Dr. Bing Wu

Review Editor

JoVE

15 October 2018

Dear Dr. Wu,

Thank you for your critical evaluation of our manuscript, entitled “A Simple Induction System for Clustered Stomata by Sugar Solution Immersion Treatment in *Arabidopsis thaliana* Seedlings” (JoVE58951). We are very grateful for your favorable reply. In accordance with the helpful comments and suggestions from the reviewers, we have revised the manuscript. Please find enclosed the revised version of our manuscript, which we would now like to re-submit for consideration. We have attached point-by-point responses to the comments.

We appreciate the critical appraisal of our manuscript from the reviewers, and have addressed all of the comments and questions as constructively as possible. We hope that the revised paper will be found to merit publication in *Journal of Visualized Experiments*.

Please address all correspondence to:

Dr. Takumi Higaki

International Research Organization for Advanced Science and Technology

Kumamoto University, Kumamoto 860-8555, Japan

Tel:+81-96-342-3404

E-mail: thigaki@kumamoto-u.ac.jp

We look forward to hearing from you at your earliest convenience.

Yours sincerely,

Dr. Takumi Higaki

Point-by-point Responses to the comments

*Editorial comments:*

*Changes to be made by the author(s) regarding the written manuscript:*

*1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

*2. Please revise the Introduction to include all of the following:*

*a) A clear statement of the overall goal of this method*

*b) The rationale behind the development and/or use of this technique*

*c) The advantages over alternative techniques with applicable references to previous studies*

*d) A description of the context of the technique in the wider body of literature*

*e) Information to help readers to determine whether the method is appropriate for their application*

*3. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*

*4. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:*

*a) Critical steps within the protocol*

*b) Any modifications and troubleshooting of the technique*

*c) Any limitations of the technique*

*d) The significance with respect to existing methods*

*e) Any future applications of the technique*

*5. References: Please do not abbreviate journal titles.*

Thank you very much for your kind instruction. We have revised the manuscript according to your instructions.

*Reviewers' comments:*

*Reviewer #1:*

*Manuscript Summary:*

*This manuscript describes a simple experimental protocol for induction of clustered stomata in Arabidopsis thaliana seedlings by immersing in a sucrose-containing solution. The protocol is properly written and the manuscript is well organized.*

*Major Concerns:*

*No major concerns.*

*Minor Concerns:*

*No minor concerns.*

Thank you very much. We appreciate your kind reviewing.

*Reviewer #2:*

*Manuscript Summary:*

*The manuscript describes a relatively simple procedure to generate clustered stomata in Arabidopsis thaliana. With "sugar solution immersion treatment" 45% of the guard cells are arranged in clusters.*

*Major Concerns:*

*My major concern is related to the paragraph on lines 162 to 171 and Figure 3 in Akita et al. 2018 ("Cortical microtubules and fusicoccin response in clustered stomatal guard cells induced by sucrose solution immersion"): The stomatal aperture in clustered guard cells is 60-80% smaller than without treatment. So to me it is unclear if the "functioning" of the guard cells has enough similarities with un-treated (guard-)cells. The authors should comment on that.*

*Please explain in more detail for which "research purposes" this treatment is useful despite reduced aperture sizes.*

Thank you for your kind suggestions. As suggested, we have revised the Discussion part (page 3, lines 168-173).

*Specifically, I would like to see a comparison of apertures in other type of clustered guard cells (clustered guard cells found in nature or obtained with other treatment): What are typical aperture values (literature) for distributed and clustered stomata? Are the immersion-treated apertures smaller?*

Thank you for kind comment. However, this manuscript is focused on the methods and the protocol. Although we keep your comment in mind, we believe that the data for the aperture comparison should not be shown here.

*Minor Concerns:*

*Line 74: "Murashige-Skoop medium salts"; please explain what it is and a potential provider*

As suggested, we have added the reference (Murashige and Skoog, 1962) in the Material table. The provider had been already shown in the previous version.

*Line 91: Could you explain how you to obtain "transgenic A. thaliana seeds" (provider?)*

We have properly cited the references for the transgenic lines expressing GFP-TUB6 or CT-GFP. Therefore, we believe that further information is not needed.

*Line 156: the authors state that 45% of guard cells are clustered. Based on how many samples did did you determine this value? What is the variation between different samples?*

As suggested, we have added the sample size (page 3, line 156-157).