmental defects (9). Similar to HUB overexpression, expression of dominant-negative forms of DRP2A has been shown to impede endocytosis and to prevent the growth of root hairs, a process heavily dependent on vesicular trafficking (10).

A small molecule ikarugamycin (IKA) that prevents budding of clathrin-coated pits from the PM has been used to inhibit CME (11). Additionally, exogenous application of plant signaling molecules like auxins (12,13) and salicylic acid (14) has also been reported to inhibit clathrin recruitment to the PM.

Apart from the key components (clathrin and dynamin), several other factors are required for CME, mainly for the assembly of clathrin-coats on the PM. Adaptor proteins through their lipid-binding abilities serve as connectors to recruit clathrin subunits to the PM. Another important function mediated by these adaptor proteins is recruiting cargo proteins to the clathrin coat. Such adaptor complexes recognize cytosolic domains on cargo proteins that bear particular tyrosine and/or dileucine motifs. Modifications like phosphorylation and ubiquitination of the cargo proteins have also been shown to play critical roles in their recognition by the adapter complexes (15). The most prominent of the adaptors are the adaptor protein complex AP2 (comprising α, β, σ, and μ-subunits) and the Adaptin-like proteins TPLATE and AP180. VA-TIRF microscopy revealed the formation of distinct endocytic foci on the PM by AP2-σ- and -μ-subunits together with clathrin (16,17). Endocytic and developmental defects have been associated with loss-of-function of AP2 complex σ-subunit (16). In addition, the small molecule inhibitor Tyrphostin-A23 (Tyr-A23) that interferes with the recognition of the Tyr-motif by the AP2 complex has been widely used to inhibit receptor endocytosis in plants (3,18). Recent work has revealed the importance of the plant-unique adapter complex TPLATE (a multiprotein complex of the proteins TPLATE, TASH3, AtEH1, AtEH2, LOLITA, TML, TWD40-1, and TWD40-2). The TPLATE complex is believed to act as an early adapter that facilitates subsequent recruitment of AP2 and clathrin on the PM. Down-regulation of TPLATE complex components is also shown to attenuate endocytosis in Arabidopsis root (19). The monomeric adapter protein AP-180 has been shown to associate with clathrin coats on the PM (20).

Among the cargo of CME, the most well-documented are the transmembrane proteins including PM receptors that initiate signaling cascades following ligand binding and the solute transporters that mediate movement of small molecules and nutrients across the PM. Although uptake in many cases is constitutive, it can be modulated by signaling ligands, external physical stimuli as well as nutrient availability. A summary of such stimulated uptake of transmembrane proteins is presented below (Table 1).

Clathrin-Independent Endocytosis

Mechanisms that do not use clathrin as the scaffold of endocytic buds have been documented in the animal kingdom. Some of the early studies of endocytosis in plants focused on the uptake of fluid-phase probes, a prominent cargo for clathrin-independent endocytosis (CIE) pathways in animals. Uptake and vacuolar transport of fluid-phase probes such as the small fluorescent dyes Alexa-568 hydrazide and Lucifer Yellow have been reported in tobacco BY-2 cells and maize inner cortex cells, respectively (37,38). However, the involvement of clathrin in these processes has not been explored.

In recent studies, using genetic (HUB overexpression) and chemical (IKA treatment) perturbation, clathrin-independent uptake of a glucose analog as well as fluorescent nanobeads has been suggested in tobacco BY-2 cells (39,40). Uptake of such fluid-phase probes remains completely unaffected in the presence of clathrin perturbations, whereas uptake of the lipid probe FM4-64 is largely abrogated. The uptake of fluid in the absence of encapsulating lipid is puzzling and reminiscent of early reports of small fluorescent molecules (generated from the cleavage of larger conjugates by the cell wall enzymes) being actively transported across the cell membrane as opposed to being taken up in endosomes (41). IKA-resistant uptake of charged gold nanoparticles has been documented in BY-2 protoplasts and tobacco pollen tubes (11,42).

The best studied clathrin-independent pathway reported in plants till date is the flotillin-mediated pathway. Flotillins are associated with sterol-rich detergent-resistant microdomains on the inner leaflet of the PM and can induce membrane curvature. VA-TIRF imaging revealed Arabidopsis Flotillin-1 (Flot-1) to form distinct foci on the PM that are separate from

TABLE 1 *Induced endocytosis of transmembrane proteins in plants*

Cargo protein	*Stimulus*	*Reference*
Boron transporter (BOR1)	High extracellular boron	21
Auxin efflux transporter (PIN2)	Salinity, gravity, and darkness	22–24
Brassinosteroid receptor-like kinase (BRI1 and BAK1)	Brassinosteroid hormones	25
Clavata 1	Clavata 3	26
Flagellin sensitive 2 (FLS2)	Bacterial flagellin protein	27
Potassium channel (KAT1)	Abscisic acid (ABA)	28
Iron transporter (IRT1)	High extracellular iron	29
Ammonium transporter (AMT1;1; AMT1;3)	High extracellular ammonium	30
EIX-inducing receptor (LeEIX2)	Ethylene-inducible xylanases (EIX)	31
Plasma membrane aquaporins (PIPs)	Salinity	32,33
Regulator of G signaling protein 1	Salinity	34
NADPH oxidase (RbohD)	Salinity, cryptogenin (fungal elicitor)	35,36

clathrin foci. Unlike clathrin, the dynamics of Flot-1 foci on the PM was sensitive to membrane microdomain disruption by the sterol-sequestering oligosaccharide methyl-beta-cyclodextrin (MβCD) but not to Tyr-A23. Interestingly, the size of Flot-1-coated vesicles (about 100 nm) is much larger than that of clathrin-coated vesicles (60 nm; ref. (43)). VA-TIRF microscopy studies revealed that some PM transporters, in addition to their predominant association with clathrin foci, partially colocalize with Flot-1 foci. Prominent examples are the aquaporin PIP2;1 (32), the ammonium transporter AMT1;3 (30), and the NADPH oxidoreductase RbohD (35). Intriguingly, under control conditions, much of the internalization of these proteins occurs through CME. The role of Flot-1 membrane microdomains becomes prominent only under conditions that require rapid internalization of these proteins, such as high ammonium concentration–induced internalization of AMT1;3. The role of salt stress on PIP2;1 and RbohD uptake will be discussed in a subsequent section.

In animal systems, glycosyl phosphatidyl inositol (GPI)-anchored proteins serve as well-characterized markers of CIE, as their internalization is mediated in most cases by the coat-independent CLIC/GEEC pathway and in some contexts by flotillin and caveolin (44). In Arabidopsis root, VA-TIRF microscopy revealed that fluorescent proteins tagged to GPI anchors form distinct foci on the PM, about one-third of which are devoid of clathrin (42). Treatment with the synthetic auxin 1-naphthalene acetic acid (NAA) drastically reduced the abundance as well as dynamics of clathrin on the PM and at the same time blocked internalization of transmembrane protein cargo, previously shown to be clathrin-dependent (PIN2, PIN1, and LTI6b) across all layers of the root (13,45). By contrast, in the presence of NAA treatment, internalization of both GPI-anchored proteins and the membrane-marking dye FM4-64 can still be observed in the epidermis, albeit to a lesser extent. Intriguingly, NAA treatment completely blocked the uptake of both these probes in internal cell layers including the stele. Uptake of FM4-64, but not transmembrane protein cargo, was observed in the epidermal cells of plants where HUB was overexpressed. Such uptake was completely blocked in internal cells of these plants (45). These observations suggest that although CME operates ubiquitously across all layers of the root, the constitutive clathrin-independent pathway seems to operate only in the epidermis under control conditions. It was also found that this epidermally restricted CIE was sensitive to sterol depletion by MβCD.

Salt Stress and Endocytic Response in Plants

High concentrations of Na^+ impose osmotic stress across the PM, causes ionic disequilibration, and the Na^+ that enters the cytosol inhibits critical enzymatic processes. High external Na^+ also lowers the water potential, subjecting the plant to drought stress (46). Leshem et al. (47) reported that salt stress induces bulk-flow endocytosis in the Arabidopsis root. Salt stress markedly increased the uptake of FM dyes as well as labeled dextran in root cells. Rapid endocytosis of PM NADPH oxidases generated intravesicular reactive oxygen species (ROS) that presumably acted as signaling molecules critical for salt stress tolerance (discussed in a later section). Several

subsequent studies confirmed the upregulation of bulk endocytosis by salt stress (32,45,48). Differential uptake of PIN2 (although endocytic rates of other transmembrane proteins remain unchanged) has been proposed to be induced by salt (22). However, recent studies (45,48) demonstrate that salt-induced augmentation of endocytosis is more of a general phenomenon and not unique for a specific protein. Enhanced uptake of PM aquaporins, as well as the NADPH oxidase RbohD in response to salt stress, has been documented (32,33,35).

The contribution of CME and CIE pathways to the salt-induced escalation of endocytosis is an area of active research. Reports of enhanced endocytosis through the clathrin-mediated pathway are based on the observed rise in clathrin recruitment to the PM (22,48), whereas others report that clathrin-independent pathways are also boosted (32,35,45). Salt-induced increase in FM4-64 uptake is observed even in the presence of genetic and chemical perturbation of clathrin although the extent of enhancement is substantially reduced. However, depletion of sterols from the PM (known to inhibit clathrin-independent pathways), together with clathrin inhibition by NAA, completely eliminates such enhanced uptake. Hence, both clathrin-dependent and -independent pathways appear to contribute to the process of endocytic augmentation (45). Contribution of CME and CIE in the uptake of two proteins, the aquaporin PIP2;1 and the NADPH oxidase RbohD, has been studied in detail. VA-TIRF microscopy revealed that under control conditions, these proteins associate both with clathrin and Flot-1 foci on the PM. However, pharmacological perturbations revealed that under control conditions, endocytic uptake of these proteins is primarily dependent on clathrin and not on CIE components such as the flotillins and sterol-rich membrane microdomains. Salt stress induced rapid uptake of both proteins from the PM, a significant proportion of the enhanced uptake is independent of clathrin. Fluorescence cross-correlation spectroscopy revealed that although salt stress enhanced the interaction of PM-localized CLC and RbohD significantly, the interaction between Flot-1 and RbohD increased even more dramatically (35).

The fate of the endocytic cargo following salt-induced augmentation of endocytosis is not clear. A study using fluorescence recovery after photobleaching has reported that along with enhanced endocytic uptake, subsequent recycling of endocytosed aquaporins to PM is augmented by salt stress (33). No report of enhanced vacuolar trafficking and degradation of proteins have been made so far.

Intracellular Trafficking and Salt Stress

Most endocytosed cargo ends up either in the vacuole and subjected to degradation or is recycled to the PM. Trafficking along either of these trajectories requires the well-concerted action of several molecular players. Among these, the roles of two types of trafficking regulators, the Rab GTPases and the Soluble *N*-ethylmaleimide-Sensitive Factor Attachment protein Receptor (SNAREs), have been explored in the context of salt stress conditions.

The first component in the vesicular trafficking machinery to be implicated in salt stress tolerance was the Rab GTPase RabG3e (a homolog of mammalian Rab7; ref. (49)). RabG3e resides in the prevacuolar compartment (PVC) and is presumed to control trafficking from the PVCs to the vacuole. Overexpression of RabG3e in Arabidopsis enhanced the uptake of endocytic tracer FM1-43 and significantly improved salt tolerance when compared with WT plants (49). This approach has been extended to other species: the overexpression of OsRab7 generates salt-resistant rice plants (50), and the overexpression of Rab7 gene from the halophyte *Prosopis juliflora* generates salt-resistant tobacco plants (51).

Another Arabidopsis Rab GTPase studied in this context is RabF1 (also termed ARA6). ARA6 is a plant-specific homolog of the animal Rab5 GTPases. However, unlike the exclusive early endosomal localization of mammalian Rab5s, ARA6 localizes mostly to PVC and also, to a lesser extent, to PM and vacuolar membranes (52). An ARA6 knockout line, apart from having normal morphology and trafficking behavior under control conditions, is mildly salt-sensitive, whereas plants overexpressing a GTP-locked constitutively active form of ARA6 (ARA6QL) have enhanced tolerance to salt stress when compared with WT. Intriguingly, salinity induces a dramatic relocalization of GFP-tagged ARA6QL protein to the PM, suggesting a function of the protein at the PM under salt stress (52). A recent study also reports an increase in the size of ARA6-positive compartments under salt stress (48), although the implications of this observation have not been followed up. Plants with a functionally compromised VPS9a protein, the Guanine nucleotide exchange factor for the Rab5 homologs, are extremely salt-sensitive (45). VPS9a services three plant Rab5 homologs (ARA6/RabF1, ARA7/RabF2b, and RHA1/RabF2a), and thus, the salt sensitivity of the VPS9a mutant could well be a cumulative effect in impairment in functions of all three Rab5/RabFs.

Although the RabG (RabG3e) and RabF (ARA6, ARA7, and RHA1) proteins described above are presumed to have some function in endocytic pathways, the RabA1 group of GTPases is presumed to coordinate the secretory trafficking from the TGN to the PM. Indeed, transgenic plants expressing a GDP-locked dominant negative form of RabA1b does not show any observable defect in endocytosis under control conditions. However, these plants are extremely sensitive to salt stress (53), suggesting that biosynthetic trafficking from TGN to PM might have an important function in salt-tolerance mechanisms.

The indispensible function of SNAREs in vesicular trafficking has led to investigations of their possible roles in salt tolerance. Screening a t-DNA insertion collection for salt sensitivity identified *osm1*, so called because of its sensitivity to osmotic as well as salinity stress. Detailed characterization revealed it to be a TGN-localized t-SNARE–SYP61 (54). In addition, mutation in TNO1, a TGN-resident protein, causes mislocalization of SYP61 and confers salt and osmolyte sensitivity analogous to the *osm1* mutant (55). A double knockout mutant of the

TGN-resident SNAREs SYP42 and SYP43 (*syp42syp43*) displayed defects in both biosynthetic delivery to the PM and vacuole. Intriguingly, in these plants, delivery to the vacuole of the auxin transporter PIN2 to the vacuole was delayed but that of endocytosed FM4-64 was faster than in WT plants (56). Like the *osm1* mutants, *syp42syp43* plants were very sensitive to salt and mildly sensitive to hyperosmolarity (57). Recently, overexpression of a Golgi-localized SNARE, AtSFT12, has been shown to confer salt tolerance in Arabidopsis (58).

Although "overexpression" of these TGN/Golgi-resident SNAREs confers salt tolerance to plants, "deletion" of two of the vacuole-localized SNAREs, VAMP711 and SYP22, has the same effect (59,60). Incidentally, the *vamp711* and *syp22* mutations seem to block later stages of endocytic transport by preventing fusion of late endosomal compartments with the vacuoles (52,60). To explain the salt tolerance of VAMP 711 knockout plants, it has been proposed that the absence of fusion of ROS-containing vesicles prevents ROS-induced alkalization of vacuoles that are thus better equipped to use the H^+ gradient to sequester Na^+ (60).

Molecular Changes Associated with Saline Stress that Modulate Endocytosis

No specific sensor for Na^+ ions has been found in any plant system. One physical feature that could tie into salinity-induced upregulation of endocytosis is the mitigation of turgor pressure by the hyperosmolarity associated with salt stress. A recent study in Arabidopsis has found that salinity, as well as hyperosmolarity, stimulates clathrin-dependent endocytosis. Hypo-osmolar conditions, on the other hand, impede endocytosis (48). Hyperosmolarity may also be expected to favor the formation of clathrin-independent vesicles such as flotillin vesicles that are of much larger size when compared with clathrin vesicles; however, this hypothesis has not been directly tested. A systematic study is required to dissect the osmotic and ionic components of saline stress on endocytosis. It may be noted that some trafficking mutants that are hypersensitive to salt stress are not labile to (53) and in some cases more resistant than WT plants (61) to hyperosmolar stress. Selective uptake of PIN2 in response to NaCl stress but not hyperosmolar or KCl stress has been reported (22). It will be intriguing to see whether proteins that selectively favor uptake by clathrin-independent mechanisms under salt stress, like PIP2;1 or RhobD (32,35), show similar behavior under hyperosmolar stress. We present below an account of some reported changes in lipid and cytoskeleton that can act as effectors of endocytic responses to salt stress.

Lipid Changes

Salinity-induced lipid changes seem to be a critical component of endocytic modulation. Multiple studies have highlighted the importance of sterols and phosphoinositides in this process. An early study reported rapid synthesis of phosphatidyl-inositol-4,5-bisphosphate (PI-4,5-P2) in a variety of plant cells following hyperosmotic stresses, including saline stress (62). The role of PI-4,5-P2 is well established for binding of clathrin adapters to the PM for effective recruitment of clathrin coat (63). The role of phosphoinositides in plant endocytosis is corroborated by the fact that treatment with the PI-3 kinase inhibitor wortmannin prevents clathrin recruitment and dynamics on the PM (64) and inhibited endocytosis in plant cells (37). Wortmannin treatment suppressed salt-induced bulk FM dye uptake and subsequent ROS generation. In addition, Arabidopsis PI3-kinase mutants were deficient in mounting a salt-induced ROS signal and were salt-sensitive (47). Furthermore, salt stress is shown to increase levels of PI-4,5-P2 on the PM and clathrin-coated vesicles, implying a role of this phosphoinositide in the increase of CME under salt stress (65). Arabidopsis plants lacking an enzyme involved in PI-4,5-P2 biosynthesis (PI-5 kinase 2) were salt-sensitive and deficient in salt-induced bulk flow endocytosis (66). Arabidopsis mutants lacking the activity of phosphatidylinositol 5-phosphatase were found to have reduced FM4-64 uptake in response to salt, reduced ROS production, and impaired viability (61). The defects in salt-induced ROS production seen in phosphatidyl-inositol 5-phosphatase deficiency can be complemented by PI-3,4-P2 supplementation (67). Cellular levels of phosphatidic acid (PA) increase under salt stress, and clathrin specifically associates with PA under salt stress (68). Salt-induced increase of clathrin recruitment to the PM and enhanced endocytosis seem to be critically dependent on phospholipase D-mediated generation of PA.

CIE pathways seem to be dependent on sterol- and sphingolipid-enriched membrane microdomains (43). Sterol depletion by MβCD disrupts membrane microdomains and lowers FM uptake (45). Sterol biosynthesis inhibition by Fenpropimorph, depletion by MβCD, or inhibition of sphingolipid biosynthesis by 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol inhibits uptake of PIP2;1 under salt stress. Salt stress increases PM sterol levels in the inner cell layers of Arabidopsis root. A sterol biosynthesis mutant (*smt2smt3*) does not display salt-induced upregulation of endocytosis (45).

Cytoskeleton Changes

The two vital cytoskeletal elements of plants, actin and microtubules (MTs), are not only intracellular scaffolds but also serve as tracks for vesicle trafficking. Cytoskeletal elements have been shown to play critical regulatory roles in endocytosis (see ref. (69) for review). Salt stress specifically and rapidly depolymerizes the well-ordered array of cortical MTs (70), whereas hyperosmolar stress does not. Furthermore, the degree of MT depolymerization is much more prominent in mutants deficient in the PM Na^+/H^+ antiporter SOS-1, where the cytoplasmic Na^+ levels are expected to be elevated under salt stress (71). Stabilization of MTs by the drug paclitaxel

enhanced cell death under salt stress, whereas depolymerizing MTs with oryzalin enhanced viability. Note that the MTs reassembled under continuing salt stress (71).

Actin filaments also display a characteristic response to salt stress. Immediately after salt stress, the long actin bundles fragmented, and incorporation of short fragments into long bundles was inhibited (72). However, long-term salt stress induced the formation of stable bundled filaments. In contrast to MTs, actin filament stabilization (by phalloidin treatment) promoted seedling viability, and actin depolymerizing agents (cytochalasin-D and latrunculin-A) rendered the plants to be salt-sensitive (73).

Integrity of both actin and MT filaments is essential for the formation of endocytic foci on the PM. Depolymerization of actin, as well as MTs, significantly altered the trajectories and dynamics of both clathrin and flotillin foci on the PM (5,43). It will be interesting to correlate the salt-induced changes of such dynamics with salt-induced cytoskeletal changes to infer the interdependence of cytoskeleton and endocytic machinery in the context of saline stress.

Possible Implication of Endocytic Upregulation Under Salt Stress

Removal of Sodium Transporter from PM to Limit Na^+ Entry into the Cell

Endocytic removal of transporters and channels from the PM followed by their degradation in the vacuole has been suggested as a common mechanism to prevent nutrient toxicity. Such mechanisms have been reported for the boron transporter BOR-1 and ammonium transporter AMT1;3 in response to high extracellular boron and ammonium, respectively (21,30). It is presumed that sodium enters the cell through potassium transporters such as the HKTs and AKTs, nonspecific cation channels, and outward rectifying potassium channels (46). However, so far, there has been no report of specific endocytic removal of any of these transporters from the PM in response to salt stress.

Facilitating Reduction of PM Area During Plasmolysis

Osmotic stress is a crucial component of saline stress, and some common survival mechanisms to both these stresses might have coevolved. The earliest response to saline stress is plasmolysis, that is, water loss leading to cell shrinkage. Cells usually counteract the osmotic stress by synthesizing intracellular osmolytes (74). However, it is conceivable that enhanced endocytosis during the initial plasmolysis phase could serve to remove membrane fragments from the PM to reduce membrane area. Indeed, internalization of Lucifer Yellow in "osmocytic" vesicles that did not fuse with the vacuole has been reported in onion epidermal cells under rapid osmotic shock (75).

Regurgitation of the internalized Lucifer Yellow through "exocytotic" vesicles from the cells has been reported during deplasmolysis (76), suggesting a plasmolysis/deplasmolysis cycle coupled with membrane uptake/regurgitation that could be an early adaptive response to salt stress (Fig. 1A).

Preventing Water Loss by Internalizing Plasma Membrane Aquaporins

Intracellular water loss through PM aquaporins could prove severely detrimental to plants under salt stress. A dramatic reduction in hydraulic conductivity of Arabidopsis roots within an hour has been reported (77). Both clathrin-dependent and -independent pathways seem to play a critical role in reducing the effective PM distribution of aquaporins under salt stress (32,33).

Intracellular ROS Generation and ROS Signaling

Salinity stress results in the production of ROS. Recent studies put forward the hypothesis that ROS signaling could contribute to turning on salt-adaptation mechanisms. Indeed, prevention of salt-induced ROS production by treatment with the NADPH oxidase inhibitor diphenyleneiodonium seems to prevent the induction of vital stress-induced genes, culminating in severe salt sensitivity (47). Rapid internalization of NADPH oxidase RbohD (35) has been suggested to be critical for ROS generation, which is specific for saline stress and not to hyperosmolar stress (61). Influx of calcium ions following saline stress might be correlated with ROS generation, as it has been shown that inhibition of ROS production by diphenyleneiodonium treatment also attenuated elevation of cytosolic calcium (67). Calcium signaling in turn has been demonstrated to be a major factor in initiating salt-tolerance mechanisms (78). The calcium surge is temporally well correlated with endocytosis and ROS generation (61). Moreover, an Arabidopsis mutant with phosphatidylinositol 5-phosphatase deficiency (*At5PTase9*) that displayed defects in endocytic response to salt stress also showed diminished ROS generation as well as calcium influx (67).

Other signaling pathways such as brassinosteroid signaling are controlled by the relative abundance of BR1 receptors on the PM and endosomes and are subjected to regulation by the rate of endocytosis (79). Modulation of endocytosis by salt stress can alter the steady-state distribution of these receptors and downstream signaling that might have implications in salt tolerance.

Halotropism: Avoiding High Salt

Halotropism refers to the ability of the plant roots to bend away from salt. Using well-defined salt gradients, it was shown that the Arabidopsis roots bend so as to avoid high salt (22). Interestingly, such bending was not seen with gradients of osmolarity or ionic stress (KCl). The authors found that when the root tip is challenged with two different NaCl concentrations on either side, endocytic internalization of PM-localized PIN2 increases in the epidermal cells facing the higher salt concentration. Differential PIN2 endocytosis results in asymmetric auxin fluxes on either side of the root causing the

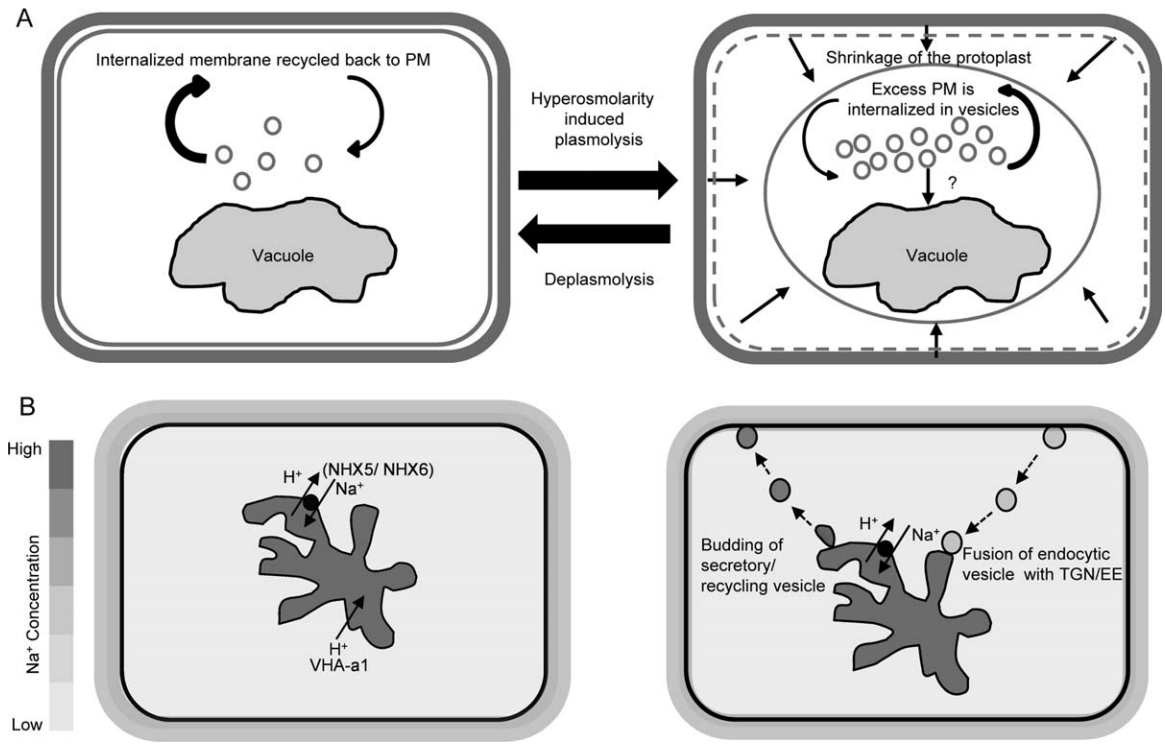

FIG 1

Two possible implications of upregulation of endocytosis by salt stress. A: Enhanced endocytosis can remove portions of plasma membrane and store in "osmocytic vesicles," thus facilitating plasmolysis under salt stress. Following osmotic adjustment, the internalized membrane pool may return to the PM during deplasmolysis. B: The TGN/EE compartments may sequester sodium ions from the cytosol by action of NHX5/NHX6 antiporters. Fusion of endocytic vesicles dilutes sodium concentration within the lumen of TGN/EE. Recycling/secretion, on the other hand, exudes the high luminal sodium of these organelles outside the cell.

epidermal cells facing higher salt to elongate more when compared with cells facing lower salt, which results in the root bending away from higher salt concentrations. These observations suggest an efficient salt-sensing mechanism that can not only sense salt at a qualitative level but can also make quantitative distinctions based on ambient Na^+ concentrations.

TGN/Recycling Endosome Mediated Sequestration of NA^+ Ions from Cytosol: Endocytosis as a Flushing Mechanism

Integrity and proper function of the TGN seems to play a critical role in salt tolerance (discussed in a previous section). Along with the efflux of cytosolic Na^+ across the PM by SOS-1 antiporters, sequestration of cytosolic Na^+ and K^+ in vacuoles by the action of vacuolar antiporters NHX1 and NHX2 could also play a critical role (80). Two isoforms of Na^+/H^+ antiporters NHX5 and NHX6 reside exclusively in the TGN/early endosome compartments. The fact that *nhx5nhx6* mutant plants are extremely salt-sensitive prompts the suggestion that sodium sequestration in TGN/EE might play an important role in salt tolerance (81). Indeed, the luminal pH of Arabidopsis TGNs is well over 1 pH unit lower than the cytosol (82), implying that H^+ gradients could drive accumulation of Na^+ to close to molar concentrations. However, the small size of such compartments limits their capacity as sodium-storing organelles. The enhanced endocytosis and recycling under salt stress could imply enhanced flux of vesicle-enclosed fluid through these compartments. Enhanced endocytosis could thus act as a flushing mechanism for these organelles, bringing in extracellular fluid to dilute the luminal sodium concentration, whereas increased recycling from these compartments can, in turn, send the concentrated brine outside the cell (Fig. 1B). Alternatively, enhanced vacuolar trafficking may also deliver the sodium to the vacuole.

Conclusion

Endocytosis in animal systems mediates vital cellular functions like the uptake of nutrient molecules, control of signaling pathways, and elimination of pathogens. The study of endocytosis in plants is relatively nascent, with molecules regulating its machinery still being characterized. Cellular mechanisms including endocytosis need to respond to environmental fluctuations and adjust accordingly. Salt stress is one of the most deleterious abiotic stresses plants have to cope with, and plants have developed unique cellular mechanisms to survive this stress. One prominent example is sequestration of sodium

in the endomembrane system to keep the cytoplasm relatively sodium-free. The body of findings discussed in this review clearly demonstrates that vesicular trafficking is perturbed by salt stress in a manner that is probably critical for survival. However, several key aspects still need detailed exploration, especially the mechanism for sensing of sodium by the cell and process of transduction of that signal to the endocytic machinery. Detailed study is also required to incorporate the existing bits and pieces of information pertaining to the functional significance of endocytic mechanisms to formulate the integrated survival strategy that is triggered by salt stress and executed by vesicular trafficking machinery.

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