Spatiotemporal Monitoring of Pseudomonas Effectors via Type III Secretion using Split Fluorescent Protein Fragments

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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.)

TPC2017-00047-BRR 1st Editorial decision – revision requested Feb. 21, 2017

We have received reviews of your manuscript entitled “Spatiotemporal monitoring of Pseudomonas effectors via type III secretion using split fluorescent protein fragments.” 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.

Thus we invite you to address the comments and concerns of the reviewers, giving special attention to the following points raised by multiple reviewers.

1. Are the effectors tagged with GFP fully functional? To assess this you may need to compare their activity to untagged effectors expressed and delivered the same way.

2. Validation of sub-cellular localization of effectors by including control experiments.

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.

Supplemental material should only be used to support data presented in the Figures and Tables. New results should not be introduced via the supplement. Please ensure that this is the case.

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

Reviewer comments:

[Reviewer comments shown below along with author responses]
Reviewer comments and author responses:

We thank the reviewers for their efforts and constructive comments.

Reviewer #1:

Overall, the manuscript is well written but should be double-checked for spelling.

We have carefully checked the manuscript text for spelling.

1) With regards to localization studies in *N. benthamiana*, I am not clear as to some of the author's conclusions. On page 3 (168-170) the authors conclude that "Transient expression of sfGFP1-OPT fused to different targeting sequences with sfGFP11 targeted to the same subcellular compartment in *N. benthamiana* leaves, reconstituted the sfGFP OPT signal in respective subcellular compartments (Figure 2)." How can this be concluded without markers for those compartments (see point 5 below)?

The subcellular organelle and subcompartment targeting sequences that we have used in this study for fusion with sfGFP1-10OPT or sfGFP11 have been published previously (see reference Nelson et al., 2007). In this paper, the authors fused the targeting sequences with four different GFP variants and found similar localization pattern. The transgenic seeds of various organelle marker lines described in Nelson et al., (2007) have been deposited and distributed through ABRC. These are popular seed set and the paper has been cited in 891 articles since its publication. Since the fluorescence patterns in our studies described here are same as shown in Nelson et al., we conclude that our GFP1-10OPT is targeted to the same organelles described in Nelson et al. In addition, the organelles that we have shown are widely described in the plant cell biology literature and the morphology of the organelles matches published reports.

On page 4 (173-175) the authors find that: "... mCherry-sfGFP11 targeted to plasma membrane, nucleus, and peroxisome successfully reconstituted the fluorescence at the targeted compartments when co-expressed with cytosolic sfGFP1-10OPT (Figure. 3)." Is the conclusion here that cytosolic sfGFP1-10OPT goes to all of these compartments (plasma membrane, nucleus, and peroxisome). Is this also true in Arabidopsis (this becomes more important for the Arabidopsis experiments with this construct-see below)? Similarly, "... expression of mitochondria-targeted mCherry-sfGFP11 with cytosolic GFP1-10OPT lead to sfGFP signal in the cytosol and in the nucleus but not in the mitochondria (Supplemental Figure. 2B)." Is the conclusion that mitochondria-targeted mCherry-sfGFP11 is also in the cytosol and nucleus?

How can the conclusion of this be that "These results indicate that proper organelle targeting of both fragments of the sfGFP is required to visualize proteins in respective organellar compartment in plant cells." if you can express cytosolic sfGFP1-10OPT and observe localization to the peroxisome? This conclusion is only true if cytosolic sfGFP1-10OPT is going to this compartment. Should there also be a statement that these constructs may detect localization to other compartments, besides those stated?

We thank the reviewer for pointing this out. In the case of the plasma membrane, sfGFP11 is tagged at the cytosolic end of the transmembrane protein PIP2;1. Thus cytosolic sfGFP1-10OPT can reconstitute with sfGFP11 but remains at the periphery of the plasma membrane.

For peroxisomal localization, there is a well-known mechanism for protein transport into peroxisome, called "piggybacking" (McNew JA and Goodman JM. J. Cell Biol. 127, 1245–1257). The peroxisomal targeting sequence (PTS) we used in this study, PTS1, can transport an additional protein with no PTS, if two proteins dimerize. We reason that sfGFP1-10OPT can interact with mCherry-sfGFP11- SKL(PTS1) and the two proteins are transported into the peroxisomes together. In the case of nucleus, cytosolic sfGFP1-10OPT diffuses into the nucleus and reconstitutes with nuclear-targeted NU-mCherrysfGFP11.

In the case of mitochondria, targeting both sfGFP1-10OPT and sfGFP11 to mitochondria does reconstitute sfGFP in mitochondria (see Supplemental Figure 5). However, we can find strong expression of MT-mCherry-sfGFP11 showing ectopic cytosolic mCherry expression (left cell in MT-mCherry-sfGFP11, Supplemental Figure 3) while
cells with less expression showed proper MT localization (right cell in MT-mCherry-sfGFP11, Supplemental Figure 3). Hence, lack of mitochondrial localization but cytosolic GFP when coexpressed with cytosolic sfGFP1-10OPT, is not due to improper localization of MT-mCherry-sfGFP11 but from overexpression of proteins over the mitochondrial import capacity. In the transgenic plants, we did not see this leaky expression. See page 4 for the revised text. We updated the text on page 4, line 173.

Figure 1C—where are the single vector controls for these constructs as in A? I would also recommend writing construct descriptors that include any fusions to mCherry and GUS as in the supplemental figures.

We have included the control data in the new Supplemental Figure 2. We have also added mCherry-sfGFP11 constructs targeted to different organelles to Supplemental Figure 1. See corresponding revised text on page 3.

2) In regards to Arabidopsis experiments: Page 4 (188-189)- "We observed the reconstitution of sfGFP signal in all the corresponding subcellular compartments (Supplemental Figure 3)." Is this true for the mitochondria (looks nuclear)? And I have difficulty seeing anything in the plastid panel. As stated for the N. benthamiana experiments, it is difficult to conclude localization without markers- it would have been nice to see the localization with Arabidopsis proteins known to go these compartments to validate their utility.

We performed additional FAST experiments using different transgenic lines expressing MTsfGFP1-10OPT. We have included a better representative image showing reconstitution of sfGFP11 in mitochondria (see Supplemental Figure 5). We have also included a new plastid localization image from a repeat experiment (see Supplemental Figure 5).

Page 4 (197-200)—"First, a transgenic line expressing sfGFP1-10OPT in the cytoplasm was infected by Pseudomonas expressing effector fused to sfGFP11 tag. This will be useful to observe the localization of effectors, whose subcellular localization is unknown (Figure 4A, left panel)." How so, since the distribution of this construct was not tested in Arabidopsis?

We thank the reviewer for pointing this out. If there is no prior information on the localization of an effector, cytosolic sfGFP1-10OPT transgenic line could be used since the first plant interface the effector will come across is plasma membrane or cytosol. Based on our results described in Figure 3, cytosolic sfGFP1-10OPT will reconstitute with plasma membrane localizing effector as well as nuclear localizing effector. However, if the effector traffic to endomembrane system, plastid, and mitochondria; cytosolic sfGFP1-10OPT will not be useful. In this case, the user could use transgenic plants expressing sfGFP1-10OPT in different organelle. These are the reasons for two possible options. We have revised the text (see page 5).

3) In regards to effector translocation experiments and constructs: Page 6 (235-238)—"We infiltrated Pst DC3000D28E with pAvrRpm1:T3SSsp::mCherry-HAsfGFP11 into the transgenic Arabidopsis plants expressing cytosolic sfGFP1-10OPT. We observed mCherry spots on the infected leaves that are presumably bacteria expressing mCherry (Figure 4C, left panel, yellow arrows)." This experiment only tests expression from one vector not sure why the authors did not conduct a western blot with all 4 constructs in hrp-inducing media to show that all vectors are functional? All contain HA-tags. (see point 5 below)

We have included bacterial secretion in plant cell for all the effectors used in this study (see Supplemental Figure 7). In addition, we have also performed the functionality test of effectors by bacterial growth assay and immunity-related cell death assay (see new Figure 4D and E). The corresponding revised text is on pages 6-8.

In Fig. 4D "We could detect AvrRPS4C-HA-2xsGFP11 from tissue infected with Pst DC3000D28E strain with pAvrRpm1:T3SSsp::AvrRPS4C-HA-2xsGFP11 (Figure 4D, lane 3), while plant infected by Pst DC3000D28E (Figure 4D, lane 1) or DC30000D28E containing mCherry-HA-2xsGFP11 (Figure 4D, lane 2) did not show any detectable proteins, suggesting that effectors tagged with a tandem sfGFP11 tag can translocate from bacteria successfully into plant cells." I am not sure why the authors do not detect bacterially expressed HA-2xsGFP11 protein? Is it because "...infected leaves were agitated in water containing 50 μg/mL of carbenicillin for 2 hours to kill remaining bacteria in plant apoplast prior to protein extraction."? Has this been shown to remove bacterial proteins? Is there any evidence that HA-2xsGFP11 is expressed? Again the author's should test in vitro expression of all constructs in hrp-inducing media as a control.
Our conclusion is based on two reasons. First, based on our experiences using the FAST method, most agrobacteria from surface of plants after co-cultivation were removed by rinsing with repeated rinsing with water and then plants are regrown on MS plates with carbenicillin (see Li et al. Plant Methods 2007). We adopted this method and tested if Pst CUCPB5500 is carbenicillin resistant. Bacterial cells cannot grow in KB media containing carbenicillin.

Second, most importantly, if mCherry can be secreted into plant cells, the protein size will be about 36 kDa (~29 kDa of mCherry plus 7 kDa of HA-2xsfGFP11tag). We were unable to detect any protein around 36 kDa in immunoblot analyses except AvrRps4C-HA-2xsfGFP11 (~19 kDa) (See Supplemental Figure 7B). Therefore we concluded that residual bacteria were removed by agitation in water containing carbenicillin and mCherry cannot be secreted through T3SS. In the revised version, we have included a new Supplemental Figure 7 that shows immunoblot detecting effector proteins in plant cells and new Figure 4D and E that show additional tests showing functionality of effectors.

Page 7 (265-266): I think the authors can include “functional effectors” into this sentence since AvrRps4 triggers cell death.

We thank the reviewer for this suggestion. We have restated the sentence to include “functional effectors” (see page 8, line 301).

Page 7 (275-276): "At 3 hours post infiltration (hpi), we observed reconstituted sfGFP signal as small foci at the plasma membrane of epidermal cells (Figure 5A)." How do the authors know that these foci are plasma membrane localized?

We have included a new image in the revised version (see Figure 5B) with propidium iodide (PI) staining to show that AvrB-sfGFP localization is at the plasma membrane.

Page 7 (287-288): “These results suggest that AvrB effector may undergo trafficking in response to plant immune responses.” Could this trafficking be a result of the HR response triggered in N. benthamiana by AvrB?

Since AvrB is known to cause cell death in N. benthamiana, it is possible that this trafficking could be a result of cell death response. As we mentioned in the Discussion, this is an interesting new observation that we will be following up with series of experiments to determine the mechanistic basis of this trafficking. The split GFP system described will provide tools for the community to study effector trafficking.

Page 8 - is the AvrRPS4N construct expressed?

Yes, it is expressed and secreted into plant cells (see new Supplemental Figure 7B).

4) Other Comments:

Figure 4E- AvrRpm1 is not referred to in the text? How do these results compare to untagged effectors? What about AvrB, which is used in Figure 5; is it functional in this vector?

In the revised version, we have performed experiments with and without the sfGFP11 tag. We have included these new results in Figure 4D and E. Our results indicate no difference in terms of bacterial growth assay and immunity-related cell death induction by the effectors tested with and without sfGFP11 tag.

Figure 4E Legend- which plants are being infected? Is this Trypan blue?

We did Trypan blue staining for the infected Arabidopsis Col-0. In the revised version, we have included images from transgenic lines expressing cytosolic sfGFP1-10OPT (see new Figure 4E). We have included more details in the figure legend and Materials and Methods section (see page 16 and page 23).

Figure 5A- there is a lot of stomatal labeling, which is interesting since AvrB can open stomata (Zhou et al. Plant Cell. 2015 Jul; 27(7): 2032-2041.) It is worth noting the stomatal localization.

The green fluorescence at the stomata is a well-known autofluorescence by 488 nm laser for GFP excitation. In Figure 5A, the stomata are more visible because the image is a projection of Z stack for better visualization. We have included a statement in the figure legend to indicate that this is due to autofluorescence (see page 24, lines 844-845).
Figure 5B- the labeling does not seem to represent foci in Arabidopsis as it did in *N. benthamiana* (Fig 5A)?

We have included a new image to show localization to foci (see Figure 5C).

Supp. Figure 3- the MT/PM/NU/PT/PX/GO-mCherry-sfGFP11 constructs are not presented in Supp. Figure 1 (*Illustration of plasmids used in this study*)

In the revised version, we have included these in Supplemental Figure 1.

5) Summary of recommendations for additional experiments. There is no doubt that the system that the authors have developed will be a valuable resource to the plant-microbe interaction community. However, I would recommend some additional experiments to confirm the quality of the resource.

a) Confirm the localization of sfGFP constructs presented in Supp. Figure 1. This could be done in *N. benthamiana*, but ideally this would be conducted on the Arabidopsis GFP fluorescence presented in Supp. Figure 3. This could be done using biochemical/staining methods (example plasmolysis for PM localization).

We have provided a significant amount of results from multiple experiments in *N. benthamiana* and Arabidopsis to show that the reconstitution occurs at proper organelle/subcompartments and hence the constructs are functional. Furthermore, we have included a new Supplemental Figure 3 in the revised version to show that the targeting sequence targets mCherry-sfGFP11 to the appropriate organelle/subcompartments.

In the revised version, we have also included propidium iodide (PI) counterstaining for cell wall and nucleus to verify plasma membrane and nuclear localization of AvrB and AvrRps4C, respectively (see Figure 5B and C; Supplemental Figure 9).

b) Confirm *P. syringae* expression from constructs in Figure 4B by western blot in hrp-inducing media. Also confirm expression of constructs used in Fig 4C,D and E.

We confirmed effector secretion in plant cells and their functionality by bacterial growth assay and cell death phenotype (see Figure 4D and E; Supplemental Figure 7).

c) How does expression of sfGFP11 affect bacterial fitness? This could be done by comparing growth of *P. syringae* expressing a T3E:sfGFP11 vs. *P. syringae* expressing the T3E in minimal hrp-inducing media or in planta.

Growth on the KB media is same with and without tag (See Supplemental Figure 7A). Functionality of the effectors tagged with sfGFP11 is same as untagged version, see Figure 4D and E; and Supplemental Figure 7.

Reviewer #2:

1. Line 152: In addition to the sfGFP tagging system, the authors also tried another sfCherry system. However, this sfCherry system has not been used in tagging any virulence proteins in this manuscript. Since the GFP and RFP are based on different protein sources, any conclusion got from the sfGFP system need to be re-examined for the sfCherry system. Since this is a resource manuscript, these constructs will be useful for the research community. We have included results in Supplemental Figure 2 (panel 4) to show that there is no cross complementation between sfCFP1-10OPT and sfCherry11.

2. Line 257-266: The authors tried to test the whether the sfGFP11 tag interferes with biological activity of effector proteins and they used increased cell death as the indicator. The programmed cell death of host cells usually results from the detection of pathogen effectors and triggered by ETI. Thus the increased cell death may only suggest that the delivered effectors could be detected by host cells. The authors need to find other lines of evidence to test whether the sfGFP11 tag interferes with biological activity of effector proteins. Moreover, they did not consider the effect of the sfGFP11-10OPT, the transgenic plants expressing sfGFP1-10OPT should be used here rather than the...
wild-type plants.

In the revised version, we have generated AvrB and AvrRps4 with no tag in the same promoter and vector backbone as used for expressing effectors fused to sfGFP11. We found no difference (see Figure 4D and E; pages 6-8 for text).

3. This manuscript described two different infection strategies by using either cytoplasmic sfGFP1-10OPT or sfGFP1-10OPT targeted to subcellular compartments. However, the author did not compare these two strategies when tagging the virulence effectors. For example, when using the CYTO-sfGFP1-10OPT, localization of AvrB-HA-2x sfGFP11 shows small dot-like structures (Figure 5A); on the other hand, when using the PM-sfGFP1-10OPT, localization pattern looks much more diffused and much less signals could be observed compared to the previous one (Figure 5B). The author failed to compare these two strategies and did not discuss what caused such kind of differences. Which one represents the real situation?

We thank the reviewer for pointing this out. Our goal here was to mimic natural conditions. Therefore, we used relatively low number of bacteria for infiltration and observed the plant cells at early time points. We generated numerous images for each line and each time point. Although fluorescence intensity varies between experiments, localization patterns are consistent, allowing us to see their localization pattern at a given time point. At later time point, we did find evidence for trafficking and this needs to be carefully examined in the future.

In this revised manuscript, we repeated the experiments with another line of PM-sfGFP1-10OPT and observed bright and thick accumulation of sfGFP at the PM (see Figure 5B and C). The resources described in this manuscript will facilitate careful evaluation of effector localization and trafficking at different time points after infection in future experiments.

4. In Figure 5D, the authors described that the AvrB showed vesicular localization. However, the images are not clear enough. And without any marker, it is not very convincing for such kind of conclusion.

Plasma membrane localization of AvrB biochemically has been well characterized in the literature. However, vesicular localization is unknown and we do not know the identity of these vesicles. Since we have seen this in multiple experiments, we have reported in this manuscript. In the future, we will be following up to determine the identity of these vesicles and their influence on pathogenicity.

5. In Figure 6A, B, C, E, the authors said the GFP signals could be detected in the nucleus. However, without any nucleus marker, such kind of conclusion is not very convincing. Could we do co-localization with nuclear TF in a N. benthamiana transient assay?

The nucleus is morphologically distinct in the cell. In the revised manuscript, we have included counter staining with propidium iodide (PI). PI is non-permeable in healthy cells, thus it stains cell wall. In dyeing cells, it is permeable and can stain nucleus. Furthermore, to increase cell permeability, we treat leaf discs with a fixative before observation. We have included results from these studies in Supplemental Figure 9 to show nuclear staining and localization of AvrRps4C.

6. In this manuscript, the authors tried to use the sfGFP system to study the temporal and spatial information of the virulence effectors. However, they did not test whether the tagging will affect the function of these secreted effector proteins. In that case, whether this temporal and spatial information obtained represent the real situation is questionable.

To show that the functionality of the effectors studied with sfGFP11 tag is same as untagged version of the effectors, we have included bacterial secretion in plant cells (see Supplemental Figure 7), bacterial growth assay (Figure 4D) and immunity-related cell death assay (Figure 4E). As suggested by Reviewer 1, the fact that the tagged versions induce cell death confirms functionality.

Reviewer #3:

This work represents the first major technical advance for the study of plant pathogen T3Es in native contexts in over a decade (Casper-Lindly in 2002). The authors have done an admirable job creating a, hopefully, robust toolset that I
predict will be rapidly adopted by the field.

We thank the reviewer for noting this. We strongly believe that the resources described here will not only be useful to study bacterial effectors but also other pathogen effectors and plant proteins.

1. Figure 4D, the western analysis has a built in assumption that the HA tagged protein present within the bacteria is not detectable and that only the protein translocated into the plant cell has built up to a degree to allow detection by western. To me this does not make sense. Also I see a trace of a cropped band in the first panel of 4D, (lane 2) that looks to be running near the expected size of mCherry. I'd like to see the uncropped gel image. The correct control for this assay would be with T3SS- strain and demonstrating that the detected bands are only present when the T3SS is present. Otherwise the interpretation doesn't hold water.

As stated in our response to Reviewer 1: Our conclusion is based on two reasons. First, based on our experiences using FAST method, most agrobacteria from surface of plants after co-cultivation were removed by rinsing with repeated rinsing with water and then plants are regrown on MS plates with carbenicillin (see Li et al. Plant methods 2007; ref #). We adopted this method and tested if Pst CUCPB5500 is carbenicillin resistant. Bacterial cells cannot grow in KB media containing carbenicillin. Second, most importantly, if mCherry can be secreted into plant cells, protein size will be about 36 kDa (~29 kDa of mCherry plus 7 kDa of HA-2xsfGFP11tag). We were unable to see any protein around 36 kDa in immunoblot analyses except AvrRps4C-HA- 2xsfGFP11 (~19kDa) (See Supplemental Figure 7B; white arrow). Therefore we reasoned that residual bacteria were removed by agitation in water containing carbenicillin and mCherry cannot be secreted through the T3SS. Attached below is the same blot with longer exposure for your reference. In the revised version, we have included a new Supplemental Figure 7 that shows immunoblot detecting effector proteins in plant cells and new Figure 4D and E that show additional tests showing functionality of effectors.

TPC2017-00047-BRR1 2nd Editorial decision – revision requested May 20, 2017

We have received reviews of your manuscript entitled “Spatiotemporal monitoring of Pseudomonas effectors via type III secretion using split fluorescent protein fragments.” On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. However, there are still some concerns with the manuscript that need to be addressed before final acceptance. One of these concerns may require further experimentation. I anticipate being able to make a final decision regarding acceptance of the revised manuscript without having to send it out for review.

In particular, please consider the following.

1. Issue related to secretion of the tagged effectors. I agree with Reviewer #3 that data shown in Supplemental Fig 7 do not demonstrate secretion of the tagged effector proteins. I believe that the tagged proteins could also be detected in infected plant tissue if they were expressed in the bacterial cell, and never secreted.

2. Apparent discrepancy with paper from Coaker lab regarding fluorescence in guard cells.

3. Additional comments and concerns of Reviewer #3.

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.

Reviewer comments:

[Reviewer comments shown below along with author responses]
Reviewer comments and author responses:

Reviewer #3:

While the new experiments conducted in Figure 4 do provide evidence of tagged effector functionality, supplemental Figure 7 is still inadequate to support the author's claims associated with this data. Expression was not validated using expression in HIM as suggested by Reviewer 1 not have they added the correct control of a T3SS-strain transformed with their constructs to validate that western-detectable protein is dependent on T3SS-mediated translocation. Particularly in the context that two of their constructs do not produce detectable protein levels by western. The authors have also failed to provide a complete uncropped gel image. I find the author's argument that carb would eliminate the presence of bacterial protein inconsistent with the mechanism of this antibiotic. P. syringae can and does display resistance to Beta lactam antibiotics. Typical use of carb for clean selection is often done with higher concentrations. To be honest S7B is detracting from the manuscript and perhaps should be removed entirely. The additional exposure provided side-by-side doesn't look like they're from the same blot.

The key point of this part of the experimentation was to show that the sfGFP11 tag does not affect the functionality of the effectors when delivered through Pst (Figure 4). In the revised version we have reworded the statement to reflect this conclusion (see page 7: lines 291-292). In the revised version we have included the data showing that there is no effect on growth in the presence of sfGFP11 tagged effectors when grown on hrp-derepressing media (see new Supplemental Figure 7B; text pages 6-7: lines 258-271). Two images shown in the previous version Supplemental Figure 7B were generated by different exposure of the same blot (not a different blot as suggested by the reviewer). We normally expose several x-ray films in multiple duration of exposure time. See below the scanned film images of the same blot with different exposure.

Since the other functionality test of bacterial effector in Figure 4 supported our hypothesis that the sfGFP11 tag does not interfere with effector function in plant cells, we have removed Supplemental Figure 7B as suggested by the reviewer.

L54. Reword. Plant pathogenic bacteria do not invade host cells

We have reworded this sentence.

We are pleased to inform you that your paper entitled "Spatiotemporal monitoring of Pseudomonas effectors via type III secretion using split fluorescent protein fragments" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor

June 14, 2017