Cell wall constrains lateral diffusion of plant plasma-membrane proteins


A cell membrane can be considered a liquid-phase plane in which lipids and proteins theoretically are free to diffuse. Numerous reports, however, describe retarded diffusion of membrane proteins in animal cells. This anomalous diffusion results from a combination of structuring factors including protein–protein interactions, cytoskeleton corralling, and lipid organization into microdomains. In plant cells, plasma-membrane (PM) proteins have been described as relatively immobile, but the control mechanisms that structure the PM have not been studied. Here, we use fluorescence recovery after photobleaching to estimate mobility of a set of minimal PM proteins. These proteins consist only of a PM-anchoring domain fused to a fluorescent protein, but their mobilities remained limited, as is the case for many full-length proteins. Neither the cytoskeleton nor membrane microdomain structure was involved in constraining the diffusion of these proteins. The cell wall, however, was shown to have a crucial role in immobilizing PM proteins. In addition, by single-molecule fluorescence imaging we confirmed that the pattern of cellulose deposition in the cell wall affects the trajectory and speed of PM protein diffusion. Regulation of PM protein dynamics by the plant cell wall can be interpreted as a mechanism for regulating protein interactions in processes such as trafficking and signal transduction.

Proteins within membranes play significant roles in signal perception and transduction, solute partitioning, and secretion. Accordingly, more than 25% of the proteome of higher plants is predicted to be membrane-associated proteins (1, 2). Proteins diffuse within the plane of a membrane through thermal agitation. Each protein diffusing freely (3) has a diffusion constant that is dependent on the protein’s hydrodynamic radius and the viscosity of the membrane and surrounding medium (4). In a hypothetical uniform membrane, proteins would be distributed randomly. However, biological membranes are spatially complex, with regions of protein and lipid concentration. Numerous reports describe retarded diffusion of membrane proteins (5–8) because of structuring factors such as protein–protein interactions (9), cytoskeleton corralling (10), and lipid organization into microdomains (11). Membrane nanostructuring is crucial for protein–protein interactions and can either segregate or colocalize membrane proteins, thus optimizing protein interactions in processes such as trafficking and signal transduction (12).

Like yeast and animal cells, plant cells have a subcompartmentalized plasma membrane (PM). Membrane rafts (reviewed in ref. 13) have been demonstrated in plant PMs by proteinomics on detergent-insoluble membranes (DIMs). DIMs are enriched in signaling, stress response, cellular trafficking, and cell-wall metabolism proteins (14–16). The Chlorella kessleri hexon-proton symporter HUP1 and Solanum tuberosum remorin StrREM1.3 have been visualized in clusters within the PM (17, 18), and the clustering localization pattern of HUP1 is disrupted in mutant yeast lines lacking typical ergosterol and sphingolipid microdomains (17). The physiological role of plant PM substructuring has been demonstrated in several studies. For instance, in the sterol mutant cyclopropylsterol isomerase-1 (cp1.1) of Arabidopsis thaliana, the asymmetric localization of PIN2 and, hence, polar auxin transport are perturbed (19). In polarized cells such as pollen tubes, perfusion with the sterol-binding toxin filipin not only perturbs membrane microdomain structure but also alters calcium gradients, production of reactive oxygen species, and normal cell elongation (20).

The relationship between membrane subcompartmentalization and protein diffusion has not been studied in detail, however, and only a few reports have quantified protein diffusion in plant-cell membranes. In the case of PM proteins, such as KAT1, PMA2 H+-ATPase, PIN2, PIP2;1, BOR1, NIP5;1, and AtFH1, only a small fraction of the protein pool is mobile (19, 21–23). In contrast, endoplasmic reticulum-associated proteins, nuclear membrane proteins, and tonoplast-associated proteins diffuse more freely within the membrane (24–26). Consequently, we are led to believe that the plant cell has specific properties that constrain PM protein diffusion.

Here, we studied PM protein diffusion in plant cells to understand better PM structure and function. Protein mobility was quantified using fluorescence recovery after photobleaching (FRAP) for a set of 13 plant PM proteins fused to fluorescent proteins. Then we developed a set of modified PM proteins that we term “minimal” because only the membrane-interacting or -spanning domains are present. Minimal PM proteins were designed to reduce the effect of protein interactions on diffusion and showed that DIM association and cytoskeleton have very little effect on protein mobility. However, PM proteins that normally are almost immobile become mobile when the cell wall is absent or when the distance between PM and cell wall is increased. Then cell-wall interaction with PM proteins was confirmed by single-molecule tracking using total internal reflection fluorescence (TIRF) microscopy. Our results show that the cell wall constrains protein diffusion, especially for proteins with larger extracellular domains, even in the absence of binding interactions between proteins and cell-wall components.


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**Results**

**PM Proteins Are Relatively Immobile.** Sutter et al. (27) demonstrated low lateral mobility of the potassium channel KAT1 and the H⁺-ATPase PMA2. These observations have been supported recently by similar results on other PM proteins (19, 21–23, 28), but systematic study of PM protein dynamics is lacking. We selected 10 *Arabidopsis* PM proteins, including examples of several different membrane-association types: transmembrane domains, lipid modifications, and peripheral membrane proteins (Table 1). These proteins were fused to fluorescent protein and transiently expressed in *Nicotiana tabacum* leaves (29). All 10 constructs marked the cell PM. We used FRAP experiments to quantify protein mobility. GFP fluorescence was bleached in a small region of PM, and fluorescence recovery within the region was monitored (Fig. 1A; see Fig. S1 for technique). Using a nonlinear curve-fitting approach, we determined the relative fraction of the protein free to diffuse within the bleached region during 60 s postbleaching (I60s). We observed large differences between constructs in this characteristic. For instance, the bleaching area of GFP-AGP4 remains visible much longer than that of GPA1-GFP (Fig. 1A). The implication is that I60s is lower for GFP-AGP4 than for these other proteins. This difference becomes clear when data are plotted (Fig. 1B). Indeed, I60s is highly variable among constructs (Table 1), indicating that a proportion of the protein potentially does not diffuse but is fixed in place, probably through interaction with other cellular components. Monitoring of this relatively short fluorescence-recovery phase was intended to exclude artifacts that would result from endo- or exocytotic removal or insertion of protein from the membrane (30). Relatively low levels of fluorescence that occur in the cytoplasm for some constructs (e.g., GFP-NPSN11 or PIP2;1-CFP) result in an apparent two-phase recovery process in which rapid initial recovery during the first few seconds is followed by a flat line indicating no subsequent recovery (Fig. 1B). The bleaching laser bleaches not only the PM but also the underlying cytoplasm, which recovers within seconds because of cytoplasmic streaming. Any subsequent change in fluorescence intensity within the bleached spot is the result of lateral diffusion within the PM.

**Protein Crowding Within the PM Has a Limited Effect on Protein Diffusion.** Protein crowding within membranes should reduce protein lateral mobility, because collision between molecules restricts diffusion (31). Consequently, we quantified diffusion of the overexpressed proteins p35S::GFP-LT16b, p35S::PIP2;1-GFP, and pUBQ10::YFP-NPSN12 in hypocotyl cells of stably transformed *Arabidopsis*. Again we observed two typical types of fluorescence-recovery curves: one-phase diffusion for GFP-LT16b and two-phase with little diffusion for PIP2;1-GFP and YFP-NPSN12 (Fig. 1C). In addition, we tested two constructs under their endogenous promoters, pFLS2::FLS2-GFP and pPIN2::PIN2-GFP, and again found that a large proportion of each protein remains immobile. To test whether the relative amount of a protein within a membrane was related to the extent of fluorescence recovery in FRAP experiments, we plotted mean prebleach intensity within a bleaching region vs. maximum recovery (I60s). We also tested whether the relative amount of protein within the PM was associated with observed FRAP levels, but no relationship was observed ($R^2$ = 0.03) (Fig. S2).

### Table 1. Fluorescence recovery after photobleaching for different PM proteins fused to GFP

<table>
<thead>
<tr>
<th>Gene (from <em>Arabidopsis</em>)</th>
<th>Type of anchoring*</th>
<th>No. of amino acids</th>
<th>Expressed in</th>
<th>Promoter</th>
<th>I60s ± SEM (%)</th>
<th>$R^2$ for curve fit</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-NPSN11</td>
<td>1 TM (type 2)</td>
<td>265</td>
<td>Tobacco</td>
<td>p35S</td>
<td>10.4 ± 0.8</td>
<td>0.75</td>
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<tr>
<td>PIP2;1-GFP</td>
<td>6TM (type 4)</td>
<td>287</td>
<td>Arabidopsis</td>
<td>p35S</td>
<td>10.8 ± 1.1</td>
<td>0.71</td>
</tr>
<tr>
<td>YFP-NPSN12</td>
<td>1 TM (type 2)</td>
<td>265</td>
<td>Arabidopsis</td>
<td>pUBQ10</td>
<td>11.8 ± 0.7</td>
<td>0.34</td>
</tr>
<tr>
<td>PIN2-GFP</td>
<td>9TM (type 4)</td>
<td>647</td>
<td>Arabidopsis</td>
<td>pPIN2</td>
<td>13.3 ± 0.8</td>
<td>0.42</td>
</tr>
<tr>
<td>AtFH1-GFP</td>
<td>1 TM (type 1)</td>
<td>1051</td>
<td>Tobacco</td>
<td>p35S</td>
<td>18.1 ± 0.5</td>
<td>0.76</td>
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<tr>
<td>FLS2-GFP</td>
<td>1 TM (type 1)</td>
<td>1173</td>
<td>Arabidopsis</td>
<td>pFLS2</td>
<td>19.0 ± 2.0</td>
<td>0.43</td>
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<td>GFP-AGP4</td>
<td>GPI</td>
<td>135</td>
<td>Tobacco</td>
<td>p35S</td>
<td>20.0 ± 2.2</td>
<td>0.52</td>
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<td>GFP-REM;3.1 †</td>
<td>Extrinsic - inner leaft</td>
<td>198</td>
<td>Tobacco</td>
<td>p35S</td>
<td>23.4 ± 1.2</td>
<td>0.46</td>
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<tr>
<td>YFP-SYP121</td>
<td>1 TM (type 2)</td>
<td>346</td>
<td>Tobacco</td>
<td>p35S</td>
<td>23.7 ± 3.4</td>
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<tr>
<td>At1g14870-GFP</td>
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<td>152</td>
<td>Tobacco</td>
<td>p35S</td>
<td>36.2 ± 2.7</td>
<td>0.74</td>
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<tr>
<td>PIP2;1-CFP</td>
<td>6TM (type 4)</td>
<td>287</td>
<td>Tobacco</td>
<td>p35S</td>
<td>43.7 ± 1.7</td>
<td>0.41</td>
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<tr>
<td>At3g17840-GFP</td>
<td>1 TM (type 1)</td>
<td>647</td>
<td>Tobacco</td>
<td>p35S</td>
<td>58.9 ± 3.8</td>
<td>0.73</td>
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<tr>
<td>GFP-LT16b</td>
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<td>54</td>
<td>Tobacco</td>
<td>p35S</td>
<td>72.4 ± 2.7</td>
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<tr>
<td>GPA1-GFP</td>
<td>Myristoylated and palmitoylated</td>
<td>383</td>
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<td>p35S</td>
<td>79.8 ± 3.9</td>
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<tr>
<td>GFP-LT16b</td>
<td>2 TM (type 4)</td>
<td>54</td>
<td>Arabidopsis</td>
<td>p35S</td>
<td>91.4 ± 3.6</td>
<td>0.99</td>
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</table>

Maximum recovery (I60s) of prebleaching fluorescence intensity during 133 s.

*TM, transmembrane domain. *Type* refers to membrane-anchoring topology.

*Gene from *Solanium tuberosum*.

Relative Immobility of Many PM Proteins Is Not Caused by Protein Interactions. Eleven of the 13 full-length PM protein constructs tested were shown to be relatively immobile (Table 1). Proteins can form larger complexes within membranes by self-association or through protein–protein interactions with other endogenous proteins. Complex formation could limit diffusion, so we designed a set of modified PM proteins as fluorescent protein fusions. These minimal PM proteins consist of only the membrane-anchoring residues, and we predicted that they would have no ability to interact with other cellular constituents. Several different membrane-anchoring types of minimal construct were generated, including a myristoylated and palmitoylated GFP (MAP-GFP), a prenylated GFP (GFP-PAP), a glycosylphosphatidylinositol-anchored GFP (GFP-GPI), a phosphatidylinositol-4-phosphate-binding protein YFP (PI-YFP) (32), and the LAMP1 transmembrane domain fused to GFP (GFP-TM23) (33) (Fig. S4). We verified the correct targeting of all constructs to the PM in leaves (Fig. S3B). Minimal FP constructs in which the FP moiety was intracellular and anchored by a single transmembrane domain were tested but lost their targeting to the PM, presumably because of the removal of a C-terminal targeting signal (Fig. S4).

As described previously (33), TM23 accumulates mainly in the PM but also is present in Golgi bodies and within the endoplasmic reticulum membrane. Then, we confirmed the predicted topology of these constructs (Fig. 2A) by incubating tobacco mesophyll protoplasts with an antibody against GFP or YFP. As expected, only GFP-GFI and GFP-TM23 showed labeling (Fig. S5). This result confirmed that in two constructs the GFP is outside the cell in the apoplastic space, whereas in MAP-GFP, GFP-PAP, and YFP-PI the fluorescent protein remains within the symplastic space. Finally, *Arabidopsis* transgenic lines were generated for each minimal construct.
FRAP experiments on constructs in which the GFP projects into the cell (i.e., MAP-GFP, GFP-PAP, and YFP-PI) showed the one-phase type of gradual but continuous recovery during 60 s postbleaching, with PM proteins finally recovering to relatively high levels [MAP-GFP I60s = 90.4 ± 3.0% (R² = 0.80); GFP-PAP I60s = 83.4 ± 3.2% (R² = 0.86); YFP-PI I60s = 80.3 ± 2.5% (R² = 0.78)] (Fig. 2B). In addition, for MAP-GFP, GFP-PAP, and YFP-PI, kymographic analysis clearly shows that recovery of fluorescence is centripetal and therefore results from lateral diffusion (Fig. 2C). The constructs in which GFP projects out of the cell (i.e., GFP-GPI and GFP-TM23) recover to significantly lower levels [GFP-GPI I60s = 26.6 ± 3.1% (R² = 0.54); GFP-TM23 I60s = 45.8 ± 2.9% (R² = 0.66)] (Fig. 2B). The shapes of their recovery curves are very similar to the ones that we describe for full-length proteins in the previous section. GFP-GPI and GFP-TM23 are relatively immobile, and kymographic analysis of their fluorescence recovery shows that recovery does not proceed from the margins of the bleached area (Fig. 2C). These results show that, even for minimal PM protein constructs, protein mobility can be limited and suggest that secondary protein association and interactions are not for the cause of limited diffusion within the PM.

Lipid Domain Organization and the Cytoskeleton Have Little Effect on Protein Diffusion. Lipid organization resulting in inhomogeneity within the membrane could induce sequestration of proteins and influence their diffusion. We used detergent fractionation to isolate DIM proteins from solubilized membrane proteins and analyzed fractions to test for the presence of minimal PM protein constructs. As a control, GFP-REM, but not PMA2-GFP, appears in DIM fractions 1, 2, and 3, confirming its previously published DIM localization (Fig. S6A) (18). Similar to GFP-REM, MAP-GFP and GFP-GPI co-occurred in DIM and non-DIM fractions. Myristoylated proteins (MAPs) and glycosylphosphatidylinositol-anchored proteins (GPIs) often are abundant in DIM fractions (15). Finally, GFP-PAP occurred mostly in non-DIM fractions, and YFP-PI and GFP-TM23 occurred only in non-DIM fractions (Fig. S6C). Surprisingly, however, minimal PM proteins that occurred in membrane microdomains (DIM fractions) had contrasting mobile fractions (I60s MAP-GFP = 90.4 ± 3.0%; I60s GFP-GPI = 26.6 ± 3.2%), indicating that there is no direct relationship between membrane subcompartmentation and mobility. Filippin III is a 3β-hydroxysterol–binding antibiotic (34) that induces changes in cholesterol organization (35). In FRAP experiments on Arabidopsis treated with 100 μM filippin III, the mobile fraction of minimal PM protein constructs did not fluctuate (control vs. filippin, P = 0.87, two-way ANOVA) (Fig. S6B).

In animal cells the cytoskeleton has been shown to corral membrane proteins within subregions (36) by forming “fences” that limit protein diffusion. To test if actin or microtubule

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**Fig. 1.** Plant PM proteins are relatively immobile. (A) FRAP examples illustrating slow (GFP-AGP4) and fast (GPA1-GFP) membrane lateral diffusion. (Scale bar: 5 μm.) (B and C) FRAP curves of PM protein GFP fusions. (B) GFP fusions expressed in N. tabacum leaf cells. GFP-LT16b and GPA1-GFP have relatively high mobility; others experience an initial rapid (2–10 s postbleaching) recovery of fluorescence that results from diffusion within the cytoplasmic fraction followed by almost no recovery during the subsequent 50 s, showing that they are highly immobile when anchored within the PM. (C) GFP fusions expressed in A. thaliana. p35S::GFP-LT16b behaves with relatively high mobility, as in tobacco leaf cells, but all others tested (including pFLS2::FLS2-GFP and pPIN2::PIN2-GFP, both of which were expressed under native promoters) were very immobile.

**Fig. 2.** Minimal FP constructs targeted to the PM have different diffusion dynamics in A. thaliana. (A) Schematic of the topology of minimal FPs. Green stars represent the fluorescent protein. (B) FRAP curves for minimal FPs. MAP-GFP, GFP-PAP, and YFP-PI are highly mobile; GFP-TM23 and GFP-GPI are relatively less mobile. (C) Kymograms of protein diffusion within the PM. MAP-GFP, GFP-PAP, and YFP-PI show a centripetal movement of fluorescence. No recovery is observable for GFP-GPI and GFP-TM23. Color scale indicates pixel intensity from 0 (black) to 255 (brightest intensity possible).
cytoskeletons might exert a similar influence on membrane-protein mobility in plant cells, we incubated minimal GFP-expressing Arabidopsis seedlings with either cytochalasin D or oryzalin to depolymerize actin microfilaments or microtubules, respectively. No increase in mobile fraction for these constructs was observed (Fig. S6C). Consequently, we believe that the cytoskeleton is not responsible for the relative immobility of GFP-GPI or GFP-TM23.

**Plant Cell Walls Have a Major Effect on Protein Lateral Mobility.** The two least mobile of our minimal PM protein constructs, GFP-GPI and GFP-TM23 (Fig. 2A), orient in the PM with GFP projecting into the cell-wall space. The remaining three minimal proteins orient in the PM so that the fluorescent protein projects into the cytoplasm. We decided to investigate whether the cell wall influences protein diffusion within the PM. To do so, cell walls either were removed by protoplasting or were separated from the PM by plasmolysis. First, we studied protein mobility during reneosynthesis of protoplast cell walls (Fig. S7). FRAP experiments were carried out both on freshly prepared protoplasts (cell-wall absent) and on protoplasts in which the cell wall had been regenerated for 24 or 48 h. GFP-GPI has a high mobile fraction in fresh protoplasts that decreases by a factor of more than 20 after cell-wall regrowth (GFP-GPI: I60s t0 = 79.9 ± 3.2%; I60s t48h = 2.6 ± 0.6%; P < 0.001, t test) (Fig. 3A and B). In contrast, the mobile fraction of MAP-GFP, in which GFP projects into the cell, did not differ between fresh protoplasts and those with regrown cell walls (I60s t0 = 76.6 ± 1.8%; I60s t48h = 75.6 ± 1.73%; P = 0.71, t test). This result suggests that the cell wall plays a role in immobilization of PM proteins that project into the cell-wall space.

These results were verified by incubating tissue in a hyperosmotic buffer to induce plasmolysis, a shrinkage of the protoplast that results in the creation of a space between the cell wall and the PM. FRAP experiments were carried out on control and plasmolysed cells expressing GFP-GPI (Fig. 3C) and GFP-TM23 and showed a significant increase in mobility of the proteins’ I60s in plasmolyzed cells (I60s GFP-GPI control vs. plasmolysis, P < 0.001, t test; I60s GFP-TM23 control vs. plasmolysis, P < 0.001, t test) (Fig. 3D).

**Single-Molecule Tracking Reveals an Effect of Cellulose Deposition on paGFP-LTI6b Diffusion.** Single-molecule imaging by TIRF microscopy has been used previously to detect and track individual proteins in plant samples (37, 38). We used this technique to observe the PM of plants expressing photoactivatable GFP (paGFP) (24, 39) fused to LTI6b. Before photoactivation, a small fraction of paGFP molecules occur naturally in the activated state, i.e., they behave as GFP without the requirement for photoactivation. This pool of autoactivated paGFP is of sufficiently low density to be useful for single-molecule tracking studies even when overexpressed. In these circumstances normal GFP is too bright, but paGFP-LTI6b has a very good signal-to-noise ratio and appears as discrete spots that are trackable over time (Fig. 4 A–C and Movie S1). paGFP molecules show a typical blinking behavior (Fig. S8) with a short time of residence (1.28 ± 0.89 s) at the PM before bleaching. Mean squared displacement (MSD) describes the average motion in a population of diffusing molecules as a function of time and is a useful means of characterizing the type of molecular motion that occurs. LTI6b is a relatively mobile PM protein (Fig. 1B) with only two residues predicted to be in the extracellular space. Its MSD in control cells has a linear dependence on time (Fig. 4D), as predicted for molecules that diffuse freely (40). When seedlings were incubated with 20 μM isoxaben for 1 h to disrupt cell-wall structure, we observed greatly restricted molecular movement and sublinear MSD, indicating constrained diffusion of paGFP-LTI6b in this condition (Fig. 4E).

**Discussion**

Measurement of protein mobility has been used in this report to get a better understanding of control mechanisms that structure the plant cell PM.

**PM Proteins Have Relatively Low Lateral Mobility.** Eleven of the 13 PM proteins fused with GFP were relatively immobile. This result is consistent with previous studies (19, 21, 23, 27). No doubt a subset of PM proteins is immobilized through specific physical interaction with cell-wall components. For instance, the potassium channel KAT1 is known to be strongly immobile at the PM (28) and forms dot patterns in the PM which coalign with cellulose microfibrils (41). The cell wall also anchors AtFH1 (23). We have demonstrated, however, that other low-mobility PM proteins have no physical interaction with cell-wall components and have tested for PM/cell-wall proximity effects that might mediate protein immobilization.

The two full-length proteins with the highest mobility were GFP-LTI6b and GPA1-GFP, which are predicted to have only two amino acids in the apoplast or to be inserted in the inner leaflet of the PM, respectively. Full-length proteins inserted only in the outer leaflet of the PM, such as GFP-GPI in which the GFP projects into the cell-wall space, were strongly immobilized by the cell wall.
A set of minimal PM proteins that retain PM targeting ability but have been modified to prevent any protein–protein interaction were used to rule out protein interactions as a diffusion constraint. Lateral mobility of minimal PM proteins within the plane of the PM was constrained, as it was for many full length proteins, so we tried to identify other cellular factors that might limit protein mobility. Protein association, membrane microdomain structure, and cytoskeletons were shown to have little effect on protein dynamics. The cell wall, however, was shown to be involved in controlling protein mobility in the PM of plants. Plasmolysis to separate the PM and cell wall physically resulted in increased protein mobility, and, conversely, cell-wall regeneration in protoplasts led to a decrease in PM protein mobility. These results show the importance of the cell wall in limiting lateral diffusion of PM proteins, but an interesting distinction was made based on the extent to which a protein projects into the cell-wall space.

**Cell Wall Corrals PM Proteins.** Electron microscopy studies show that the distance between cell wall and PM must be smaller than a few tens of nanometers. This close association is highlighted by three factors. The first is the cellulose synthesis mechanism, which is mediated by the cellulose synthase complex (42). The cellulose synthase complex is a rosette protein complex inserted which is mediated by the cellulose synthase complex (42). The cellulose microfibril meshwork and associated components of the cell wall such as pectins and hemi-celluloses no doubt are appressed directly to the lipid bilayer. As a consequence, this meshwork might form bounded regions that serve to constrain the diffusion of PM proteins and phospholipids.

A cell-wall meshwork that acts as a constraint to PM protein lateral mobility is analogous with the animal cell anchored-protein picket model of Fujiwara et al. (36). Those authors used single-molecule tracking experiments to demonstrate that the actin cytoskeleton corrals proteins and limits protein and lipid lateral mobility in NRK cells. Our experiments show that actin and microtubule cytoskeletons do not perform this same function in plant cells but that a mechanism to stabilize proteins is conserved at the PM/cell-wall interface. Maintenance of protein localization and association within the PM is vital for cell signaling and transport and is a key component in the mechanism that maintains asymmetric distribution of PM proteins such as the PIN auxin efflux facilitators (22, 46).

The herbicide isoxaben inhibits cellulose synthases 2, 3, 5, and 6 (47) and therefore has a very detrimental effect on cell-wall structure. In single-molecule tracking experiments, paGFP-LT16b, with only two residues in the apoplastic, experienced a significant change to a more constrained movement pattern after very short (1 h) isoxaben treatment. Short-duration treatment of mature cells with isoxaben is unlikely to alter cell-wall structure dramatically, as occurs when seedlings are grown in the drug. In the short term, slight alteration to cellulose microfibril patterning might result from isoxaben treatment, but deposition of other cell-wall components such as pectins might be affected also. That the mobility of LT16b is constrained by short-term isoxaben treatment shows not only that interactions at the PM/cell-wall interface have an effect on proteins with extracellular domains but also that cell-wall organization universally influences protein diffusion.

**Materials and Methods.** A full discussion of materials and methods can be found in SI Materials and Methods. FRAP and single-molecule tracking methods are presented briefly here.

**FRAP Experiments.** The relative mobile fraction at time 60 s postbleaching (60s) of different fluorescent proteins was assessed by FRAP following the technique of Martinière et al. (23) (Fig. S1). Circular regions of interest (ROIs) (radius 4.3 μm) were bleached in median optical sections of the fluorescent PM. Recovery of fluorescence was recorded during 60 or 120 s with a delay of 1.5 s between frames. Fluorescence intensity data were normalized using the equation:

\[ I_{\text{t}} = \left( \frac{I_{\text{tmin}}}{I_{\text{tmax}}} \right) \times 100 \]

where \( I_{\text{t}} \) is the normalized intensity, \( I_{\text{tmin}} \) is the intensity at any time \( t \), \( I_{\text{tmax}} \) is the minimum intensity postphotobleaching, and \( I_{\text{tmax}} \) is the mean intensity before photobleaching.

Nonlinear regression was used to model the normalized FRAP data. In this case, a two-phase exponential association equation was used:

\[ Y_{\text{t}} = A + B \left( 1 - \exp \left( -\frac{t}{C_{1}} \right) \right) + C \left( 1 - \exp \left( -\frac{t}{C_{2}} \right) \right) \]

where \( Y_{\text{t}} \) is normalized intensity, \( A, B, C, K_{1}, \) and \( K_{2} \) are parameters of the curve, and \( t \) is time.

For each treatment, 10–20 cells were analyzed. The value of the fluorescence intensity recovery plateau was calculated for \( t = 60 \) and was used as an approximation of the relative mobile fraction (60s).

**TIRF Microscopy and Single-Molecule Tracking.** TIRF imaging was performed using a custom-built microscope equipped with a 100× objective (e-Plan-Fluar, NA = 1.45; Zeiss), 491-nm laser excitation (Cobolt), HQ525/50-nm emission filter (Chroma), and an electron-multiplication CCD (iXon; Andor) (48).

Cells expressing nonphotoactivated paGFP-LT16b have a population of naturally fluorescent paGFP molecules that is at a suitable density for TIRF microscopy and single-particle tracking. Tracks were calculated as described in Rolfe et al. (49). We measured 399 spots in six repetitions. Mean time of PM residence and track length were recorded. The MSD was calculated for all molecules:

\[ \text{MSD}(\Delta t) = \langle r_{i}(t + \Delta t) - r_{i}(t) \rangle^{2} \]

where \( r_{i}(t + \Delta t) - r_{i}(t) \) is the distance traveled by molecule \( i \) between time \( t \) and time \( t + \Delta t \), and the expectation value is over all pairs of time points separated by \( \Delta t \) in each molecular track.

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Supporting Information

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SI Materials and Methods

Constructs and Cloning Procedures. Fusion of 35S Full-length Protein-GFP fusions. All genes tested were from Arabidopsis. For generating GFP-AGP4, cDNA of AtAGP4 [Arabidopsis Biological Resource Center (ABRC) clone number U12380] was used as the PCR template. The signal peptide of AtAGP4 was removed by amplifying the fragment between amino acids 66–704. This DNA was fused by chimeric PCR to the C terminus of sp-mGFP5. The GFP-AGP4 fragment then was inserted into pVKH186En6 using XbaI-SacI restriction sites. cDNA of AtGPA1 (ABRC clone number U12585) and GFP-NPSN11 (1) were used as templates to amplify ORFs of GPA1 and NPSN11, which then were cloned into pENTR/d-TOPO (Invitrogen). pB7WG2 and pB7FWG2 Gateway (Invitrogen) binary vectors were used to generate GPA1-GFP and GFP-NPSN11 by LR reaction. The other full-length protein fusions with fluorescent protein included At3g17840-GFP and Atglg1870-GFP (2), GFP-LTI6b (3), AtFH1-GFP (4), PIP2;1-CFP (5), YFP-SYP121 (1), and GFP-StREM13 (6).

Construction of 35S minimal fluorescent proteins. To produce MAP-GFP, the first 36 aa of AtGPA1 were amplified and fused to the N terminus of EGFP by chimeric PCR and were cloned into pBIB using BamHI and SacI restriction sites. For GFP-PAP, nucleotides 243–297 of AtAGG1 (ABRC clone number U82084) were fused to the C terminus of EGFP and were cloned into pBIB using BamHI and SacI restriction sites. For GFP-GPI, the sporamin signal peptide and mGFP5 were amplified from GFP-HDEL and fused to nucleotides 318–405 of AtAGP4. The generated fragment was transformed into pVKH186En6 using XbaI and SacI restriction sites. Other minimal fluorescent proteins (FPs) included GFP-TM23 (7), and YFP-PHAPP1 (called “YFP-PI” in this study (8)).

Arabidopsis Stable Transformation and Tobacco Transient Transformation. Stably transformed Arabidopsis thaliana lines used include p35S::PIBP2-GFP (9), p35S::GFP-LTI6b (2), pUBQ10::YFP-NPSN11 (10), pFLS2::FLS2-GFP (11), p35S::paGFP-LTI6b (12), and pPIN2::PIN2-GFP (13). To transform minimal FP constructs stably, A. thaliana cv. Columbia plants were transformed using floral-dip transformation (14). Homozygous T1 seedlings expressing MAP-GFP, GFP-PAP, GFP-GPI, and YFP-PI were used for the analysis. For GFP-TM23, we used T1 plants. Nicotiana tabacum cv. Petit Havana plants were grown and used in transient transformation experiments as described by Sparkes et al. (15).

Drug Treatments, Cell-Wall Labeling, and Plasmolysis Experiments. A. thaliana seedlings were immersed in 25 mM latrunculin B (Calbiochem) (60 min) to remove the actin cytoskeleton, 20 μM oryzalin (Dow Elanco) (60 min) to remove microtubules, or 100 μM filipin III (Sigma-Aldrich) (30 min) to disrupt sterol in the plasma membrane. In protoplast experiments (see below), primary cell-wall regeneration was monitored by staining with 1 μg/mL of Calcofluor White M2R (Sigma-Aldrich). For plasmolysis assays, tobacco leaf sections (approx. 0.25 cm²) were incubated in a hypertonic solution of 0.5 M mannitol for 30 min and then were mounted in this solution for microscopy. In single molecule tracking experiments, some seedlings were immersed in 20 μM isoxaben (Sigma-Aldrich) for 1 h prior to observation.

Fluorescence Recovery After Photobleaching Experiments. The relative mobile fraction at time 60 s post bleaching (I60s) and relative diffusion rate (Dv) of different FPs was assessed by fluorescence recovery after photobleaching (FRAP) following the technique of Martinière et al. (4) (Fig. S1). Five scans of the entire field of view were made to establish the prebleach intensity of the FP, and then a circular region of interest (ROI) of approximately 57 μm² (radius 4.3 μm) was bleached in a median optical section of the fluorescent plasma membrane. Recovery of fluorescence was recorded during 60 or 120 s with a delay of 1.5 s between frames. We confirmed that the energy of the laser used to record postbleach data had no bleeding effect by recording a control region outside the bleaching ROI. Fluorescence intensity data were normalized using the equation:

$$ I_n = \left( \frac{I_t - I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} \right) \times 100 $$

where $I_n$ is the normalized intensity, $I_t$ is the intensity at any time $t$, $I_{\text{min}}$ is the minimum postphotobleaching intensity, and $I_{\text{max}}$ is the mean intensity before photobleaching.

Nonlinear regression was used to model the normalized FRAP data. In this case, a two-phase exponential association equation was used:

$$ Y_i = A + B \left( 1 + \exp^{\left(-K_1t\right)} \right) + C \left( 1 - \exp^{\left(-K_2t\right)} \right) $$

where $Y_i$ is normalized intensity, $A$, $B$, $C$, $K_1$, and $K_2$ are parameters of the curve, and $t$ is time.

For each treatment, 10–20 cells were analyzed. The value of the fluorescence intensity recovery plateau was calculated for $t = 60$ and was used as an approximation of the relative mobile fraction (I60s).

Values of $D_v$ and I60s were compared using two-tailed $t$ tests or ANOVA followed by Tukey’s Honestly Significant Difference (HSD) test using Microsoft Excel and SPSS software.

Total Internal Reflection Fluorescence Microscopy and Single-Molecule Tracking. Total internal reflection fluorescence (TIRF) imaging was performed on a custom-built microscope, equipped with a 100x objective (o-Plan-Fluar, NA = 1.45; Zeiss), 491-nm laser excitation (Cobolt), HQ525/50 nm emission filter (Chroma), and an electron-multiplication CCD (iXon; Andor) (16). Five-day-old seedlings were mounted in a drop of 1/2 strength Murashige and Skoog plant growth medium, pH 5.7, between a slide and a #0 coverslip. The angle of incidence of the excitation beam is continuously adjustable to optimize image contrast. Images were integrated for 30 ms.

Cells overexpressing nonphotoactivated paGFP-LTI6b have a population of naturally fluorescent paGFP molecules that is at a suitable density for TIRF microscopy and single-particle tracking. Plasma membranes were imaged for 30 s at 30 ms per frame.

A Bayesian segmentation-based feature-detection algorithm (17) was used to analyze single-molecule images of paGFP-LTI6b. Spots exhibited the blinking behavior typical for single molecules. Tracks were calculated as described by Rolfe et al. (18). Briefly, features are linked between frames into tracks according to connection probabilities that consider both spatial and temporal proximity, thus allowing tracks of blinking features to be determined. We measured 939 spots in six repetitions. Mean time of plasma membrane (PM) residence and track length were recorded. The mean squared displacement (MSD) was calculated for all molecules:

$$ \text{MSD}(\Delta t) = \left( \frac{1}{n} \sum_{i=1}^{n} \left( r_i(t + \Delta t) - r_i(t) \right)^2 \right) $$

where $r_i(t + \Delta t) - r_i(t)$ is the distance traveled by molecule $i$ between time $t$ and time $t + \Delta t$, and the expectation value is over all pairs of time points separated by $\Delta t$ in each molecular track.
Detergent-Insoluble Membrane Purification and Western Blot Analysis. Membrane fractionation was done following the protocol of Laloi et al. (18). *A. thaliana* seedlings were homogenized in the presence of 10 mM KH$_2$PO$_4$ (pH 8.2) with 0.5M sorbitol, 5% (wt/vol) polyvinylpyrrolidone 40 (Sigma-Aldrich), 0.5% (wt/vol) BSA, 2 mM salicylhydroxamic acid (Sigma-Aldrich), and 1 mM PMSF (Sigma-Aldrich). Homogenates were subjected to successive centrifugations at 1,000×g for 10 min, 10,000×g for 10 min, and 150,000×g for 60 min. Microsomal pellets were suspended in 25 mM HEPES buffer with 3 mM EDTA and 150 mM NaCl (pH 7.4). Microsomal membranes were incubated with 1% Triton X-100 (Sigma-Aldrich) for 30 min at 4 °C (detergent-to-protein ratio of 3) and then were mixed with an equal volume of 72% sucrose (wt/vol), transferred to centrifuge tubes, and overlaid with 40%, 35%, and 30% sucrose solutions. Sucrose gradients were centrifuged 16 h at 190,000×g at 4 °C. Gradients were collected and the Detergent-Insoluble Membrane (DIM) fractions were recovered. Detergent-insoluble membranes (DIM) were found in the top 3 fractions of the gradients. Proteins of each fraction were separated by SDS/PAGE on 12% polyacrylamide gels. Bands were blotted onto PVDF membranes (Biorad). For immunolabeling with an anti-GFP (Abcam), PVDF membranes were incubated sequentially with a saturated solution of PBS (1% BSA) for 1 h, with anti-GFP IgGs (Millipore) (1/4,000) for 2 h, and then with the anti–IgG-peroxidase conjugate (Biorad) (1/10,000) for 30 min.

**Protoplast Preparation and Immunofluorescence.** Tobacco leaf pieces were incubated for 15 h in a solution containing 0.4 M mannitol, 1% cellulase (Sigma-Aldrich) R10, 0.1% macerozyme (Sigma-Aldrich), 1 mM CaCl$_2$, 20 mM KCl, and 10 mM MES at pH 5.6. Protoplasts were used directly for imaging or labeled for immunofluorescence. Rabbit anti-GFP IgG (1/5,000 dilution) (Abcam), which also detects YFP, and anti-rabbit Texas Red (1/1,000 dilution) (Molecular Probes) were incubated sequentially for 1 h each with two rinsing steps between antibodies and after the secondary antibody.

Cell-wall regeneration experiments were carried out using *A. thaliana* leaf protoplasts. An aliquot of cells was imaged immediately as a time 0 sample. Protoplasts were left at room temperature in light for imaging after 48 h.
Fig. S1. Detailed analysis of FRAP curves for GPA1-GFP (A–D) and GFP-NPSN11 (E–H). (A and E) Analyzed ROI: (1) the bleached spot, (2 and 3) nonbleached plasma membrane regions, and (4) outside the cell. (B and F) Mean normalized pixel intensities during prebleach and 60-s postbleach recovery phases. Intensity drops sharply within the bleached region and then recovers quickly in the case of GPA1-GFP (B) or slowly in the case of GFP-NPSN11 (F). Fluorescence intensity within ROIs 2, 3, and 4 remains constant over time illustrating a lack of photobleaching during data-acquisition scanning. (C and G) Best-fit two-phase exponential curve to model the normalized data set. (D and H) Distribution of residuals from curve fitting is random.
Fig. S2. Mean prebleach intensity within a bleaching region vs. maximum recovery (I60s) during FRAP experiments. No relationship was observed ($R^2 = 0.03$), suggesting that the amount of protein free to diffuse is independent of the amount of that protein within the PM.
Fig. S3. Minimal FP constructs. (A) Schematic of minimal FPs. (B) All minimal FPs localized to the PM in tobacco leaf epidermal cells. The GFP signal (green) in cells expressing cytosolic eGFP can be seen surrounding the chloroplasts (red). PM localization means that the GFP always is exterior to chloroplasts for MAP-GFP, GFP-PAP, YFP-PI, GFP-GPI, and GFP-TM23. (Scale bar: 5 μm.)
Attempts to generate a minimal FP construct consisting of an intracellular FP moiety anchored by a single transmembrane domain (TMD). Three soluble NSF attachment protein receptors (SNAREs), SYP121, NSPN11, and NSPN12, were tested as a source of the TMD. (A) All three SNAREs were found to possess small extracellular domains as indicated in hydrophobicity plots. The TMD is indicated by shading, and the region to the right of the shading must project into the extracellular space. Plots generated using ARAMEMNON software (1) (http://aramemnon.uni-koeln.de/). (B) Three GFP fusions were made with the C-terminal region of NSPN11. The putative TMD is indicated in uppercase. The extracellular domain was shortened successively. (C) The putative extracellular tail of NPSN11 is required for proper targeting to the PM. The minimal construct targeted the PM only when the entire C terminus was present. (D) FRAP experiments on GFP-NSPN11-TM-C-ter in control and plasmolyzed cells. Deletion of most of the cytoplasmic region of NSPN11 resulted in a small increase in protein mobility (compare with Fig. 1A), and plasmolysis increased GFP-NSPN11-TM-C-ter mobility slightly but nonsignificantly.

Fig. S5. Immunofluorescence on living tobacco protoplasts expressing minimal FPs. Protoplasts were incubated with an unlabeled rabbit anti-GFP antibody (the antibody also labels YFP; see Fig. S6), which was detected using a goat anti-rabbit antibody coated with Cy5 fluorophore. GFP or YFP (green) marks the PM of all protoplasts, but the PM is labeled by the secondary antibody (red) only when the FP projects into the cell-wall space as for GFP-GPI and GFP-TM23. (Scale bar: 20 μm.)
Lipid domain structure and cytoskeleton have a limited effect on protein mobility in *A. thaliana*. (A) DIM fractionation of minimal FP-expressing *A. thaliana* followed by Western blotting with anti-GFP (lanes 1–8 represent position within the fractionation gradient). MAP-GFP and GFP-GPI are present in both DIM and non-DIM fractions, whereas GFP-PAP, YFP-PI, and GFP-TM23 occur primarily in non-DIM fractions. GFP-REM occurs in both DIM and non-DIM fractions, as expected. In contrast, PMA-GFP, a non-DIM protein, is absent from the first five fractions. (B) Fluorescence recovery (160s) of each minimal FP construct in the presence or absence of the sterol-disrupting drug filipin III. Filipin treatment had no effect on protein mobile fraction ($P = 0.87$, two-way ANOVA). (C) Fluorescence recovery (160s) of each minimal FP construct in the presence or absence of cytochalasin D, an actin-depolymerizing drug, or oryzalin, a microtubule-depolymerizing drug. Disruption of cytoskeletons had either no significant effect or induced a decrease in fluorescence recovery (160s MAP-GFP control vs. MAP-GFP cytochalasin D, $P = 0.003$, Tukey’s HSD test; 160s MAP-GFP control vs. MAP-GFP oryzalin, $P = 0.02$, Tukey’s test; 160s GFP-TM23 control vs. GFP-TM23 cytochalasin D, $P < 0.001$, Tukey’s test; 160s GFP-TM23 control vs. GFP-TM23 oryzalin, $P < 0.001$, Tukey’s test).
Fig. S7. Cell-wall regeneration in A. thaliana protoplasts. Calcofluor white M2R (green) was used to stain cellulose of freshly prepared protoplasts or of cells after 48 h of cell-wall neosynthesis. No cellulose was detectable in freshly prepared protoplasts. Chlorophyll autofluorescence is shown as red. (Scale bar: 10 μm.)

Fig. S8. Single-molecule tracking. (A) Fluctuations in intensity of a single molecule during a 2.5-s track. Such blinking is a typical phenomenon for single fluorochrome molecules. (B) Quantification of the exocytosis events that mark the appearance of single fluorescent molecules. Nontransformed Col0 cells had virtually no molecules, whereas control cells expressing paGFP-LTI6b had 50 ± 10 molecules per 50 μm². Sodium azide treatment, which inhibits exocytosis, significantly reduced the number of molecules.

Movie S1. Hypocotyl cells expressing nonphotoactivated paGFP-LTI6b observed using TIRF microscopy with 30-ms time integration and 0.16 μm per pixel. Single particles are visible as short-lived spots brighter than the background.

Movie S1