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Cell-to-cell connectivity assays for the analysis of cytoskeletal and other regulators of plasmodesmata

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Abstract

The actin cytoskeleton has close, but so far incompletely understood connections to plasmodesmata, the cell junctions of plants. Plasmodesmata are essential for plant development and responses to biotic and abiotic stresses, and facilitate the intercellular exchange of metabolites and hormones, but also macromolecules such as proteins and RNAs. The molecular size exclusion limited of plasmodesmata is dynamically regulated, including by actin-associated proteins. Therefore, experimental analysis of plasmodesmal regulation can be relevant to plant cytoskeleton research. This chapter presents two simple imaging-based protocols for analysing macromolecular cell-to-cell connectivity in leaves.

Key words Plasmodesmata, size exclusion limit, microprojectile bombardment, *Arabidopsis thaliana*, *Nicotiana benthamiana*, acto-myosin

1 Introduction

Plasmodesmata are the intercellular junctions of plant cells, and are vital for developmental, physiological and defence signalling, as well as distribution of photosynthate and nutrients [1]. Structurally, plasmodesmata differ from the cell connections of other eukaryotes in that they not only consist of a plasma membrane-lined cytoplasmic channel spanning the cell wall, but also contain a

central strand of highly constricted tubular endoplasmic reticulum (called the desmotubule) that is continuous between cells. The desmotubule and plasma membrane are connected by tethering proteins inside the plasmodesmal channels (Fig. 1a) [1,2]. Transport of small and macro-molecules occurs mostly through the cytoplasmic compartment of plasmodesmata, though the endoplasmic reticulum membrane and lumen have also been observed to allow for limited intercellular exchange of molecules [3,4]. Plasmodesmata are extremely small, with an overall cross-section of ~50 nm, and an estimated free space between the two membranes and the tethering proteins of only ~3 nm. The molecular size exclusion limit (SEL) of plasmodesmata, which is the maximum molecular weight, or more accurately, the maximum (hydrated) Stoke's radius of molecules that can pass through the channels, is dynamically regulated. The most well-characterised mechanism of regulation is the deposition and removal of callose (β-1,3-glucan) in the cell wall surrounding plasmodesmata entrances, which is thought to narrow the channel aperture by bringing the plasma membrane closer to the desmotubule. However, there may be additional regulatory mechanisms, possibly linked to the complex architecture of the channels [1].

A number of proteins associated with the actin cytoskeleton have been found to be associated with plasmodesmata, including actin itself, plant-specific myosin VIII, formins, the plant-specific actin binding protein NETWORKED 1, and an unidentified protein recognised by antibodies against animal tropomyosin (reviewed in [1,4-6]). Several studies have also shown that some of these proteins, as well as chemical inhibitors of actin and myosin, influence the SEL [7,8]. Immuno-electron microscopy suggests actin and myosin may be structural components within plasmodesmata [9,10] and myosin VIII has been suggested as a candidate for the tethering proteins connecting the desmotubule and plasma membrane [11]. On the other hand, it is unclear how microfilaments or the very large myosins might physically fit into the tight internal space of plasmodesmata, or if the actin cytoskeleton is instead associated with the channel entrances [4,9,12].

Due to this intricate, yet only partially understood connection of acto-myosin with plasmodesmata, it may be of interest to researchers studying the plant cytoskeleton to analyse functional links between cytoskeletal proteins and plasmodesmata. The most direct way to do this is by probing cell-to-cell

connectivity using fluorescent reporters. This can be done using small fluorophores [13,14], however, fluorescent proteins like Green/Red Fluorescent Protein (GFP/RFP; 27 kDa, Stoke's radius ~3 nm) are closer to the typical plasmodesmal SEL and more representative of mobile macromolecules such as transcription factors and small RNAs. Due to the temporally dynamic and spatially heterogenous fluctuation of plasmodesmal regulation, such tracer studies using a reporter close to the SEL usually do not yield clear yes/no answers regarding mobility, but rather reveal differential regulation, where the reporter moves in a certain percentage of cases and across a certain range of cells/cell boundaries. This allows for identification of subtle changes in plasmodesmal permeability [15].

In order to use a fluorescent protein as a marker for intercellular mobility the fluorescence needs to be initially confined to single cells. One elegant way of achieving this is to use photoactivatable or photoswitchable fluorescent proteins which can be selectively activated or switched in single cells. When the reporter is stably expressed throughout the plant, plasmodesmata permeability can theoretically be measured at any cell interface that is accessible to microscopy [16]. Transient expression without the generation of stably transformed reporter plant lines is quicker, but limited to tissues that can be transiently transformed, usually leaves. Single-cell expression can easily be achieved by microprojectile bombardment. Alternatively, very low-density *Agrobacterium* infiltration can also produce single transformed cells. Here, we describe two alternative cell-to-cell connectivity assays which can be used for analysis of Arabidopsis transgenic lines as well as *Nicotiana benthamiana* leaves transiently transformed by *Agrobacterium* infiltration.

2 Materials

Novel and published plasmids described in this chapter are available upon request for non-commercial academic research.

2.1 Cell connectivity assay by microprojectile bombardment

1. Growth facilities appropriate for handling of transgenic plants.

2. *Arabidopsis thaliana* stable transformant lines for overexpression or knock out / knock down of protein(s) of interest; grown for ~3 weeks on soil until large rosette but before first bolt.
3. Plasmid prep of pRTL2.GFP [17], pRTL2.GFP-sporamin [17], pRTL2.mCherry (Z. Barr, unpublished), or pRTL2.mCherry-sporamin (Z. Barr, unpublished), as appropriate (*see* **Note 1** and **Note 2**).
4. Gold suspension: 1 μm gold particles (BioRad 1652263) in ethanol (*see* Subheading 3.1.1).
5. Gold-plasmid mix (*see* Subheading 3.1.2).
6. Swinnex syringe filter holder 13 mm (Sigma-Aldrich SX0001300).
7. Hand-held particle bombardment device (Gene-gun) [18].
8. Plasmid miniprep kit.

2.2 Cell connectivity assay by *Agrobacterium* infiltration

1. Growth facilities appropriate for handling of transgenic plants.
2. *Nicotiana benthamiana:* grown for 4 weeks on soil under suitable conditions.
3. Glycerol stock of *Agrobacterium tumefaciens* transformed with pGWB402Ω.GFP (J. Tilsner, unpublished).
4. Optional: Glycerol stock of *A. tumefaciens* transformed with relevant expression construct of interest.
5. Optional: Glycerol stock of un-transformed *A. tumefaciens*.
6. LB agar plates (prepared using LB liquid medium with the addition of 15 g/L agar) with appropriate selection antibiotics (for pGWB402Ω.GFP: 100 μg/mL spectinomycin; 50 μg/mL rifampicin for most *Agrobacterium* strains).
7. LB liquid medium (10 g Tryptone, 10 g NaCl, and 5 g yeast extract per 1 L, pH 7.0) containing appropriate selection antibiotics (*see* Subheading 2.2, **item 6**), but omitting rifampicin.
8. Infiltration medium: 10 mM $MgCl_2$, 10 mM MES (2-(N-morpholino)ethanesulfonic acid), 0.15 mM acetosyringone. You will require 2 mL for every culture to resuspend and 2 mL for every leaf to infiltrate.

9. 28 °C static incubator.
10. 28 °C shaking incubator.
11. 25G needle.
12. 1 mL syringe.

2.3 Imaging

1. Upright confocal laser scanning microscope equipped with 488 nm or 561 nm light source for GFP or RFP excitation, respectively, and 10 x long-distance and 40 x water immersion lenses.
2. Glass slides.
3. Double sided sticky tape.
4. Prepared leaf (*see* Subheadings 3.1 or 3.2, respectively).
5. Data sheet.

3 Methods

Select appropriate (*see* **Note 3**) choice for transformation (*see* Subheadings 3.1 and 3.2). The following presupposes bombardment of *A. thaliana* and (co-)infiltration of *N. benthamiana*. The subsequent imaging and data analysis (*see* Subheading 3.3) are identical for both assays

3.1 Cell connectivity assay by microprojectile bombardment

This assay can be used to analyse the effect of stably overexpressed or knocked down / knocked out proteins of interest on cell-to-cell connectivity in *A. thaliana*. It can also be used on stable transgenic or transiently transformed *N. benthamiana* (but *see* **Note 3**).

3.1.1 Preparing the gold suspension

1. Weigh out 50 mg of 1 μm gold.
2. Add 1 mL ethanol and vortex.
3. Gently pellet the gold (1 min 1 krpm), remove the supernatant.

4. Resuspend in 950 µL ethanol and 50 µL H_2O, vortex and store at -20°C (*see* **Note 4**).

3.1.2. Preparing the gold-plasmid mix

1. Calculate the number of shots required (*see* **Note 5**) and select the appropriate tracer construct (*see* **Note 1**).
2. For 10 shots, mix 14 µL plasmid, 14 µL ethanol, 22 µL Gold suspension and vortex thoroughly.

3.1.3. Microprojectile bombardment

1. Set the gene-gun with the nitrogen-gas pressure at 20-25 psi and the trigger intensity dial at approximately 20. These settings should be adjusted so that the trigger only just causes a pressure blast, whilst minimising leaf damage.
2. Vortex the gold-DNA suspension in an open tube held below the rim while pipetting to ensure the best resuspension possible and immediately pipette 5 µL (1 shot) on to the Swinnex filter holder. Attach the filter holder to the gene-gun.
3. Select the 4th true leaf of an *N. benthamiana* plant (*see* **Note 6**). Position the end of the filter holder approximately 2 cm above the leaf. Hold the leaf upper epidermis up and place your fingers to support underneath.
4. Trigger two nitrogen gas bursts ('shots') above different leaf positions before re-filling the Swinnex filter holder. Shoot *A. thaliana* leaves with one 5 µL aliquot/leaf and *N. benthamiana* leaves with two 5 µL aliquots/leaf.

3.1.4. Slide preparation

1. Keep plants in the growth facility for 3 days before imaging (*see* **Note 7**).
2. Detach leaf and position with double sided sticky tape on a glass slide with the upper epidermis facing upwards

3.2 Cell connectivity assay by *Agrobacterium* infiltration

This assay can be used to analyse the effect of transiently overexpressed proteins of interest on cell-to-cell connectivity in *N. benthamiana*. It can also be used on stable transgenic *N. benthamiana* lines with overexpressed or knocked down/knocked out proteins of interest.

When testing transgenic or wild-type plant lines, only *Agrobacterium* transformed with the reporter construct pGWB402Ω.GFP is infiltrated. When a candidate protein needs to be overexpressed transiently, *Agrobacterium* transformed with the candidate protein expression vector is co-infiltrated with pGWB402Ω.GFP.

3.2.1 Culture preparation

1. Streak *A. tumefaciens* transformed with pGWB402Ω.GFP from the glycerol stocks on an LB agar plate with appropriate selection antibiotics (*see* Subheading 2.2, **item 6**). If testing the effect of a candidate protein that is overexpressed by *Agrobacterium* infiltration, do the same separately with *A. tumefaciens* carrying the relevant construct of interest, as well as untransformed *Agrobacterium*. Grow plates at 28 °C for 2-3 days. Plates can be kept in the fridge for 2 weeks.
2. Separately inoculate 4 mL LB liquid medium containing appropriate antibiotics for plasmid selection (*see* Subheading 2.2, **item 6**), but omitting rifampicin which slows down growth, from a colony from each *A. tumefaciens* plate. Incubate overnight at 28 °C with shaking.
3. Pellet *A. tumefaciens* cultures by centrifugation at 4 krpm for 15 min.
4. Resuspend each pellet in 1.5 mL infiltration medium and incubate suspensions in the dark, at room temperature for 1 hour.
5. Use a spectrophotometer to measure the optical density at 600 nm (OD_{600}) of the *Agrobacterium* suspensions.

3.2.2 Infiltration

6. Prepare 2 mL of (co-)infiltration suspension per leaf for infiltration. Dilute *Agrobacterium* suspension with infiltration medium to a final OD_{600} of 0.0001 for pGWB402Ω.GFP agrobacteria. If a candidate protein for plasmodesmal regulation is overexpressed by agro-infiltration, the infiltration mixture should contain pGWB402Ω.GFP agrobacteria at a final OD_{600} of 0.0001 and agrobacteria carrying the candidate protein expression construct at a final OD_{600} of 0.1. For the negative control, agrobacteria carrying pGWB402Ω.GFP (OD_{600} of 0.0001) are co-infiltrated with

un-transformed agrobacteria (OD_{600} of 0.1) to balance any potential direct effects of bacterial density.

7. Select the 4th true leaf of an *N. benthamiana* plant (*see* **Note 6**). Pierce the lower epidermis gently with the needle in approximately 6 well-spaced places. Avoid piercing through to the opposite side.
8. Fill a 1 mL syringe with (co-)infiltration suspension. Place the syringe at the site of a needle prick and firmly hold in place with a finger on the other side of the leaf. Slowly depress the syringe plunger. The airspace of the leaf will fill and the colour change will indicate the extent of spread. Repeat to infiltrate the entire leaf.

3.2.3. Slide preparation

9. Keep plants in the growth facility for 3 days before imaging (*see* **Note 7**).
10. Detach leaf and position with double sided sticky tape on a glass slide with the lower epidermis facing upwards

3.3 Imaging and cell counting

1. Image 3 days post bombardment (*see* Subheading 3.1) or post (co-)infiltration (*see* Subheading 3.2) (*see* **Note 7**)
2. Use a long-distance lens with large field of view. X10 lens for *N. benthamiana*, X20 lens for *A. thaliana*
3. Select imaging parameters to remain constant throughout (*see* **Note 8**). GFP excitation and detection is at 488 nm and 495–525 nm, respectively. mCherry excitation and detection is at 594 nm and 600–630 nm, respectively.
4. Locate and image every cluster of fluorescent cells (*see* **Note 9**). Collect a large z-stack spanning the entire depth of the brightest cell. Produce a maximum Z-projection (*see* **Note 10**).
5. Count total cells in cluster and number of cell layers (Fig. 1c) (*see* **Note 11**)
6. Record data (*see* **Note 12**)

7. Perform statistical analysis comparing treatments using an appropriate non-parametric test (*see* **Note 13**)

4 Notes

(1) Tracer choice: GFP is often easier to identify and therefore our default choice but the mCherry construct is utilised when transgenic lines already express GFP constructs. Instead of free GFP/mCherry (27 kDa), we sometimes use GFP/mCherry-sporamin fusions, which at 47 kDa result in more limited movement [17]. The reasoning for this is that a clearer 'yes/no' result may be obtained if the fluorescent tracer is closer to the plasmodesmal SEL. However, the smaller reporters will also be generally suitable, as SEL fluctuations even well above 27 kDa will result in alterations in diffusive flow that affect the size of fluorescent clusters [15,19].

(2) Prepare a large plasmid prep to use one prep at a consistent concentration of approximately 200 ng/uL for all biological repeats. Plasmid concentration will determine expression levels because expression is episomal. For ease prepare a glycerol of the transformed *E. coli* for repeated colony growth.

(3) Following infiltration, *N. benthamiana* leaves remain too delicate for reliable bombardment and the autofluorescence from leaf damage makes cluster counting difficult. Therefore, if the conditions of interest have to be achieved by agro-infiltration of *N. benthamiana* we recommend single-cell tracer transformation by co-infiltration (*see* Subheading 3.2). To clearly identify the initially transformed cell, it may be beneficial to use a dual-expression reporter construct that expresses a non-fluorescent marker of the initially transformed cell alongside free GFP, e.g. RFP targeted to the endoplasmic reticulum or the nucleus [15].

(4) A clumpy gold suspension will result in fewer bombardment sites and needs to be thoroughly vortexed or sonicated, and should be washed with ethanol again before use.

(5) Prepare one 5 µL aliquot per *A. thaliana* and two aliquots per *N. benthamiana* leaf. For each independent experiment, bombard 2-3 leaves per line/condition.

(6) To minimise developmental and physiological variation influencing cell-to-cell connectivity, always use the same leaf position for experiments.

(7) Fluorescent protein levels influence potential for leakage and thus, absolute cluster size. We recommend imaging 3 days post infiltration/bombardment, but the priority is that the time interval remains constant across experiments.

(8) Gain and brightness settings must be high enough to identify nuclei of the outermost cells of the cluster. The brightness will therefore appear very high for the brightest initial single transformed cell.

(9) Avoid bias by imaging and counting every cluster at chosen bombardment site. The larger clusters are more visible and therefore easy to preferentially select. When first looking under the eye-piece, identify a starting point and move logically across the leaf for example clockwise around the circle of tissue damage resulting from bombardment.

(10) Count from maximum projection. The faintest transformed cells will frequently be missed if counting only under the eye piece. The physical shot of bombardment causes areas of damage close to transformed cells. Taking images aids unequivocal distinction between the auto-fluorescence of damaged cells and the true fluorescent cells of the cluster.

(11) Freely diffusive fluorescent proteins such as GFP/mCherry and their respective sporamin fusions distribute freely between the cytoplasm and nucleoplasm. Compared with the very thin peripheral layer of cytoplasm in epidermal cells, the nucleus usually appear much brighter. Therefore, look for weak nuclear fluorescence to identify the outermost cells of the cluster. Cell count should include total cluster size and number of cell layers passed (Fig. 1c). Because the majority of clusters often consist of 1-2 cells, but some clusters will be much larger (20+ cells), the data is not normally distributed. Therefore, counting cells and cell layers crossed provides a better quantification of cell permeability than just categorising clusters into single or multiple cells. A cluster of two may occasionally result from transformation of adjacent cells, in which case there may not be a clear primary transformed brighter cell. We just count these as a cluster of two regardless, as they are equally likely to occur across different biological treatments.

(12) We find ease of data recording and later reference back to images is improved with a spreadsheet listing image name, cluster size and number of cell layers that is continuously updated during image acquisition, rather than doing the counting separate from the imaging.

(13) Repeat and collect data for high numbers of clusters (>50). A non-parametric test is required because the data is not normally distributed. We utilise the Wilcox test. For a more detailed discussion and a bootstrap dependent statistical test see [20].

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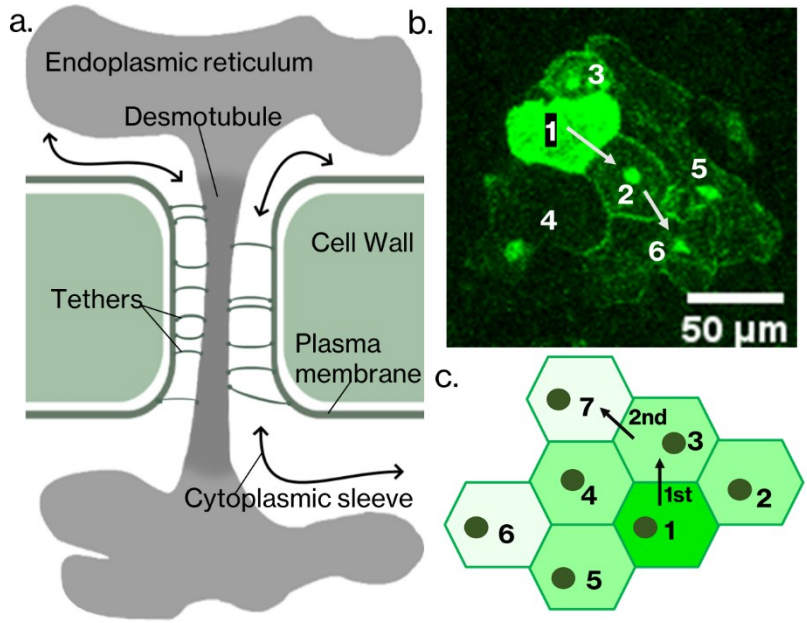

Figure 1. a) Schematic diagram of plasmodesma structure. See Introduction for details. b) Example of a cluster of GFP-sporamin fluorescent Arabidopsis leaf epidermal cells resulting from initial transformation of cell 1 by microprojectile bombardment. c) Schematic of a fluorescent cell cluster illustrating counting of individual cells (numbered) and cell boundaries crossed by GFP movement (arrows).