A PtdIns(4)P-driven electrostatic field controls cell membrane identity and signalling in plants

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Many signalling proteins permanently or transiently localize to specific organelles. It is well established that certain lipids act as biochemical landmarks to specify compartment identity. However, they also influence membrane biophysical properties, which emerge as important features in specifying cellular territories. Such parameters include the membrane inner surface potential, which varies according to the lipid composition of each organelle. Here, we found that the plant plasma membrane (PM) and the cell plate of dividing cells have a unique electrostatic signature controlled by phosphatidylinositol-4-phosphate (PtdIns(4)P). Our results further reveal that, contrarily to other eukaryotes, PtdIns(4)P massively accumulates at the PM, establishing it as a critical hallmark of this membrane in plants. Membrane surface charges control the PM localization and function of the polar auxin transport regulator PINOID as well as proteins from the BRI1 KINASE INHIBITOR1 (BKII)/MEMBRANE ASSOCIATED KINASE REGULATOR (MAKR) family, which are involved in brassinosteroid and receptor-like kinase signalling. We anticipate that this PtdIns(4)P-driven physical membrane property will control the localization and function of many proteins involved in development, reproduction, immunity and nutrition.

Each membrane compartment has a unique identity and thereby recruits a specific set of proteins. It has been established for decades that these identities are acquired by the combined presence of specific lipid and protein molecules that act as biochemical landmarks on each membrane. For example, small GTPases from the Rab and ADP ribosylation factor (ARF) families as well as the Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor (SNARE) family are important components that contribute to membrane identity. On the lipid side, major determinants that distinguish one membrane from another belong to the phosphatidylinositol phosphate family (also known as phosphoinositides). These phospholipids have an inositol head group that can be phosphorylated at various positions on their polar head. Many organelles contain a specific combination of phosphoinositides, which therefore attract proteins containing phosphoinositide-interacting stereospecific domains. Furthermore, it is well established that phosphoinositide production, dynamics and localization are regulated by Rab, ARF and SNARE proteins and, conversely, that the activity and localization of these regulators is under the control of phosphoinositides.

More recently, it has also been recognized that each membrane can additionally be distinguished by its own biophysical properties, including lipid packing, curvature and electrostatics. Although the importance of these parameters was acknowledged long ago by biophysicists using theoretical modelling and artificial membrane systems, tools to probe these particular membrane properties in vivo have only recently been developed and have to date seldom been used in plants. Plant membranes share many characteristics with other eukaryotes; nonetheless, they have singular features, including the presence of unique lipids (for example, phytosterols, highly polar phytosphingolipids, galactolipids) and a drastically different endomembrane system (for example presence of chloroplasts, plasmodesmata, several vacuoles, a unique compartment that serves as the trans-Golgi network (TGN) and early endosome).

Here, we begin to address how membrane biophysical properties contribute to membrane identity in plants. In particular we have analysed the importance of membrane electrostatics in organelle identity and protein localization. Electrostatic interactions with negatively charged membrane contribute to the localization of many proteins containing polybasic clusters or cationic domains. Membrane surface charges (MSCs) are carried by anionic phospholipids. MSCs are not evenly distributed throughout the cell but are organized in specific cellular territories. How MSCs are distributed and organized in plants is unknown. Using a set of surface charge biosensors, we found that the plant PM and the cell plate of dividing cells are highly electronegative compared with endomembranes. Our results further revealed that the specific electrostatic field of the PM is lost upon chemical or genetic depletion of PtdIns(4)P and that it contributes to the PM localization and function of several proteins involved in hormone and receptor-like kinase signalling.

Results
The plant PM has a specific electrostatic signature. To address the importance of membrane electrostatics in plants, we set out to map MSCs in vivo in Arabidopsis root epidermis. We raised a set of transgenic lines that constitutively express an mCITRINE (cYFP) fluorescent protein fused to a carboxy (C)-terminal farnesyl anchor in conjunction with an adjacent unstructured peptide of varying net positive charges (Supplementary Fig. 1). The least cationic probe (8Q-Farn, 0+) was localized in numerous endomembrane compartments (Fig. 1a,p and Supplementary Fig. 1). Increasing electrostatic interactions by the gradual addition of lysines (cationic charges) targeted the probes to the PM at the expense of endomembrane localization (Fig. 1a–p and Supplementary Fig. 1). Probes of intermediate charges (4+ to 6+) clearly associated with the PM and labelled few endomembrane compartments (Fig. 1c,d,p and Supplementary Fig. 1), which presumably are of intermediate electronegativity (Fig. 1q and Supplementary Fig. 1). The most

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Figure 1 | The plant PM and the cell plate are highly electronegative. **a–o.** Confocal images of membrane surface charge (MSC) probes in Arabidopsis root epidermis. Probes are indicated at the bottom and net charges in the top right corner of each image. **p,** Tukey boxplot showing the distribution of intracellular compartments (spots) per cell for each MSC reporter. Different italicized letters indicate significant differences among means (P < 0.0001, Kruskal–Wallis test). **q,** A schematic representation of MSC organization in plants. **r,** Dual-colour imaging during cytokinesis in Arabidopsis root epidermis. Plants are co-expressing 2×CyPet–1×PHFAPP1 (r, top) and cYFP–2×PHPLC (r, bottom) or 2×mCHERRY–1×PHFAPP1 (s, top) and cYFP–KA1MARK1 (s, bottom). Arrows indicate the earliest time point at which the cell plate can be detected in each channel. Confocal images are colour-coded with respect to pixel intensity based on the scale shown in the top right corner. Scale bars, 5 µm.
Figure 2 | PI(4)K activity is required to maintain the PM electrostatic signature. (a) A schematic representation of the drugs used to perturb phosphoinositide production and lipid sensors used as read-out. WM, Wortmannin. For 2×FYVE<sup>PESI</sup> see Supplementary Fig. 4. (b–i) Dual-colour imaging of plants treated for the indicated time with the indicated drug concentration. PS (phosphatidylserine), PtdIns(4,5)P<sub>2</sub> and MSC images are pseudo-coloured in green (left) and 1×PHFAPP1 images are pseudo-coloured in purple (middle). Colocalizations are shown in white in the merged channel (right). (b, c) Plants co-expressing 2×mCHERRY–C<sub>2</sub>Lact and cYFP–1×PHFAPP1. (d, e, h, i) Plants co-expressing cYFP–KA1MARK1 and 2×mCHERRY–1×PHFAPP1. (f, g) Plants co-expressing cYFP–KA1MARK1 and 2×mCHERRY–1×PHFAPP1. (j–s) Confocal images of cYFP–KA1MARK1 MSC reporter treated for the indicated time with the indicated drug concentration. t, The corresponding dissociation index (mean ± s.e.m.) for j–s, LY, LY294002. Drug concentration is given in μM. Different italicized letters indicate significant differences among means (P < 0.0001, Kruskal–Wallis test). Scale bars, 5 μm.
**Figure 3 | PtdIns(4)P is a hallmark of the plant PM.** a-c, FRAP analyses of 1×, 2× and 3×PHFAPP1 sensors. a, Representative confocal images. b, Kymograms of protein diffusion within the PM. c, Traces of fluorescence intensity during FRAP analyses. d-h, Confocal images of PtdIns(4)P probes in Arabidopsis root epidermis. Probes are indicated above each image. Scale bars, 5 μm. i, Tukey boxplot showing the distribution of intracellular compartments (spots) per cell for each PtdIns(4)P reporter. Different italicized letters indicate significant differences among means (P < 0.0001, Kruskal–Wallis test). j-o, Confocal images of PtdIns(4)P probes in N. benthamiana leaf epidermis. Probes are indicated above and mutations in PHFAPP1 at the bottom of each image. The panels below show schematic representations of PHFAPP1 membrane recruitment mechanisms according to the different mutations used. Orange arrows indicate endosomal localization of 1×PHFAPP1. Scale bars, 20 μm.

cationic probe (8K-Farn, 8+) was strictly localized at the PM (Fig. 1e,p and Supplementary Fig. 1). To confirm the importance of the charges in the localization of our biosensors, we substituted the lysines within the cationic stretch with either arginine (8R-Farn, 8+, Fig. 1f) or glutamic acid (6K2E, 4+; 7K1E, 6+, Fig. 1g,h). Consistently, the probes with identical net charges showed a similar localization (Fig. 1c–h,p).

Next, we tested the effect of adding an adjacent polybasic sequence to different lipid anchors (Supplementary Fig. 1). Probes that were either geranylated (8Q-Gege, 0+) or myristoylated (Myr-8Q, 0+) were mainly localized in endomembrane compartments (Fig. 1i,j,p, Supplementary Fig. 1). By contrast, the 8+ probes (8K-Gege and Myr-8K, 8+) were specifically localized to the PM, supporting the notion that strong electrostatic interactions provide PM specificity regardless of the lipid anchor (Fig. 1k,l,p, Supplementary Fig. 1). Next, we expressed two cationic amphipathic helices that do not rely on a lipid anchor for membrane association (the synthetic KRΦ sequence, 8+ and the C-terminal tail of the human GTPase Rítí (Rítí-tail), 9+, Supplementary Fig. 1)16,17. Both probes were strictly localized to the PM (Fig. 1m,n,p). We also assayed the localization of the Kinase Associated-1 (KA1) domains of the human microtubule affinity-regulated kinase (MARK)1 (KA1MARK1) and yeast Kcc4p proteins (KA1Kcc4p)20. KA1 is a folded domain that lacks stereo-specificity and associates non-specifically with every anionic lipid21,21. Consistent with our peptide-based probes, both KA1 domains localized specifically to the PM (Fig. 1o, p and Supplementary Fig. 1). Altogether, our results indicate that the PM of Arabidopsis root epidermal cells has a strong electrostatic field, a unique and intrinsic property of this membrane that contributes to its identity (Fig. 1q). Interestingly, both 8K-Farn and KA1Kcc4p MSC probes were insensitive to the cycling inhibitor

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Brefeldin A (BFA) (Supplementary Fig. 1), suggesting that high PM electrostatics does not require endocytic recycling. In addition, this property is not restricted to the root epidermis and was confirmed in other Arabidopsis cell types and in Nicotiana benthamiana (Supplementary Fig. 2).

PtdIns(4)P is present early during cell plate formation, which correlates with the acquisition of a distinctive electrostatic state at the surface of this membrane. In animal cells, PM MSCs are controlled by phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂), a phosphoinositide that localizes specifically at this membrane3,17,21. However, PtdIns(4,5)P₂ is necessary but not sufficient to maintain PM electrostatics17,21. Indeed, depletion of PtdIns(4,5)P₂ alone does not perturb PM MSCs17,21. However, concomitant depletion of PtdIns(4,5)P₂ together with phosphatidylinositol-4-phosphate (PtdIns(4)P)30 or phosphatidylinositol-3,4,5-bisphosphate (PtdIns(3,4,5)P₃)37 triggers loss of PM MSC. PtdIns(3,4,5)P₃ does not exist in plants3,22, but PtdIns(4,5)P₂ and PtdIns(4)P both localize at the PM13,22–24 and are therefore potential anionic phospholipid candidates that might regulate PM electrostatics either alone or in combination. PtdIns(4)P and PtdIns(4,5)P₂ have both been reported to localize preferentially on the apical and basal poles of root epidermal cells rather than on their lateral sides25,26. To analyse whether the localization of our MSC reporters correlates with the reported polar localization of phosphoinositide reporters25,26, we determined their polarity indices (Supplementary Fig. 3). However, contrarily to previous reports25,26, our analysis suggested that phosphoinositides reporters are not differentially localized compared with non-polar controls (Supplementary Fig. 3). We favour the hypothesis that confocal images of root cells might be biased in favour of the apical/basal signal over the lateral signal because of the topology of these cells (see Supplementary Fig. 3).

Figure 4 | PM PtdIns(4)P drives the electrostatic field of the cell membrane. a, A schematic representation of the genetic system used to specifically deplete PM PtdIns(4)P. b–c, mTURQUOISE2 imaging of MAP-mTU2-SAC1DEAD (b) and MAP-mTU2-SAC1 (c) in N. benthamiana leaf epidermis. d–m, cYFP imaging of the lipid or MSC reporter indicated at the top in N. benthamiana leaf epidermis, co-expressed with MAP-mTU2-SAC1DEAD (d–h) or MAP-mTU2-SAC1 (i–m). n, Quantification of localization observed in d–m. o, A schematic representation of PtdIns(4)P and MSC organization in non-perturbed cells (left) or cells with reduced PM PtdIns(4)P (right). Scale bars, 20 μm.
PtdIns(4)P and/or PtdIns(4,5)P2, we analysed MSCs and the localization of these phosphoinositides during cytokinesis. In tobacco BY-2 cells, PtdIns(4)P is present early during cell plate formation, and localization of these phosphoinositides during cytokinesis. In tobacco BY-2 cells, PtdIns(4)P is present early during cell plate formation, and localization of these phosphoinositides during cytokinesis.

Figure 5 | PINOID and BKI1/MAKRs are effectors of the PM electrostatic field. a, Lipid overlay assays with recombinant PID-Flag, PID9Q-Flag, PID9Q-MAKR1-Flag, HA-MAKR2, HA-MAKR3, MAKR4 and the HA-KA1MARK1 control. Anionic lipids are indicated in blue. LPA, lysophosphatidic acid; SIP, sphingosine-1-phosphate; LPC, lysophosphatidylcholine; PA, phosphatic acid; PE, phosphoethanolamine; PC, phosphatidylcholine. b, Representative confocal images showing the localization of the indicated GFP-fused protein in WT and cho1Δ yeast. Scale bars, 5 μm.

Next, to analyse whether MSCs could be regulated preferentially by PtdIns(4)P and/or PtdIns(4,5)P2, we analysed MSC and the localization of these phosphoinositides during cytokinesis. In tobacco BY-2 cells, PtdIns(4)P is present early during cell plate formation, and localization of these phosphoinositides during cytokinesis. In tobacco BY-2 cells, PtdIns(4)P is present early during cell plate formation, and localization of these phosphoinositides during cytokinesis.

PtdIns(4) kinase activity is required to maintain PM surface charges. To test this importance of PtdIns(4)P in membrane electrostatics, we used short-term treatment of phenylarsine oxide (PAO), a PtdIns(4) kinase (PI(4)K) inhibitor (Fig. 2a)21,23,28. We found that PAO triggers dissociation of PtdIns(4)P-biosensors from the PM into the cytosol, but it had no or little effect on the PM localization of phosphatidylserine (PS) and PtdIns(4,5)P2 biosensors19,22 (Fig. 2b–c.i and Supplementary Fig. 4). This later result suggested that short-term inhibition of PI(4)Ks did not have a strong impact on PtdIns(4,5)P2 synthesis, although we found, as expected, that longer term PAO treatment dissociated partially PtdIns(4,5)P2 from the PM (Supplementary Fig. 4). Likewise, short-term PAO treatment in mammalian cells inhibits PtdIns(4)P production without severely affecting the PtdIns(4,5)P2 level21. Similar to PtdIns(4)P biosensors, PAO triggered the dissociation from the PM of our MSC sensor KA1MARK1 (Fig. 2f,g,i–o,t) in a time- and dose-dependent manner. In addition, PAO also dissociated the MSC reporters Rist-Tail and KRCP from the PM (Supplementary Fig. 4). These results suggest that PI(4)K activity plays a critical role in the PM electrostatic field. We confirmed these results using Wortmannin, an inhibitor of PtdIns(3)Ks (Fig. 2a)31. We exploited our lipid biosensors22 to assess the effect of these drugs on PtdIns(3)P, PtdIns(4)P, PtdIns(4,5)P2 and PS sensors (Supplementary Fig. 4). Treatments at 30 μM Wortmannin dissociated both our PtdIns(4)P biosensors and our MSC probe KA1MARK1 from the PM (Fig. 2a,t and Supplementary Fig. 4), albeit less effectively than PAO, but they had no effect on the PM localization of PtdIns(4,5)P2 and PS sensors (Supplementary Fig. 4). However, neither LY294002 nor 1 μM Wortmannin dissociated KA1MARK1 from the PM, confirming that PI(4)K but not PI(3)K activity is required for PM MSCs (Fig. 2p–r,t and Supplementary Fig. 4). Live imaging in dividing cells together with our pharmacological approaches suggest that PtdIns(4)P, which is produced by PI(4)Ks, might be critical for PM electrostatics.

PtdIns(4)P massively accumulates at the plant PM. PtdIns(4,5)P2 in mammals and PS in yeasts are major determinants of PM electrostatics. In both cases, these lipids specifically localize at the PM, thereby providing a specific electrostatic signature to this membrane. In plants, PtdIns(4)P accumulates at the PM and endomembranes, as visualized by the PtdIns(4)P biosensor 1xPHFAPP1 (Fig. 2b,d,f,i,t) and PtdIns(4,5)P2 biosensors (Supplementary Fig. 4). This raised the question of how PtdIns(4)P might specifically control PM electrostatics while harbouring such a binary localization. To probe whether PtdIns(4)P preferentially accumulates at the PM, we compared the localization of these biosensors with increasing PtdIns(4)P avidity. Increasing the number of PHFAPP1 domains increases the dwell time of the sensor in PtdIns(4)P-riched membranes (Supplementary Fig. 5)22, as confirmed by fluorescence recovery after photobleaching (FRAP) experiments (Fig. 3a–c). Accordingly, 3xPHFAPP1, and to a lesser extent 2xPHFAPP1, preferentially localize to the PM rather than endosomes (Fig. 3d–f). Consistently, we previously found that PHFAPP1, another PtdIns(4)P-binding protein, had strict PM localization22 (Fig. 3g,i). To
confirm these findings, we used the recently described P4M domain from the Legionella pneumophila SidM protein, which was elegantly demonstrated to be an exquisitely specific PtdIns(4)P biosensor in vivo. In mammalian cells, P4MSidM highlights several PtdIns(4)P pools, including a main pool in the Golgi/TGN, and two relatively minor pools at the PM and endolysosomes. In contrast, P4MSidM was strictly localized at the PM in Arabidopsis and in Nicotiana benthamiana leaf epidermis (Fig. 3h–j and Supplementary Fig. 6). The localization of the PH domain of FAPP1 relies on coincidence binding with both PtdIns(4)P and ARF1 small GTPase. In plants, ARF1 localizes to endosomes and might account for the endomembrane localization of 1×PHFAPP1. However, the fact that 1×PHFAPP1 also accumulates at the PM in plants, a compartment that lacks the ARF1 proteins, further suggests that PtdIns(4)P accumulates to a significant extent in this membrane. Moreover, PHFAPP1 has two distinct binding sites for PtdIns(4)P and ARF1, which can be mutated independently to specifically impair binding in vitro. We tested these mutants in vivo using transient expression in N. benthamiana leaf epidermis (Fig. 3k–o). We found that mutants impaired in PtdIns(4)P

Figure 6 | PM targeting by PID cationic membrane hook is required for function. a, A schematic representation of the PID protein. b, Confocal images of pUBQ10::PID-cYFP and pUBQ10::PID02-cYFP in Arabidopsis root meristem epidermis. Scale bars, 5 µm. c–g, Representative images of root hair phenotypes (left) and localization of the indicated construct (right; magnifications shown in insets). Each image was taken with identical settings, indicating that each transgenic line expressed comparable levels of PID protein. Blue arrowheads indicate elongated root hairs and yellow arrows indicate root hairs with inhibited growth. Scale bars, 100 µm. h, Tukey boxplot showing the quantification of root hair length in the following lines: pEXP7::PID-cYFP (P-Y); pEXP7::PID02-cYFP (P0Q-Y); pEXP7::PID02-cYFP9Q-Q5K3Q-Farn (P0Q-Y5K-F); pEXP7:2×cYFP8K-Farn (Y8K-F) and WT. Different italicized letters indicate significant differences among means (P < 0.0001, Kruskal-Wallis test).
binding did not localize at the PM but in the cytosol and endomembranes, which is likely to be because of binding to ARF1 (Fig. 3l,m). In contrast, PTHFK3Q variant version mutated in their ARF1-binding interface had a similar localization as P4MΔNΔΔ, being specifically localized at the PM and excluded from endomembranes (Fig. 3n,o). This result further exemplifies that PtdIns(4)P is highly enriched at the PM in plants, which contrasts with other eukaryotic cells in which PtdIns(4)P predominantly localizes to Golgi/TGN membranes and to a lesser extent at the PM.

The pool of PtdIns(4)P at the PM controls the surface charge signature of this membrane. Next, we built a genetic system to specifically deplete the plant PtdIns(4)P pool and test its importance in PM MSC. In this system, we fused the active or inactive (DEAD) catalytic domain of the yeast Sac1p protein (a PtdIns(4)P phosphatase) with the MAP (myristoylation and palmitoylation) sequence, which induces PM targeting in plants (Fig. 4a)37. To verify that our chimeric proteins were specifically targeted to the PM, we fused them to the cyan fluorescent protein mTURQUOISE2 and expressed them transiently in N. benthamiana (Fig. 4b,c). Next, we transiently co-expressed our chimeric enzymes together with our cYFP-tagged MSC probes or phosphoinositide markers. We found that MAP–SAC1, but not a catalytic mutant (MAP–SAC1DEAD), displaced PtdIns(4)P sensors to endosomes, suggesting that our approach efficiently decreases PtdIns(4)P concentration at the PM (Fig. 4d,e,i,j,n). However, we could not see any effects on the localization of our PtdIns(4,5)P2 biosensors (Fig. 4f,k,n). Importantly, we found that MAP–SAC1 perturbed the PM localization of the KAI1MAKR1 and 8K–Farn MSC markers, which were also found in endosomes in this condition (Fig. 4g,h,l,n). This experiment confirms two predictions: (1) PtdIns(4)Ps are much more concentrated at the plant PM than in endosomes and PtdIns(4)P binding proteins localize to endosomes only when PtdIns(4)P concentration at the PM is reduced, and (2) PM PtdIns(4)Ps are required to establish the high electrostatic property of the PM compared with endomembranes. Our data therefore suggest that in plants PtdIns(4)P will confer endosomal localization to proteins that bind concomitantly to PtdIns(4)P and to another endosome-localized partner (for example, ARF1). However, it will target strict PtdIns(4)P binders specifically to the PM. Together, our results establish PtdIns(4)P as a hallmark of the plant PM and a driving force behind the PM electrostatic field.

The PM electrostatic field drives the localization and function of a hormone signalling proteins. Next, we asked whether endogenous Arabidopsis proteins might rely on the PM electrostatic field for their localization. The auxin transport regulator PINOID (PIND) binds anionic lipids in vitro38,39 and is targeted to the PM by means of a polybasic unstructured loop within its kinase domain (PIND membrane hook, PIND9+). The negative regulator of the brassinosteroid receptor kinase, BKI1, relies on a lysine–arginine–lysine membrane hook for PM localization and function60,61. We found that the cationic stretches in PID and BKI1 contribute to the interaction with anionic phospholipids in vitro and to their PM localization in yeast (Fig. 5a,b). Likewise, BKI1 family members (MAKR1 to MAKR4) also interacted with anionic lipids in vitro (Fig. 5a and Supplementary Fig. 7). Next, we took advantage of the yeast choltA mutant, which is impaired in PS biosynthesis19 and therefore lacks a strong PM electrostatic field59. As a result of this loss of PM MSC in choltA, endomembranes become more electronegative than the PM and cationic proteins relocalize to endomembranes at the expense of their PM localization59 (Supplementary Fig. 8). We confirmed that PID, BKI1 and MAKR1 to MAKR4 localized in the endomembrane rather than the PM in choltA, whereas they associated with the PM in WT yeasts (Fig. 5b and Supplementary Fig. 8). In planta, MAKR1 to MAKR4 also associated with the PM by means of their polybasic amino termini (Supplementary Fig. 9). Next, we visualized MAKR2–cYFP and PID–YFP under the control of their endogenous promoter and found that they were targeted to the PM, although PID was also present in endomembrane compartments59 (Fig. 5c). These results are consistent with the notion that PID, MAKR2 and possibly other family members might localize to the PM by reading out the electrostatic field. Consistently, PID localization and MAKR2 localization were sensitive to PAO (Fig. 5c), indicating that in their case localization relies on 4K activity.

Next, we tested the functional requirement of PID targeting at the PM by electrostatics. We took advantage of the PID9Q membrane hook mutant (0+, Fig. 6a) that localizes to endosomes but not at the PM (Fig. 6b). We adopted a gain-of-function strategy by specific overexpression of PID–cYFP (9+) in root hair cells, which inhibits root hair elongation43,44 (Fig. 6c,h). By contrast, PIDΔN–cYFP (0+) overexpression in these cells had elongated root hairs (Fig. 6d,h), this phenotype resembled wild type (WT), PID205N overexpression in these cells had elongated root hairs (Fig. 6d,h). We added a 5K3Q–Farn tail (5+, Supplementary Fig. 1) at the C-terminus of cYFP to target PID9Q at the PM and endosomes (Fig. 6e). This construct induced a short root hair phenotype that was statistically different from PID9Q overexpression (Fig. 6d,h), suggesting that PID9Q is a functional kinase and that, similar to BKI140,41, PID PM association by its cationic membrane hook is required for its function. This result further confirmed that PID is active at the PM rather than endomembranes59.

Discussion
In this study we found that PtdIns(4)P biosensors accumulate specifically at the PM in various cell types and in two plant species (Arabidopsis thaliana and Nicotiana benthamiana). Previous studies, using the PH domain of FAPP1, identified a pool of PtdIns(4)P in endomembranes22,23. We show that PHTFK3Q localization in endomembranes is due to coincidence binding of this domain with endosomal ARF1. This raised the question of whether PtdIns(4)P does accumulate in endosomes in plants. Multiple lines of evidence suggest that it does: (1) PI(4)Kβ localizes in endosomes45,46 and (2) several Arabidopsis proteins that bind PtdIns(4)P also localize in endomembranes47,48. However, similar to PHTFK3Q, these proteins also bind both PtdIns(4)P and endosomal small GTPase52 proteins. In addition, we show that PtdIns(4)P-binding domains that localize specifically to the PM, also localize to endomembranes on depletion of the PtdIns(4)P PM pool. This experiment suggests that there are two PtdIns(4)P pools in plant cells that compete for the localization of PtdIns(4)P-binding proteins: a major pool at the PM and a minor pool in endosomes (Fig. 4o). As a result, proteins that bind only to PtdIns(4)P localize to the PM, and proteins that bind concomitantly to PtdIns(4)P and an endomembrane protein are targeted to intra-cellular compartments. This organization of PtdIns(4)P in two quantitatively different pools might therefore allow differential targeting of PtdIns(4)P-binding proteins based on whether or not they bind additional molecules. It is important to bear in mind that PtdIns(4)P biosensors can only associate with lipids that are not constantly occupied by endogenous PtdIns(4)P-binding proteins (‘unoccupied’ PtdIns(4)P pool)3. It is therefore possible that a massive pool of PtdIns(4)P is present in endomembranes but not available to target lipid sensors to this compartment (‘occupied’ PtdIns(4)P pool)49. However, such an occupied PtdIns(4)P pool does not generate negative membrane charges, and is therefore not relevant for the generation of membrane electrostatic fields. In any case, in plant cells, the localization of ‘unoccupied’ PtdIns(4)Ps that are labelled by biosensors is drastically different from other eukaryotes, in which relatively equal pools of PtdIns(4)P are detected at the PM and endomembrane inner surfaces59.
We found that accumulation of PtdIns(4)P at the PM is essential to generate a high electrostatic field at this membrane. Our analyses in dividing cells suggests that PtdIns(4,5)P2 is dispensable for PM MSC. As such, PM MSC is differentially regulated in plants and animals17,30,21. In the latter, PtdIns(4,5)P2 is required for the PM electrostatic field, but PtdIns(4)P and/or PtdIns(3,4,5)P3 are also important for the generation of high PM MSC21,27. By contrast, in plants, loss of PM PtdIns(4)P is sufficient to perturb membrane electrostatic properties. However, we cannot exclude that other anionic phospholipids such as PS or phosphatidic acid (PA) might also contribute to the PM electrostatic field. PA is not normally present at the PM in yeast and animal cells, but it has been visualized in this membrane in plant tip-growing cells90. Given that PA has two net negative charges, it could also be important for PM MSC. PS is the major anionic phospholipid at the yeast PM20, but is also involved in PM electrostatics in animals39, in a non-redundant manner with phosphoinositides7,21. Future studies will reveal whether PA and/or PS are involved, together with PtdIns(4)P, in PM MSC.

We have described several proteins involved in auxin, brassinosteroid and/or RLK signalling that rely on PM MSC for localization and function. There is a broad signalling potential behind this electrostatic localization mechanism18, since these interactions might be rapidly modulated by variations in lipid composition (for example, activation of phospholipases), the local cytosolic environment (for example, ion influx) or modification of the protein itself. For example, phosphorylation of a conserved tyrosine within BKI1 membrane hook triggers PM dissociation40, which is likely to be by acting as an electrostatic switch18. Here, we provide several examples of MSC effectors in Arabidopsis, but we expect that many more proteins will rely on this particular PM physical property for localization and function.

Methods

Growth condition and plant materials. The following transgenic lines: pUBQ10::cYFP, pUBQ10::YFP, pUBQ10::cYFP::2×PHFAPP1, pUBQ10::cYFP::PHFAPP1, pUBQ10::cYFP::PHFAPP1+1024B camera (Princeton Instruments, http://www.princetoninstruments.com/) were used. GUS expression was determined as the frame preceding the dissociation index. For quantification of the number of intracellular compartments (spots) per cell. The intracellular compartments (spots) per cell were counted in a double-blind set-up. One hundred cells were counted per condition, in at least 20 independent roots imaged during the course of at least three independent experiments.

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Author contributions
M.S. and M.C.C. carried out the membrane experiments. V.B. performed the PAO and Wortmannin experiments. M.S. and M.C.C. performed cytokinesis and transgenic line collection for cell type-specific functional genomics in Arabidopsis. Methods Mol. Biol. 536, 85–109 (2012).

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