Published in final edited form as:

Methods Mol Biol. 2017 ; 1531: 141–153. doi:10.1007/978-1-4939-6649-3_12.

Subcellular Localization of *Pseudomonas syringae* pv. *tomato* Effector Proteins in Plants

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Summary

Animal and plant pathogenic bacteria use type III secretion systems to translocate proteinaceous effectors to subvert innate immunity of their host organisms. Type III secretion/effector system is a crucial pathogenicity factor in many bacterial pathogens of plants and animals. *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 injects a total of 36 protein effectors, which target a variety of host proteins. Studies of a subset of *Pst* DC3000 effectors demonstrated that bacterial effectors, once inside the host cell, are localized to different subcellular compartments, including plasma membrane, cytoplasm, mitochondria, chloroplast, and Trans-Golgi network, to carry out their virulence functions. Identifying the subcellular localization of bacterial effector proteins in host cells could provide substantial clues to understanding the molecular and cellular bases of the virulence activities of effector proteins. In this chapter, we present methods for transient or stable expression of bacterial effector proteins in tobacco and/or *Arabidopsis thaliana* for live cell imaging as well as confirming the subcellular localization in plants using fluorescent organelle markers or chemical treatment.

Keywords

Plant pathogen; Bacterial pathogenesis; Type III secretion; Plant immunity; Tobacco; *Arabidopsis thaliana*; Agrobacterium; Confocal microscopy

1. Introduction

Pseudomonas syringae pv. *tomato* (*Pst*) DC3000 is a gram-negative bacterium, which infects tomato as well as the model plant *Arabidopsis thaliana* (1). Similar to most phytopathogenic bacteria, *Pst* DC3000 is an extracellular pathogen. To successfully manipulate host cells and infect plant tissues, *Pst* DC3000 translocates 36 proteinaceous effectors, via the type III secretion system, into host cells (2). Once inside the host cell, bacterial effectors might travel to different subcellular compartments to function. Indeed, several *Pst* DC3000 effectors have been shown to localize to different cellular compartments of plant cells, including plasma membrane (PM) (AvrE; (3)), trans-Golgi network (TGN)/early endosome (EE) (HopM1; (4)), endoplasmic reticulum (HopD1; (5)), chloroplast (HopI1, HopK1, and HopN1; (6-8)), mitochondria (HopG1; (9)), and nucleocytoplasm (HopU1 and HopQ1-1; (10, 11)). In this

chapter, we describe live cell imaging approaches using fluorescent microscopy to study the subcellular localization of *Pst* DC3000 effector proteins in the host cell.

1.1 Selection of Appropriate Fluorescent Proteins

Identification of subcellular localization of bacterial effectors in plants is greatly advanced by utilizing fluorescent proteins for live cell imaging. The different variants of fluorescent proteins have provided a wide range of selection (12). Two major classes of fluorescent proteins have been widely used in plants: green fluorescent protein (GFP) and red fluorescent protein (DsRed) variants. Others and we have successfully used color variants of GFP in determining the subcellular localization of bacterial effector proteins in plants. Among the GFP variants, yellow fluorescent protein (YFP) and Venus have been routinely used as they have brighter signals and faster maturation compared to original GFP. In addition, YFP and Venus can be distinguished from another GFP variant, cyan fluorescent protein (CFP) in confocal microscopy. Thus, the YFP vs. CFP or Venus vs. CFP combination is ideal to be imaged together, providing a valuable tool to accurately confirm the subcellular localization of a bacterial effector through co-localization with various known organelle markers in plants.

1.2 Selection of Appropriate Cloning Vectors

To select the right cloning vector for expressing bacterial effector proteins in plants, two key features should be considered: (i) use of a constitutive promoter or inducible promoter and (ii) fuse of a fluorescent protein to the amino (N-) or carboxyl (C-) terminus of the effector protein. Several binary vectors, containing replicons for both *Escherichia coli* and *Agrobacterium tumefaciens*, for plant transformation are publicly available (13, 14). As some of the bacterial effectors are toxic to plant cells, it is crucial to express these effector proteins under the control of an inducible promoter for live cell imaging; however, most effectors can be constitutively expressed in plants. We have successfully expressed several toxic effector proteins in plants using dexamethasone (DEX)-inducible (3, 4) or β -Estradiol-inducible vectors (3). To constitutively express fusion proteins, we routinely use several Gateway-compatible vectors (15-17).

Tagging a bulky fluorescent protein like GFP or YFP, with a molecular weight of ~25 kDa, at either end of an effector protein might affect the function and/or localization of the fusion protein in plants. Thus, it is important to examine the subcellular localization of both N- or C-terminally tagged fusion proteins. For toxic effectors, the functional fusion proteins can be determined by observing whether the expression of the fusion proteins induces toxic effect, often indicated by tissue necrosis or chlorosis, in plants or not. As for non-toxic effectors, it is rather tricky to determine whether the fusion protein remains functional or not. In that scenario, further confirmation and functional characterization are required to address the issue. A systemic subcellular localization of 31 *Pst* DC3000 effectors, conducted in our lab, have shown that tagging a fluorescent protein at the C-terminal end of bacterial effectors is more likely to yield the functional fusion proteins (unpublished); however, a small subset of effectors required a fluorescent protein to be tagged at the C-terminus (e.g., HopAA1-1, see Figure 1A and 1B). HopAA1-2, on the other hand, does not induce cell death in tobacco leaf, but HopAA1-2-YFP is localized to specific cellular compartments, mitochondria (see

Figure 1A and 1B), suggesting that HopAA1-2-YFP might result in functional fusion protein. If the N- or C-terminal fusion does not reveal the correct subcellular localization of the fusion proteins, internal tagging of fluorescent protein (18) or immunolocalization (19) approaches should be considered.

In addition, for making stable transgenic plants to further examine the subcellular localization of the fusion protein, especially using plant lines that expressing fluorescent organelle markers, it is important to properly choose the transgene selection marker for screening the transgenic plants expressing two or more transgenes.

1.4 Selection of fluorescent organelle markers or fluorescent dyes

Initial examination of fluorescent signals in plant cells expressing fusion proteins often suggest a putative subcellular localization(s) based on the knowledge of the spatial pattern, morphology, size, and numbers of specific organelles. However, for a more definitive identification, it is necessary to perform co-localization experiments using known fluorescent organelle markers. A comprehensive set of fluorescent organelle markers is available in plants (20, 21); they provide excellent tools to confirm the subcellular localization of any proteins. In addition to these *in vivo* protein markers, several organelle-specific fluorescent dyes have been demonstrated to be useful to determine the subcellular localization of proteins (22).

Below, we describe methods for (i) growing tobacco and *Arabidopsis thaliana* plants, (ii) transient expression of bacterial effectors in tobacco using *Agrobacterium*-mediated transformation, (iii) transient expression of bacterial effectors in *Arabidopsis* using the microparticle bombardment method, (iv) stable expression of bacterial effectors in *Arabidopsis* using *Agrobacterium*-mediated transformation, and (v) examination of subcellular localization of bacterial effectors using confocal microscopy.

2. Materials

2.1 Growth of Tobacco and Arabidopsis Plants

1. Potting soil. Suremix perlite (Michigan Grower Products, Inc.) for growing tobacco plants and Arabidopsis mix (1 part suremix, 1 part medium vermiculite, and 1 part perlite) for growing Arabidopsis plants.
2. 4-inch square pots.
3. Growth chamber set to 23°C/18°C day/night temperature, 18-h/6-h photoperiod, and light intensity of ~40 $\mu\text{mol}/\text{m}^2/\text{s}$ for tobacco (see **Note 1**) and 22°C, 12-h/12-h photoperiod, and light intensity of 80-100 $\mu\text{mol}/\text{m}^2/\text{s}$ for Arabidopsis.

2.2 Agrobacterium-Mediated Transient Expression in Tobacco Leaves

1. 6-8-week-old tobacco plants.
2. *A. tumefaciens* cells carrying the plasmid of interest.
3. 5-ml culture tube.

4. Luria-Bertani (LB) medium with appropriate antibiotics.
5. Shaking incubator set to 28°C.
6. 1-ml syringe without needle.
7. Sterile ddH₂O.

2.3 Microparticle Bombardment-Mediated Transient Expression in Arabidopsis

1. Plasmid DNA harboring the gene of interest.
2. Leaves of 4- to 6-week-old Arabidopsis.
3. Biolistic® PDS-1000/He Particle Delivery System.
4. Gold microparticles, 1.0 µm.
5. Macrocarrier.
6. Rupture Disc, 1,100 psi.
7. Stopping screen.
8. 2.5 M CaCl₂.
9. 0.2 M Spermidine.
10. Sterile ddH₂O.
11. 70% EtOH.
12. 100% EtOH.
13. Isopropanol.
14. Vortexer.
15. Microcentrifuge.
16. Sterile 1.5 ml microcentrifuge tubes.
17. Whatman filter paper, 9.0-cm diameter.
18. Parafilm.
19. Insect screen (Phifer Inc., Tuscaloosa, AL, USA).
20. Petri dish, 100 mm × 15 mm.
21. Kimwipe.

2.4 Agrobacterium-Mediated Stable Expression in Arabidopsis

1. 6-week-old Arabidopsis plants. (see **Note 2**).
2. *A. tumefaciens* cells carrying the plasmid of interest.
3. 5-ml sterile culture tube and 500 ml sterile flask.
4. LB medium with appropriate antibiotics.

5. Shaking incubator set to 28°C.
6. 500-ml centrifuge bottle.
7. Centrifuge.
8. ddH₂O.
9. Sucrose.
10. Silwet-L77.

2.5 Confocal Imaging

1. Leaves transiently or stably expressing the fusion protein of interest.
2. ddH₂O.
3. Glass slide and coverslips
4. Agrobacterium carrying fluorescent organelle markers for plant cells.
5. NaCl.
6. Confocal laser scanning microscope

3. Methods

3.1 Growth of Tobacco and Arabidopsis Plants

1. Prepare pots with the Suremix perlite or Arabidopsis mix for growing tobacco and Arabidopsis, respectively, by lightly packing the pots with moist soil.
2. Place pots in trays and fill tray bottom with water to soak the soil.
3. Sow seeds on pots. With 4-inch square pot, sow ~10 Arabidopsis seeds at four corners of a pot and ~10 tobacco seeds at the center of a pot.
4. Place a transparent plastic dome to keep high humidity for germination.
5. Place the tray in growth chamber as mentioned above (2.1).
6. Remove extra seedlings 7 days and 14 days after germination of Arabidopsis and tobacco, respectively, so that only four or one seedling remains in each pot of Arabidopsis and tobacco, respectively.
7. Grow Arabidopsis for another 3-5 weeks and tobacco for 5-7 weeks in growth chamber without a plastic dome.

3.2. Plant Transformation

3.2.1 Transient Expression in *Nicotiana tabacum* or *Nicotiana benthamiana*

1. Inoculate Agrobacterium carrying plasmid DNA in 2 ml of LB medium with appropriate antibiotics.
2. Grow cells at 28°C for ~20 h in a shaking incubator set to 200 rpm.
3. Measure cell density (A_{600}) using a spectrophotometer.

4. Adjust the final concentration of Agrobacteria to A_{600} of 0.02 using sterile ddH₂O (see **Note 3**). For co-localization experiments, mix two *Agrobacterium* strains containing a pair of compatible fluorescent proteins (e.g., GFP and RFP) at the 1:1 ratio, after adjusting the A_{600} of each strain.
5. Infiltrate the *A. tumefaciens* suspension into a small sector ($\sim 2\text{ cm}^2$) of a tobacco leaf from the abaxial side. (see **Note 4**).
6. Put plants back to the same growth condition for 2 days.

3.2.2 Transient Expression in *A. thaliana*—Agrobacterium-mediated transient expression does not work well in Arabidopsis. Particle bombardment provides an alternative approach to transiently express a gene in plants (see Figure 1D). Here, we present a method to transiently express bacterial effector proteins in Arabidopsis.

3.2.2.1 Preparation of Microparticles

- 1 To prepare gold particles for coating, weight 20 mg of 1.0 μM gold particles, transfer into a 1.5 ml microcentrifuge tube, add 70% EtOH, vortex at maximum speed for 5 min, and soak in 70% EtOH for 15 min.
- 2 To rinse the particles, spin the particles at 3,000 rpm for 5 sec, remove and discard supernatant, add 1 ml sterile ddH₂O, vortex at maximum speed for 1 min, settle for 1 min, spin at 3,000 rpm for 5 sec, remove supernatant, and discard.
- 3 Repeat step 2 for 2 more times.
- 3 Add 333 μl of 50% glycerol (see **Note 5**).
- 4 To coat plasmid DNA on the particles, vortex the prepared particles in 50% glycerol at maximum speed for 5 min, transfer 25 μl of particles into a new microcentrifuge tube, add 5 μl (1 $\mu\text{g}/\mu\text{l}$) of plasmid DNA (see **Note 6**), vortex at maximum speed for 5 sec, add 25 μl of 2.5 M CaCl₂, vortex at maximum speed for 5 sec, add 5 μl of 0.2 M spermidine, vortex at maximum speed for 3 min, settle for 1 min, spin at 3,000 rpm for 5 sec, remove supernatant, add 100 μl of 100% EtOH, spin at 3,000 rpm for 5 sec, remove supernatant, repeat 100% EtOH rinse for two more times, and add 25 μl of 100% EtOH (see **Note 7**).

3.2.2.1 Particle Bombardment

1. Place a macrocarrier disc in a macrocarrier holder, pipette particles up and down to suspend particles evenly, and place 8 μl of particles on macro carrier (see **Note 8**).
2. To prepare Arabidopsis leaves, put a layer of filter paper on the lid of a petri dish, wet with ddH₂O, place 8 to 10 fully developed leaves on top of the wet filter paper with the abaxial side facing up, place a window screen on top of leaves, and put two heavy metal blocks on top of the screen without blocking the leaves to stabilize the leaf samples during bombardment (see Figure 1C).

3. To set up the biolistic chamber, soak rupture discs (1100 psi) in 100% isopropanol, properly place them in the rupture disk retaining cap, and screw the retaining cap to the unit.
4. To assemble the launch unit, place a stopping screen, put the macrocarrier with the macrocarrier disc facing downward, screw on the macrocarrier cover lid, and place the unit right below the retaining cap.
5. Put the sample prepared at step 2 on the target shelf and place the target shelf on the third position from the bottom.
6. To deliver the particles, turn on Helium gas supply, biolistic chamber and vacuum pump, build up the vacuum in the chamber to 27 in. of Hg by pressing the middle button at VAC position, switch to the HOLD position when the pressure reach ~27 in. of Hg, and press and hold the FIRE button until He pressure gauge reach 1,100 psi to burst the rupture disc (see **Note 9**).
7. Take the sample out when the pressure of biolistic chamber is back to 0, cover the petioles of leaves with wet kimwipe (see Figure 1C), cover the petri dish, seal with parafilm, and put the plate back into the same growth chamber overnight (see **Note 10 and 11**).

3.2.3 Stable Expression in *A. thaliana*

1. Inoculate *A. tumefaciens* cells carrying the plasmid of interest in 2 ml of LB with appropriate antibiotics, place the culture tubes in 28°C shaking incubator for overnight and refresh the overnight culture in 200 ml of LB with appropriate antibiotics at 28°C for another overnight.
2. Spin down cells at 6,000 rpm for 10 min, discard supernatant, and suspend the pellet in 500 ml of 5% sucrose with 0.08% of Silwet L-77 to a final concentration of A₆₀₀ of 0.8.
3. To dip the plants, transfer the cell suspension into an autoclavable plastic container or polypropylene pan, and dip the entire inflorescence into the bacterial suspension for 30 sec with gentle agitation.
4. Gently shake off excess bacterial suspension from plants, place the plants in a tray with a plastic dome or plastic wrap, and keep the tray under dark at room temperature overnight.
5. Place the plants back in the same growth condition to set seeds (see **Note 12**).
6. Collect seeds when they are mature and dry.
7. Select the transgenic plants using an appropriate selection marker (see **Note 13**).

3.3 Confocal imaging

3.3.1 Examining the subcellular localization of effector proteins—To visualize fluorescent signals, epifluorescent microscope with optimal filters or confocal microscope

can be used; however, confocal microscopy will enable higher resolution imaging for better determining the correct subcellular localization of the fluorescent proteins.

1. Tobacco leaves transiently expressing the fusion proteins can be imaged 24-48 h after *Agrobacterium*-infiltration. *Arabidopsis* leaves transiently expressing the fusion proteins can be imaged ~16-20 hours after the particle bombardment. As for *Arabidopsis* transgenic plants, cotyledons or true leaves of ~14-day-old seedlings are optimal for imaging, but plants up to 4-week-old are usable.
2. To prepare the sample for imaging, use a sharp razor blade or scissor to cut a small piece (~4 mm²) of leaf tissues right before imaging, mount the tissue with water on a glass slide with the abaxial side facing upward, and top with a cover slip. For plasmolysis, put one or two drops of 0.5 M NaCl solution on the glass slide, cut small pieces of leaves and place them in the NaCl solution for 5-10 minutes, and top with a cover slip (see **Note 14**).
3. For confocal imaging, we use Zeiss Laser Scanning Microscopes 510 or Olympus FluoView 1000 Spectral-based Laser Scanning Confocal Microscope. For Zeiss confocal microscope, we used 488-, 458-, 514-nm and 543-nm lasers to excite GFP, CFP, YFP, and RFP, respectively. Fluorescent signals were collected using an emission filter of a 505- to 530-nm band pass for GFP, a 465- to 510-nm band pass for CFP, and a 530- to 600-nm band pass for YFP and 560- to 615-nm band pass for RFP (see **Note 15**).

3.3.2 Confirming subcellular localization using organelle markers or PM

plasmolysis—Here we show the localization patterns of two *Pst* DC3000 effector proteins, HopM1 and AvrE, as examples of confirming the subcellular localization by co-localizing with TGN/EE organelle markers (HopM1) or PM plasmolysis (AvrE). HopM1 and AvrE are highly toxic to plant cells, including tobacco and *Arabidopsis thaliana*. Therefore a DEX-inducible promoter for HopM1 and a DEX and β -estradiol inducible promoter for AvrE were used for the expression of the two effectors. Fluorescent protein-tagged HopM1 and AvrE were first confirmed to retain the toxicity in plant cells, suggesting the fusion proteins are functionally active (3, 4). Following protocols described above, localization of HopM1 and AvrE after transient expression in *N. benthamiana* and/or stable expression in *A. thaliana*, was examined. Because HopM1 and AvrE cause cell death, a right imaging time point after protein induction needs to be determined, to allow enough time for protein to accumulate before plant cell death.

3.3.2.1 TGN/ EE localization: A DEX-inducible HopM1 expression construct (DEX: *hopM1-GFP*) was generated and mobilized to *Agrobacterium*. Twenty-four to forty-eight hours after *Agrobacterium* infiltration in *N. benthamiana* leaves, 30 μ M DEX was sprayed to induce *hopM1-GFP* expression. As shown in Figure 2B, HopM1-GFP showed intracellular punctuate signals, resembling the localization pattern of intracellular vesicles. To determine to which organelle HopM1-GFP is targeted, co-localization imaging of HopM1-GFP with various organelle markers, including VHA-a1 (marker for TGN/EE), Ara6 (marker for late endosome) and ST (marker for Golgi), was performed (4, 21), and HopM1-GFP was found

to co-localize with VHA-a1-RFP only (see Figure 2A). This suggests that HopM1-GFP is localized to TGN/EE in the plant cell.

3.3.2.2 PM localization: For the localization of AvrE effector, two inducible constructs with AvrE tagged with YFP/Venus) at either N- or C- terminus (DEX:*His:YFP:avrE* and β -estradiol:*avrE:Venus*; (3)) were generated and used for imaging. Both fusion proteins showed high toxicity upon induction, justifying their use to reveal the right localization pattern of AvrE. Transient expression of His:YFP:AvrE (data not shown) and AvrE-Venus (see Figure 2B) in *N. benthamiana* was first performed and the two proteins showed the same localization pattern at the cell periphery. Transgenic Arabidopsis lines expressing DEX: *His:YFP:avrE* were then generated, and localization of His:YFP:AvrE in Arabidopsis leaf cells was examined. Similarly to transient expression, His:YFP:AvrE is localized at the cell periphery, likely PM, in Arabidopsis cells (see Figure 2C). To confirm the PM localization, plasmolysis with 0.5 M NaCl, followed by confocal imaging, was carried out. As shown in Figure 2D, Hechtian strands, a characteristic of plant PM proteins, were observed after plasmolysis, supporting that AvrE is indeed localized on the PM in the plant cell.

4. Notes

1. Under this growth condition, tobacco plants produce thinner leaves, which are better for confocal imaging.
2. Remove the primary inflorescence of 5-week-old plants to facilitate the emergence of secondary bolts.
3. Inocula ranging from A₆₀₀ 0.02 to 0.1 works well; high inocula may increase the chance of detecting lowly-expressed proteins. In most cases, directly diluting inocula with sterile ddH₂O work as well as using infiltration buffer containing 50 mM MES (pH 5.6), 2 mM Na₃PO₄, 0.5% glucose, and 100 μ M Acetosyringone.
4. If it is difficult to infiltrate cell suspension into the leaf, a needle can be used to poke the surface of the leaf at the infiltration site before infiltration.
5. The prepared gold particles can be stored at -20°C for up to 2 months.
6. We have successfully used the same plasmid DNA (~11 kb) prepared for Agrobacterium-mediated transient expression. The efficiency is as good as using a smaller a plasmid DNA (~5 kb). For co-localization assays, plasmid harboring the gene of interest and plasmid carrying the organelle marker gene can be mixed together to coat gold particles.
7. The coated particles can be stored at 4°C for ~2 weeks, but we normally prepare freshly coated particles.
8. To keep the humidity low, place the macrocarrier holder with macrocarrier disc in a petri dish with desiccant.

9. If the He pressure gauge doesn't move, check whether the rupture disk is properly placed in the retaining cap. If the rupture disk doesn't burst at 1,100 psi, check whether more than one rupture disk are placed in the retaining cap.
10. To avoid the bombarded leaves from wilting, keep the humidity in the plate high and have the petioles in touch with wet filter paper or kimwipe (see Figure 1C).
11. In general, one or two leaves are heavily bombarded, showing metallic glow on the leaf surface. In most cases, at least 100 bombardment events can be observed in the heavily bombarded leaves.
12. If the humidity of the growth chamber is low, place the plastic dome with ~3 cm opened for 2-5 days after moving the plants back into the growth chamber.
13. For imaging purpose, T₁ generation plants can be used. To avoid non-specific effects due to locations of the transgene in the plant genome, it is recommended to analyze at least three independent transgenic lines.
14. Plasmolysis will continue during imaging and cells will gradually die, so it is recommended to use fresh samples and collect images quickly.
15. In most cases, we capture single focal plane images. When necessary, a series of Z-plane images for Z-stacking can be collected. For co-localizing fusion proteins with dynamic organelles, which move rapidly within cells, it is recommended to fix the tissue for 10 min using fixation buffer (2% formaldehyde, 10 mM EGTA, 5 mM MgSO₄, and 50mM PIPES, pH7.0) before imaging.

Acknowledgments

This work was supported by funding from the National Institutes of Health R01 GM109928; the Chemical Sciences, Geosciences, Department of Energy DE-FG02-91ER20021 (support of research infrastructure); U.S. Department of Agriculture/National Institute of Food and Agriculture AFRI-004412; and the Gordon and Betty Moore Foundation GBMF3037. SYH is a Howard Hughes Medical Institute-Gordon Betty Moore Foundation Investigator.

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Figure 1.

Transient expression of bacterial effectors tagged with fluorescent proteins in *N. tabacum* and *A. thaliana*. (A) A tobacco leaf infiltrated with *Agrobacterium* carrying a relevant plasmid expressing an effector fusion protein. The infiltrated areas are outlined. The images were taken 48 h after infiltration. Grey area indicated tissue necrosis induced by YFP-HopAA1-1. (B) Confocal images of epidermal cells of tobacco leaves expressing effector fusion proteins were captured at 48 h after *Agrobacterium* infiltration, except YFP-HopAA1-1 was imaged 36 h after infiltration. Scale = 10 μ m. (C) Arabidopsis leaves before and after the microparticle bombardment. (D) Confocal images of epidermal cells of Arabidopsis leaves expressing effector fusion proteins. Confocal images were taken at 16 h after bombardment. Scale = 10 μ m.

Figure 2.

Subcellular localization of HopM1 and AvrE. (A) Co-localization of HopM1-GFP and VHA-a1-RFP in *N. benthamiana* leaf. Confocal images of epidermal cells co-expressing HopM1-GFP and VHA-a1-RFP (a TGN/EE marker) were collected 8 h after 30 μ M DEX spray. (B) Localization of AvrE-Venus in epidermal cells of a *N. benthamiana* leaf. Images were collected 4 h after 20 μ M β -estradiol infiltration. (C) Localization of His:YFP:AvrE in the epidermal cells of transgenic Arabidopsis. Images were collected 3.5 h after 10 μ M DEX spray. (D) Localization of His:YFP:AvrE in the epidermal cells of transgenic Arabidopsis after plasmolysis by 0.5 M NaCl. Images were collected 4 h after 10 μ M DEX spray. Scale = 10 μ m.