August 9, 2019

Dear *Plant Direct* Editors,

We are resubmitting our manuscript entitled, "Efficient and Modular CRISPR-Cas9 Vector System for *Physcomitrella patens*". We thank the reviewers for their careful reading of the manuscript and their suggestions. We have included a revised manuscript with track changes as well as a clean version of the manuscript. Below we append a detailed point-by-point response to the reviewers.

I hope with the changes that the manuscript will now be acceptable at *Plant Direct*.

Sincerely,
Magdalena Bezanilla, for the authors

Response to reviewers

Reviewer 1:

1. References:
PtU6 promoter was previously used for driving gRNA in Pp. please add references.
Please add references for more crops (line 205-208)

> We apologize for the oversight. We have added the references to lines 138-139 and lines 209-212 in the revised manuscript.

2. Sentence that starts at line 234 is confusing. In general, using 2 guides definitely increase the chance of getting a knock out mutation. If both guides are very efficient one will get easily mutations with one guide only however, without previous knowledge about guides efficiency it is recommended to use 2 guides per gene. (Pauwels, Laurens, et al. "A dual sgRNA approach for functional genomics in Arabidopsis thaliana." G3: Genes, Genomes, Genetics 8.8 (2018): 2603-2615.)

> We have edited the text (lines 257-259 in the revised manuscript) to clarify that targeting adjacent sites may not necessarily generate large deletions at a high frequency since both sites could repair separately.

3. Please add photos of the plants with seamless integration of GFP between the coding region and 3'UTR. Is the GFP shows the same cellular localization that is predicted for this protein?

> Yes, we observed the expected localization for this protein and we have added an image of a cell from one of the plants that has integrated GFP between the coding sequence and the 3'UTR. This is now Supplemental Figure 3.

4. Please remove this sentence in line 440:
"The simple and modular design of our vector system allows fast and economical vector construction"
Multisite gateway is quite expensive and researchers in many countries can't afford it. In addition, the efficiency of LR reactions with multisite GW (3-4 inserts) is low so it's not simple.

> We agree with the reviewer that Multisite Gateway is expensive. Thus, we have removed "economical" from this sentence (line 473 in the revised manuscript). However, the rest of the sentence has remained as we respectfully disagree that the LR reactions are inefficient. We routinely use this method and have had no problems with efficiency in generating multisite constructs. Due to the speed of generating constructs, less personnel time is used.

5. Figure 3B, the table is a bit confusing, In the text the authors describe screening of 24 plants by T7 for site 4 and only 15 were positive (cleaving) however they only talk about 6 plants that had frameshift mutations (are the other 9 were in-frame mutants)? Then some of the sites in some of the plants were sequenced but not all? For example, Plant2 site 6, how do they know it's a WT? plant 22 site 3 how do they know its 200bp deletion?

> Yes, only six plants had frameshift mutations and the other nine plants were in-frame mutants. We apologize for the confusion. We have fixed the table in the revised figure 3. All of the sequenced plants are correctly indicated now. We know it is a ~200bp deletion because the amplified PCR product is smaller by 200 bp. We have now indicated this in the figure legend (lines 913-914 in the revised manuscript).

6. Figure 5. Please label the lanes in the gel. And in your figure legend I think there is a mistake. The color for 5'UTR and Coding is opposite.
Lines 901-902: The 5' UTR, the coding region, and the 3' UTR are represented by blue, green, and purple, respectively.

> Thank you for pointing this out. We have fixed these issues in the revised Figure 5 by changing the 5' UTR to orange so that it is not confusing with the gene model shown in panel (D). We have also added labels to the gel image.

Reviewer 2:
116, 178 and 363—the authors mention being able to edit 12 sites. Only 4 were successfully edited. Speaking of the capability of 12 in the introduction and discussion seems misleading since it was not achieved. Additionally, no sources are listed to support any other research successfully completing 12 edits.

> The vector system has the capacity to do this, but as the reviewer points out we did not demonstrate this in the paper. Thus, we have modified the language to indicate that this is the capacity of the system in the first two instances where this was mentioned (in the introduction and the results, lines 120 and 190-191 in the revised manuscript) and have removed it entirely from the discussion.

443—Line 103 states that moss has a higher success rate at homologous recombination. This should be at least addressed by stating efficiency may decrease when mentioned in conclusion in line 443 that these CRISPSR tools are transferable between organisms.

We modified the final sentence to address this concern (lines 477-478 in the revised manuscript).

140 and 571—contradictory selection periods. Which was used?

We have modified the materials and methods (line 621-623 in the revised manuscript) to include the longer selection period that was used in the experiments to test editing efficiency with the rice U3 promoter versus the moss U6 promoter.

184-186—was there any reason for choosing these 6 genomic sites other than PAM sequence convenience? Is there a significance to these genes?

We were targeting redundant members of a gene family. There is no particular significance of these genes to this manuscript.

365 and 374—is "protospacer efficiency" quantifiable? Some protospacers seemed to edit more than others, but were there enough plants monitored to make this claim? Is there any data suggesting the edits are not purely random?

Protospacer efficiency may be quantifiable, but we did not screen enough plants to generate a number for the efficiency. We have not included data in this paper about the frequency with which we uncover specific edits. We do have data from other work in the lab suggesting that certain protospacers/genomic sites lead to non-random edits, but those data are beyond the scope of this particular manuscript.