

# Perforated-tape Epidermal Detachment (PED): A simple and rapid method for isolating epidermal peels from specific areas of *Arabidopsis* leaves

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**Abstract** Leaf epidermal stomata play pivotal roles in gas exchange and transpiration in higher plants. The regulatory mechanisms of the opening and closing of stomata (stomatal movement) and stomatal morphogenesis have been intensively studied. Such studies require the collection of detached epidermal cell layers (“peels”) for the detailed observation of stomata under the microscope. However, there are drawbacks to the conventional techniques for preparing and handling epidermal peels, i.e., a certain level of skill or an apparatus (e.g., a Waring blender) is required. In this report, we present a simple and rapid method for preparing epidermal peels called **Perforated-tape Epidermal Detachment (PED)**. For PED, Time Tape is adhered to the adaxial epidermis of an *Arabidopsis* leaf, and the abaxial epidermal layer is detached using Scotch Tape perforated with a small hole. The area inside the hole is suitable for microscopic observation because the Scotch Tape does not mask the detached epidermal layer there. PED can also be used to prepare epidermal peels from tobacco and legume leaves. The crucial advantage of PED over the conventional blender method is that the epidermal cells prepared by PED are rarely damaged, and we demonstrate that PED can be used to obtain material for physiological assays of stomatal responses to blue light and externally applied ABA. We thus believe that the PED method is suitable for preparing epidermal cell layers for physiological studies.

**Key words:** *Arabidopsis thaliana*, epidermal layer, stomata, guard cell, perforated tape.

Epidermal stomata are essential organs for gas exchange and transpiration between the plant interior and the environment. Stomata are able to open/close in response to external factors, including light intensity, CO<sub>2</sub> concentration and relative humidity (Raschke 1979; Sharkey and Raschke 1981), and exhaustive studies have been performed to better understand the mechanisms that regulate such stomatal movements. One such study found that blue light-dependent stomatal opening is controlled by the blue light photoreceptors phototropin 1 and 2 (phot1 and phot2, respectively) (Kinoshita and Shimazaki 1999; Kinoshita et al. 2001). Stomatal guard cells also respond to phytohormones such as abscisic acid (ABA) (Finkelstein et al. 2002; Leung and Giraudat 1998). Several different methods have been applied to indirectly assess the stomatal pore apertures in leaves, for instance by measuring the transpiration rate with a porometer or detecting the leaf surface temperature by thermography (Jones 1999). Although these techniques are powerful and convenient, microscopic observation of the leaf epidermis with direct quantification of the degree of stomatal aperture is indispensable for detailed studies.

However, one of the technical drawbacks of microscopic observation is that it requires preparation of epidermal cell layers, which must be performed with certain skills or equipment. For instance, leaves are briefly blended using a commercial Waring blender, and the fragmented leaf epidermis is collected using a nylon mesh (Pei et al. 1997). In this procedure, the epidermal peels are derived from both the abaxial and adaxial sides of leaves, and thus researchers need to distinguish between them for the measurement of the stomatal aperture under the microscope (see main text for more details). Young et al. reported an improved method that uses medical adhesive to attach the abaxial epidermis to a coverslip (Young et al. 2006). This method requires a certain level of skill to remove the upper cell layers with a razor blade, and the medical adhesive directly contacts the epidermal layer. To avoid these technical disadvantages, we developed an easy and efficient method for isolating *Arabidopsis* leaf epidermal peels called Perforated-tape Epidermal Detachment (PED), a technique that was inspired by the “Tape-*Arabidopsis* Sandwich” method reported by Wu et al. (2009).

## Materials and methods

### Plant material and growth conditions

The *Arabidopsis thaliana* strains used here were all derived from the wild type of ecotype Columbia. For examining light-induced stomatal opening, a blue light-insensitive *phot1 phot2* double mutant (*phot1/2*) and its parental strain (*glabra 1: gl1*) were used, and for examining ABA-induced inhibition of stomatal opening, *conditional chlorina: cch* (a Mg-chelatase H subunit mutant that is an ABA-insensitive mutant of guard cells) (Mochizuki et al. 2001; Shen et al. 2006) was used. The plants were grown on vermiculite watered with 1,000-fold-diluted Hyponex under a 16-h fluorescent light ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ )/8-h dark cycle at 23°C. The plants were grown in a plastic container with a transparent plastic lid to maintain the humidity (approximately 85% RH). *Nicotiana tabacum* was grown on rockwool under continuous light conditions. *Pisum sativum*, *Vicia faba* and *Panicum virgatum* were grown on soil in a greenhouse. *Oryza sativa* and *Zea mays* were grown in a test field at Kyoto University.

### Preparation of epidermal peels

For the experiments, fully expanded rosette leaves were harvested from 4- to 6-week-old *Arabidopsis* plants. The epidermal cell layer was prepared in two ways. For the conventional blender method, approximately 5 leaves were fractured in cold water using a Waring commercial blender (model 7011S with MC1 mini-container, FMI, Tokyo, Japan)

and then filtered through nylon mesh (N-No. 305T,  $48 \mu\text{m}$ , NBC, Tokyo, Japan) to collect the fragmented tissues, as previously described (Allen et al. 1999; Doi and Shimazaki 2008; Ichida et al. 1997). For PED, Time Tape (Hirasawa, Tokyo, Japan) was used to hold the adaxial surface of the leaf, and Scotch Magic Tape (3M, Tokyo, Japan; referred to as Scotch Tape here) perforated with a hole (see below) was used to detach the abaxial epidermis of *Arabidopsis* and young tobacco leaves. Scotch Tape was used unless otherwise noted, and Blue tape (SLIONTEC, Kanagawa, Japan), Time Tape, or duct tape was used for difficult samples, such as mature tobacco and legume leaves. The perforated tape was prepared as follows. In the first step, Scotch Tape was attached to a plastic frame (e.g., exposed X-ray film) with an opening of approximately  $1.5 \text{ cm} \times 5 \text{ cm}$ , and the tape was punched at regular intervals using a Biopsy Punch (Kai Industries, Gifu, Japan) and then cut into strips (approx.  $1.5 \text{ cm} \times 4 \text{ mm}$ ) containing a single hole (Figure 1, Supplemental Figure 1). One of the most important aspects of the preparation is that the adhesive surface of the tape (particularly around the hole) must be left untouched during all the steps of this procedure. Next, the adaxial side of a dark-adapted leaf was fixed on a strip of Time Tape, and the perforated Scotch Tape was gently attached to the lower epidermal surface (Figure 1). The Scotch Tape was then carefully pulled away from the leaf surface, with the lower epidermal cell layer within the hole becoming detached together with the peripheral layer that was firmly affixed to the Scotch Tape (see Supplemental Movie). The epidermal

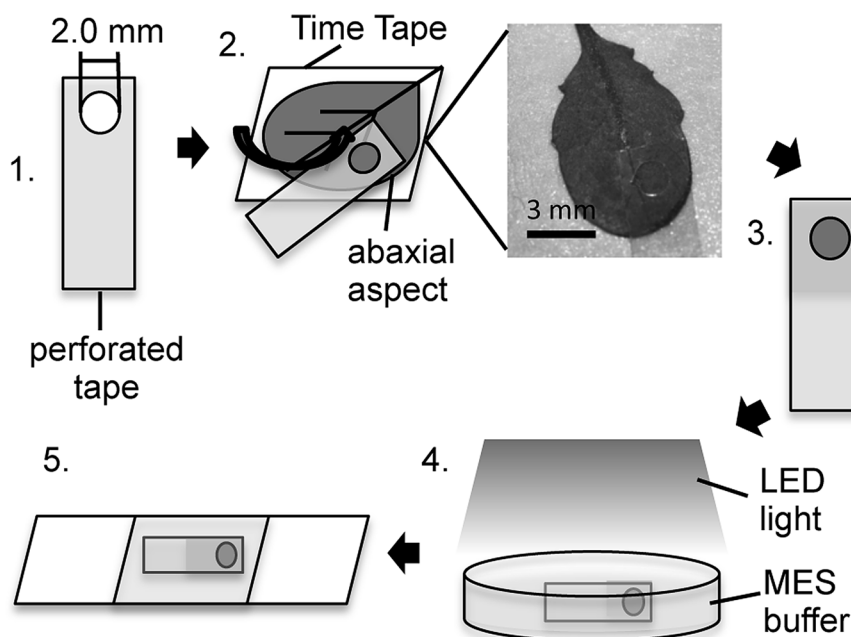


Figure 1. Schematic protocol of the PED method for epidermal tissue isolation. 1. Perforate the tape using a Biopsy Punch. 2. Affix the adaxial epidermal surface to a strip of Time Tape, and adhere the perforated tape to the abaxial epidermal surface. Then carefully pull the perforated tape away. 3. The abaxial epidermal tissue surrounded by the edge of the hole is thereby detached together with the rest of the abaxial epidermal tissue that is affixed to the perforated tape. (The following is the protocol for stomatal aperture measurement.) 4. Soak the tape-affixed abaxial epidermis in MES buffer (5 mM MES-BTP [pH 6.5], 50 mM KCl and 0.1 mM  $\text{CaCl}_2$ ) and irradiate with an LED for 2.5 h. 5. Place the tape-affixed abaxial epidermis on a glass slide and observe the stomata within the perforated area using a microscope.

peel affixed to the tape was immediately submerged in an appropriate buffer or solution for the subsequent experiments.

### Measurement of stomatal aperture

Stomatal apertures were measured according to Inoue et al. (2008), with some modifications. The leaves were soaked in 3 ml of 1/2 MS buffer (a half-strength solution of Murashige and Skoog Plant Salt Mixture [Wako, Tokyo, Japan]) and incubated in the dark for 16 h at 23°C to cause closing of their stomata (dark treatment). The epidermal layers were prepared by PED or the blender method, as described above, and the layers were maintained in 3 ml of basal reaction buffer (5 mM MES-BTP (MES, 2-(*N*-morpholino)ethanesulfonic acid, and BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane), pH 6.5, 50 μM KCl and 0.1 mM CaCl<sub>2</sub>). The samples were irradiated with red light at 50 μmol m<sup>-2</sup> s<sup>-1</sup> (Red), or blue light at 10 μmol m<sup>-2</sup> s<sup>-1</sup> superimposed on a red background (Blue) or kept in the dark for 2.5 h at 23°C in the presence or absence of 20 μM ABA. The images of the epidermal peels were captured with a microscope (BX51, Olympus, Tokyo, Japan) that was equipped with a digital camera (C11440, HAMAMATSU, Shizuoka, Japan). The images were analyzed with ImageJ (ver. 1.42q, National Institutes of Health, USA) software to obtain the stomatal aperture data. Only visibly intact guard cells were subjected to measurement of the stomatal aperture. For each strain, the size of the stomatal aperture is expressed as the mean of 25 stomata with the standard deviation (SD).

### Observation of GFP fluorescence

The fifth or sixth rosette leaf of *Arabidopsis* transgenic lines expressing either plastid-localized GFP (CT-GFP, Köhler et al. 1997) or cytosol-localized GFP ("GFP", Mano et al. 2002) was used to observe GFP fluorescence in epidermal layers prepared by the PED method. Fluorescence from GFP-tagged proteins was observed under a confocal laser-scanning microscope (FV300+BX60; Olympus, Tokyo, Japan) with a band-pass filter (510–530 nm).

### Evans Blue staining

Evans Blue (Fluka, France) staining, which stains dead cells, was performed to assess the viability of the epidermal cell layers (Baker and Mock 1994). The epidermal layers were incubated with 0.25% Evans Blue (diluted in MES buffer) for 20 min at 23°C. The tissues were rinsed with sterile water until no further blue dye eluted from the samples, and the images were captured with a microscope (BX51, Olympus, Tokyo, Japan) that was equipped with a digital camera (DFC300FX, Leica).

## Results and discussion

### PED: a simple method to prepare epidermal peels

We used a piece of Scotch Tape perforated with a single hole (approx. 2 mm in diameter) to detach the epidermal tissue layer from an *Arabidopsis* rosette leaf. Using PED, we could prepare an epidermal peel of approximately

3 mm<sup>2</sup>, an area that contained approximately 100 stomata (Figure 2D). In contrast, the epidermal peels prepared using the blender method were fragmented into small pieces (approximately 0.25–0.3 mm<sup>2</sup>, 5–15 stomata per piece), and thus several pieces were necessary for adequate microscopic observation (Figure 2A). The epidermal layer prepared by PED is held in place by tape such that the epidermis never bends over itself and can be handled quite easily. This method allows the observer to distinguish the side of the epidermal layer, which is necessary for some experimentation, such as

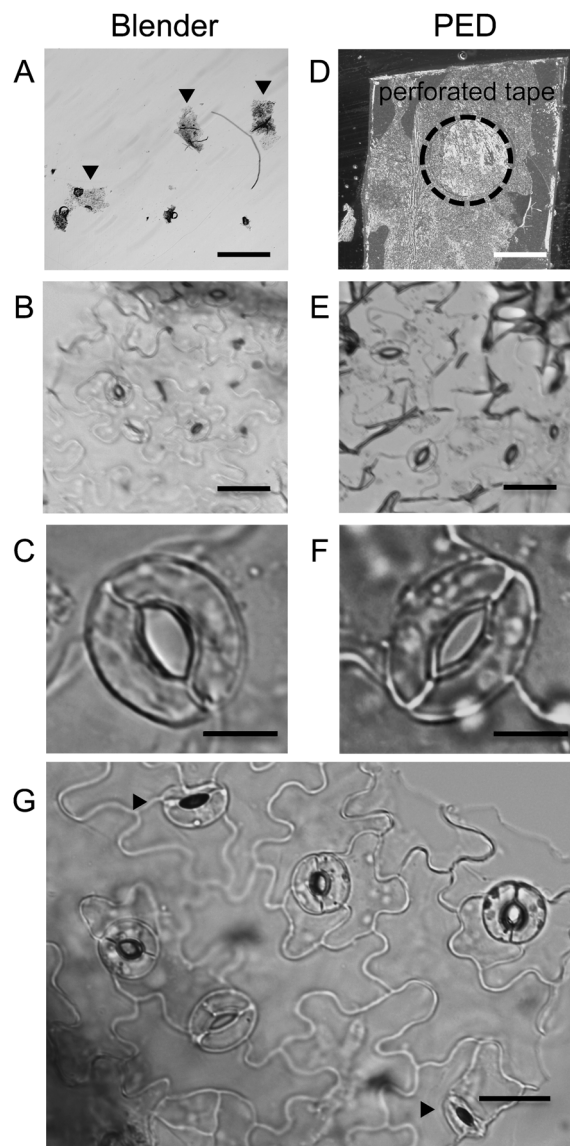


Figure 2. Comparison of epidermal layers and stomatal images obtained using two different methods. Microscopic images of the epidermal cell layers and stomata prepared with the blender method (A–C and G) and the PED method (D–F). (A) The epidermal fragments prepared using the blender method are indicated by arrowheads. (D) The epidermal layer area in the perforated area of the tape obtained using the PED method is indicated by a dashed circle. (G) The deflated stomatal guard cells are indicated by arrowheads. Bar = 1 mm (A and D), 100 μm (B and E), 10 μm (C and F), and 20 μm (G).

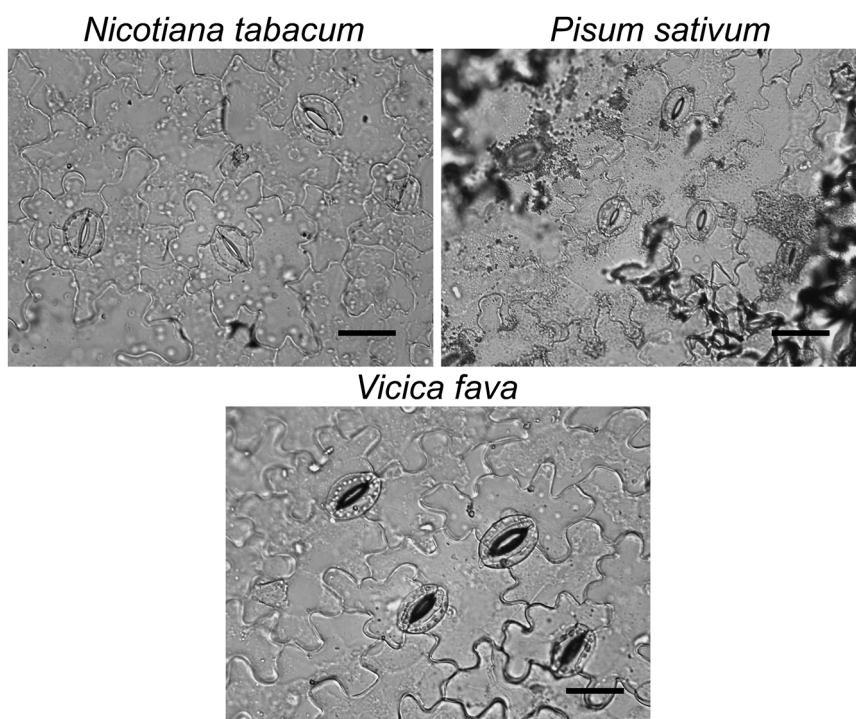


Figure 3. Application of PED to other plant species. DIC images of epidermal layers from *Nicotiana tabacum* (left), *Pisum sativum* (middle) and *Vicia faba* (right) are shown. The epidermal layers were detached with the PED method using perforated Scotch Tape (*N.t.*), Time Tape (*P.s.*) or duct tape (*V.f.*). The bar indicates 50  $\mu\text{m}$ .

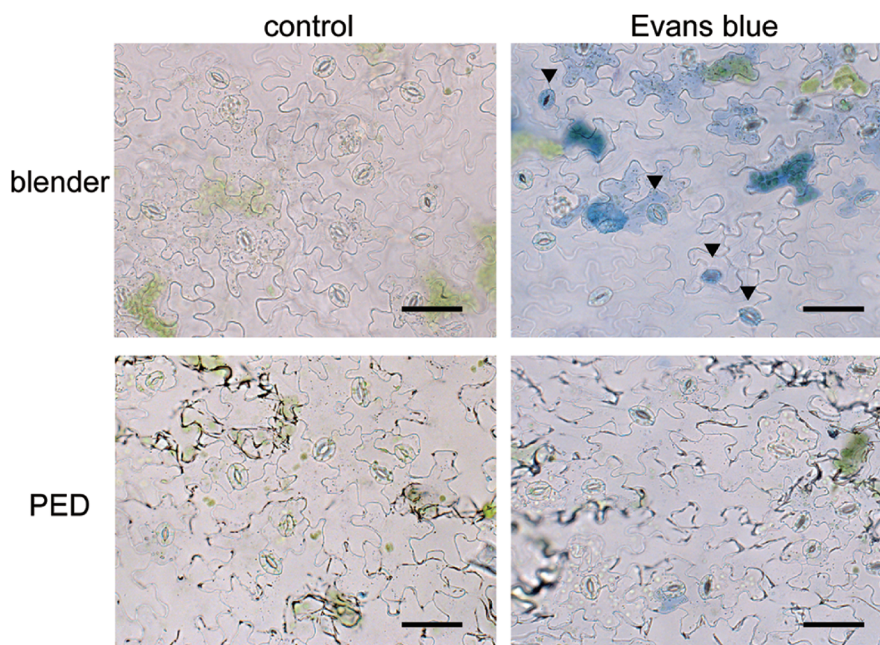


Figure 4. Comparison of cell viability between the epidermal cell layers prepared by the PED and blender methods. The epidermal cell layers were isolated from 4-week-old rosette leaves of Columbia (WT) grown at 23°C under continuous white light ( $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The layers were incubated with (right column) or without (left column) 0.25% Evans Blue in MES buffer and washed as described in Materials and methods. The arrowheads indicate dead guard cells stained with Evans Blue. The heavily stained cells with blurry shape observed in the sample obtained using the blender method are mesophyll cells that were detached along with the epidermal layer. The bar indicates 100  $\mu\text{m}$ .

photographing microscopic images. Another advantage is that one can obtain peels from a specific area of interest on the leaf. Additionally, epidermal peels can be either floated on the surface or submerged in buffer by adhering

the tape-peel to the bottom of a container, such as a Petri dish. This is particularly convenient when the response of the same peel to successive treatments (e.g., applications of different light qualities or different chemicals) is

observed. We also emphasize that this method does not require any special equipment or skills, and it enables one to prepare epidermal peels without laborious procedures.

We compared microscopic images of epidermal peels that were prepared using the blender method and PED and found that clear images of the stomatal guard cells and the pore as well as pavement cells could be observed in both cases (Figure 2B, C, E, F). However, it should be noted that there were a substantial number of deflated guard cells in the blender samples (Figure 2G). We describe this point in greater detail in the next section. In addition, because epidermal peels that were prepared using the blender method were fragmented into small pieces and dispersed among other types of tissues, it was laborious to identify peels suitable for observation (Figure 2A). As shown in Figure 3, we also successfully applied the PED method to *Nicotiana tabacum*, *Pisum sativum* and *Vicia faba*, though it was necessary to use perforated tape with stronger adhesiveness, such as Time Tape or duct tape, to detach the epidermal layers from these leaves, which have a stronger tissue architecture or denser trichomes than *Arabidopsis*. We were, however, unable to prepare a substantial quantity or good quality of epidermal peels from *Panicum virgatum* (switch grass), *Oryza sativa* or *Zea mays* using PED. These grass species have tougher leaf tissue architecture (stronger conjunction of epidermal and mesophyll layers) and an uneven surface structure (striped vascular patterns) and wax, which prevent the effective adhesion of the tape to the epidermal layer. Using material obtained by PED, we were able to observe GFP-tagged proteins expressed in different organelles (plastids and cytoplasm in guard cells and cytoplasm in pavement cells) (Supplemental Figure 2). Furthermore, the epidermal cell layers prepared by PED can be used for physiological experiments, as described in the following sections.

#### **PED is a non-damaging method for isolating epidermal peels**

Epidermal tissues prepared using the blender method often contain deflated guard cells, whereas such cells were rarely found in our PED samples. Indeed, it has previously been reported that, typically, approximately 25% of the guard cells in epidermal tissues isolated by the blender method are dead (Hayashi et al. 2011). Therefore, we compared the epidermal cell viability between the blender and PED methods. The epidermal cells were treated with Evans Blue, by which dead cells are stained blue in color. As shown in Figure 4, the blender-treated epidermal tissue contained stained guard cells (approx. 7.8% dark-stained cells among 900 cells, Supplemental Table 1) and pavement cells, which is consistent with the above-mentioned report (Hayashi et al. 2011). In contrast, dark-stained guard cells and pavement cells were seldom found, and only a few light-stained cells

were observed, in the PED preparations (Figure 4). These data strongly suggest that PED is a non-damaging method suitable for physiological studies.

#### **PED can be used for studying stomatal responses to light and phytohormones**

To examine whether this method could be used for physiological experiments, we compared blue light-

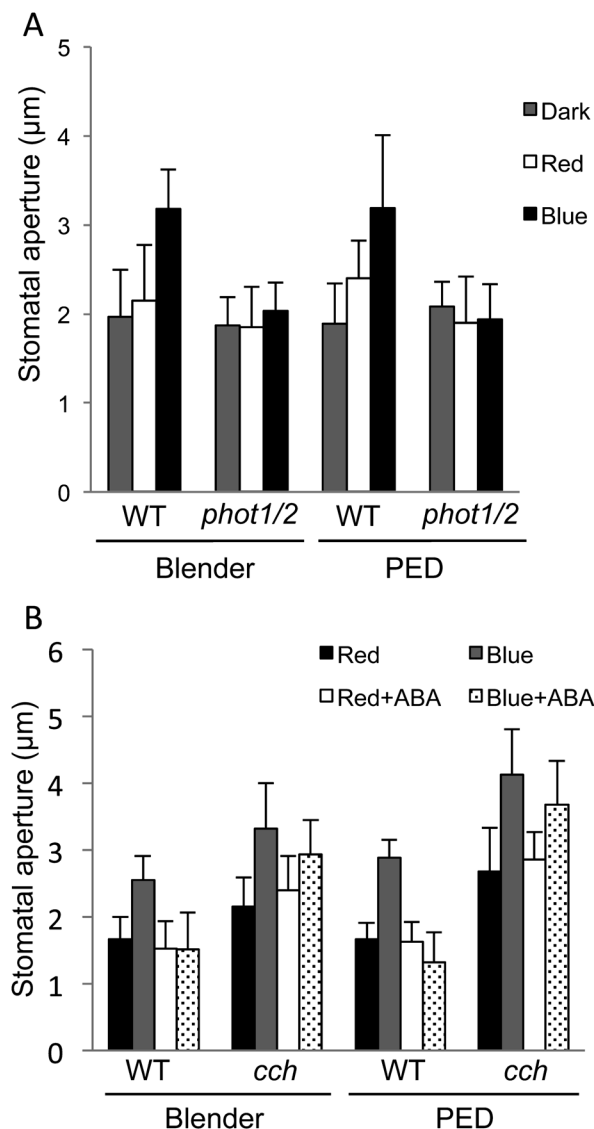


Figure 5. Comparison of blue light-dependent stomatal opening and the inhibitory effect of ABA on light-induced stomatal opening between the PED and blender samples. (A) *gli* (WT) and *phot1/2* leaves were incubated under the following light conditions for 2.5 h: continuous dark (Dark), red light at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Red) or blue light at  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  with background red light at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Blue). The data are shown as the mean  $\pm$  SD ( $n=25$ ). (B) Columbia (WT) and *cch* (Mg-chelatase H subunit mutant) were tested for ABA-responsiveness of stomatal opening. The leaves were incubated under the following conditions for 2.5 h: red light at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Red) or blue light at  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  with background red light at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$  (Blue) with or without  $20 \mu\text{M}$  ABA (+ABA). The data are presented as the mean  $\pm$  SD ( $n=25$ ).

dependent stomatal opening responses in epidermal peels that were prepared with the PED and blender methods. Epidermal peels prepared from wild type (*g11*) and *phot1/2* were used in this analysis (Figure 5A). The rosette leaves of 4-week-old plants were adapted to the dark for 16 h and then illuminated with red (red light at  $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) or blue (blue light at  $10 \mu\text{mol m}^{-2} \text{s}^{-1}$  superimposed on a red background) light for 2.5 h. For wild-type specimens, the stomatal apertures from the dark-adapted samples were equivalent (approx.  $2 \mu\text{m}$ ) for the PED and blender methods, with both showing a comparable aperture increase in response to blue light. Using the PED method, we could also reproduce the previously reported finding that *phot1/2* is defective in stomatal opening in response to blue light (Figure 5A).

As another example of PED application, we tested epidermal peels that were prepared by PED for sensitivity to exogenously applied ABA, a hormone that inhibits light-induced stomatal opening and induces stomatal closure. Dark-adapted leaves were incubated with or without  $20 \mu\text{M}$  ABA under red or blue light for 2.5 h as described in Materials and methods. As expected, blue light-dependent stomatal opening was greatly inhibited by ABA in the wild type (Columbia) (Figure 5B). In contrast, the stomata of the ABA-insensitive *cch* mutant showed no significant response to ABA ( $p > 0.05$ , Student's *t*-test), as previously reported (Figure 5B) (Shen et al. 2006; Tsuzuki et al. 2011). These results clearly suggest that PED can be used to prepare epidermal peels for physiological experiments.

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**Supplemental Table 1 Comparison of cell viability between the epidermal cell layers prepared by the PED and blender methods.** Four-week-old rosette leaves of Columbia (WT) were used. Epidermal peels were prepared by the PED or the blender method and stained with Evans Blue as described in Materials and Methods. Dark-stained guard cells were judged to be “dead cells”. Experiments were repeated three times (#1 - #3), and 300 stomatal guard cells were observed in each test.

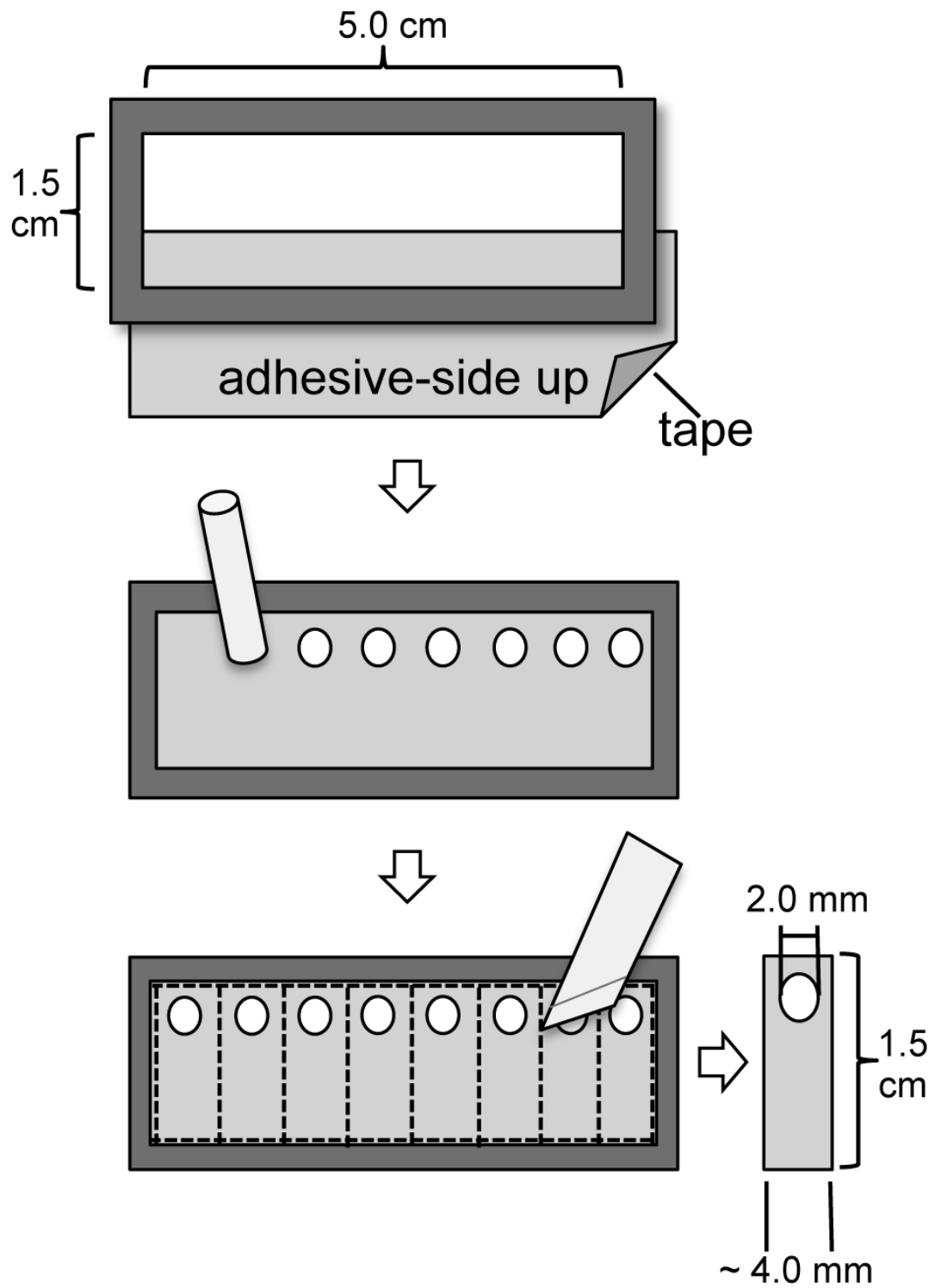
**Supplemental figure 1 Procedure to prepare the perforated tape for PED method.** Scotch Tape (3M, Tokyo) or Blue tape (SLIONTEC, Kanagawa, Japan) (pale blue square) was applied to a plastic frame (e.g. used X-ray film), and perforated with holes using a Biopsy Punch (Kai Medical, 2 mm in diameter). Then the tape was cut to make perforated strips as shown in the figure. It is necessary to keep a sufficient area of tape around the hole for successful detachment of the epidermis. The adhesive side of the tape must be kept facing upward, and contact with it must be avoided throughout the process described above.

**Supplemental Figure 2 Confocal microscope images of GFP-tagged proteins in epidermal peels prepared by PED method.** The epidermal cell layers were isolated from 2-week-old rosette leaves of transgenic *Arabidopsis* lines expressing GFP-tagged proteins by the PED method. GFP fluorescence and DIC images of CT-GFP (chloroplasts, left) and “GPF” (cytoplasm, right) lines, respectively, are shown. The bar indicates 50  $\mu\text{m}$ .

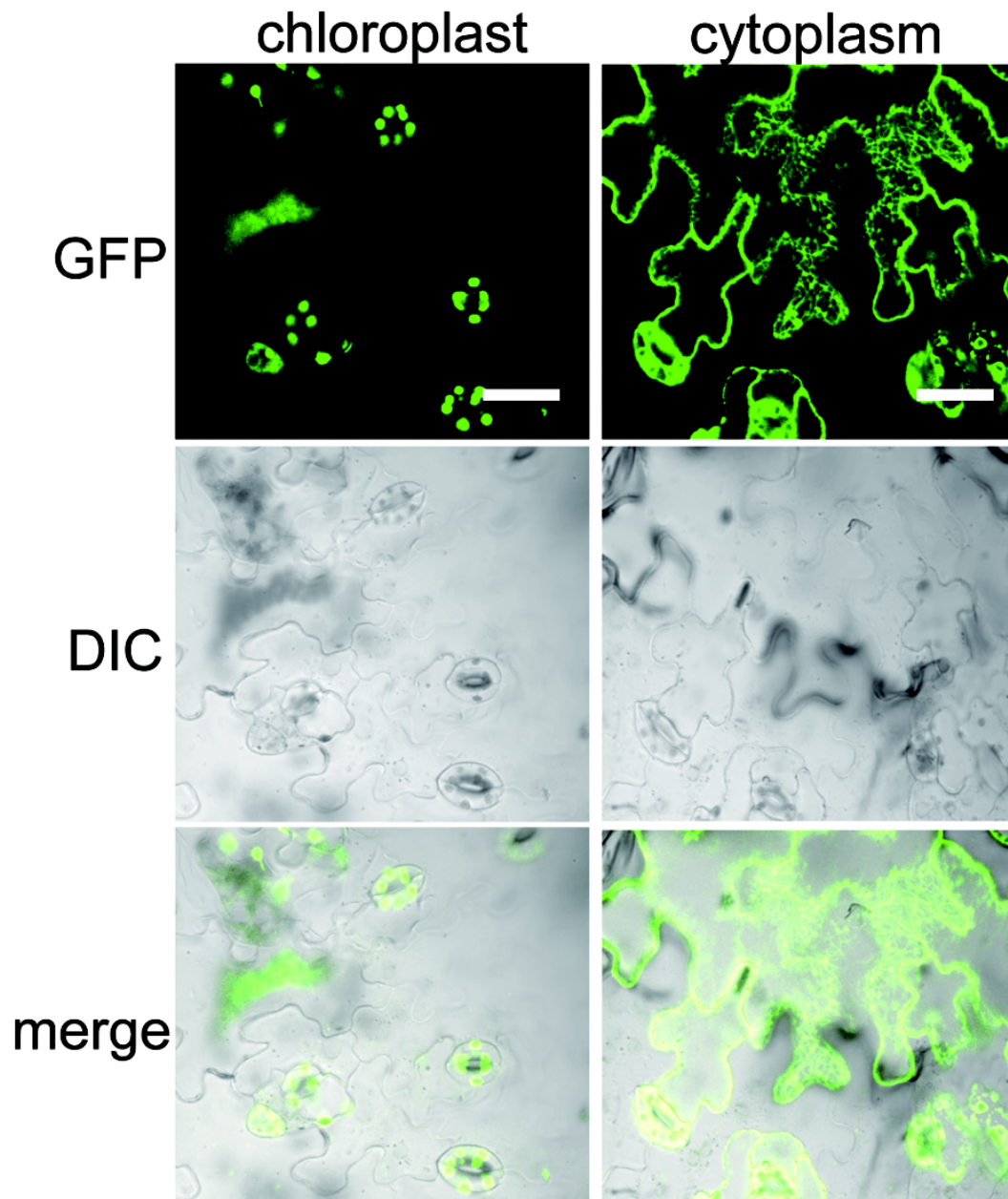
experiment	dark-stained guard cells (n = 300)		cell death rate (%)	
	PED	Blender	PED	Blender
#1	0	21	0	7
#2	0	23	0	7.7
#3	0	26	0	8.7

Supplemental Table 1 Ibata et al.





Supplemental Figure 1 Ibata et al.



Supplemental Figure 2 Ibata et al.

**Supplemental Movie.** Procedure to prepare epidermal peels by the PED method. The movie shows the procedure from step 2 to 4 described in Figure 1.

