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An Induction System for Clustered Stomata by Sugar Solution Immersion Treatment in Arabidopsis thaliana Seedlings

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TITLE:

An Induction System for Clustered Stomata by Sugar Solution Immersion Treatment in *Arabidopsis thaliana* Seedlings

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KEYWORDS:
Arabidopsis thaliana, Chloroplasts, Fluorescent proteins, Guard cells, Microtubules, Plant cell biology, Stomata, Sucrose, Sugar

SUMMARY:
The goal of this protocol is to demonstrate how to induce clustered stomata in cotyledons of *Arabidopsis thaliana* seedlings by immersion treatment with a sugar-containing medium solution and how to observe intracellular structures such as chloroplasts and microtubules in the clustered guard cells using confocal laser microscopy.

ABSTRACT:
Stomatal movement mediates plant gas exchange, which is essential for photosynthesis and transpiration. Stomatal opening and closing are accomplished by a significant increase and decrease in guard cell volume, respectively. Because shuttle transport of ions and water occurs between guard cells and larger neighboring epidermal cells during stomatal movement, the spaced distribution of plant stomata is considered an optimal distribution for stomatal movement. Experimental systems for perturbing the spaced pattern of stomata are useful to examine the spacing pattern's significance. Several key genes associated with the spaced stomatal distribution have been identified, and clustered stomata can be experimentally induced by altering these genes. Alternatively, clustered stomata can be also induced by exogenous treatments without genetic modification. In this article, we describe a simple induction system for clustered stomata in *Arabidopsis thaliana* seedlings by immersion treatment with a sucrose-containing medium solution. Our method is easy and directly applicable to transgenic or mutant lines. Larger chloroplasts are presented as a cell biological hallmark of sucrose-induced clustered guard cells. In addition, a representative confocal microscopic image of cortical microtubules is shown as an example of intracellular observation of clustered guard cells. The radial orientation

of cortical microtubules is maintained in clustered guard cells as in spaced guard cells in control conditions.

INTRODUCTION:

The plant stoma is an essential organ for gas exchange for photosynthesis and transpiration, and stomatal movement is accomplished by significant changes in guard cells through ion-driven uptake and release of water. Under a microscope, we can observe a spaced distribution pattern of stomata on the surfaces of leaves and stems. This spaced distribution of stomata is considered to help stomatal movement, which is regulated by ion and water exchange between guard cells and neighboring epidermal cells[1,2]. Experimental induction systems for clustered stomata are useful for investigating the importance of the spaced distribution of stomata.

It has been reported that spatial clustering of stomata can be induced by genetic modification of key genes for guard cell differentiation[3,4] or treatment with a chemical compound[5]. We also reported that immersion treatment with a medium solution supplemented with sugars including sucrose, glucose, and fructose caused stomatal clustering in cotyledons of *Arabidopsis thaliana* seedlings[6]. Reduced callose in new cell walls separating meristemoids and epidermal cells was observed in the sucrose-treated cotyledon epidermis, suggesting that sucrose solution immersion treatment negatively affects the cell wall, which prevents the leakage and ectopic action of key gene products for guard cell differentiation (*e.g.* transcription factors) towards adjacent epidermal cells[6]. A similar mechanism was suggested from studies on *gsl8/chor* mutants[7,8]. Our experimental system for reproducible induction of clustered stomata using sucrose-containing medium solution is quite easy and cheap. It can also be used to investigate intracellular structures such as organelles and the cytoskeleton in the clustered guard cells when applied to transgenic lines expressing fluorescent markers that label intracellular structures[9,10].

PROTOCOL:

1. Preparation of 3% Sucrose-containing 1/2 Murashige-Skoog Medium Solution

1.1. Add 1.1 g of Murashige-Skoog medium salts and 15 g of sucrose to a beaker.

1.2. Add 490 mL of distilled water and mix well using a stir bar.

1.3. Adjust the pH to 5.8 using KOH.

1.4. Dilute to 500 mL with distilled water and transfer the solution into a medium bottle.

1.5. Sterilize the solution by autoclaving (121 °C, 20 min). If not used immediately, this solution may be kept at 4 °C after sterilization.

2. Induction of Clustered Stomata by Sucrose-Containing Medium Solution Immersion Treatment

2.1. Sterilize the seeds.

2.1.1. Prepare the sterilization solution by adding 500 µL of 5% active chlorine NaClO solution and 1 µL of 10% Triton X-100 to 500 µL sterile water.

2.1.2. Place ca. 50 transgenic *A. thaliana* seeds carrying a fluorescent marker such as CT-GFP[11] or GFP-TUB6[12] into a 1.5-mL tube.

2.1.3. Add 1 mL of 70% ethanol solution and mix well by inverting five times. Leave for 1 min.

2.1.4. The seeds will sink to the bottom of the tube. On a clean bench, gently remove the 70% ethanol using a micropipette, and add 1 mL of sterilization solution. Mix well by inverting five times and leave for 5 min.

2.1.5. Wash the seeds. Still working under aseptic conditions on a clean bench, gently remove the solution using a micropipette, and add 1 mL of sterile water. Repeat this step five times.

2.2. Add 1.5 mL of sterilized 3% sucrose-containing 1/2 Murashige-Skoog medium solution to each well of a 24-well plate on a clean bench.

2.3. Add two sterilized seeds into each well. Tape the lid onto the 24-well plate using two layers of parafilm.

2.4. Transfer the 24-well plate to a growth chamber set at 23.5 °C with a 12-h/12-h light-dark cycle using 100 µmol m^{-2} s^{-1} white light and incubate for 14 days.

3. Microscopic Observation of Clustered Stomata

3.1. Place 30 µL of 3% sucrose-containing 1/2 Murashige-Skoog medium solution from a well of the 24-well plate onto the center of a glass slide (size: 76 × 26 mm, thickness: 1.0–1.2 mm).

3.2. Remove a cotyledon from a 14-day-old seedling using dissecting scissors. Float the cotyledon with the observation side facing up on the solution drop.

3.3. Prepare the cotyledon specimen according to our previous method[13]. Essentially, place 30 µL of the solution on the center of a cover glass (size: 18 × 18 mm, thickness: 0.12–0.17 mm). Turn the cover glass upside down and place it on the cotyledon gently. Wipe off excess buffer using a lint-free tissue.

3.4. Set a specimen on the stage of a confocal laser microscope and select clustered guard cells for observation using bright field illumination.

3.5. Acquire confocal images of fluorescently labelled intracellular structures according to the microscope manufacturer's instructions.

REPRESENTATIVE RESULTS:

Here, the protocol for a simple method of inducing stomatal clustering with sucrose-containing medium solution in *A. thaliana* seedlings has been presented. The clustered guard cells grown in sucrose-containing medium solution (**Figure 1B**) have larger chloroplasts than guard cells grown in sucrose-free control conditions (**Figure 1A**). The enlargement of chloroplasts was confirmed with CT-GFP[11], a chloroplast stroma marker, and chlorophyll autofluorescence (**Figure 1C–F**), suggesting that sucrose treatment resulted in starch grain accumulation in the chloroplasts *via* sucrose solution uptake. In addition, confocal observation of GFP-TUB6[12] revealed that cortical microtubules were radially oriented even in sucrose-treated clustered guard cells, like those in spaced guard cells in sucrose-free control conditions (**Figure 2**). These observations suggest that the sucrose-induced clustered guard cells have a normal orientation for cortical microtubules and cellulose microfibrils to enable stomatal opening in response to environmental cues[9].

FIGURE AND TABLE LEGENDS:

Figure 1: Chloroplasts in clustered guard cells treated with sucrose-containing medium solution. Bright field **(A, B)**, chloroplast stroma marker CT-GFP **(C, D)**, and chlorophyll autofluorescence **(E, F)** images of guard cells grown in sugar-free control conditions **(A, C, E)** and 3% sucrose conditions **(B, D, F)**. Scale bars = 10 µm.

Figure 2: Cortical microtubules in clustered guard cells treated with sucrose solution. Cortical microtubules labelled with GFP-TUB6 of guard cells in the sugar-free control **(A)** and clustered guard cells in 3% sucrose conditions **(B)**. Scale bars = 10 µm.

DISCUSSION:

We have presented protocols for induction of clustered stomata in *A. thaliana* seedlings by immersion treatment with a sucrose-containing medium solution. As shown here, this method is very simple and requires no specialized skill but can efficiently induce clustered stomata. More than 45% of guard cells are clustered with 3% sucrose-containing medium solution (mean values of more than 20 independent observations)[6]. Moreover, this experimental system can be directly applied to transgenic or mutant lines as shown for transgenic lines expressing CT-GFP (**Figure 1**) or GFP-TUB6 (**Figure 2**). Although only snapshot images are shown here, it would also be possible to perform time-sequential observations during stomatal development.

Note that this method is based on an artificial exogeneous treatment, so we cannot exclude the possibility that phenomena that are not directly related to the stomatal distribution are caused by sucrose solution immersion treatment. In fact, guard cell chloroplasts are enlarged by the treatment (**Figure 1**). This might be due to starch grain accumulation in the chloroplasts *via* sucrose solution uptake. In addition, a smaller stomatal aperture was observed in the sucrose-induced clustered guard cells[9], suggesting that sucrose-mediated hyperosmotic stress suppressed stomatal opening. Nevertheless, the radial orientation of cortical microtubules was maintained (**Figure 2**). In addition, the stomatal aperture of the clustered guard cells significantly increases in response to fusicoccin treatment, as in the case of spaced stomata[9]. Thus, although it will be necessary to carefully judge whether this experimental model system is useful depending on your research purposes, our system would provide insightful information

concerning relationship between stomatal distribution and response.

As mentioned in the Introduction, sugar solution treatment might decrease the cell wall integrity, resulting in leakage of key gene products for stomatal differentiation (*e.g.* transcription factors) to adjacent epidermal cells. We assume that the sucrose-induced ectopic localization of these gene products causes clustered stomata. However, this working hypothesis is not sufficiently supported by molecular biological evidence. Screening for sugar-insensitive mutants would be a promising way to clarify the molecular mechanisms underlying sugar solution-induced stomatal clustering.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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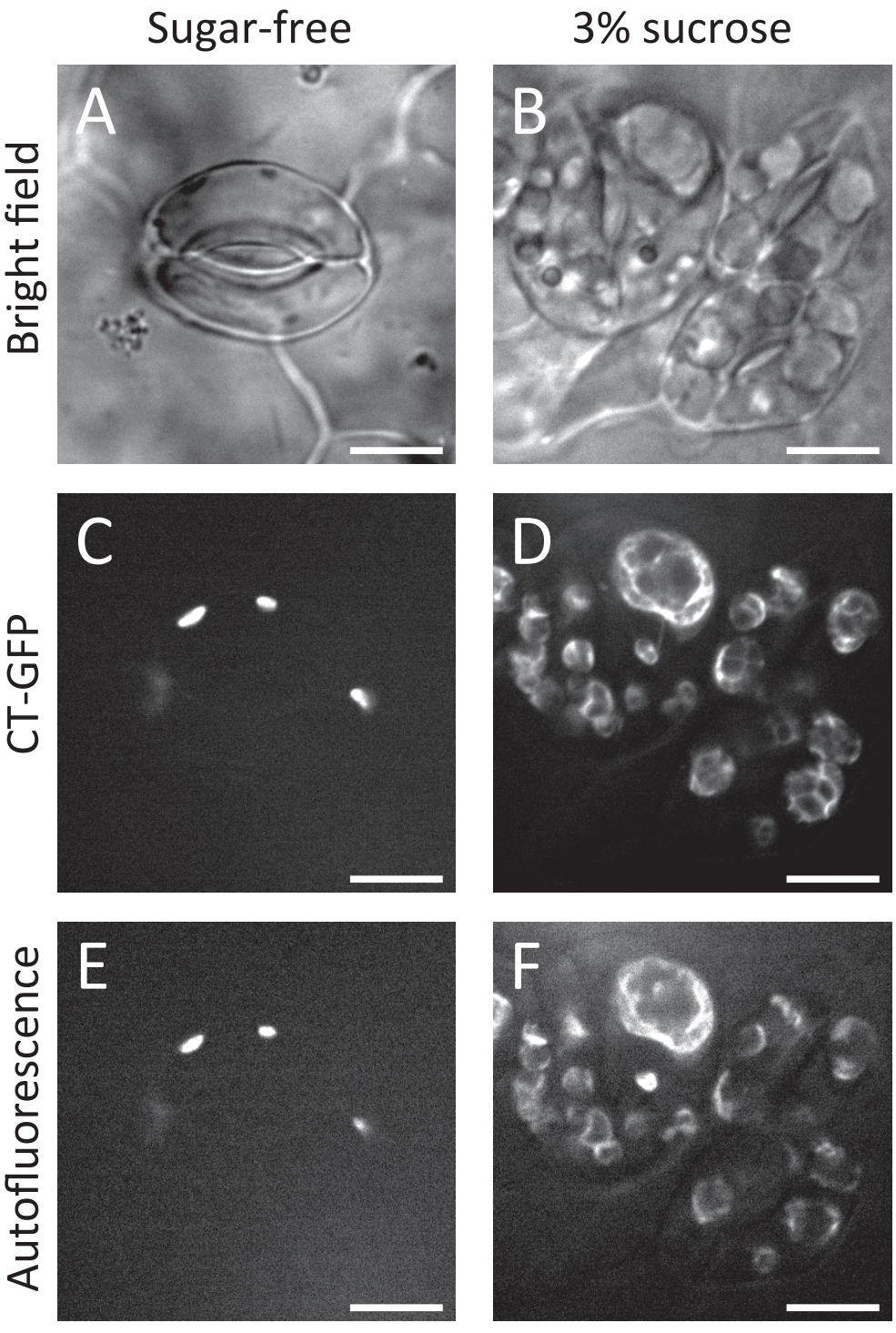

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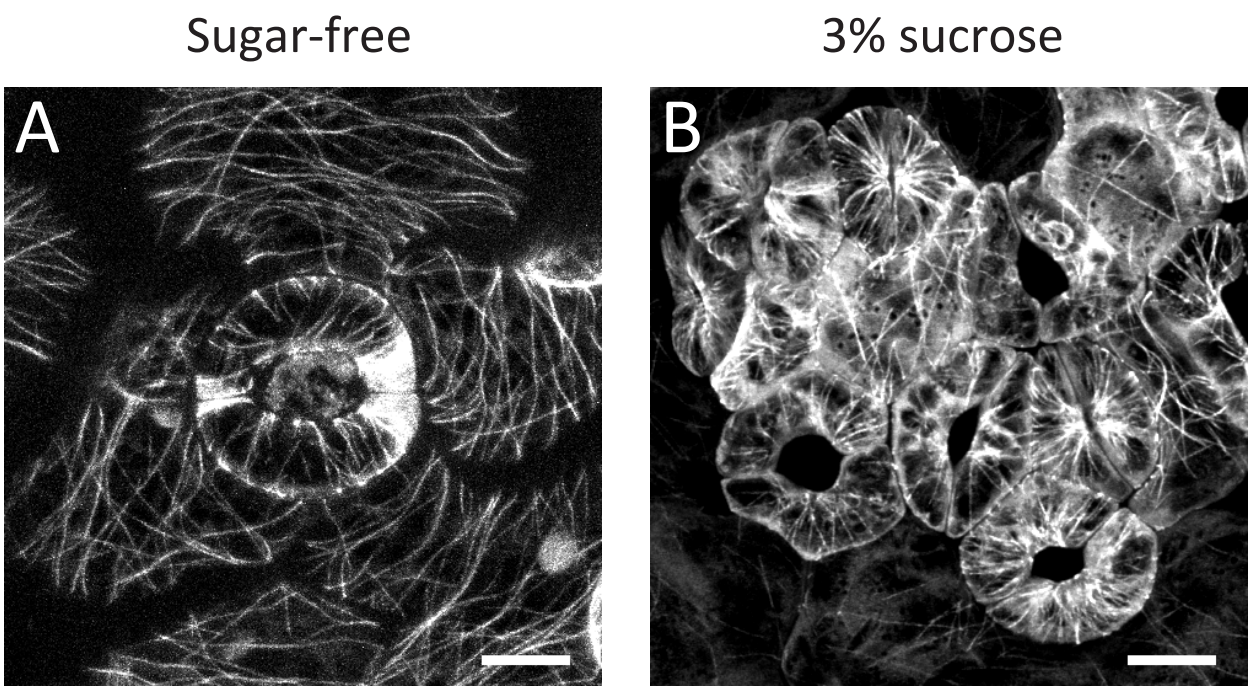

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
24-well plate	Sumitomo Bakelite	MS-0824R	
488 nm laser	Furukawa Denko	HPU-50101-PFS2	
488 nm laser	Olympus	Sapphire488-20/O	
510 nm long-pass filter	Olympus	BA510IF	
524 - 546 nm band-pass filter	Semrock	FF01-535/22-25	
530 nm short-pass filter	Olympus	BA530RIF	
561 nm laser	CVI Melles Griot	85-YCA-025-040	
604 - 644 nm band-pass filter	Semrock	FF01-624/40-25	
Confocal laser scanning head	Yokogawa	CSU10	
Confocal laser scanning head	Olympus	FV300	
Cooled CCD camera	Photometrics	CoolSNAP HQ2	
Image acquisition software	Molecular Devices	MetaMorph version 7.8.2.0	
Image acquisition software	Olympus	FLUOVIEW v5.0	
Immersion oil	Olympus	Immersion Oil Type-F	ne = 1.518 (23 degrees)
Inverted microscope	Olympus	IX-70	
Inverted microscope	Olympus	IX-71	
Murashige and Skoog Plant Salt Mixture	FUJIFILM Wako Pure Chemical Corporation	392-00591	Murashige T and Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15(3), 473-497.

Objective lens	Olympus	UPlanApo 100x / 1.35 NA Oil Iris 1.35	NA = 1.35
Objective lens	Olympus	UPlanAPO 40x / 0.85 NA	NA = 0.85
Sucrose	FUJIFILM Wako Pure Chemical Corporation	196-00015	

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Date:	2018/08/16

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Rebuttal Letter
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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:
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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).