Chapter 12

Transient Gene Expression as a Tool to Monitor and Manipulate the Levels of Acidic Phospholipids in Plant Cells

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Abstract

Anionic phospholipids represent only minor fraction of cell membranes lipids but they are critically important for many membrane-related processes, including membrane identity, charge, shape, the generation of second messengers, and the recruitment of peripheral proteins. The main anionic phospholipids of the plasma membrane are phosphoinositides phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 4,5-bisphosphate (PI4,5P_2), phosphatidylserine (PS), and phosphatidic acid (PA). Recent insights in the understanding of the nature of protein–phospholipid interactions enabled the design of genetically encoded fluorescent molecular probes that can interact with various phospholipids in a specific manner allowing their imaging in live cells. Here, we describe the use of transiently transformed plant cells to study phospholipid-dependent membrane recruitment.

Key words Microscopy, *Nicotiana benthamiana*, *Nicotiana tabacum*, Phosphoinositides, Phospholipid-binding domains, Pollen tube, Transient expression

1 Introduction

All living cells are surrounded by membranes, which help to define their spatial identity and create the semipermeable boundary between intracellular and extracellular space [1]. Typical cellular membrane is composed of a bilayer of lipids and proteins, whose organization and interactions are crucial for its function as organizing platforms for cellular processes. Historically, cellular membrane studies were once dominated by a protein-centric view, where proteins executed majority of membrane-related functions and the membrane lipids were often regarded only as passive players whose role was to provide structural support for bilayer formation [2]. It is now generally accepted that both lipids and proteins play indispensable active roles in the various functions of cellular membranes [3].

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Among plant plasma membrane lipids, negatively charged (anionic) phospholipids, phosphoinositides like phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PI4,5P_2), together with phosphatidylserine (PS) and phosphatidic acid (PA), constitute low-abundant but essential component [4–6]. They possess many important roles, which include defining membrane identity, generation of downstream signaling molecules, generating membrane negative charge, modulating membrane curvature, and creating binding sites for the targeting of effector proteins [7–12].

The realization of the important roles of anionic phospholipids has created a need for methods that would enable their noninvasive spatiotemporal monitoring in living cells. The identification and characterization of protein modules that specifically bind to various anionic phospholipids led to the idea that these protein modules might be used to detect phospholipids in living cells. This resulted in the development of genetically encoded phospholipid sensors that consist of the specific phospholipid-binding domains (either single or in tandem) fused with various fluorescent proteins, enabling the live cell imaging of phospholipid dynamics [13–15]. Especially in the past decade, this approach has been successfully used in plants to generate sensors for a wide variety of phospholipids including PI4P [16–18], PI4,5P_2 [17, 19, 20], PA [21, 22], and PS [17, 22]. Concomitantly, many enzymes involved in the production and degradation of anionic lipids were identified and the phenotypes of their knockout or overexpressing mutant lines described (for plants *see* for example refs. 23, 24). This also led to the development of tools allowing the manipulation of phospholipid levels in the cell upon generic or targeted overexpression of phospholipid-modifying enzymes [25].

The recruitment of phospholipid-binding peripheral proteins to cell membranes is essential for many cellular processes. The targeting of proteins to specific phospholipids or to the membranes of particular lipid compositions, mediated by lipid-binding domains, allows their recruitment to be precisely controlled in spatiotemporal fashion. Despite the manifold biological consequences associated with the targeted recruitment of peripheral protein to their target membranes, only a basic understanding of the interactions of proteins with membrane surfaces exists because these questions are inaccessible by commonly used structural techniques [26, 27]. Therefore, the selective colocalization of peripheral proteins (or individual protein domains, protein deletions, point mutations etc.) with the particular lipid marker together with its relocalization after coexpression with corresponding phospholipid-modifying enzymes may bring valuable information about the nature of protein–membrane interface. Transient gene expression approaches are particularly beneficial, since they enable quick screening of many proteins or protein variants and allow for

easy manipulation of expression level. Here, we describe the protocols allowing transient coexpression of proteins of interest with genetically encoded lipid markers or phospholipid-modifying enzymes in two different plant cell types: biolistics-mediated transformation of growing tobacco pollen tubes (that show spatially separated plasma membrane domains enriched with distinct phospholipids, refs. 21, 22), and agroinfiltration of *Nicotiana benthamiana* leaf epidermal cells (where high transformation efficiency can be achieved).

2 Materials

Prepare all solutions using ultrapure water and store at room temperature (RT), unless stated otherwise.

2.1 Particle Bombardment Solutions

1. Gold particles: resuspend 30 mg of 1.6 μm gold microcarriers (Bio-Rad, #1652264, *see* **Note 1**) in 1 ml absolute ethanol, vortex vigorously for 3 min, and spin down at table top centrifuge (1 min, maximum speed). Wash twice with H_2O and resuspend in 1 ml of 50% glycerol (sterile). Store at 4 °C.
2. 2.5 M $CaCl_2$: dissolve 3.675 g $CaCl_2 \cdot 2H_2O$ (Sigma, #C7902) in 10 ml H_2O. Filter-sterilize and keep 1 ml aliquots at −20 °C. Working aliquot can be kept at 4 °C for several months.
3. Protamine: dissolve 10 mg of protamine sulfate (Sigma, #P4505) in 10 ml H_2O. Filter-sterilize and keep 0.5 ml aliquots at −20 °C. Working aliquot can be kept at 4 °C for several weeks.

2.2 DNA

1. For tobacco pollen particle bombardment, dilute DNA stock with H_2O to 0.25–1 μg/μl working solution. In order to achieve good transformation frequency and expression levels, target construct expression must be driven by promoters active in pollen (typically LAT52p or UBQ10p). 35S promoter is not recommended. Usually, clean miniprep is enough for several transformations. Store at −20 °C.
2. For *N. benthamiana* leaves infiltration, use DNA plasmid concentrated at 0.25–1 μg/μl to transform *Agrobacterium*. Expression of the construct is usually driven by *UBQ10* or *35S* promoters.

2.3 Biological Materials

1. Pollen: flowers of outdoor- or glasshouse-grown tobacco plants (*N. tabacum* cv. Samsun) are collected in warm and dry weather conditions before opening; anthers are taken out and kept in laboratory conditions on a filter paper for 1 day to let anthers open and dehydrate (anthers might be surface sterilized and dried in the laminar box to harvest sterile pollen). Dried

pollen grains are sifted through to remove anthers. Harvested pollen can be kept frozen at −20 °C without apparent loss of the germination capacity for several years. 1 mg of pollen is usually used per transformation.

2. *N. benthamiana* leaves: use leaves from 2 to 3 weeks old plants. For infiltration, select leaves that are heart shaped. If the plant has already flowered, it is too late to perform the infiltration.
3. Agrobacterium: the electrocompetent *A. tumefaciens* C58pmp90 strain is used for tobacco leaf infiltration.

2.4 Cultivation Media

1. 2× pollen tube medium (2×PTM): 10% w/v sucrose, 25% w/v PEG-6000, 2 mM $CaCl_2$, 2 mM KCl, 1.6 mM $MgSO_4$, 3.2 mM H_3BO_3, 60 μM $CuSO_4$, 0.06% w/v casein acid-hydrolysate, 0.6% w/v MES, pH 5.9. Prepare 10× stock solution for salts (1470 mg/l $CaCl_2 \cdot 2H_2O$, 746 mg/l KCl, 1972 mg/l $MgSO_4 \cdot 7H_2O$, 989 mg/l H_3BO_3, 75 mg/l $CuSO_4 \cdot 5H_2O$) and 50× stock for casein hydrolysate (1.5% w/v). Store the stocks at −20 °C. Dissolve appropriate amounts of sucrose, MES, salt, and casein hydrolysate stocks in H_2O, adjust pH to 5.9 with 4 M KOH, add PEG-6000 and make up to the final volume with H_2O. Store 50 ml aliquots at −20 °C.
2. Solid pollen tube medium: 1×PTM solidified with 0.25% Phytagel (*see* **Note 2**). Thaw 50 ml aliquot of 2×PTM and warm it in water bath to at least 60 °C. Prepare 0.5% Phytagel (Sigma, #P8169) solution: weigh 0.25 g of Phytagel into 50 ml H_2O and resuspend. Dissolve Phytagel by carefully heating up to the boiling point while stirring. Mix 2×PTM and Phytagel solutions and keep the 1×PTM/0.25% Phytagel medium in hot water bath. In laminar box, prepare plates with solidified medium by rapid pouring of 4 ml of hot PTM/Phytagel solution onto 5 cm petri dishes. Let dry and store up to 1 month at 4 °C.
3. LB medium: Agrobacterium are grown on LB liquid medium (Difco™ LB Broth, Lennox, #240230, 20 g/l) and LB plate (Difco™ LB Broth, Lennox, #214010, 20 g/l, 15% w/v agar, Difco™ Bacto Agar).
4. Infiltration medium: 10 mM MES (Sigma-Aldrich, #D8250-250G), 10 mM $MgCl_2$ (Sigma-Aldrich, #M2670), 0.15 mM acetosyringone (Sigma-Aldrich, #D134406). Prepare a stock solution of 100 mM MES. Weight and dissolve the appropriate amount of MES in H_2O and adjust the pH to 5.7 using KOH solution. Autoclave the MES solution for 30 min and store it at RT. Prepare a stock solution of acetosyringone at 100 mM in EtOH and store it at −20 °C. Just before performing the infiltration, dilute the MES solution in H_2O, add the $MgCl_2$ and the acetosyringone to a final concentration of 10 mM and 0.15 mM, respectively.

3 Methods

Carry out all procedures at RT, unless otherwise specified (*see* **Note 3**).

3.1 DNA Macrocarrier Preparation for Pollen Particle Bombardment

1. Add following to the 1.5 ml microcentrifuge tube to prepare one macrocarrier (the sample should be mixing continuously, keep sequence and timing): 25 μl of gold particles suspension, vortex 1 min, add 2.5–7 μl of plasmid DNA (~1–10 μg, *see* **Note 4**), 25 μl of 2.5 M $CaCl_2$, and 10 μl of protamine (1 mg/ml) solution.
2. Vortex vigorously for at least 3 min. Spin down in tabletop centrifuge for 30 s at max speed. Remove the supernatant carefully using yellow pipette tip or vacuum and discard it.
3. Resuspend the pellet completely (*see* **Note 5**) in 200 μl of absolute ethanol and vortex for 3 min. Spin down again and discard the supernatant.
4. Resuspend the pellet in 18 μl of absolute ethanol, vortex for 1 min and load the suspension on the macrocarrier (Bio-Rad, #1652335). Keep the suspension dispersed by constant pipetting. For future manipulations, it is better to have macrocarrier fitted in the steel macrocarrier holder (Bio-Rad, #1652322) before loading.
5. Let the macrocarrier dry in a vibration-free environment. One should obtain evenly distributed layer of gold particles without any visible clumps. Although dried macrocarriers can be stored in dry chamber at RT for several hours, it is better to perform the transformation immediately after preparation.

3.2 Pollen Plating

1. For one transformation, resuspend 1 mg of tobacco pollen per 5 ml of 1×PTM. Pour the suspension on the prewetted nylon 47 mm (0.8 μm) filter disc (Whatman, #Z746282) placed on filtration apparatus (e.g., Millipore, #XX1004720) and remove medium using vacuum.
2. Transfer the pollen to the solidified PTM by placing the filter disc upside-down briefly. Repeat for the next DNA sample and/or proceed to the particle bombardment immediately (*see* **Note 6**).

3.3 Particle Bombardment

1. Set up the PDS-1000/He system (Bio-Rad, #1652257) according to the instruction manual using standard settings.
2. Put the rupture disc (1100 psi, Bio-Rad, #1652329) in place and tighten gently with the screwdriver.
3. Assemble macrocarrier and stopping screen (Bio-Rad, #1652336) into microcarrier launch assembly and insert it into the uppermost position.

4. Use the second free slot from above for the pollen plated on solidified medium.
5. Evacuate the chamber to 28 inHg and perform the bombardment.
6. Release the vacuum immediately, seal the sample plate with Parafilm and store it at RT.

3.4 Agrobacterium Transformation

1. For each construct, add 1 μl of DNA plasmid into 50 μl of electrocompetent Agrobacterium on ice.
2. Transfer the Agrobacterium into cold 1 mm wide electroporation chamber (Eurogentec, #CE00150). Put the electroporation chamber into the MicropulserTM (Bio-Rad, #165-2100) and give a pulse of 2 kV, 335 Ω, 15 μF, for 5 ms.
3. Add 1 ml of liquid LB medium and transfer the bacteria into a new tube and incubate them at 29 °C for at least 2 h.
4. Plate the Agrobacterium onto LB plates containing the appropriate antibiotics to select the Agrobacterium strain (gentamycin 20 μg/ml and rifampicin 50 μg/ml) and the target construct. Incubate the plate at 29 °C for 48 h.

3.5 Nicotiana benthamiana Infiltration

1. Scoop transformed Agrobacterium from the transformation plate with a tip and resuspend the bacteria into 2 ml infiltration medium (*see* Subheading 2.4) by pipetting. Measure the OD_{600} using a spectrophotometer (Biophotometer, Eppendorf). Adjust the OD_{600} to 1 by adding infiltration medium. For coinfiltration of several constructs, mix the same quantity of each transformed Agrobacterium to obtain a final OD_{600} of 1.
2. Using 1 ml syringe (Terumo, #125162229), press the infiltration solution with the Agrobacterium onto the abaxial side of the chosen tobacco leaf keeping your finger on the other side of the leaf. The solution must spread into the leaf (*see* **Note 7**).
3. Mark the place where the infiltration has been made with a permanent marker. Put the plant back to the growth chamber for 2–3 days.

3.6 Data Acquisition, Analysis, and Quantification

Images can be exported from the microscope-specific acquisition software and analyzed with suitable analysis software. We use Fiji for this purpose, which is a distribution of well-known software ImageJ [28, 29], bundling a lot of plugins which facilitate scientific image analysis, and which is freely available at https://fiji.sc/. A number of basic and advanced tools are available within this software, including subtraction of background, and measurements of intensities, both based on the definition of a region of interest.

1. Particle-transformed pollen tubes: for the initial evaluation of protein overexpression on pollen tube growth and polarity,

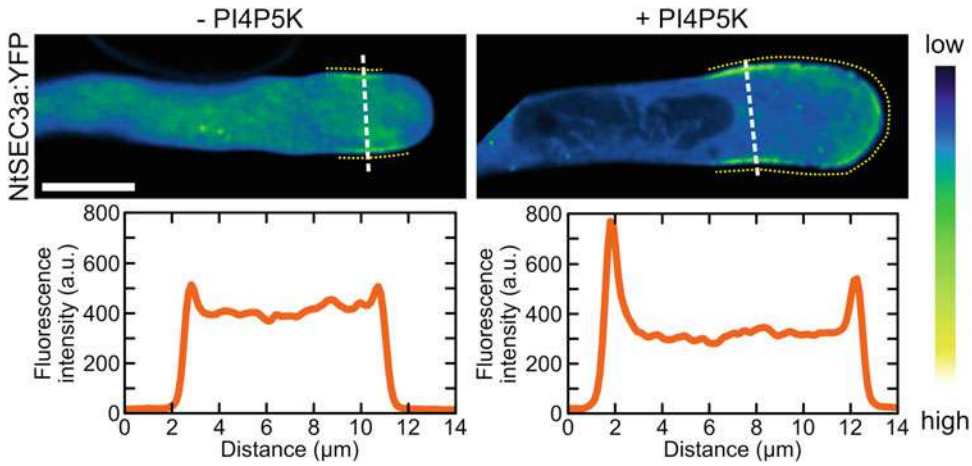

Fig. 1 Elevation of PI4,5P_2 levels by the overexpression of the CFP-tagged PI4P-5 kinase (PI4P5K) results in increased NtSEC3a:YFP recruitment to the plasma membrane in tobacco pollen tubes. Pollen tubes with comparably low expression level of NtSEC3a:YFP were selected. For coexpression with PI4P5K, cells expressing high levels of PI4P5K:CFP (not shown) and displaying characteristic PI4PK5-overexpression phenotype were selected. White dashed lines mark the site of intensity profiles and yellow dotted lines indicate length of the membrane fluorescence signal. Micrographs are shown using a color intensity code in order to display local enrichment of the YFP fluorescence

observe the cells 12–24 h after transformation with 5–10 × lenses. Identify transformed cells based on FP fluorescence and take images using the same acquisition settings. Mean pollen tube length, pollen tube width, tip swelling and cell "curviness" (calculated as the ratio of the distance between the pollen grain/pollen tube tip and the pollen tube length, is close to 1 for straight pollen tubes) are good parameters for the initial quantitative assessment. Several simple measures can be used to monitor the binding of protein of interest to the plasma membrane (e.g., measuring of the membrane- and cytoplasmic-associated intensities from the line scan (Fig. 1, *see* also refs. 21, 30) and calculating the ratio as a proxy for membrane recruitment index, and/or measuring the length of membrane signal (in the case of asymmetric localizations, *see* Fig. 1)). For the quantitative assessment of colocalization, Pearson or Spearman rank correlation coefficients can be calculated from the data (e.g., with Coloc 2 plugin available in Fiji).

2. Confocal observation of *N. benthamiana* leaves: cut 5 mm^2 regions of the leaf that surround the place where the infiltration has been made. Place the piece of leaf into water between slide and coverslip with the abaxial side of the leaf facing the coverslip. It may be convenient to tape the slide and coverslip together to maintain the coverslip on the slide as the leaf sample is thick. Using the appropriate wavelength, an epifluorescent microscope and the smallest objective (10×), screen the

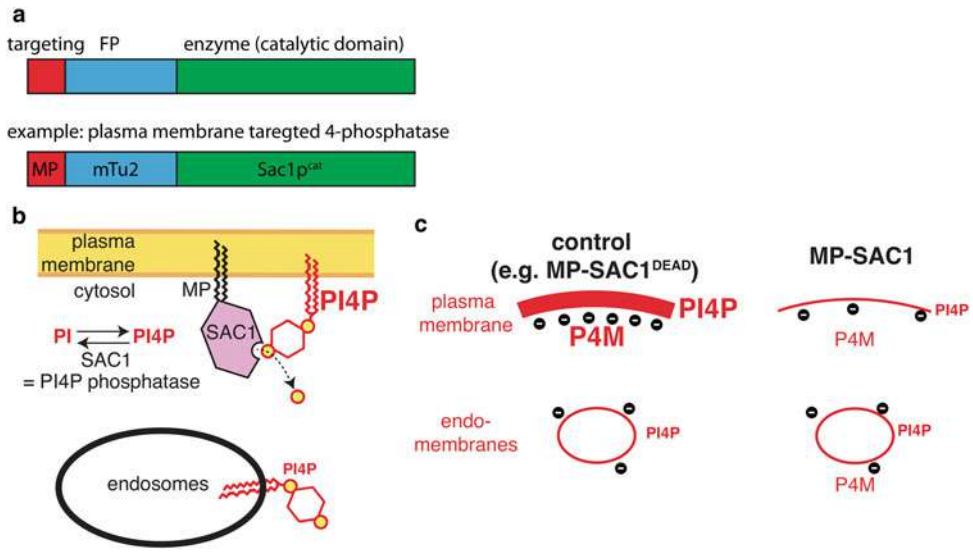

Fig. 2 Organelle-targeted phosphatase as a way to locally interfere with acidic phospholipids. (**a**) The top panel represents a schematic representation of a possible construct for organelle-specific targeting of a lipid modifying enzyme. Such system include an organelle-specific targeting anchor/protein (which may be at the N- or C-terminal end of the synthetic chimeric protein), a fluorescent protein to verify localization specificity, and the isolated catalytic domain of a lipid modifying enzyme. An important criterion for the use of the catalytic domain is that it should be free of any endogenous targeting capacity. The bottom panel represents an example of construct for the specific depletion of PI4P at the plasma membrane (i.e., MP-Sac1). (**b**) Schematic representation of MP-Sac1 action at the PM but not endosome and (**c**) effect of MP-Sac1 on PI4P accumulation. In the control condition (left, for example with expression of a catalytically dead MP-Sac1 enzyme), there is much more PI4P at the PM than in endosomes and as a result, a PI4P biosensor such as P4M is localized preferentially at the PM. Upon expression of MP-Sac1 (right), the pool of PI4P at the PM is reduced, which triggers the redistribution of the P4M PI4P sensor to both PM and endosomes

surface of the leaf to find the transformed cells. Then, switch to confocal microscope and 63× objective to look at the subcellular localization of the fluorescent protein.

3. Analysis of the effect of PM-targeted phosphatases on anionic phospholipid localization: in order to perturb anionic phospholipid production with subcellular accuracy, it is possible to target an isolated phosphatase (or kinase) domain to a specific organelle. For example, the 4-phosphatase SAC domain of the yeast Sac1p protein is targeted to the PM using a myristoylation and palmitoylation anchor (MP) (ref. 18; Fig. 2). This MP-Sac1 construct was fused to a mTurquoise2 (mTu2) protein to monitor protein localization (MP-mTu2-Sac1) in order to verify that this synthetic enzyme was indeed efficiently targeted to the PM. Cotransfection with genetically encoded anionic phospholipid sensors allowed to determine the effect of the 4-phosphatase activity on the production of a given phospholipid. Quantification of the effect of the phosphatase activity may be performed using the analyses mentioned above

for pollen tubes. Typically, three behaviors may be anticipated for the biosensors following coexpression with an organelle targeted phosphatase: (1) no effect, (2) redistribution of the sensor from a membrane to a cytosolic pool, and (3) redistribution of the sensor to a different organelle. For example, MP-Sac1 expression induced the redistribution of PI4P biosensors from the PM to endosomes (Fig. 2, *see* also refs. 18, 22, 31). This can be quantified qualitatively, as a percentage of cells with endosomal labeling by the PI4P sensor, as compared to the total number of cells analyzed. It can also be quantified by making a ratio of membrane vs. soluble signal, but this later quantification method is difficult given the reduced cytoplasm of *N. benthamiana* leaf cells. Once validated, such heterologous transient assay may be used to probe the importance of a given lipid for targeting a protein. It may also be used to validate in vivo catalytic activity of a phosphoinositide phosphatase of unknown specificity.

4 Notes

1. Different sizes of particles (0.6 or 1.0 μm) may be also used; this will however affect the amount of coatable DNA. Alternatively, cheaper tungsten particles may be used, their size distribution is however much more variable, resulting in yet greater variability in expression levels.
2. Do not use agar or agarose as they would cause the precipitation of pollen tube medium.
3. For the details of PDS-1000/He assembly and operation, consult the PDS-1000/He Particle Delivery System Instruction Manual (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/M1652249.pdf) and watch the YouTube tutorial (https://www.youtube.com/watch?v=dfD95gsEdrg&t=90s).
4. When transforming with more than one construct, premix the DNA before coating. Use only small amount (0.5–1 μg of plasmids expressing phospholipid markers to prevent the perturbation of phospholipid signaling due to overexpression of lipid-binding domain).
5. This is crucial for obtaining good transformation frequency. The more DNA is added the longer it takes to resuspend the pellet completely.
6. We routinely transform up to 12 plates in a row. If more transformations are needed, split the plating/bombardment into batches of ten.
7. The infiltration might not work if the stomata are closed. To get around this problem, make small holes with a needle.

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