A Protocol to Measure the Extent of Cell-to-cell Movement of RNA Viruses *in Planta*

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[Abstract] Here, we present a simple and rapid protocol to measure the extent of cell-to-cell movement of RNA viruses *in planta*. To do that, the green fluorescent protein (GFP) gene was incorporated into the genome of *Melon necrotic spot virus* (MNSV) as a coat protein (CP) fusion protein using the *Thosea asigna virus* 2A catalytic peptide (TaV 2a) (Serra-Soriano *et al*., 2014). TaV 2a allows the co-translational cleavage of the fusion protein resulting in the independent expression of both proteins (Kim *et al*., 2011). Viral infection was initiated by agro-infiltration of *Cucumis melo* leaves*.* At 6-7 days post-infiltration, fluorescent infection foci images were taken with a fluorescent stereo microscope and infection areas were measured using FIJI software.

Materials and Reagents

1. 3-4 weeks old *Cucumis melo L. subsp. melo cv. Galia* plants
2. *Agrobacterium tumefaciens* (*A. tumefaciens*) strain C58C1, or similar, transformed with the binary vector harboring the GFP-tagged viral genome
   In our case, pMNSV(Al)/GFP encoding GFP-tagged *Melon necrotic spot virus* (MNSV) genome under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and the potato proteinase inhibitor terminator (PoPit) (Figure 1).
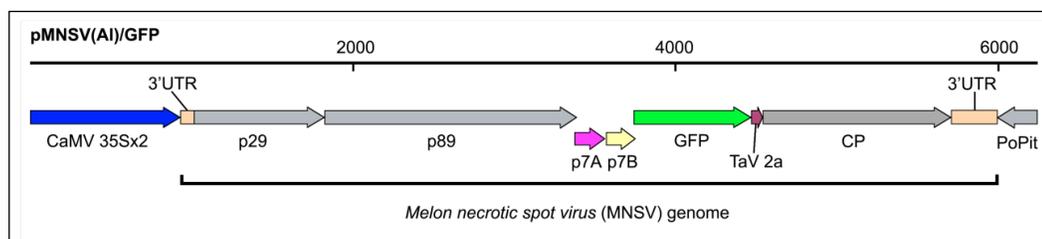
Figure 1. Schematic representation of the recombinant infectious clone pMNSV(Al)/GFP used in this assay

3. Yeast extract (Difco, catalog number: 212750)
4. Tryptone (Difco, catalog number: 211705)

5. Sodium chloride (NaCl) (Panreac Applichem, catalog number: 131659)

6. Acetosyringone (Sigma-Aldrich, catalog number: D134406)

7. MES (Sigma-Aldrich, catalog number: M8250)

8. Magnesium chloride (MgCl$_2$) (Sigma-Aldrich, catalog number: M9272)

9. Antibiotics

    a. Kanamycin (50 mg/ml) (Sigma-Aldrich, catalog number: K4000)

    b. Rifampicin (50 mg/ml) (Duchefa Biochemie, catalog number: R0146)

10. LB medium (see Recipes)

11. *Agrobacterium* infiltration buffer (see Recipes)

Equipment

1. 28 °C growing chamber

2. 15 ml culture tubes

3. Swinging centrifuge rotor for 15 ml tubes

4. 1.5 ml tubes (standard Eppendorf tubes or similar)

5. BioPhotometer plus (Eppendorf, catalog number: 6132000008)

6. 1 ml syringes without needle

7. Plant growing chamber

8. Leica MZ12 fluorescent stereo microscope

Software

1. Adobe Photoshop CS5 or higher

2. ImageJ, FIJI or similar software

3. MS Excel

Procedure

A. Viral inoculation by agro-infiltration

    1. Inoculate a single colony of *A. tumefaciens* transformed with pMNSV(Al)/GFP in 5 ml of LB medium with Kanamycin (50 µg/ml) and Rifampicin (50 µg/ml). Incubate the culture overnight (about 12-16 h) at 28-30 °C with vigorous shaking.

    2. Measure the A$_{600}$ of the culture.

    3. Collect the bacteria by slow-speed centrifugation (2,000 *x g*, 15 min) in a swinging rotor. Remove the supernatant.

4. Resuspend the pellet with *Agrobacterium* infiltration buffer. Since some viruses replicate and then move faster than others, the final A_{600} need to be adjusted depending on the virus. Large inoculum of a fast-moving virus may generate a high number of viral infection foci so close between them that they could be quickly merged. Therefore the resulting average size of the infection foci could be overestimated. If this occurs, reduce the final A_{600} until you get infection foci physically separated from each other. If, instead, only few infection foci are observed, the final A_{600} must be increased.

5. Leave at room temperature for 2 h before infiltration.

6. Infiltrate the cultures with 1 ml syringe into the lower side of the leaves. Use 3-4 week-old *Cucumis melo* plants and avoid non fully-expanded leaves. Use your fingertip to apply gentle counter pressure to the other side of the leaf. A fully infiltrated part of the leaf gives a water-soaked appearance.

7. Label de infiltrated leaves with a ring of adhesive one-sided tape placed around the petiole.

8. Keep the plants in growth chambers in 16 h light at 25 °C and 8 h dark at 22 °C.

B. Image acquisition

1. Several days after infiltration check the appearance of fluorescent infection foci with a Leica MZ12 fluorescent stereo microscope or similar device. The exact time will depend on which virus is assayed (5-8 days after infiltration).

2. Place the melon leaves beneath the objective (Planachromatic 0.5x) and engage the lowest magnification factor (0.71x)

3. Select GFP1 filter set [excitation filter 425/60 nm (395-455 nm), barrier filter 480 nm].

4. Look into the eyepieces and bring the fluorescent foci into focus using the rotatory knob.

5. Correct the values for "exposure", "gain" and "gamma" until you obtain the desired results for the image and capture it. Don't forget to add a scale bar.

C. Image analysis

1. Open the image with Adobe Photoshop CS5 or higher.

2. If necessary, enhance the image brightness and contrast until infection foci have well-defined borders.

3. Use the magic wand selection tool to select the infection foci. This tool selects similarly colored non-contiguous areas. If the magic wand selects less area than you want, increase the tolerance setting.

4. Fill selection with black color by selecting "Fill" in the "Edit" menu.

5. Invert selection by choosing "Invert" in the "Select" menu and fill with white color. If you have infection foci that have merged together separate them by drawing a white line were it feels the division should be. Save the resulting black and white image (Figure 2).
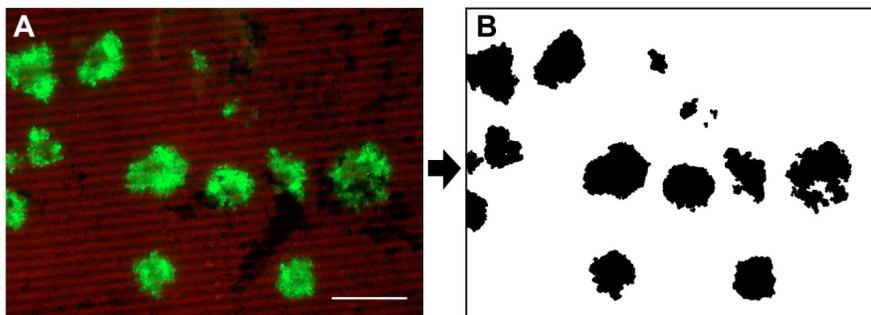
Figure 2. Images of after B and before A the Adobe Photoshop editing

6. Open the black and white image with ImageJ.
7. Select "Binary" in the "Process" menu and "Make binary".
8. Check the spatial calibration of the image. If the image is calibrated, the number of pixels in each dimension and dimensions with a unit of length will be visible at the top of the image window. If the image is not calibrated, only the number of pixels will be written and you will need to set the dimension of the image manually. Then, open an image with a scale bar and using the "Straight line" tool, draw a line along the scale bar. In the "Analyze" menu select "Set scale". A dialog box will open and the distance in pixels will be automatically adjusted. By choosing global, you set this calibration for all images you open during the ImageJ session.
9. Select "Analyze particles" in the "Analyze" menu. In the new dialog box, set size range by trial and error to exclude tiny background particles. Choose "Exclude on edges" option to avoid counting partial infection foci at the edges of the image. Check "Include holes" if you want it to fill in holes and include that area in the measurement. Under "Show" select "Overlay outlines" to number the infection foci that are measured. These numbers correspond to data for individual particles that will be listed in the "Results" window if you check the "Display results" and click "Ok".
10. Save results as MS Excel file and calculate the average size of infection foci in MS Excel.

Recipes

1. LB medium
   Mix 5 g of yeast extract with 10 g of tryptone, 10 g of NaCl

Add dH$_2$O to 1,000 ml

2. *Agrobacterium* infiltration buffer

   10 mM MES (pH 5.6)

   10 mM MgCl$_2$

   150 µM acetosyringone

Acknowledgments

References

1. Kim, J. H., Lee, S. R., Li, L. H., Park, H. J., Park, J. H., Lee, K. Y., Kim, M. K., Shin, B. A. and Choi, S. Y. (2011). High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *PLoS One* 6(4): e18556.

2. Serra-Soriano, M., Pallas, V. and Navarro, J. A. (2014). A model for transport of a viral membrane protein through the early secretory pathway: minimal sequence and endoplasmic reticulum lateral mobility requirements. *Plant J* 77(6): 863-879.