Direct and Indirect Visualization of Bacterial Effector Delivery into Diverse Plant Cell Types During Infection

Elizabeth Henry, Tania Y Toruño, Alain Jauneau, Laurent Deslandes, Gitta Laurel Coaker


**Corresponding author:** Gitta L. Coaker, glcoaker@ucdavis.edu

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**REPORT:** (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

**TPC2017-00027-BRR  1st Editorial decision – revision requested**  
Feb. 20, 2017

We have received reviews of your manuscript entitled "Direct visualization of bacterial effector delivery during plant infection." Thank you for submitting your best work to *The Plant Cell*. The editorial board agrees that the work you describe is substantive, falls within the scope of the journal, and may become acceptable for publication pending revision, and potential re-review.

In addition to addressing the concerns and comments of the reviewers, please also address the issue of whether T3S effectors genes expressed under the control of their "native promoters" from a plasmid are properly expressed (see Reviewer Editor comments below).

Note that the sampling and nature of "biological replicates" should be described precisely (i.e. different plants, parts of plants, pooled tissue, independent pools of tissue, sampled at different times, etc). The reader should know exactly what was sampled; what forms the basis of the calculation of any means and statistical parameters reported. This is also necessary to ensure that proper statistical analysis was conducted.

Please contact us if there are ambiguous comments or if you wish to discuss the revision.

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Reviewer comments:

[Reviewer comments shown below along with author responses]

**TPC2017-00027-BRR1  1st Revision received**  
April 16, 2017

Reviewer comments and **author responses:**

We thank the reviewers and editor for their positive evaluation and detailed comments on the manuscript. We have addressed reviewer comments below with new experimentation as well as textual changes.
Reviewer #1:

The manuscript by Henry et al. describes an exciting new technology to visualize type III effector secretion into plant tissues. The authors clearly demonstrate that the split GFP system can be used to visualize effector from two different pathogens (P. syringae and Ralstonia) which differ in their infection process (roots vs. leaves) and in which types of cells they inject effectors. Overall the experiments are thorough and for the most part support the author's conclusions.

1) Page 3, line 62 and 63- the authors state that "Despite the importance of bacterial effectors in the modulation of host-microbe interactions, direct TTSS effector delivery has not been visualized in whole organisms." However, VirE2 translocation through the T4SS has been visualized into whole plant tissues using the split GFP system (Li et al., 2014; The Plant Journal (2014) 77, 487-495.). The authors should reference this study.

   We have referenced this study in the introduction on page 3, lines 65-69.
   "Recently, delivery of the virulence protein VirE2 by Agrobacterium tumefaciens through the type IV secretion system was successfully visualized using the GFP strand system in yeast, Arabidopsis and tobacco (Li et al., 2014; Yang et al., 2017). The type IV secretion system is responsible for delivery and uptake of proteins and DNA, with a conduit diameter of about 18.5 nm (Wallden et al., 2010)."

2) Page 7, line 190- the authors state that "Bacterial growth assays demonstrate that AvrB-GFP11 is delivered and recognized by RPM1...", the authors would need an rpm1 mutant to confirm that AvrB-GFP11 is recognized by RPM1 (see point 3).

   We have now performed bacterial growth assays on Col-0 and the rpm1-3 mutant side-by-side in Figure 3E. AvrB-GFP11 is not recognized in rpm1-3, but is recognized in Col-0. P. syringae DC3000 EV and AvrB-GFP11 grow to a similar level in rpm1-3. We have also included AvrB-FLAG as a side-by-side comparison with AvrB-GFP11. These experiments demonstrate that both AvrB-FLAG and AvrB-GFP11 can elicit HR after high density inoculation on Col-0 (Figure 3C), restrict bacterial disease symptoms after low density inoculation on Col-0 (Figure 3D), and are specifically recognized by RPM1 (Figure 3E).

3) In Figure 3D the authors show that P. syringae DC3000 expressing AvrB-GFP11 causes reduced disease symptoms in wild type Arabidopsis Col-0 relative to the DC3000 empty vector control; this is corroborated by bacterial growth in Figure 3E. The authors should compare this to an rpm1 mutant. This would test whether the recognition of the AvrB-GFP11 fusion is RPM1-dependent and whether the in planta fitness of P. syringae is compromised by AvrB-GFP11 expression?

   We have performed these experiments, as described in the response to point 2, above. We do not see that the in planta fitness of P. syringae is compromised by AvrB-GFP11 expression. P. syringae DC3000, P. syringae DC3000 + AvrB-FLAG, and P. syringae DC3000 + AvrB-GFP11 all grow to similar levels three days post-inoculation on the susceptible genotype rpm1-3 (Figure 3E).

4) In Figure 4 are the effectors AvrB and AvrPto membrane localized as predicted, the labeling seems quite diffuse?

   Effectors are delivered at low levels during natural infection. In order to specifically test membrane localization of AvrB and AvrPto, we would need to co-transform with a membrane localized fluorescent marker. We have not constructed these lines and it is not possible to complete this experiment in the time requested for a revision. Both AvrPto and AvrB are membrane localized in Nicotiana (Figure 2) as well as based on previous investigations. The more diffuse localization of AvrB and AvrPto in Figure 4 could be due to the low level of effector delivery into plant cells as well as initial cytosolic localization after injection from the type III secretion system.

5) The hrcC mutant negative control included in Figure 4A and B for AvrPtoB is important to support that the observed fluorescence requires type III effector translocation. Why was this not included for AvrB and/or AvrPto in the surface inoculation in Fig 5?

   We have now included the hrcC mutant negative control for AvrB and AvrPto in the surface inoculation in Figure 5. We did not observe GFP fluorescence in the hrcC mutant control, demonstrating that the GFP signal is specific for effector delivery.
6) The authors should be cautious about comparing effector delivery across cell types since they do not know the relative expression of their 35S::GFP1-10 in each of the cell types.

We agree. We have been more cautious when comparing effector delivery across cell types and included the following statement:

"Expression of GFP1-10 under the control of 35S promoter within individual cell types may vary and contribute to an observational bias for effector delivery in cells with higher expression of the transgene."

7) The authors should state that a limitation of their system is that visualization of effectors is limited to the distribution of their 35S::GFP1-10 construct. As such, effector delivery to organelles and some tissues could be overlooked.

We agree and have included the following statement on page 12, lines 338-340:

"Effectors delivered to the cytoplasm, plasma membrane, and nucleus can be visualized with the 35S::GFP1-10 system. However, effector visualization is dependent on the subcellular distribution of GFP1-10 and it is likely that specific organelle-targeted variants of GFP1-10 will be necessary to visualize effector in other cellular organelles."

Reviewer #2:

1. In the first part of the manuscript, the authors tried to show that different cell types of Arabidopsis leaves recognized and showed defense response to the bacterial effector (Figure 1). However, this is not very relevant to the rest part or the main idea of the manuscript. This part should be removed or moved to the supplementary information.

We have included Figure 1 as a main manuscript figure as it was considered to be an important addition for other reviewers. Reviewer 3 found cell specific complementation particularly relevant to the manuscript. The experiments in Figure 1 also support subsequent findings that effectors can be delivered into diverse leaf cell types (pavement, guard, and mesophyll cells).

2. In Figure 3A and B, the authors claimed that GFP11 tagging does not affect the biological function of both AvrPto and AvrPtoB. Although the quantitative assay shows DC3000ΔavrPto/ΔavrPtoB with either AvrPto-GFP11 or AvrPtoB-GFP11 shows recovered bacterial titers, this only suggests the recovery of host recognition and resistance rather than the virulence protein function. Based on the images in Figure 1A lower panel, the symptoms look similar for the RG prf3. Quantitative studies are needed for the virulence assay, otherwise the conclusion that the virulence is recovered is not very convincing.

We have provided whole leaf images in Figure 3A, which now more clearly illustrate the ability of AvrPto-GFP11 and AvrPtoB-GFP11 to be recognized on 76R as well as enhance bacterial symptoms on the susceptible line 76R prf3. We have also quantified growth of the different bacterial genotypes on 76R and 76R prf3 in Figure 3B. DC3000ΔavrPto/ΔavrPtoB + AvrPto-GFP11 exhibited significantly higher bacterial titers than DC3000ΔavrPto/ΔavrPtoB on 76R prf3, demonstrating that AvrPto-GFP11 also enhances bacterial virulence. Although DC3000ΔavrPto/ΔavrPtoB + AvrPtoB-GFP11 enhanced bacterial disease symptoms compared to DC3000ΔavrPto/ΔavrPtoB, there was no reproducible increase in bacterial titers four days post-inoculation. These findings are in line with a previous publication which also found AvrPtoB can enhance bacterial symptoms, but not bacterial titers at 4 days post-inoculation (MPMI Vol. 18, No. 1, 2005, pp. 43–51). It is possible that we did not capture the appropriate time to see a slight increase in bacterial growth in the presence of AvrPtoB-GFP11. We have now more clearly explained these results on page 8.

3. Similarly, in Figure 3C-E, the authors show that the Pst DC3000 (avrB-GFP11) elicit robust host defense response compared to empty vectors. First, a positive control is missing here. Second, the robust host defense responses only suggest that the delivery of the AvrB-GFP11 is not affected. However, other experiments are needed to show that the biological function of AvrB-GFP11 is not affected (For example, nonfunctional effectors may also be recognized by the host cell and elicit defense responses due to conserved structure.)
We have now included the rpm1-3 mutant in the bacterial growth assays, demonstrating that AvrB-GFP₁₁ recognition is specifically mediated by RPM1. Please see response to Reviewer 1, points 2 and 3.

4. In Figure 6B-D, the authors claimed that the GFP signals accumulated in the nuclei of host cells. However, without any nucleus marker, this claim is not convincing enough.

DAPI staining can be achieved in root hairs, but is exceedingly difficult to visualize in other intact root and nearby xylem tissues. PopP2-GFP₁₁ was not detected in root hairs. Therefore, we were unable to co-localize the PopP2-GFP₁₁ signal with a nucleus marker in intact roots. However, PopP2 has been demonstrated to be nuclear localized, possesses a nuclear localization signal required for function, targets WRKY transcription factors in the nucleus, and is recognized by nuclear localized RRS1-R in the Ws-0 ecotype. We have more clearly explained this supporting evidence in the text.

Reviewer #3:

This work represents one of the first major advances in the study of plant pathogenic T3Es in well over a decade. In addition, I would like to say that the use of cell-type specific HR complementation is both elegant and clever and should be given equal billing in the title and abstract
I would suggest: Direct and Indirect detection of bacterial effector delivery into diverse plant cell types during infection

Thank you for the detailed and positive evaluation of the manuscript. We have changed the title as suggested to highlight the different approaches used to visualize effector delivery into different cell types.

Major concerns, ranked
1. L69: I don't think you can exclude out of hand the potential for effectors to move through plasmodesmata. In fact the work of Khang et al. directly supports such a model in Magnaporthe

We have corrected this oversight and updated the text to state:

"However, due to the spread of defense signaling by apoplastic ROS and potentially other small molecules that can move through plasmodesmata (including some pathogen effectors), it is impossible to determine which cells are direct recipients of bacterial effectors, or which host cells are capable of directly recognizing effectors (Allan and Fluhr, 1997; Torres et al., 2002; Greenberg and Yao, 2004; Khang et al., 2010)."

3. Fig 4D, 5C, 5D: Is this the average between replicates? The combined numbers among 4 replicates? Include in methods section how the number of cells positive for effector delivery was determined. How was the number of cells normalized between samples? How much tissue was used for the counts? How was biased remove from selecting sections of leaves with more fluorescence for counting?

We have provided experimental details for these figures in the figure legends as well as the methods section. We have included the number of micrographs quantified in the figure legends and methods section on page 19. In order to identify effector delivery events, negative control images were first scanned and to set thresholds, followed by scanning two entire leaves per plant to detect delivery events.

Figure 4D represents the sum of all positive cells over four individual plant replicates after both syringe and surface inoculation.

The graph in Figure 5C indicates the total number of cells where effector delivery was detected after surface inoculation, n = 4 individual plants per treatment and time point. Numbers reflect the sum of all positive cells at 24h or 48h post-inoculation.

The graph in Figure 5D indicates cell-type specific distribution of effector delivery, contrasting syringe and surface inoculations n = 4 individual plants per treatment and time point. Numbers reflect the sum of all positive cells at both 24h and 48h post-inoculation.

4. Fig 1D: guard cells do not possess plasmodesmatal linkages. Offer an explanation of how macroscopic HR is developing in the GC-specific complements when presumably only GCs are undergoing cell death. Is there a reason the GC HR leaf is half the size of the other leaves?
We have updated the image in Figure 1D to include a more representative, and larger GC leaf.

We have now provided some potential alternative explanations for how macroscopic HR could develop, and included the following statement:

"Together, these results indicate that discrete cell types within the leaf tissue are capable of responding to recognized effectors and may propagate cell death signals across tissues either through plasmodesmata linkages or by apoplastic ROS signaling. Alternatively, there may be a small amount of RIN4 expressed in other cells in CSP lines that is sufficient to induce cell death after high density inoculation."

5. Fig 3A. ΔΔ symptoms don't appear attenuated in the closeup. Please add images of whole leaves or plants.

We have included images of whole leaves in Figure 3A, which demonstrate more clearly the attenuation of symptoms in ΔΔ. Also see response to Reviewer 2, point 2.

6. Fig 3. These bacterial load results should be compared with untagged effector complements

We have compared bacterial titers results with DC3000, which possesses AvrPto and AvrPtoB in its genome. We have now compared bacterial titers with a well-characterized plasmid containing AvrB-FLAG that was previously demonstrated to specifically elicit RPM1-mediated responses (The Plant Cell 2015 27:2042-2056). AvrB is not present in DC3000, but was cloned from P. syringae pv. glycinea.

7. L179: describe avrptoB-GFP11 partial complementation

We have now described the AvrPtoB-GFP11 partial complementation on page 8. Also see response to Reviewer 2, point 2.

8. L348 Neither avrPto nor avrPtoB have associated T3E-chaperones nor does AvrB to my knowledge so this seems like a poor argument to support the variation observed in this report.

We have removed this statement as AvrPto, AvrPtoB, and AvrB do not possess chaperones.

Minor concerns
Supp figures: add MW marker sizes to the blot images and in Figure 3 indicate that *** is associated with two tailed t-tests with alpha = 0.001

We have updated this information.

Figure 4 C should have scale bar

We have included a scale bar for Figure 4C.

Line 132 list source that also uses ion leakage as a proxy for cell death

We have cited a source for this.

L146: does not interfere with bacterial effector delivery in Salmonella

We have updated this statement.

Line 306 include a reference for the disease severity scale used in the R. solanacearum root infection.

We have included a reference for the disease severity scale.

L407: cite Radics et al, 2014

We have included this citation.

Review Editor Comments:

I have questions regarding whether the T3S effectors genes expressed under the control of their "native promoters" from a plasmid are being expressed properly. What is meant by "native promoter is not described for each gene. Further, these constructs are expressed from pBBR1, a freely replicating plasmid that is presumably maintained in multiple copies within the cell. Ideally each T3S-GFP fusion construct would be integrated into the genome at their
endogenous gene locations, to ensure that transcription of the fusions are regulated by their endogenous regulatory regions. Given the approach for expressing these genes in Pst DC3000 in your study, caution should be used when interpreting these results, especially when wanting to draw conclusions related to comparing effector delivery between different effector constructs.

We have now included a description of the promoter regions used for each effector on pages 16-17.

We agree with the editor that ideally each fusion construct would be integrated into the genome at their endogenous gene locations for detailed investigations. We are currently working to assemble bacterial strains with endogenous integrations, but have not completed their construction. For this breakthrough report, we focused on proof-of-concept of the GFP strand system to visualize type III effector delivery into diverse cell types. We have demonstrated that this system can be used to visualize effector delivery during natural infection, which we anticipate will open up new avenues of research and pave the way to a deeper understanding of pathogen infection.

An integrative plasmid was used for PopP2 effector delivery by Ralstonia solanacearum cells. For AvrB, AvrPto, and AvrPtoB, we used the same pBBR1 plasmid backbone and inoculated at the same time under identical conditions. Thus, it is reasonable to assume a similar copy number would be present for different effectors. We have included more caution when interpreting results, specifically noted the use of the pBBR1 plasmid, and discussed the goal of integration of the GFP11 coding sequence into endogenous gene locations in the discussion.

The white space in 2B could be more uniform.

The white space has been fixed.

The subscripts in 2A, 3A are too small.

The subscripts have been enlarged.

Figure 3, minus sign should match plus sign in size.

We have corrected font issues in Figure 3.

All fonts must be Arial or Helvetica and within a figure the fonts should be similar size for all panels (see Fig. 3B vs E)

We have fixed font issues.

You might consider whether you want a different color scheme for different variables. Compare key in 3B vs E. The key should also be similar sized. The superscript 2 in 3BE axes labels is too small.

Figure 5CD should match size better.

We have fixed font issues for the key and superscripts in Figure 3.

See also attached file for supplemental figures. I wonder if the legends should be on the same page as the figures.

The instructions for authors did not specifically state that that supplemental figure legends should be on the same page, but I noticed that they are frequently on the same page for online early publications. Supplemental figure legends are now included on the same page to facilitate manuscript flow.

We have updated supplemental figures as noted in the file attached after review.

TPC2017-00027-BRR1 2nd Editorial decision – accept with minor revision May 12, 2017

We have received reviews of your manuscript entitled "Direct and indirect visualization of bacterial effector delivery into diverse plant cell types during infection." On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on you making a few additional minor revisions, based on the comments of our reviewers. In particular, please consider the following:
1. There is an apparent discrepancy between your results and an observation reported in the co-submitted manuscript by the Dinesh-Kumar lab (Park et al), related to guard cells fluorescence. In your manuscript you interpret GFP signal in guard cells as indication of delivery of Type III secreted proteins. However, Park et al. attribute fluorescent signal in guard cells to autofluorescence. This may be confusing to people who read both the manuscripts. Is this something you could address or reconcile prior to publication?

2. Several small editorial changes suggested by Reviewer #2.

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**Reviewer comments and author responses:**

We thank the reviewers and editors for their positive evaluation and comments. We have addressed all comments, below.

**Editor Comments:**

1. There is an apparent discrepancy between your results and an observation reported in the co-submitted manuscript by the Dinesh-Kumar lab (Park et al), related to guard cells fluorescence. In your manuscript you interpret GFP signal in guard cells as indication of delivery of Type III secreted proteins. However, Park et al. attribute fluorescent signal in guard cells to autofluorescence. This may be confusing to people who read both the manuscripts. Is this something you could address or reconcile prior to publication?

   We have spoken to the Dinesh-Kumar lab and read their latest manuscript. The Dinesh-Kumar lab did not specifically analyze effector delivery into stomata. All effectors used in that manuscript are recognized by corresponding NLRs in the Col-0 GFP1-10 transgenic line. Therefore, the fluorescence signal was weak. They do show one image that illustrates strong autofluorescence in guard cells at the inner lip (Fig 5A), along with other images with low/no autofluorescence (Fig 6). Surface inoculation was not investigated, which is where we were able to detect the most guard cell delivery events.

   In contrast, we used effectors and/or plant genotypes that are not recognized by corresponding NLRs, enabling effector detection at later time points (and possibly at higher levels). We did detect guard cell autofluorescence at the inner wall flanking stomatal pores in some cases, but not in the outer edge flanking pavement cells in negative controls. A description of how we were able to detect delivery into guard cells is now more clearly described on page 9, lines 265-268:

   "Guard cells can exhibit autofluorescence in their inner walls flanking the stomatal pore. Therefore, guard cells only exhibiting fluorescence in their inner walls were not included in the quantification of effector delivery. Effector delivery was detected when fluorescence occurred at the guard cell outer edge, which is adjacent to the surrounding epidermal pavement cells."

2. Several small editorial changes suggested by Reviewer #2.

   We have made these changes.

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**Reviewer #1:**

The author's have satisfactorily addressed my concerns in their revised manuscript.

**Reviewer #2:**

The following points should be addressed before publication:

   We have included this citation on page 2, line 68.

2. In Figure 2B top panel, the labeling is not correct, should be "GFP1-10"?

   We have corrected this error.

3. Figure 2B legend, the "left panel" should be "right panel"?

   We have corrected this error.

4. Figure 3B labeling looks not correct; the black bar should be 76Rpf3 while the grey bar is 76R?

   We have corrected this error.

5. In Figure 3, the authors claimed that "the bacterial growth and disease symptoms of Pst DC3000 (avrB-GFP11) were attenuated at 4 days post-inoculation compared to DC3000 EV on Col-0 but not the rpm1-3 mutant line". But why they did not show the disease symptom of rpm1-3 mutant line? They should incorporate this data in Figure 3D.

   We have removed this statement on page 7, line 205 and refer only to the decrease in bacterial growth.

Reviewer #3:
My previous concerns have been adequately addressed through revisions and author comments.

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**TPC2017-00027-BRR2 3rd Editorial decision – acceptance pending** May 25, 2017

We are pleased to inform you that your paper entitled "Direct and indirect visualization of bacterial effector delivery into diverse plant cell types during infection" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

**Final acceptance from Science Editor** June 8, 2017