Ionic stress enhances ER–PM connectivity via phosphoinositide-associated SYT1 contact site expansion in *Arabidopsis*

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Edited by June B. Nasrallah, Cornell University, Ithaca, NY, and approved December 6, 2018 (received for review October 23, 2018)

The interorganelle communication mediated by membrane contact sites (MCSs) is an evolutionary hallmark of eukaryotic cells. MCS connections enable the nonvesicular exchange of information between organelles and allow them to coordinate responses to changing cellular environments. In plants, the importance of MCS components in the responses to environmental stress has been widely established, but the molecular mechanisms regulating interorganelle connectivity during stress still remain opaque. In this report, we use the model plant *Arabidopsis thaliana* to show that ionic stress increases endoplasmic reticulum (ER)–plasma membrane (PM) connectivity by promoting the cortical expansion of synaptotagmin 1 (SYT1)-enriched ER–PM contact sites (S-EPCSs). We define differential roles for the cortical cytoskeleton in the regulation of S-EPCS dynamics and ER–PM connectivity, and we identify the accumulation of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] at the PM as a molecular signal associated with the ER–PM connectivity changes. Our study highlights the functional conservation of EPCS components and PM phosphoinositides as modulators of ER–PM connectivity in eukaryotes, and uncovers unique aspects of the spatiotemporal regulation of ER–PM connectivity in plants.

ER–PM contact sites | SYT1 | ionic stress | phosphoinositides | cytoskeleton

Being sessile, plants cannot avoid the exposure to changing environmental conditions. As an adaptive response, plants have evolved robust regulatory mechanisms that sense extracellular signals and promote highly regulated responses to stress (1–3). At the cellular level, the stress responses in plants involve the coordinated action of different organelles, which is achieved by either vesicular trafficking using the endomembrane system (4–6) or by nonvesicular communication at interorganelle membrane contact sites (MCSs) (7–12). In plants, stress responses mediated by vesicular trafficking, such as the up-regulation of bulk flow endocytosis during salt and osmotic stresses, have been well characterized (13–15). However, relatively little is known about the stress responses mediated by nonvesicular communication at MCSs. The MCSs are widespread across eukaryotic lineages and can be structurally defined as narrow (10–30 nm) interorganelle junctions stabilized by protein tethers (10–12). In yeast and mammals, MCSs regulate basic eukaryotic functions such as lipid homeostasis maintenance and interorganelle Ca²⁺ exchange (7–9). In plants, MCS function has been associated with the coordination of cell-to-cell communication at plasmodesmata (16–18), the lipid transfer at ER–plastids and ER–mitochondria contact sites (19–21), and the integration of environmental and developmental signals at cortical endoplasmic reticulum (ER)–plasma membrane (PM) contact sites (EPCSs) (22–28), which is the focus of this study.

The EPCSs are a morphologically diverse set of cellular microdomains that physically connect the cortical ER (cER) and the PM. In *Arabidopsis*, the cER–PM connectivity is controlled by evolutionarily conserved MCS components such as the *Arabidopsis* synaptotagmins (SYTs) (23, 28), orthologs of the mammalian extended synaptotagmins (E-Syts) and yeast tricalbins (11), and the vesicle-associated membrane-associated protein 27s (VAP27s) and VAP27-related proteins (24, 26, 27), which are orthologs of the mammalian VAPs and yeast suppressor of choline sensitivity (Scs2) EPCS components (29). Plant EPCSs also contain unique components, such as the actin binding protein Networked 3C (30), that links VAP27-enriched EPCS (V-EPCS) with the cortical cytoskeleton, and could regulate plant-specific V-EPCS functions (22). To explain the mechanisms by which EPCSs control ER–PM connectivity, two nonexclusive mechanisms, namely the tethering arrangement and the tethering signature models, have been proposed (28).

Significance

Interorganelle connectivity and nonvesicular information transfer are hallmarks of biological systems. These processes facilitate communication between organelles, allowing them to adapt to changing cellular environments. In plants, the endoplasmic reticulum (ER)–plasma membrane (PM) contact sites (EPCSs) physically connect the cortical ER and the PM, and act as general platforms for Ca²⁺ homeostasis regulation and the cellular adaptation to environmental stresses. Our identification of ionic stress and PM phosphoinositides as enhancers of ER–PM connectivity advances our understanding of how stress influences interorganelle communication. Furthermore, our analyses of the spatiotemporal regulation of EPCS expansion highlights unique mechanisms that plants activate to maintain interorganelle communication during long-term exposure to environmental stress, not described in other eukaryotes.

Author contributions: A.R. designed research; E.L., J.P.-S., F.B.-F., M.S., and A.P.M. performed research; S.V. and A.P.M. contributed new reagents/analytic tools; E.L., S.V., J.P.-S., M.A.B., J.F., and A.R. analyzed data; and A.R. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1818099116/-DCSupplemental.

Published online January 4, 2019.

The tethering arrangement model states that ER–PM connectivity is controlled by the differential organization of EPCS tethering complexes in response to developmental cues and/or intracellular signals (e.g., Ca^{2+} or lipids). This model postulates that changes in the electrostatic interaction between the SYT1 lipid binding C2 domains and negatively charged phospholipids at the PM (23, 31) could change the SYT1-enriched EPCS (S-EPCS) morphology and influence ER–PM connectivity (28). The tethering signature model proposes that ER–PM connectivity is controlled by the presence of different tethering complexes and/or molecular interactions at individual EPCSs. This model is supported by the spatial separation of S-EPCS and V-EPCS within the same cell (32), and by protein–protein interaction studies showing that the SYT1 tethers establish transient and/or stable interactions with endomembrane trafficking components (25), cER structural regulators (33), phytosterol binding proteins (34), and receptor-like kinases (26).

In recent years, the functional characterization of EPCS components in *Arabidopsis* has uncovered their critical roles in essential cellular processes, including the activation of immune secretory pathways (25), the regulation of viral movement at plasmodesmata (16, 17, 35), the stabilization of the cER network (32), the Ca^{2+} -dependent responses to environmental stresses (23, 36, 37), endocytosis (35, 38), and pollen, seeds, and root hairs development (24). However, the environmental triggers and molecular mechanisms that regulate ER–PM connectivity at those EPCS microdomains remain unexplored. In this report, we used the model plant *Arabidopsis* to analyze the influence of long-term exposure to ionic stress, an environmental condition to which plants are often exposed to in nature, on the dynamics of S-EPCS components and ER–PM connectivity. Using stable transgenic lines expressing SYT1-GFP and the nonregulated membrane-attached peripheral ER (MAPPER)-GFP EPCS markers (39), we show that ionic stress enhances ER–PM connectivity by inducing reversible S-EPCS expansions along cER tubules. Using pharmacological approaches, we established that the stress-induced changes in ER–PM connectivity are cytoskeleton independent, and by using fluorescent lipid sensors we uncovered that the dynamics of S-EPCS expansion closely match the dynamics of phosphatidylinositol 4,5-bisphosphate [$\text{PI}(4,5)\text{P}_2$] accumulation at the PM. These results support the tethering arrangement model of S-EPCS organization, and highlight temporal and mechanistic differences between the fast (within minutes) stress-induced changes in ER–PM connectivity described in mammalian cells (40–42) and the slow (within hours) stress-induced increase in ER–PM connectivity observed in *Arabidopsis* seedlings. We propose that the changes in ER–PM connectivity in response to long-term exposure to ionic stress modulate the activity of EPCS-localized components (e.g., SYT1) and facilitate long-term plant adaptive responses to stress (e.g., exchanges of membrane lipids between the cER and the PM).

Results

Generation of a Constitutive EPCS Marker to Study ER–PM Connectivity in *Arabidopsis*. In plants, the study of ER–PM connectivity is limited by the small size of the EPCSs (100–300 nm long/10–30 nm wide) (12) and by the small number of bona fide EPCS-localized fluorescent markers. To partially overcome these limitations, we adapted the MAPPER marker, originally developed in mammalian cells (39), for use in *Arabidopsis*. The original MAPPER is a constitutive EPCS marker that nonselectively labels ER–PM junctions without significantly affecting their size, density, and functions (39, 40). The *Arabidopsis* version of MAPPER includes an internal GFP localized within the ER lumen, and maintains the original flexible linker of ~25-nm length and the C terminus lysine-rich polybasic domain that enables nonselective electrostatic interactions with negatively charged PM phosphoinositides (39) (Fig. 1A).

The subcellular localization of the MAPPER-GFP marker in stably transformed *Arabidopsis* epidermal cells was different from that of luminal ER marker GFP-HDEL (43) or the ER-membrane resident marker cinnamate 4-hydroxylase (C4H)-GFP (44), and strongly resembled that of the EPCS marker SYT1-GFP (23) (Fig. 1B–E). Indeed, colocalization analyses using a transgenic *Arabidopsis* line coexpressing SYT1-RFP and MAPPER-GFP markers showed that ~88% of the SYT1-RFP-labeled EPCSs were also labeled by MAPPER-GFP, and only ~12% of the SYT1-RFP-labeled EPCSs were devoid of MAPPER-GFP signal. The colocalization

Fig. 1. MAPPER-GFP labels EPCSs in *Arabidopsis*. (A) Schematic representation of the proposed topology and localization for MAPPER-GFP in *Arabidopsis* EPCSs. (B–E) Confocal microscopy images of *Arabidopsis* transgenic lines expressing the luminal ER marker GFP-HDEL (B), the ER membrane marker C4H-GFP (C), the artificial EPCS marker MAPPER-GFP (D), and the *Arabidopsis* EPCS marker SYT1-GFP (E). The confocal images were acquired in the cortical region of 5-d-old cotyledon epidermal cells. (F) Confocal microscopy image showing the colocalization of the SYT1-RFP and MAPPER-GFP EPCS markers in 5-d-old cotyledon epidermal cells. (G) Quantification of the SYT1-RFP, MAPPER-GFP, and SYT1-RFP/MAPPER-GFP fluorescent signals. The colocalization results show the percentage of SYT1-RFP and/or MAPPER-GFP signal identified on each individual channel ($n = 1,894$ signals from 28 independent cells). (Scale bars: B–E, 15 μm ; F, 20 μm .)

analyses also show that ~25.5% of the MAPPER-GFP-labeled structures were devoid of SYT1-RFP, supporting the presence of an EPCS fraction with different tether composition (32) (Fig. 1 *F* and *G*). Based on these results, we conclude that MAPPER-GFP, in *Arabidopsis*, is a nonselective EPCS marker that can be used to track EPCS responses and changes in ER–PM connectivity.

The SYT1-GFP and MAPPER-GFP Markers Redistribute Along cER Tubules in Response to NaCl Stress. In the presence of NaCl, the phenotype of the *syt1-2* (hereafter *syt1*) seedlings is characterized by a Ca^{2+} -dependent reduction in root elongation (36) (*SI Appendix*, Fig. S1*A*) and delayed cotyledon expansion (*SI Appendix*, Fig. S1*B*). Both phenotypes are linked to the SYT1 function as they are partially rescued in a SYT1-GFP complementation line generated by crossing the SYT1-GFP marker (23) with the *syt1* allele in Columbia background (36) (*SI Appendix*, Fig. S1*A* and *B*). Previous reports have shown that SYT1 acts as a regulator of the stability of the cER network in leaf epidermal cells (32) and controls the EPCS responses to mechanical stress in cotyledon epidermal cells (23); we questioned whether SYT1-mediated NaCl responses could also be observed in this cell type. To study these responses, we analyzed the effect of long-term NaCl exposure (16 h/100 mM NaCl) in the subcellular localization of the SYT1-GFP marker in the Col and *syt1* backgrounds. These

experiments were performed in 5-d-old cotyledon epidermal cells as they (*i*) display mechanical and NaCl hypersensitivity in the *syt1* background, (*ii*) have a fully reticulated cER network that enables accurate localization of SYT1-GFP within specific cER subdomains, and (*iii*) have a flattened surface that is amenable for EPCS imaging. Our results show that the NaCl treatment has a general effect on S-EPCS organization as the localization of the SYT1-GFP marker transitions from an open “beads-and-strings” pattern, which likely labels cER subdomains as described in *Nicotiana benthamiana* (23) (Fig. 2 *A* and *B*), to an expanded “closed-reticule” arrangement that resembles that of the ER membrane marker C4H-GFP shown in Fig. 1*C* (Fig. 2 *E* and *F*). The shift from the beads-and-strings to the expanded closed-reticule configuration was also observed in the MAPPER-GFP/Col background, indicating that the S-EPCSs expand in response to NaCl stress (Fig. 2 *C* and *G*), and was also observed in the MAPPER-GFP/*syt1* background, indicating that additional molecular components are involved in the NaCl-induced S-EPCS remodeling and that the NaCl hypersensitivity of *syt1* is not directly linked with S-EPCS expansion defects (Fig. 2 *D* and *H* and *SI Appendix*, Fig. S2). It is worth noting that the NaCl treatment not only altered the ER–PM connectivity but also had an effect on the morphology of the underlying cER. This effect was characterized by a dosage-dependent remodeling of

Fig. 2. NaCl promotes S-EPCS expansion and increases ER–PM connectivity. (*A–H*) Effect of 100 mM NaCl treatments in the cortical localization of the SYT1-GFP and MAPPER-GFP markers stably expressed in either Col-0 or *syt1* mutant backgrounds. Before imaging, 5-d-old seedlings were treated in liquid 1/10-strength MS (mock) (*A–D*) or liquid 1/10-strength MS supplemented with 100 mM NaCl for 16 h (100 mM NaCl) (*E–H*). Confocal images were acquired at the cortex of cotyledon epidermal cells. (*I* and *J*) Schematic representation of the subcellular localization of the GFP-HDEL/SYT1-RFP (*I*) and RFP-HDEL/MAPPER-GFP (*J*) markers in the double-transgenic lines used for colocalization. (*K–N*) Colocalization pattern of the GFP-HDEL/SYT1-RFP and RFP-HDEL/MAPPER-GFP markers in mock and NaCl treated seedlings. Before imaging, 5-d-old transgenic GFP-HDEL/SYT1-RFP or RFP-HDEL/MAPPER-GFP seedlings were treated in liquid 1/10-strength MS (mock) (*K* and *L*) or liquid 1/10-strength MS supplemented with 100 mM NaCl for 16 h (*M* and *N*). (Scale bars: *A–H*, 10 μm ; *K–N*, 15 μm .)

the cER network that increases the average surface area of the closed cER reticules labeled by GFP-HDEL (*SI Appendix, Fig. S3*).

To visualize the NaCl-induced EPCS expansion within the context of the cER network, we performed colocalization analyses of a transgenic line coexpressing the SYT1-RFP and GFP-HDEL markers (Fig. 2*J*), and a transgenic line coexpressing MAPPER-GFP and RFP-HDEL markers (Fig. 2*J*). The colocalization results show that the SYT1-RFP and MAPPER-GFP beads-and-strings pattern marks discrete EPCSs at cER subdomains (Fig. 2*K* and *L*), and that the SYT1-RFP and MAPPER-GFP closed-reticule configuration represents EPCS expansions along the cER tubules (Fig. 2*M* and *N*). Combined, these results indicate that NaCl stress increases ER-PM connectivity by redistributing SYT1 and likely additional EPCS tethers along cER tubules, and that the *sytl* phenotypes are the result of an uncharacterized SYT1 function within the expanded S-EPCS that goes beyond its described role as cER-PM tether.

Once we established that NaCl increases cER-PM connectivity and promotes S-EPCS expansion, we inquired whether this effect was a specific cellular response to NaCl toxicity or a general response to osmotic and/or ionic imbalance. To address this question, we analyzed the effect of different osmotic stress generators and ionic stress-inducing agents in the subcellular localization of the SYT1-GFP marker. Treatments with the osmotic stress generators mannitol and sorbitol largely maintained the beads-and-strings configuration and had limited effect in SYT1-GFP distribution (*SI Appendix, Fig. S4 A–C* and *J*). In contrast, treatments with ionic stress generators induced a slow transition (within hours) of the SYT1-GFP marker from the beads-and-strings to the closed-reticule configuration (*SI Appendix, Fig. S4 D–J*). We conclude that ionic imbalance within the cell is responsible for the SYT1-mediated increases in ER-PM connectivity.

The SYT1-GFP Dynamics Within the cER Network Are Cytoskeleton Dependent. In *Arabidopsis* cells, ionic stress negatively affects cytoskeletal dynamics because they promote microtubule disassembly and actin filament reorganization (45). Based on this effect, we hypothesized that either the NaCl-induced S-EPCS expansion requires cytoskeleton disassembly or it is a cytoskeleton-independent process. Both hypotheses, however, seemingly contradict reports showing that interactions between EPCS components and the cortical cytoskeleton are critical for proper EPCS dynamics (22, 24). To address this apparent dichotomy, we aimed at dissecting the specific roles of the cortical cytoskeleton in the control of SYT1-GFP dynamics, and in the regulation of the NaCl-induced S-EPCS expansion. First, we analyzed the dynamics of the SYT1-GFP marker at the cER using fluorescence recovery after photobleaching (FRAP) assays. In these experiments, we compared the fluorescence recovery rate of the SYT1-GFP marker in 5-d-old seedlings transferred to 1/10× Murashige and Skoog (MS) medium, with that of the SYT1-GFP marker in seedlings transferred to 1/10× MS medium supplemented with either 100 mM NaCl, 25 μM of the microtubule polymerization inhibitor oryzalin (46), or 1 μM of the F-actin depolymerizing drug latrunculin B (LatB) (47). In these experiments, we used the ER-membrane marker C4H-GFP as an EPCS-independent control. The FRAP results show that the fluorescence recovery rates of SYT1-GFP and C4H-GFP were similar in the 1/10× MS medium used as control (60–70% recovery after 300 s) (Fig. 3*A*), and that the 100 mM NaCl treatment had little influence in the recovery rate of the C4H-GFP signal (50–60% recovery after 300 s) but strongly reduced the recovery rate of the SYT1-GFP signal (0–5% recovery after 300 s) (Fig. 3*B*). Similarly, the FRAP results in the presence of 25 μM oryzalin showed that the microtubule depolymerization induced by oryzalin had little effect on the fluorescence recovery rate of the C4H-GFP marker (50–60% recovery after 300 s) but completely

abolished the fluorescence recovery of the SYT1-GFP signal (0–5% recovery after 300 s) (Fig. 3*C*). Last, the FRAP results in the presence of 1 μM LatB show that the disassembly of the actomyosin cytoskeleton delayed the fluorescence recovery of the C4H-GFP signal (25–35% recovery after 300 s) and completely abolished the fluorescence recovery of the SYT1-GFP signal (0–5% recovery after 300 s) (Fig. 3*D*). These results collectively support a model where functional microtubule and F-actin networks are required to maintain the dynamics of the SYT1-GFP marker within the cER, and where the reduced delivery and/or lateral diffusion of SYT1 to/at S-EPCS upon NaCl stress could be explained by defects in microtubule assembly and actin filament organization.

The Stress-Induced S-EPCS Expansion Is Cytoskeleton Independent.

The FRAP experiments provide important information about the regulation of the SYT1-GFP dynamics under NaCl stress, but they are not sufficient to establish whether a functional cortical cytoskeleton is required for S-EPCS expansion. After NaCl treatment, the expanded S-EPCSs labeled by SYT1-GFP (Fig. 4*A, B, I*, and *J*) were present in a cortical environment where the microtubules are largely depolymerized, as indicated by the microtubule marker GFP-TUA6 (48) (Fig. 4*E* and *F*), and the actin filaments are bundled, as indicated by the actin cytoskeleton marker GFP-ABD2 (49) (Fig. 4*M* and *N*). Based on this observation, we hypothesized that the cytoskeleton disassembly and/or remodeling could be a trigger for S-EPCS expansion. However, our results show that the cytoskeleton disassembly induced by oryzalin (Fig. 4*C* and *G*) or LatB (Fig. 4*K* and *O*) was not sufficient to induce the redistribution of SYT1-GFP along the cER tubules. Remarkably, our results also show that a pretreatment of SYT1-GFP seedlings with oryzalin (Fig. 4*D* and *H*) or LatB (Fig. 4*L* and *P*) before the 16-h NaCl treatment did not abrogate the NaCl-induced S-EPCS expansion. Based on these results, we conclude that the NaCl-induced S-EPCS expansion can take place in the absence of a functional cortical cytoskeleton, but this approach cannot establish whether the cytoskeleton disassembly/remodeling is one of the prerequisites for S-EPCS expansion (see next section). It is also important to mention that the LatB treatment used in this study is known to cause severe defects in the organization of the perinuclear ER (23) that could, as a secondary effect, influence S-EPCS expansion at the cell cortex.

The Accumulation of Phosphoinositides at the PM Is Associated with S-EPCS Expansion. Our results suggest that NaCl stress enhances ER-PM connectivity using a cytoskeleton-independent mechanism. However, the molecular components regulating the NaCl-induced SYT1 redistribution along the cER tubules remain uncharacterized. The current model of ER-PM tethering at S-EPCS postulates that SYT1 is anchored to the cER via its transmembrane domain, and it connects the PM through electrostatic interactions between its C2 phospholipid binding domains and PM-localized phosphoinositides (23, 31). Given that NaCl stress induces the accumulation of negatively charged phosphoinositides at the PM (50–52), we hypothesized that the accumulation of specific phosphoinositide species at the PM could promote the docking of SYT1 to the PM, effectively increasing the cER-PM connectivity. To test this hypothesis, we examined the effect of NaCl stress in the PM accumulation of two phosphoinositides, namely phosphatidylinositol 4-phosphate (PI4P), which is the larger contributor to the PM electrostatic field in *Arabidopsis* (53), and PI(4,5)P₂, which is a known interactor of the mammalian E-Syts tethers (42) and also binds SYT1 in vitro (23). As proxies to measure endogenous PI4P and PI(4,5)P₂ levels in *Arabidopsis*, we used the fluorescent phosphoinositide sensors CITRINE 1xPH^{FAPP} (for PI4P) and CITRINE 2xPH^{PLC} [for PI(4,5)P₂] (52). In these measurements, an increase in the

Fig. 3. The dynamics of the SYT1-GFP marker at the cER is cytoskeleton dependent. (A–D) Representative FRAP experiments using the ER membrane marker C4H-GFP and the S-EPCS marker SYT1-GFP subject to different treatments. Five-day-old *Arabidopsis* seedlings were mock-treated with 1/10-strength MS medium (A) or treated with 1/10-strength MS medium NaCl supplemented with 100 mM NaCl, 16 h (B), 25 μ M oryzalin, 16 h (C), or 1 μ M LatB, 2 h before FRAP. In the graphs in A and B, the dotted lines represent the fluorescence signal recovery for the mock treatment (1/10 MS), and the solid lines represent the fluorescence signal recovery for the 100 mM NaCl treatment. In the graphs in C and D, the dotted lines represent the fluorescence signal recovery for the mock treatment (1/10 MS + DMSO), and the solid lines represent the oryzalin and LatB treatments. All fluorescence recovery data points represent mean $\pm$ SD from at least six independent FRAP experiments. The resulting mean values were subject to nonlinear curve fitting. (Scale bars: 10 μ m.)

fluorescent intensity of the sensor represents an increase in their residence time at the target membrane due to the accumulation of its lipid partners (52). Using these sensors, we showed that a NaCl treatment that induced S-EPCS expansion (100 mM NaCl/16 h) did not change the fluorescence intensity of the CITRINE-1xPH^{FAPP} (PI4P) sensor (Fig. 5 A, B, and G), but induced a fourfold to fivefold increase in the fluorescence of the CITRINE-2xPH^{PLC} [PI(4,5)P₂] sensor (Fig. 5 D, E, and G).

Since the fluorescent intensity of the CITRINE-2xPH^{PLC} sensor increases upon NaCl stress, we hypothesized that the accumulation of PI(4,5)P₂ in the PM facilitates the docking of SYT1 and underlies the SYT1-mediated S-EPCS expansion. To

test this hypothesis, we generated artificial accumulations of PI(4,5)P₂ at the PM using the phosphoinositide-dependent phospholipase C (PI-PLC) inhibitor U-73122 (SI Appendix, Fig. S5) (54, 55) and evaluated its effect in the subcellular localization of the SYT1-GFP and MAPPER-GFP markers. Similarly to the NaCl treatment, the U-73122 treatment did not change the fluorescence intensity of the CITRINE-1xPH^{FAPP} sensor (Fig. 5 A, C, and G) but induced threefold to fourfold accumulations of the CITRINE-2xPH^{PLC} sensor (Fig. 5 D, F, and G). Remarkably, the U-73122 treatment mimicked the effect of NaCl in S-EPCS expansion, as indicated by the transition of the SYT1-GFP (Fig. 5 H and I) and the MAPPER-GFP (Fig. 5 J and

Fig. 4. The S-EPCS expansion follows cytoskeleton-independent dynamics. (A–H) Confocal images of *Arabidopsis* seedlings carrying the SYT1-GFP and GFP-TUA6 markers subject to different treatments. Five-day-old transgenic seedlings grown in 1/10× MS were transferred to liquid 1/10× MS, 16 h (A and E), or the same media supplemented with 100 mM NaCl, 16 h (B and F), 25 μ M oryzalin, 16 h (C and G), or sequentially treated with 25 μ M oryzalin, 16 h, followed by 100 mM NaCl, 16 h (D and H) before imaging. (I–P) Confocal images of *Arabidopsis* seedlings carrying the SYT1-GFP and GFP-ABD2 markers subject to different treatments. Five-day-old transgenic seedlings grown in 1/10× MS were transferred to liquid 1/10× MS, 16 h (I and M), or the same media supplemented with 100 mM NaCl, 16 h (J and N), 1 μ M LatB, 2 h (K and O), or sequentially treated with 1 μ M LatB, 2 h, followed by 100 mM NaCl, 16 h (L and P) before imaging. (Scale bars: 20 μ m.)

K) markers from the open beads-and-strings configuration to the closed-reticulate arrangement (*SI Appendix, Fig. S6*) without causing cytoskeleton disassembly (*SI Appendix, Fig. S7 A–D*). Based on these results, we deduce that (i) the accumulation of PI(4,5)P₂ is associated with the S-EPCS expansion, and (ii) the cytoskeleton disassembly is not a prerequisite for S-EPCS expansion.

The Changes in ER–PM Connectivity Are Reversible and Correlate with the Kinetics of PI(4,5)P₂ Accumulation at the PM. In mammalian cells, the stress-induced changes in ER–PM connectivity take place within minutes and critically depend on the concentration of PI(4,5)P₂ at the PM membrane (42). Since our results show that, in *Arabidopsis*, the stress-induced changes in ER–PM connectivity are slow (within hours) and can be mimicked by artificial accumulations of PI(4,5)P₂ at the PM, we asked whether the kinetics of the NaCl-induced changes in connectivity correlate with the kinetics of PI(4,5)P₂ accumulation in vivo. To answer this question, we estimated the percentage of epidermal cells in which SYT1-GFP acquires the closed-reticulate configuration (*SI Appendix, Fig. S8*) and the accumulation of PI(4,5)P₂ at different time points. Our results show that both NaCl-induced changes in ER–PM connectivity and PI(4,5)P₂ accumulation are relatively slow processes (within hours). Thus, the T₅₀ for reticulation (the time in which 50% of the cells display the closed reticulate configuration) is 6.2 ± 1.8 h, and the T₅₀ for PI(4,5)P₂ accumulation [the time in which the cells reach 50% of the maximum PI(4,5)P₂

accumulation] is 8.5 ± 2.7 h (Fig. 6A and B). Remarkably, NaCl did not induce PI4P accumulation at any time point (Fig. 6C), which precludes the presence of transient accumulations of PI4P as potential trigger for S-EPCS expansion. In our final experiment, we asked whether the effects of NaCl in ER–PM connectivity and PI(4,5)P₂ accumulation were reversible. To answer this question, we performed a 24-h NaCl washout experiment using the SYT1-GFP marker and the CITRINE-2xPH^{PLC} sensor. Our results show, that the NaCl-induced increase in ER–PM connectivity is a reversible process as the original beads-and-strings configuration and basal PI(4,5)P₂ accumulation were almost fully recovered 24 h after the NaCl stress removal (Fig. 6D–F).

Combined, the results of this study provide evidence for a model in which the cortical cytoskeleton is essential for the dynamics of the SYT1-GFP marker within the cER (Fig. 7A), and where NaCl induces reversible and cytoskeleton-independent S-EPCS expansion that increase in ER–PM connectivity. We postulate that the S-EPCS expansion is mediated by the accumulation of PI(4,5)P₂, which facilitates the docking of SYT1 to the PM (Fig. 7B). As indicated previously, this study cannot rule out that additional EPCS tethers, and negatively charged lipid species (e.g., phosphatidylserine or phosphatidic acid), could be important regulators of ER–PM connectivity in plants.

Discussion

The Increase in ER–PM Connectivity as a General Response to Ionic Stress in Plants. The cellular adaptation to a changing ionic status requires coordinated interorganelle responses aimed at transducing the stress signals and maintaining the integrity of cellular membranes (1–4). Our study supports a model in which S-EPCSs act as stress-sensitive microdomains, whose expansion facilitates the exchange of information between the ER and the PM. Given that the molecular components controlling ER–PM connectivity in mammalian (E-Syts) and plant (SYTs) cells are evolutionarily conserved, we hypothesize that these components could carry out equivalent biochemical functions. Thus, the mammalian E-Syts are multifunctional proteins that regulate ER–PM connectivity in response to stress-derived Ca²⁺ signals (40–42), facilitate the nonvesicular transfer of glycerolipids and phosphoinositides between the ER and the PM (56–59), and control the receptor abundance during signal transduction (60). Likewise, the *Arabidopsis* SYT1 may be a multifunctional protein that participates in the regulation of ER–PM connectivity by controlling the Ca²⁺-dependent activity of its phospholipid binding C2 domains (23, 42), facilitates the nonvesicular transfer of lipids via its putative Synaptotagmin-like mitochondrial lipid-binding protein domain (SMP) (56, 57), and regulates receptor-mediated signaling pathways through physical interactions with receptor-like kinases (26). Furthermore, our results indicate that SYT1 is not required for S-EPCS expansion during NaCl stress and suggests additional SYT1 functions beyond cER–PM tethering, as the underlying cause for the NaCl hypersensitivity observed in the *syt1* mutant. Nevertheless, we cannot exclude that SYT1 tethering and redistribution along the cER tubules provide the positional information that is required to regulate those SYT1 functions during NaCl stress.

Notably, both the EPCS components and the lipid species controlling ER–PM connectivity seem to be evolutionarily conserved in eukaryotes. For instance, the phosphoinositide-associated S-EPCS expansion triggered by ionic stress in *Arabidopsis*, strongly resembles the phosphoinositide-driven E-Syts EPCS expansion in mammalian cells (42). However, our study uncovers fundamental differences in the regulation of ER–PM connectivity between mammalian and plant cells. For example, in mammalian cells, the stress-induced E-Syt1 EPCS expansions occur within minutes and are triggered by store-operated Ca²⁺ entry (SOCE) (40–42). In contrast, the slow ER–PM connectivity changes in *Arabidopsis*

Fig. 5. The PI(4,5)P₂ accumulation at the PM is associated with S-EPCS expansion. (A–F) Confocal images of *Arabidopsis* cotyledon epidermal cells expressing CITRINE-tagged 1xPH^{FAPP1} (A–C) and CITRINE-tagged 2xPH^{PLC} (D–F) treated with 1/10× MS, 16 h (A and D), or the same media supplemented with 100 mM NaCl, 16 h (B and E), or 50 μM U-73122, 16 h (C and F). The pictures are color-coded in pixel intensity following the LUT scale shown in C. (G) Quantification of the CITRINE-tagged 1xPH^{FAPP1} and CITRINE-tagged 2xPH^{PLC} fluorescent signal relative to mock conditions. In the plot, the center line represents the median fluorescence intensity fold increase relative to mock, the cross represents the mean fluorescent intensity, the top and bottom edges are the 25th and 75th percentiles of the distribution, and the ends of the whiskers are set at 1.5 times the interquartile range (IQR). All values outside the IQR are shown as outliers. At least 100 regions of interest (ROIs) were measured for each treatment. The blue asterisks indicate statistically significant differences with the mock treatment, Student's *t* test (*P* < 0.05). (H–K) Effect of the U-73122 treatment in S-EPCS expansion. Confocal images of 5-d-old *Arabidopsis* cotyledon epidermal cells expressing the SYT1-GFP (H and I) or the MAPPER-GFP (J and K), markers incubated for 16 h in liquid 1/10× MS media, or the same media supplemented with 50 μM U-73122 before imaging. (Scale bars: A–F, 40 μm; H–K, 20 μm.)

(within hours) are not consistent with a direct regulation by (Ca²⁺)_{cyt} as the transient (Ca²⁺)_{cyt} signature induced by ionic stress generally occur within minutes (61) and essential SOCE components, such as homologs of the mammalian STIM proteins, are not encoded in the *Arabidopsis* genome (62). Still, we cannot rule out that the S-EPCS expansion is a downstream response to the transient (Ca²⁺)_{cyt} increases induced by NaCl stress.

Cortical Cytoskeleton Requirements for S-EPCS Dynamics and ER–PM Connectivity. In plant cells, the remodeling of the cER network is regulated by biophysical processes (63, 64), cER–actomyosin interactions (64–66), close associations between the cortical cytoskeleton and EPCS components [e.g., VAP27/NET3C complexes (22, 32)], and, to a minor extent, cER–microtubule interactions (67). In this study, we assign specific roles for the cortical cytoskeleton in the remodeling of S-EPCS and ER–PM connectivity in response to NaCl stress. In agreement with previous reports that describe close associations between V-EPCS tethers and the cortical cytoskeleton (22, 32), our study shows that the cortical cytoskeleton is indispensable for the proper delivery and/or lateral diffusion of SYT1 tethers to S-EPCS. However, our pharmacological results also reveal that the increase in ER–PM connectivity can occur in a depolymerized cytoskeleton environment, suggesting that the mechanisms controlling S-EPCS expansion are largely cytoskeleton independent. The limited role for the cytoskeleton on S-EPCS expansion is also supported by the slow dynamics of the process (minimum lag of 4–8 h) that are difficult to reconcile with the short time frames in which ionic stress evokes cytoskeleton-mediated cER remodeling (minutes) (45, 68, 69). In analogy with the mammalian system, we propose that the fast (within minutes)

cytoskeleton-dependent delivery of SYT1 tethers to the cell cortex, is the plant equivalent of the F-actin–dependent dynamics of E-Syt tethers in mammalian cells (70), and that the cytoskeleton-independent S-EPCS expansion represents a plant-specific response to facilitate ER–PM communication and/or regulate signaling events associated to the long-term exposure to ionic stress.

A Role for PM Phosphoinositides in S-EPCS Expansion and cER Remodeling. In plants, multiple components involved in cER tubulation and cisternalization have been already identified (71, 72), but questions remain as to how the cER regulates its plasticity and modifies the interorganelle contacts in response to environmental stress. In mammals, it has been proposed that stress-induced PM depolarization triggers cER remodeling and EPCS expansion (41). In *Arabidopsis*, this electrostatic model could partially explain our observation that S-EPCS expansion occurs in the presence of ionic osmolytes that induce strong PM depolarization (e.g., NaCl; ref. 73), but not in the presence of nonionic osmolytes that induce PM hyperpolarization (e.g., mannitol; ref. 74). At the molecular level, local changes in the PM electrostatic field could facilitate the molecular interaction between negatively charged phosphoinositides at the PM inner leaflet and the K/R-rich polybasic clusters that potentially confer electropositive potential to the SYT1 C2 domains (75). This electrostatic mechanism is not exclusive for EPCS components as changes in the PM phospholipid composition are sufficient to determine the PM localization of other plant proteins with cationic stretches (76). Against a purely electrostatic model of ER–PM connectivity, we could argue that PI(4,5)P₂, the only phosphoinositide associated to S-EPCS expansion, is present in very

Fig. 6. The NaCl-induced S-EPCS expansion is reversible, and its kinetics resembles that of the PI(4,5)P₂ accumulation at the PM. (A–C) Time course analyses of SYT1-GFP reticulation (A), PI(4,5)P₂ accumulation (B), and PI4P accumulation (C) in response to NaCl treatments. At least 100 ROIs from 10 independent seedlings were used to determine the percentage of cells undergoing S-EPCS expansion and the relative accumulations of PI(4,5)P₂ and PI4P at any given time point. (D–F) S-EPCS expansion and PI(4,5)P₂ washout experiment, 5-d-old SYT1-GFP and CITRINE-tagged 2xPH^{PLC} seedlings grown on 1/10× MS solid media were transferred for 16 h to 1/10× MS liquid media (D) or the same media supplemented 100 mM NaCl (E). Upon treatment, the seedlings were transferred to fresh 1/10× MS liquid media for an additional 24 h (F). Images are from cotyledon epidermal cells. Percentages represent the fraction of reticulated structures ($n = 30$ cells) and the relative fluorescent intensity of the CITRINE-tagged 2xPH^{PLC} marker ($n = 300$ ROIs). (Scale bars: 20 μm.)

low abundance at the PM and likely has little influence in the polarization status of the PM (53). Thus, if changes to the PM electrostatic field underlie S-EPCS expansion, the contribution of additional phospholipids to the binding of the SYT1 C2 domains in vivo [e.g., PA or PS (23, 36)] must be required. The requirement for additional phospholipids during S-EPCS expansion could also explain the observed lag in the timing of PI(4,5)P₂ accumulation compared with the timing of S-EPCS expansion, and the Ca²⁺-dependent affinity of the SYT1 C2 domains for different negatively charged phospholipids such as PS and PA observed in vitro (23, 36). Therefore, a more detailed characterization of phospholipid accumulation and S-EPCS expansion in individual cells is needed to unequivocally establish the individual contribution of different lipid species in S-EPCS expansion.

Finally, our study highlights the importance of protein–lipid interactions for the regulation of ER–PM connectivity; however, the downstream mechanisms controlled by this process in plants are not known. In mammalian cells, changes in ER–PM connectivity are required for the homeostatic regulation of PI(4,5)P₂ signaling (77). Interestingly, PI(4,5)P₂ signaling is controlled by a C2-containing PI-PLC in *Arabidopsis*, and it is possible that the SYT1 C2 domains modulate this signaling pathway by promoting competitive binding for the PI(4,5)P₂ substrate. Alternatively, the enhanced ER–PM connectivity could be required to facilitate the nonvesicular transfer of signaling lipids as described in *Drosophila* photoreceptor EPCSs (78), or to regulate the transfer of glycerolipids between the cER and the PM as described in

mammalian cells (79). These disparate possibilities highlight the importance of further exploring the functional relationships that link ionic stress and ER–PM connectivity to understand the cellular mechanisms that regulate the plant responses to environmental stress.

Materials and Methods

Plant Materials and Growth Conditions. *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild-type and *syt1-2* mutant allele as the background of the SYT1-GFP complementation line. Previously published lines used in this study are SYT1-GFP (23), GFP-HDEL (43), C4H-GFP (44), GFP-TUA6 (48), GFP-ABD2 (49), CITRINE-2xPH^{PLC} (52), and CITRINE-1xPH^{FAPP} (52). Plants were grown on half-strength of MS media (Caisson Labs) or soil (Sunshine mix #4; Sun Gro Horticulture Canada) at 22 °C with a 16-h light/8-h dark cycle.

Cloning and Transformation. The MAPPER coding sequence was cloned into pDONR221 via BP cloning using a PCR fragment generated using ATTB1-MAPPER and ATTB2-MAPPER primers (SI Appendix, Table S1). The MAPPER clone that served as a template for the PCR was kindly provided by Dr. Jen Liou (University of Texas Southwestern). The resulting pEN_MAPPER was sequence confirmed and subcloned with pEN-L4-UBQ10-R1 into pB7m24GW (80) to generate UBQ10::MAPPER. To generate *proUBQ10::SYT1-tagRFP*, the 1,737 bp of SYT1 cDNA was amplified via PCR using RT-PCR product as a template using the cSYT1-F and cSYT1-R primers (SI Appendix, Table S1), followed by cloning PCR products into pENTR/TOPO (Life Technologies) and then recombined into a UBQ10 promoter-driven C-terminal tagRFP fusion destination binary vector (81). All constructs were introduced into wild-type or GFP-HDEL plants using floral dipping (*Agrobacterium* strain GV3101) (82). Transgenic lines were selected on half-strength MS medium containing 15 μg/mL glufosinate-ammonium (Sigma-Aldrich).

Chemical Applications. Five-day-old seedlings were incubated in liquid 1/10-strength MS media containing 100 mM NaCl, 100 mM KCl, 50 mM MgCl₂, 50 mM Na₂SO₄, 50 mM K₂SO₄, 200 mM mannitol, 200 mM sorbitol, 50 μM U-73122, or 25 μM Oryzalin for 16 h. The 1 μM LatB treatment was performed for 2 h. All reagents were purchased from Sigma (<https://www.sigmaaldrich.com/>) except for LatB (Abcam; <https://www.abcam.com/>).

Image Acquisition and Quantitative Analyses. Living cell images were obtained using a Nikon C1 confocal laser-scanning microscope, an Olympus FV1000 multiphoton confocal laser-scanning microscope (www.olympus-lifescience.com/de/), and a Perkin-Elmer spinning-disk confocal microscope (www.perkinelmer.com). The Nikon C1 confocal laser-scanning microscope was equipped with 488- and 515/30-nm emission filter and Nikon Plan Apochromat oil immersion objectives (40×, 1.0 N.A., and 60×, 1.4 N.A., respectively) (<https://www.nikoninstruments.com>). The Olympus FV1000 was equipped with 405-, 473-, and 559-nm lasers and a 60× oil Planon (60×, 1.4 N.A.), and the Perkin-Elmer spinning-disk confocal microscope was equipped with 488- and 561-nm lasers. Images were captured using Nikon-EZ C1, Olympus FV1000, and Velocity software, respectively. For colocalization analyses, we applied the pixel intensity correlation over space method, automatic thresholding, and statistical significance testing of the Fiji's COLOC2 plugin (83). To quantify the change from the beads-and-strings configuration to the closed-reticulate configuration, 5-d-old *Arabidopsis* seedlings harboring the SYT1-GFP or MAPPER-GFP marker were incubated for 16 h in liquid 1/10-strength MS medium (Mock) or liquid 1/10-strength MS medium supplemented with various chemicals inducing ionic or osmotic stresses, or with U-73122. For each treatment, the number of closed reticules labeled by SYT1-GFP or MAPPER-GFP in the cortex of cotyledon epidermal cells was scored in at least 50 (15 × 15 μm) regions of interest (ROIs) from four to six independent seedlings using the cell counter tool of Fiji (ImageJ) (NIH; <https://imagej.nih.gov/ij/>) (83). To compare the fluorescent intensity of the CITRINE-2xPH^{PLC} or CITRINE-1xPH^{FAPP} sensors between control and treated samples, confocal laser-scanning images of 5-d-old epidermal cotyledon cells were acquired from at least 10 individual seedlings. For each data point, the fluorescent intensity data were scored from at least 100 (20 × 15 μm) ROIs using the integrated density measurement tool of Fiji (83). In this analysis, stomatal lineage cells were excluded from the quantification. The fluorescent data were normalized using the equation: $\Delta F/F = (F - F_0)/F_0$, where F_0 is the mean intensity of background fluorescence. The data were subject to Student's *t* test to identify statistical differences among treatments. All statistical analyses were performed using the GraphPad Prism 5.0b software (<https://www.graphpad.com/>).

Fig. 7. NaCl stress induces phosphoinositide-mediated S-EPCS expansion and increases ER-PM connectivity. (A) In normal conditions, the SYT1 tether is delivered to S-EPCSs in a cytoskeleton-dependent manner and S-EPCSs are formed by the interaction of SYT1, and likely other tethering proteins with negatively charged phosphoinositides already present at the inner leaflet of the PM. (B) NaCl stress induces microtubule depolymerization and F-actin bundling that inhibits the delivery of SYT1-GFP to the cell cortex. The NaCl stress also induces PI(4,5)P₂ accumulations at inner leaflet of the PM (red). The accumulation of PI(4,5)P₂, alone or in coordination with other phospholipids (e.g., PS), facilitates the binding of the SYT1 C2 domains. The binding of SYT1, and likely additional S-EPCS tethers, along cER tubules promotes S-EPCS expansion and effectively increases the ER-PM connectivity as a subcellular response to sustained exposure to NaCl stress.

FRAP Experiments. The FRAP analyses were performed as described in ref. 23; a single bleaching scan using a 405-nm diode laser was employed and the images were collected before and after bleaching using low-laser intensities and monitored FRAP during 15 min with a delay of 20 s between frames. Fluorescent recovery of the rectangle ROI was quantified using the integrated density measurement tool of ImageJ (NIH; <https://imagej.nih.gov/ij/>). Representative images were generated using Adobe Photoshop and Adobe Illustrator (<https://www.adobe.com/>). Fluorescence intensity data were normalized using the following equation: $I_n = ((I_t - I_{min}) / (I_{max} - I_{min})) \times 100$, where I_n is the normalized intensity, I_t is the intensity at any time t , I_{min} is the minimum intensity postphotobleaching, and I_{max} is the mean intensity before photobleaching (84). All quantitative values represent means from at least six cells. All nonlinear curve fitting and the statistical comparisons were performed in GraphPad Prism 5.0b (<https://www.graphpad.com/>).

ACKNOWLEDGMENTS. We thank our undergraduate students Melissa Ko, Geety Hafizi, and Shelley Wu for technical support. We thank Dr. Geoffrey Wasteneys (University of British Columbia) for the UBQ10 promoter-driven C-terminal *tagRFP* fusion destination binary vector, Dr. Jen Liou (University of Texas Southwestern) for providing the MAPPER-GFP template, and Dr. Lacey Samuels and Dr. Mathias Schuetz (University of British Columbia) for their scientific insights and the gift of C4H-GFP and RFP-HDEL markers. We also thank Elizabeth Samuels and Laryssa Halat for help proofreading the manuscript. This research was undertaken thanks to funding from Natural Sciences and Engineering Research Council of Canada Discovery Grant RGPIN-2014-06468 (to E.L. and A.R.), a postdoctoral research fellowship of the Research Foundation-Flanders (to S.V.), a Formación del Personal Investigador Fellowship from Ministerio de Economía y Competitividad FPI-BES 2012-052542 (to J.P.-S.), Ministerio de Ciencia y Tecnología Grant BIO2017-82609-R (to M.A.B.), the Chinese Academy of Sciences and the Chinese 1000 Talents Program (A.P.M.), and the Canada Research Chairs Program (A.R.).

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