Evaluating molecular movement through plasmodesmata

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Abstract

Plasmodesmata are membrane-lined cytoplasmic passageways that facilitate the movement of nutrients and various types of molecules between cells in the plant. They are highly dynamic channels, opening or closing in response to physiological and developmental stimuli or environmental challenges such as biotic and abiotic stresses. Accumulating evidence supports the idea that such dynamic controls occur through integrative cellular mechanisms. Currently, a few fluorescence-based methods are availale that allow monitoring changes in molecular movement through plasmodesmata. In this chapter, following a brief introduction to those methods, we provide a detailed step-by-step protocol for the Drop-ANd See (DANS) assay, which is advantageous when it is desirable to measure plasmodesmal permeability non-invasively, in-situ and in real-time. We discuss the experimental conditions one should consider to produce reliable and reproducible DANS results along with troubleshooting ideas.

Keywords: molecular movement, fluorescent reporter, carboxyfluorescein diacetate, cell communication, plasmodesmata

Introduction

Plasmodesmata (singular, plasmodesma) are nanostructures embedded in the cell walls between neighboring plant cells. Plasmodesmata are lined by the plasma membrane and usually have an appressed endoplasmic reticulum (historically called the desmotubule) held at the core of each cytoplasmic pore. Plasmodesmata are primarily formed within the newly developing cell wall that separates two daughter cells in land plants. Once formed, primary plasmodesmata undergo various types of structural modifications depending on the growth and developmental fate of those cells. Besides the primary plasmodesmata formed in dividing cells, de novo plasmodesmata can be formed across existing cell walls of mature cells. These types of changes allow plants to support the development of cells needing more, fewer, or sometimes no plasmodesmal connections with their adjacent cells (Reviewed by Amsbury et al., 2017; Bayer et al., 2014; Burch-Smith et al., 2011; Cheval & Faulkner, 2018; Nicolas et al., 2017; Sager & Lee, 2018; Sager & Lee, 2014).

Plasmodesmata are highly dynamic. They facilitate the diffusion of not only soluble molecules smaller than 1 kDa, but also the movement of macromolecules such as proteins and ribonucleic acids. They also vary in gating status in response to cellular and environmental stimuli or challenges, and the changes in gating status can occur rapidly and transiently, or slowly and sustainably. It is now well established that the primary mechanism underlying plasmodesmal regulation is through counteracting enzyme activities involved in the synthesis and degradation of callose, a β-1,3-glucan cell wall polymer deposited around the channel. Measuring callose accumulation levels using an aniline blue stain or antibodies has been extensively used to inversely correlate plasmodesmal permeability with callose levels. To visually monitor the extent to which molecules move between cells and evaluate dynamic plasmodesmal gating, researchers have developed various fluorescence-based techniques. These include microinjection techniques, the fluorescent recovery after photobleaching (FRAP) method, or a genetic encoding approach using fluorescent proteins (see Table 1).

An in-situ assay method we named Drop-And-See (DANS) is useful to measure plasmodesmal permeability non-invasively in real-time. It involves a few simple steps (Fig. 1). First, a droplet of the membrane-permeable, non-fluorescent dye carboxyfluorescein diacetate (CFDA), is loaded onto the upper surface of an intact leaf of an *Arabidopsis* plant. Immediately upon loading, CFDA is absorbed into the epidermal cells in direct contact with the droplet and hydrolyzed by cellular esterases, releasing carboxyfluorescein molecules. Because carboxyfluorescein is a hydrophilic molecule, it can no longer diffuse cell-to-cell through the plasma membrane, but only through plasmodesmata. Hence, the degree to which the carboxyfluorescein dye diffuses, determined by quantifying the size of the fluorescent area on the underside of the leaf using confocal microscopy, represents plasmodesmal permeability in that plant genotype under a given experimental condition.

In addition to its conceptual simplicity, the DANS assay offers other advantages. It could be used to report how various plant species and mutants might differ in relative plasmodesmal permeability without needing to have genetically encoded tracers. It could also be used to monitor in real-time how plants differently regulate cell-to-cell movement in response to cellular stimuli or environmental challenges such as biotic and abiotic stresses. Using DANS assays, we recently identified novel *Arabidopsis* mutants that exhibit altered plasmodesmal permeability due to the inability to synthesize callose at plasmodesmata (Cui & Lee, 2016). Moreover, using the method, we demonstrated how plasmodesmal dynamics in *Arabidopsis* plants change in response to hormones or abiotic or biotic stresses (Lee et al., 2011; Lim et al., 2016; Wang et al., 2013). The DANS method has been also applied by other research groups to such plant species as tobacco and maize (Gui et al., 2014; Tran et al., 2019; Yang et al., 2019) as well as to *Arabidopsis* seedlings to monitor plasmodesmal response to metals in the root tips (O'Lexy et al., 2018).

In this chapter, we first provide a summary of various techniques used to monitor plasmodesmal permeability and cell-to-cell movement (see Table 1), followed by a detailed protocol for performing DANS assays. In addition, we offer a practical guide to troubleshooting and optimization of the method based on the lessons we learned over the years and numerous inquiries we received about the DANS protocol. We present a step-by-step protocol of the DANS assay and instructions on how to prepare and handle plants and the tracer dye for successful experimental outcomes. We describe the critical experimental procedures along with important cautions, such as how plants need to be cared for and handled adequately immediately before and during DANS assays.

1. Techniques used to measure plasmodesmal permeability

1.1 Microinjection

The microinjection technique has been predominantly used to measure plasmodesmal permeability in real-time. Using this method, fluorescent tracers are applied either through a wound site or injected directly into cells of interest. Although invasive, it is most advantageous in terms of the almost unlimited types of molecules one can introduce as a tracer (Table 1). A small fluorescent molecule (~0.5 kDa), such as fluorescein or rhodamine, can be conjugated to variously sized dextrans to evaluate the size exclusion limit of plasmodesmata. It can also be conjugated to macromolecules, such as viral movement proteins, or plant non-cell-autonomous proteins or nucleic acids, to examine the mobility of those macromolecules. A free tracer can also be introduced as a mixture with a molecule that might alter plasmodesmal permeability.

1.2 Genetically-encoded fluorescent proteins

A common approach to assess plasmodesmal permeability relies on the use of freely diffusible monomeric fluorescent proteins (FPs). This method also allows one to estimate the size exclusion limit and can help to determine if a protein of interest is non-cell autonomous. Popular FPs include green or red fluorescent proteins (GFP or RFP), which can be expressed freely or fused to a non-cell-autonomous protein of interest. Free FPs or FP fusions are expressed in leaf tissues transiently using biolistic or *Agrobacterium*-mediated DNA delivery methods or in stable transgenic plants. In transient expression, it takes a couple of days before the cell-to-cell movement of the FP can be evaluated. When used in stable transgenic lines, free FPs or FP fusions can be expressed in cell- or tissue-specific manners under chosen promoters.

1.3 Other techniques

Additional techniques used to measure plasmodesmal gating status or molecular movement between cells include monitoring photoswitchable FPs, FRAP, and pair correlation analysis (Table 1). These techniques are primarily used to measure molecular mobility within a cell but are found applicable to track molecular movement between cells. Photoswitchable FPs are used to monitor the distribution of fluorescence after photoactivation, while FRAP focuses on the recovery of fluorescence after photobleaching. Pair correlation analysis technique is used to track the diffusion of single molecules from one cell to another across cell wall barriers.

1.4 Callose staining

Callose staining does not in itself report plasmodesmal permeability but is a useful complementary method to inform if changes in plasmodesmal permeability are linked to alterations in callose accumulation levels. Callose levels at plasmodesmata can be detected using the stain aniline blue, or by using an immuno-fluorescence or -gold labeling approach, then quantified using confocal or transmission electron microscopy. Aniline blue-based callose measurement can be done in both live and fixed tissues, and the procedure is relatively simple. However, the dye is highly prone to photo-bleaching, hence imaging needs to be done with extra caution.

Table 1. Techniques used to monitor or evaluate cell-to-cell movement.

Technique	Tracer used	Plant examples and references	Comments
DANS	CFDA	Mature *Arabidopsis* (Cui et al., 2015; Lee et al., 2011; Lim et al., 2016; Wang et al., 2013) Seedling *Arabidopsis* (O'Lexy et al., 2018) *Nicotiana benthamiana* leaves (Gui et al., 2014; Yang et al., 2019) Maize leaves (Tran et al., 2019)	Non-invasive, in situ & real-time, and quantitative.
Dye loading through wounding or cut surface	CF, HPTS, LYCH, F-dextrans	*Arabidopsis* seedling roots (Carlotto et al., 2016; Duckett et al., 1994; Stonebloom et al., 2009; Wright & Oparka, 1996) Wheat grains (Fisher & Cash-Clark, 2000; Wang Ning & Fisher, 1994) Barley stems, *Abutilon pictum* flowers (Wright & Oparka, 1996) *N. tabacum* leaves (Crawford & Zambryski, 2001)	Can be used to address size-exclusion questions; cell-to-cell or long-distance movement; invasive; application easy but difficult to quantify.
Microinjection	CF, HPTS, LYCH, F-dextrans Fluorescently labelled proteins or nucleic acids	*Arabidopsis* root epidermis (Radford & White, 2001) *Tradescantia virginiana* (Radford & White, 2001) Potato tuber tissue (Oparka & Prior, 1988) *N. tabacum* leaves (Ding et al., 1996; Iglesias & Meins, 2000; Storms et al., 1998; Wolf et al., 1989) *Egeria densa* leaves (Goodwin et al., 1990) *N. cleuelandu* leaves (Oparka et al., 1991) *N. benthamiana* leaves (Su et al., 2010; Kragler, 2015) Brown alga (*Halopteris congesta*) (Nagasato et al., 2017)	Can be used to address size-exclusion limit questions; cell-to-cell movement in real-time; invasive; most versatile in types of probes to choose from.
Biolistic DNA delivery	Free FPs or proteins fused to FP	*N. clevelandii* leaf epidermis (Oparka et al., 1999) *N. tabacum* leaf epidermis (Crawford & Zambryski, 2001) *N. benthamiana* leaf epidermis (Liarzi & Epel, 2005) *Arabidopsis* leaf epidermis (Diao et al., 2019; Guseman et al., 2010; Lee et al., 2011)	Reports cell-to-cell movement of 27 kDa or larger; reproducible and quantitative; invasive; response not real-time.
Genetically encoded free FPs or FP fusions	Free FPs or proteins fused to FP, Photoinducible FPs	Various tissues in *Arabidopsis* (Imlau et al., 1999; Kim et al., 2005) Tobacco plants (Imlau et al., 1999; Roberts et al., 1997; Voinnet et al., 1998) *Arabidopsis* shoot apical meristem (Kim et al., 2002; Wu et al., 2003) *Arabidopsis* seedling roots (Benitez-Alfonso et al., 2013; Gallagher et al., 2004; Gerlitz et al., 2018; Rutschow et al., 2011; Sager et al., 2020; Stadler et al., 2005; Vatén et al., 2011; Wu & Gallagher, 2013)	Can be used to report cell-to-cell movement of 27 kDa or larger within or out of specific cellular domains; expression can be constitutive or inducible; plant transformation required.
Callose staining	Aniline blue or anti-callose	*Arabidopsis* leaves (Cui & Lee, 2016; Lee et al., 2011; Wang et al., 2013; Zavaliev & Epel, 2015) *Arabidopsis* seedling roots (Pendle & Benitez-Alfonso, 2015; Vatén et al., 2011) *N. benthamiana* and *N. tabacum* (Zavaliev & Epel, 2015) Cotton leaves (Ruan et al., 2004) Wheat (Danila et al., 2016; Sivaguru et al., 2000) Rice (*Oryza sativa*)(Danila et al., 2016) Maize (*Zea mays*)(Danila et al., 2016)	Can provide complementary information to plasmodesmal permeability; quantitative.

2. DANS assays: Plant care, handling, dye preparation, and validation

We cannot overemphasize how crucial it is to have healthy and stress-free plants in order to obtain accurate results using the DANS assay. If control plants are already compromised in cell-to-cell movement due to biotic or abiotic stress, one cannot compare their plasmodesmal permeability to those of the mutant or treated experimental plants with any confidence. Based on our experience, plants neglected from timely watering, consistent temperature, lighting and diurnal cycles, or grown in an environment subject to pest or mechanical stresses, fail to produce meaningful results in DANS assays. Furthermore, it is advisable to perform pilot DANS experiments on a small scale to establish optimal conditions for the plant species of choice. Specifically, it is helpful to determine the optimal developmental stage of the plant, leaf maturity, the region to load the dye within the selected leaf, number of samples per experiment, and time of day to perform assays.

Below we describe the plant growth conditions we found optimal for DANS assays in Arabidopsis, some criteria we used to produce and select healthy experimental plants, and a guide for proper storage and handling of CFDA.

2.1 Optimizing plant growth conditions

In our laboratory, we routinely perform DANS assays using *Arabidopsis thaliana* Col-0 plants that are grown for approximately 3.5 weeks under long-day conditions, with the daily cycle of 16 hr light (22°C) and 8 hr dark (20°C) (setting A, Table 2). By that time, the plants are vegetatively mature but not yet bolting. When grown in these conditions, Col-0 plants tend to produce rosette leaves that are optimal in size and shape to perform DANS assays (Fig. 2). They have a few fully expanded, flat leaves ideal to load the dye droplet. They are also more receptive to hormones or other mist-sprayed chemicals. Usually, the fourth or fifth true leaf gives the most reliable results, though the sixth leaf can also be used depending on plant size.

Compared to plants grown under the above conditions, plants grown under a slightly higher temperature tended to be relatively fast-growing and larger in 3 weeks (setting B). These plants had rosette leaves that were fully expanded but curled slightly at the leaf margins. This condition tended to slightly lower the trichome density, which helps to even out the absorption of the dye droplet at the contact surface. On the other hand, plants grown under short-day conditions grew slowly, producing rosettes that had flat and thick leaves (setting C). These plants tended to exhibit slower dye movement than plants grown in other settings.

We routinely transfer the healthiest 4-day-old seedlings from pots with multiple seedlings to individual pots; alternately, we sow several seeds per pot, then thin them out several days later, leaving one healthy seedling per pot to grow. After thinning, we ensure that the seedling left in the pot is carefully repositioned at the center. To ensure even growth, we periodically rotate the pots and flats around in the growth chamber every few days until they are ready for assays, and immediately remove plants showing any symptoms of stress from the chamber.

Table 2. Plant growth conditions and dye movement.

Growth condition					Plant morphology 3-4 week post-germination		Dye movement
Setting	**Temperature**	**Day length**	**Light Intensity**	**Humidity**	**Rosette**	**Leaves**	
A	22 °C Day & 20 °C Night	16 h Light & 8 h Dark	120-160 µmole/m^2/s	40-50%	Compact. Leaves with short petioles.	Thin, flat & fully expanded.	The dye moves into the lower leaf surface within 5 minutes.
B	24 °C Day & 21 °C Night	16 h Light & 8 h Dark	120-160 µmole/m^2/s	55-65%	Loose. Leaves with longer petioles.	Thin, long & fully expanded. Curled margins.	Tend to be faster in the dye movement than in A.
C	22 °C Day & 20 °C Night	12 h Light & 12 h Dark	120-160 µmole/m^2/s	40-50%	Compact. Leaves dark green in color.	Thick & hairy. Short and not fully expanded	Little dye movement in 5 minutes.

2.3 Number of plants needed for a set of DANS experiments

For each set of DANS assays, we routinely collect at least 20 images per tested genotype/treatment for data analysis. Ideally, if two CFDA spots are loaded on the assayed leaf per plant, a total of at least 10 plants, for each genotype or treatment, should be prepared. Therefore, each set of DANS assay experiments requires approximately 15-20 healthy plants. We repeat each experimental set at least two times. Plants must have grown under the same conditions and reached the same developmental stage by the time they are selected for assays. This way, one can ensure that individual plants are as close to identical in size and appearance as possible. To secure a pool of healthy, mature plants that are optimal for DANS assays in our experimental conditions, we apply the following criteria:

a. Plants exhibit no visible sign of the shift from vegetative to reproductive phase (infloresence development or bolting).
b. Leaves have no visible chlorosis or signs of disease.
c. Leaves have no soil debris, insect eggs or larvae on the top or bottom surfaces.
d. Leaves exhibit no obvious mechanical damage, such as tears or crushed areas.
e. Roots of the plants have not severely outgrown the pot.

2.4 CFDA storage, preparation, and handling

Chemical: 5(6)-Carboxyfluorescein diacetate (CFDA) (Sigma: cat# 124387-19-5) is a colorless, non-fluorescent chemical. It is a hydrophobic molecule permeating intact cell membranes. Once it enters the cytoplasm of a cell, CFDA is enzymatically hydrolyzed by cellular esterases into carboxyfluorescein, which is fluorescent and hydrophilic, thus making it membrane-impermeant.

Storage: CFDA is a highly sensitive light, which easily autolyzes it and turns it from a colorless powder or solution to a greenish-yellow form. The powdered form is stored in the dark at -20°C, whereas stock solutions are stored at 4 or −20°C. These stocks are stable for several months or longer ONLY if kept from any exposure to light.

Stock solution: CFDA is made into a 50 mM stock solution for long-term storage by dissolving the CFDA powder in dimethyl sulfoxide. The stock solution is made into 1-5 µL aliquots for single use.

Working solution: The concentration of the CFDA working solution we make is 1 mM. For each set of DANS experiments, a fresh CFDA working solution is prepared by diluting the stock solution at 1:50 in sterile nano-water under dim light. To delay autolysis, the working solution tube is wrapped with a piece of foil and kept on ice in the dark throughout the experiment.

Additional materials needed to perform DANS assays include the Nunc Lab-Tek chamber, nano water, a water-filled small petri dish, micropipettes, sharp tweezers, and retention-free, siliconized pipette tips.

2.5 Validation of symplasmic carboxyfluorescein movement

Whenever evaluating molecular movement between cells using a fluorescent tracer, it is critical to validate that the tracer has moved symplasmically, i.e., through plasmodesmal pores, not across cellular membranes. In DANS assays, this validation step involves imaging both loading and unloading sites of the leaf at high magnification (Fig. 3). In the epidermal cells of the upper leaf surface at the loading site, both epidermal pavement cells and immature or mature guard cells exhibit carboxyfluorescein signals indiscriminately. In contrast, in the epidermal cells of the lower leaf surface, fluorescent signals should be detected in the cytoplasm of those cells that are in symplasmic connection and excluded from mature guard cells.

3. DANS assays: image acquisition, processing, and data analysis

3.1 Confocal image acquisition

Any confocal microscopes equipped with 488-nm Argon laser excitation with a 505-550nm band-pass emission filter for CF detection are suitable for this DANS protocol. If desired, one can add a 560-nm laser line with a 636-754-nm emission filter to detect chloroplast autofluorescence simultaneously; seeing the leaf tissue gives context to the images and facilitates the imaging process by help find the desired focal plane. A low magnification lens is sufficient for the objective lens; we have found that a 5x/0.25 FLUAR objective lens is suitable for DANS assays performed on mature *Arabidopsis* leaves.

3.2 Image filtering, processing, and quantification

After each dye loading session, we process and quantify collected DANS images in Zeiss LSM Image Examiner (or Zen software), using the intensity distribution profiles function. Under the “Profile” function, graphic elements can be overlaid over fluorescent images. Draw a line across the center of the fluorescent area, and intensity values for each point that falls onto this line will be shown as a histogram. Then, using a threshold intensity and uniform cut-off, subtract the background fluorescence. If necessary, the brightness and contrast of fluorescent channels can be adjusted using the histogram player under “Display”; this will only modify image appearance on-screen, not alter fluorescence intensities for later quantification. Based on the pre-set cutoff, the length of the drawn line that exceeds the threshold intensity level is recorded. This length is the diameter of the fluorescein diffusion area for this spot. In the case of oval-shaped dye spots, more than one measuring line can be drawn across the longest and shortest diameters of the oval; the average of these two lines is then used as a dye diffusion radius. Measure the diameters using the same threshold for all collected images, and the yielded average value will be used as the mean for the assayed sample set. Alternatively, relative dye movement can be compared using the areas of the fluorescent spots, rather than the diameters. Any image analysis software (such as ImageJ) that converts confocal images into numeric pixel intensity data could also be used for these measurements.

After you have determined all the images you will use for your measurements, compare the individually-measured fluorescent spread radii and the mean values of each dataset between each background. Significance among data could be pairwisely compared via standard Student’s t-test. Multi-sample comparisons could be done by the Least Significant Difference (LSD) test following one-way ANOVA. Final data can be easily presented by bar graphs, box plots, or other graphic designs, depending on what best suits your purposes.

4. A step-by-step guide to the DANS assay procedure

4.1 Dye loading

1. Prepare a CFDA working solution fresh and keep it on ice in the dark.
2. Gently transfer one or two pots of 3-4-week-old plants from the growth chamber to the confocal microscope room immediately before the dye loading [a].
3. Determine the leaves that will be used for dye loading by counting the leaf number [b].
4. Without touching the leaf surface, gently release a 1 µL droplet of CFDA cleanly onto the upper surface of each half leaf blade (Fig. 2b) [c].
5. Wait for 5 minutes [d, e].
6. Withdraw the CFDA droplet from the leaf surface using a pipette without touching the leaf itself with the tip.
7. Carefully detach the loaded leaf by snipping the petiole using a pair of sharp forceps and rinse the dye-loaded area quickly by floating the leaf upside down on a dish filled with nano-pure water (2-3 seconds).

8. Immediately following the rinsing step, mount the detached leaf in nano-water in a microscope glass chamber. Gently place a coverslip over the leaf sample [f].

4.2 Image acquisition

1. Through the eyepiece, quickly locate a fluorescent spot in the lower epidermal surface using a 5x/0.25 Fluar objective lens mounted on a confocal microscope (see Section 3.1 for more details).
2. Upon locating a spot of dye diffusion, quickly take a single 5x scan image of the area [g].
3. Locate the second spot and take the image again.
4. Repeat steps 1-3 for every loaded leaf.
5. Collect at least 20 DANS images from 10 or more plants per genotype or treatment.

4.3 Image processing and quantification

1. Open each confocal image in Zeiss LSM Image Examiner (or Zen software), and select the Intensity Distribution Profile.
2. Open the FITC or an appropriate channel.
3. Open the "Profile" function and draw a line across the center of the fluorescent area in the image.
4. Use the "Intensity-Distance" profile to determine a threshold intensity value, which represents the background fluorescent level.
5. Calculate the length of the drawn line that exceeds the threshold intensity level. This length is considered to be the diameter of the CF diffusion area.
6. Repeat image quantification steps (1-5) until all collected images are processed and calculated for fluorescent diameters.
7. Perform desired statistical analysis on diameter values.

4.4. Footnotes and troubleshooting ideas

a. As you load the CFDA onto a leaf, use enough light to accurately see what you are doing, but try not to work in an excessively bright room. Plan for each DANS assay experimental session to last no more than 2 hours.
b. A pilot experiment can be performed to quickly assess which leaf is most suitable. Once determined, pick the leaf of the same number from each remaining plant to perform one set of DANS assays.
c. Up to four droplets can be loaded on a single leaf, two per half leaf blade. Take extra caution that only the droplet, not the pipette tip, should touch the leaf surface. If the pipette tip touches the leaf surface, disregard that leaf and move onto the next plant

sample, as the tip might have damaged the leaf. Also, it is important to release the droplet without any air bubbles. If an air bubble is formed in the droplet, disregard this loading from recording; the image resulting from this loading would not be usable.

d. Keeping the incubation time consistent is absolutely critical as the dye would keep moving as long as plasmodesmata remain open. If dye moves too fast or incubation time is too long, there will not be enough detectable fluorescent signal left to capture by imaging.
e. Cover the dye-loaded plant from a light source by placing it under a box with a wet paper towel to help maintain humidity and keep the droplet from drying out. Use a large enough box so that the covering does not disturb the dye droplets.
f. If the whole leaf is not mounted flat enough (and if time permits), the mid-vein can be swiftly trimmed off using a sharp razor blade, to mount the sample flatter for easier focusing and imaging. Also, make sure that the leaf sample is mounted such that the lower leaf surface will be imaged.
g. Images that show a single fluorescent area that is either round or slightly oval in shape represent successful loading. Other aberrantly shaped or multi-spot images should not be counted toward the quantification. If the latter occurs frequently, inspect the loading area of the leaf more closely to avoid loading on veins or near trichomes, and ensure no air bubbles are caught in the droplet during dye loading.

Figure legends

Figure 1. An overview of DANS assay. A diagram depicts the time and process involved in performing DANS assay. A total time needed for collecting two confocal images from one leaf sample from the dye preparation to image acquisition is approximately 15 minutes.

Figure 2. Mature Arabidopsis ready for DANS assay and dye loading. A. Mature *Arabidopsis* at 3.5 weeks post-germination. Leaf numbers 4 and 5 are most suitable for dye loading. The 6th leaf can also be used. **B.** Time-lapse photos show a dye loading process. *Approaching*: A pipette tip loaded with one µL dye is approaching the middle region of the right half of the leaf. *Loading*: When the tip reaches right above the leaf surface, slowly discharge the solution, letting the growing droplet immediately adheres to the

leaf surface. *Releasing*: Once the full droplet forms, carefully retract the tip, releasing the droplet to completion without disturbing it.

Figure 3. Validation of symplasmic dye movement. A. A confocal image shows a close-up view of epidermal cells of the upper leaf surface, with which the CFDA droplet is in direct contact. Note that green fluorescent signals, resulting from free carboxyfluorescein molecules, are detected indiscriminately across different cell types, including mature guard cells, which are symplasmically isolated. **B**. A close-up view of epidermal cells in the lower leaf surface into which the fluorescent tracer moved symplasmically. Mature guard cells (red darts) lack green fluorescence, indicating dye movement occurred via plasmodesmal connections between cells, not across cellular membranes. **C.** & **D.** Confocal images of a site on the upper leaf surface loaded with dye (**C**) and the area on the lower surface positioned directly opposite to the loading site, exhibiting symplasmic dye unloading (**D**). The red background represents autofluorescence from chloroplasts. The circle in red color is drawn over the fluorescent area to evaluate the extent to which dye has moved. Size bars, 20 μm in **A** and **B**; 200 μm in **C** and **D**. [Images are adopted from the Figure 6 included in the report published by Lee et al. (2011) and used with the author's permission.]

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