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Analysis of Guard Cell Readouts Using *Arabidopsis thaliana* Isolated Epidermal Peels

Rosario Pantaleno, Paula Schiel, Carlos García-Mata and Denise Scuffi*

Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Consejo Nacional de Investigaciones Científicas y Técnicas (IIB-UNMdP-CONICET), Mar del Plata, Argentina
*For correspondence: dscuffi@mdp.edu.ar

Abstract

Stomata are pores surrounded by a pair of specialized cells, called guard cells, that play a central role in plant physiology through the regulation of gas exchange between plants and the environment. Guard cells have features like cell-autonomous responses and easily measurable readouts that have turned them into a model system to study signal transduction mechanisms in plants. Here, we provide a detailed protocol to analyze different physiological responses specifically in guard cells. We describe, in detail, the steps and conditions to isolate epidermal peels with tweezers and to analyze i) stomatal aperture in response to different stimuli, ii) cytosolic parameters such as hydrogen peroxide (H_2O_2), glutathione redox potential (E_{GSH}), and $MgATP^{-2}$ in vivo dynamics using fluorescent biosensors, and iii) gene expression in guard cell–enriched samples. The importance of this protocol lies in the fact that most living cells on epidermal peels are guard cells, enabling the preparation of guard cell–enriched samples.

Key features

- Isolation of epidermal peels as a monolayer enriched in guard cells.
- Measurement of cytosolic guard cell signaling component dynamics in isolated epidermal peels through fluorescent biosensor analysis.
- Gene expression analysis of guard cell–enriched isolated tissue.

Keywords: Guard cells, Epidermal peels, Stomatal aperture, Biosensors, RNA, *Arabidopsis thaliana*

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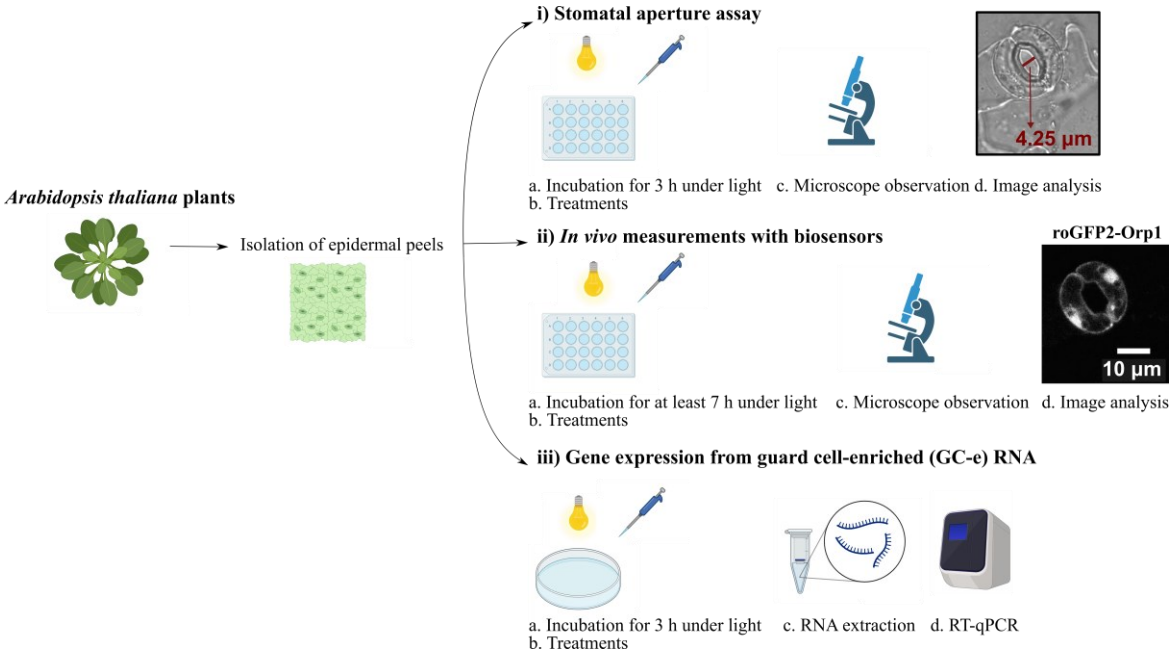
Graphical overview



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Background

Stomata are microscopic pores located in the epidermis of aerial tissues of most land plants, delimited by specialized cells known as guard cells. Given that the plant epidermis is covered by the cuticle, an external impermeable layer, almost 95% of gas exchange occurs through stomatal pores. Thus, the regulation of the stomatal pore area controls the uptake and release of CO_2 and O_2 and the maintenance of hydric homeostasis by the regulation of H_2O vapor loss, through the transpiration stream [1].

Guard cells continuously sense internal and external cues and integrate them into a complex signaling network that produces modifications in guard cell volume to control the stomatal pore width [2,3]. The isolation of epidermal peels, cellular monolayers mainly formed by functional guard cells, has become a widely used and validated experimental system to study signaling mechanisms in plants [4].

Several protocols have been developed in order to study guard cell physiology [5–7]. Although all of them converge in the isolation of epidermal peels, there are different approaches including the use of adhesive tape [8–10], resin imprints where leaf surface is copied [11–13], blender and filters [14–16], tape and razor blade [17–19], and striping with fine-tip tweezers [20–22].

For the last twenty years, our research group has reproducibly employed fine-tip tweezers to prepare epidermal strips, where almost 90% of living epidermal cells are guard cells (Figure 1A and B) [20,23–27]. Isolation of epidermal peels allowed us to perform stomatal aperture assays and in vivo measurements of endogenously encoded fluorescent proteins (biosensors) that specifically detect hydrogen peroxide (H_2O_2,), the glutathione redox potential (E_{GSH}), and the biologically relevant portion of adenosine 5'-triphosphate ($MgATP^{2-}$) [25,27]. In addition, the methodological procedure presented here has been employed to obtain guard cells–enriched RNA (Figure 1B) to analyze transcript levels of different target genes [23,26]. Although peeling produces mechanical stress, evidenced by an increase in reactive oxygen species (ROS) in guard cells, we set up the conditions to recover the strips to basal redox status levels without affecting guard cells' viability or their physiological responses to different signals [27].

In this protocol, we describe how to prepare isolated epidermal peels excised from the abaxial side of *Arabidopsis*

thaliana leaves and how to perform different experiments using this biological system to study real-time and single-cell physiological processes.

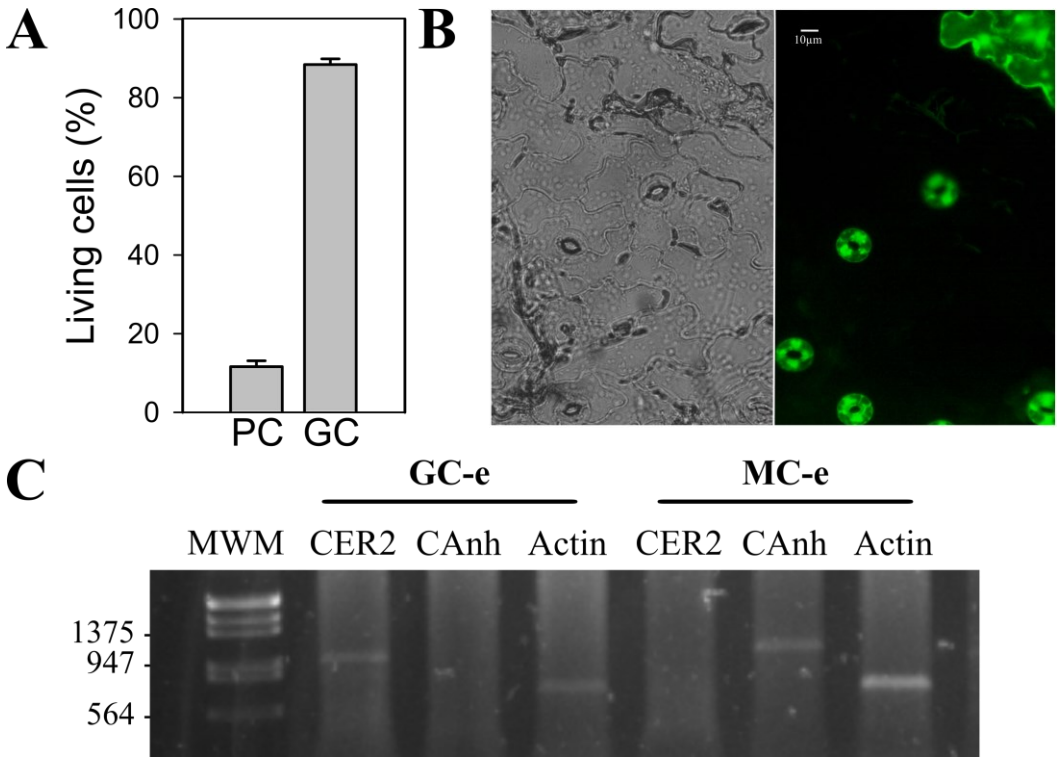

Figure 1. Living cells in epidermal peels. A. *Arabidopsis thaliana* wild-type ecotype Col-0 epidermal peels were incubated in opening buffer (OB, 5 mM MES pH6.1, 50 mM KCl) under light for 30 min and then loaded with 5 µM of the viability fluorescent dye fluoresce in diacetate (FDA) for 5 min. Peels were washed three times with OB and then mounted on a coverslip, and analyzed by epifluorescence microscopy. Fluorescent cells were quantified, and the total cell/fluorescent cell percentage was calculated for each cell type. PC: pavement cells, GC: guard cells. B. Representative image of an epidermal peel stained with FDA taken with a 40 × objective. Scale bar: 10 µm. C-RT-PCR analysis using guard cell–specific gene ECIREFERUM2 (*CER2*) (930 bp) and mesophyll cell–specific gene carbonic anhydrase 1 (*Canh1*) (1,000 bp) was performed in RNA prepared from guard cell–enriched (GC-e) or mesophyll cell–enriched (MC-e) extracts. The amplification of *ACTIN* (651 bp) transcripts under identical conditions was used as a constitutive expression control. Data from Scuffi et al. [26].

Materials and reagents

Biological materials

4–6 weeks-old wild-type *Arabidopsis thaliana* (ecotype Col-0) and transgenic *Arabidopsis thaliana* lines expressing the following biosensors: roGFP2-Orp1 [28], Grx1-roGFP2 [29], and ATeam1.03-nD/nA [30]. See Table 1 to find detailed information regarding the fluorescent biosensor features.

Table 1. Biosensor characteristics

Biosensor name	roGFP2-Orp1		Grx1-roGFP2		ATeam 1.03-nD/nA	
Parameter detected	H_2O_2		E_{GSH}		$MgATP^{2-}$	
Fluorescent protein	EGFP		EGFP		mse CFP, cp173-mVenus	
Promoter	35S (CaMV)		UBQ10 (from Arabidopsis)		35S (CaMV)	
Plasmid	pH2GW7		pBinCM		pH2GW7	
Selection in plants	Hygromycin		Kanamycin		Hygromycin	
Rationing principle	Dual excitation, Single emission		Dual excitation, Single emission		Single excitation, Dual emission (FRET)	
Emission maximum	511 nm		511 nm		475 nm (mseCFP), 527 nm (cp173-mVenus)	
Microscopy type	**Epifluorescence**	**CLSM**	**Epifluorescence**	**CLSM**	**Epifluorescence**	**CLSM**
Excitation wavelength/filter	450–490 nm, 385–425 nm	405 nm, 488 nm	450–490 nm, 385–425 nm	405 nm, 488 nm	426–446 nm	458 nm
Emission range	505–530nm	505–530 nm(*)	505–530 nm	505–530 nm(*)	467–499 nm, 528.5–555.5 nm(**)	465–500 nm, 526–561 nm(*)
References	(15)	(14), (22)	(15)	(11): (22)	(15)	(3)

(*) A 650–695 nm filter can be used for chlorophyll fluorescence collection.
(**) Use a dichroic 510–440 nm mirror for simultaneous acquisitions.
CSLM: Confocal scanning laser microscopy.

Reagents

1. Soil (Antoniucci, https://viveroantoniucci.mitiendanube.com/productos/tierra-negra/)
2. Vermiculite (Terrafertil, https://www.terrafertil.com/productos_jardin/acondicionadores_vermiculita.html)
3. Perlite (Terrafertil, https://www.terrafertil.com/productos_jardin/acondicionadores_vermiculita.html)
4. 2-(N-Morpholino) ethane sulfonic acid (MES) (Sigma, CAS: 4432-31-9)
5. Potassium chloride (KCl) (Sigma, CAS: 7447-40-7)
6. Potassium hydroxide (KOH) (Sigma, CAS: 1310-58-3)
7. Trizol (Invitrogen, catalognumber: 15596026)

Solutions

1. Opening buffer (see Recipes)

Recipes

1. Opening buffer

Reagent stock	Final concentration	Amount
MES (50 mM, pH 6.1)*	5 mM	10 mL
KCl (1M)	50 mM	5 mL
H_2O	n/a	85 mL
Total	n/a	100 mL

(*) MES is adjusted to pH with KOH

Laboratory supplies

1. Precision tweezers, Dumont #5 (Fine Science Tool, catalog number: 11251-10)
2. Scalpel n° 4 (KLS Martin, catalog number: 10-100-04)
3. 1 mL syringe with needle with the tip bent, n° 27 g (Bremen Seiseme, catalog number: 7791914003029)
4. Pipette tips [Deltalab, catalog number: 327-34 (2-200 µL), 200070 (1,000 µL)]
5. Pipettes [Gilson, Pipetman, catalog number: F144056M (P20), F144058M (P200), F144059M (P1000)]
6. 24-well plate (BIOFIL, catalog number: TCP001024)
7. Petri dish 55 × 14 mm (Deltalab, catalog number: 200201)
8. 1.5 mL tubes (Deltalab, catalog number: 200400)
9. Microscope slides (Deltalab, catalog number: D100010)
10. Coverslips 18 × 18 mm nº 1 (Deltalab, catalog number: D101818)
11. Neubauer chamber (BOECO Germany, catalog number: BOE01)
12. Mortar (Günther Argentina, catalog number: 14660) and pestle (Günther Argentina, catalog number: 154200)
13. Liquid nitrogen

Equipment

1. Brightfield microscope (Olympus, model: CKX53) coupled to a digital camera (AmScope, model: MU1000) and 40 × objective (LUC Plan FLN, numerical aperture: 0.6)
2. Confocal laser scanning microscope (CLSM) (Carl Zeiss Microscopy, model: LSM980) with a 40 × water immersion objective (C-Apochromat, 1.2 numerical aperture)
3. Epifluorescence microscope (Nikon, model: Ti-E) coupled to a double digital camera (Hamamatsu, model: ORCA-D2 Dual CCD) and 60 × oil immersion objective (CFI Plan APO Lambda, 1.4 numerical aperture)

Software and datasets

1. NIS-Elements AR (https://www.microscope.healthcare.nikon.com/products/software/nis-elements/nis-elements-advanced-research)
2. Fiji analysis software (ImageJ), National Institutes of Health, Version 1.53t [31]

Procedure

Stratificate *Arabidopsis thaliana* seeds in water at 4 °C and in darkness for 48 h. Sow the seeds in a proportion of soil:vermiculite:perlite (3:1:1, v/v/v) and grow them for 4–5 weeks (for stomatal aperture and biosensor experiments) or 5–6 weeks (for RNA extraction) under short-day conditions (8/16 h light/dark photoperiod, 200 µmol photons $m^{-2}s^{-1}$).

A. Stomatal aperture

1. Before removing the epidermal peels, prepare the 24-well plate by adding 500 µL of opening buffer (OB) in as many wells as treatments to be performed, including the control. Then, pipette 50 µL of OB drops onto a clean flat surface, previously washed with deionized water (surface sterilization is not needed) (Figure 2A). Excise epidermal peels from the abaxial side of fully expanded leaves of *Arabidopsis thaliana* plants. Place the leaf on the index finger and hold it with the thumb and the middle finger. Detach the epidermis layer piercing the leaf with tweezers and slowly pull upwards at a 45° angle (Figure 2B).

2. Immediately after, place the peels outside up on the OB drops pipette in step A1 (Figure 2C) and remove the mesophyll tissue with a scalpel (Figure 2D).
   Note: An epidermal layer of approximately 4 mm^2 is enough to image stomata in different microscope fields.
3. Lift the epidermal peels with a 1 mL syringe with a needle bent (at an approximate 120° angle) (Figure 2E) and put them in each well of the plate prepared in step A1 (Figure 2F). Put about four peels per well to have enough tissue for documentation. Maintain the epidermal peels in OB under white light for 3 h to promote the maximal opening of most of the stomata.
4. Pipette the respective treatments into each well from a 100 × stock solution and incubate the peels for the desired time. Then, take the peels from each well and mount them in a microscope slide with an OB drop. Observe stomata in an optical brightfield microscope with a 40 × objective (LUC Plan FLN, numerical aperture: 0.6) (Figure 2G).
5. Take a photo of the 50 um^2 squares from a Neubauer chamber, or a calibrated slide, in each experiment to have a reference scale.

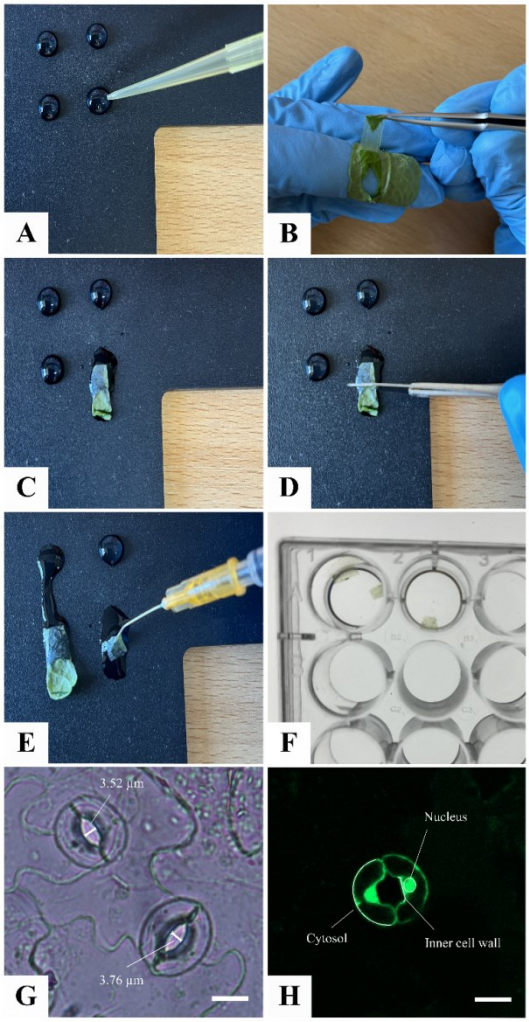

Figure 2. Epidermal peeling protocol. A. Opening buffer (OB) drops. B. Peeling with tweezers. C. Epidermal peel on an OB drop. D. Mesophyll dissection. E. Lifting peels with the needle. F. Incubation in a 24-well plate. G. Brightfield microscopic image of stomata, taken with a 40× objective, showing the stomatal aperture values measured as the maximal distance between the inner walls of guard cells. H. Fluorescence image of stomata expressing roGFP2-Orp1, taken with a 40× objective, where the nucleus, inner cell walls, and a portion of the cytosol from guard cells are indicated by arrows. Scale bars: 10 µm.

B. Biosensors

1. Excise epidermal peels with tweezers from the abaxial side of *Arabidopsis thaliana* leaves expressing the genetically encoded proteins roGFP2-Orp1, Grx1-roGFP2, or ATeam 1.03-nD/nA (as described in steps A1–A3).
2. Place epidermal peels in a 24-well plate containing OB under white light in the growing chamber for at least 7 h and up to a maximum of 12 h [27].
3. Add desired treatments to epidermal peels directly in the incubation well.
   Note: For the redox-sensitive roGFP2-Orp1 and Grx1-roGFP2 sensors, include 10 mM H_2O_2 and 20 mM DTT treatments for 10 min to fully oxidize or reduce the sensors [22,27]. On the other hand, to avoid the effects of active photosynthesis on the ATP levels detected, ATeam 1.03-nD/nA peels should be pre-incubated for at least 1 h in the dark, before treatment addition [30,32].
4. Image peels in a CLSM or an epifluorescence microscope with 40× (C-Apochromat, 1.2 numerical aperture) or 60× (CFI Plan APO Lambda, 1.4 numerical aperture) objectives, respectively, and take photos. For roGFP2-Orp1 and Grx1-roGFP2 imaging using a CLSM, excite samples sequentially at 488 nm and 408 nm (line-switching mode) and collect fluorescence at 505–530 nm. For epifluorescence microscopy, excite roGFP2-Orp1 and Grx1-roGFP2 sequentially with 450–490 nm and 385–425 nm and collect the emissions using a 505–530 nm band pass filter (GFP-specific filter). For ATeam 1.03-nD/nA imaging with CLSM, excite samples at 458 nm and collect the fluorescence at 465–500 nm (mseCFP) and 526–561 nm (cp173-mVenus). For epifluorescence microscopy, excite ATeam 1.03-nD/nA using a 426–446 nm filter and collect fluorescence with 467–499 nm (mseCFP) and 528.5–555.5 nm (cp173-mVenus) filter with a dichroic 510 nm mirror (Hamamatsu Photonics) for simultaneous acquisitions.
5. Analyze fluorescence intensity in the cytosol of guard cells. Define the region of interest (ROI) using Fiji software, as described in Data Analysis section, avoiding the cytosolic portion adjacent to the inner cell walls, since these structures usually emit significant autofluorescence (Figure 2H).

C. RNA

1. For guard cell–enriched (GC-e) RNA extraction, collect epidermal peels from the abaxial side of fully expanded *Arabidopsis* leaves (as described in step A3) and place them in an OB-containing Petri dish until the surface of the dish is fully covered with peels. Incubate the peels for 3 h in white light and then perform the desired treatments.
   Note: Use twelve 5–6-week–old Arabidopsis *plants for each treatment to collect enough epidermal peels to completely cover the surface of a Petri dish with 15 mL of OB (diameter: 9 cm, surface: 56,7 cm^2).*
2. For mesophyll cell–enriched (MC-e) RNA extraction, incubate the rest of the leaf, after detaching the abaxial epidermal peel, in an OB-containing Petri dish (as described in step C1). The leaf surface without epidermis must be in direct contact with OB.
   Note: Obtain these MC-e samples just if you are interested in comparing gene expression in different cell types.
3. Remove the buffer with a pipette and harvest the peels with a needle until obtaining a “peel pellet” and dry it with a blotting paper to remove the remaining buffer.
4. Following the manufacturer instructions, grind the tissue samples (CG-e and MC-e) in a mortar with liquid nitrogen and extract RNA with the TriZol method. For qRT-PCR, synthesize cDNA from 1 ug of total RNA. Represent data in a graph (Figure 3).
   Note: AtACT2 (At3g08510) gene is recommended as a housekeeping gene for GC-e and MC-e samples.

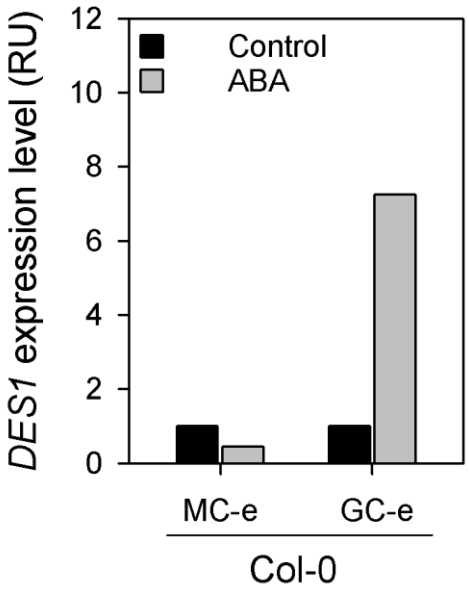

Figure 3. Analysis of the expression levels of *DES1* gene in guard cell–enriched (GC-e) and mesophyll cell–enriched (MC-e) RNA extracts of wild-type plants in response to abscisic acid (ABA). Epidermal peels (GC-e) and mesophyll tissue (MC-e) prepared from *Arabidopsis thaliana* wild-type ecotype Col-0 were pre-incubated for 3 h in opening buffer (OB, 5 mM MES, pH 6.1, 50 mM KCl) and then treated with or without 50 μM of ABA under light. After 90 min of treatment, qRT-PCR analysis of *DES1* gene expression was performed. The values are expressed as relative units (RU) to the control treatment. Data from Scuffi et al. [26].

Data analysis

A. Stomatal aperture

1. Open the file from the Neubauer chamber in Fiji (ImageJ) software to set the scale. Using the *Straight Line* tool, measure the length of the squares (50 μm) by clicking on *Analyze* > *Measure*, or with the M key. Repeat at least ten times to reduce the error. Copy this data into a spreadsheet and average all the measurements. Set the scale to this value by clicking on *Analyze* > *Set Scale*. Complete the field *Distance in pixels* with the averaged pixel number and fill the *Known distance* with the grid known size of the Neubauer chamber. Click the option *Global* to set the same scale for all images.
2. Once the scale is set, open the image files of the epidermal peels. Measure the width of the stomatal pore by drawing a straight line between the inner wall so the guard cells at the middle of the stomatal pore (Figure 2G and H). Press M after the line is drawn.
3. Copy the values obtained in the *Results* window into a spreadsheet and represent data in a plot (Figure 4).

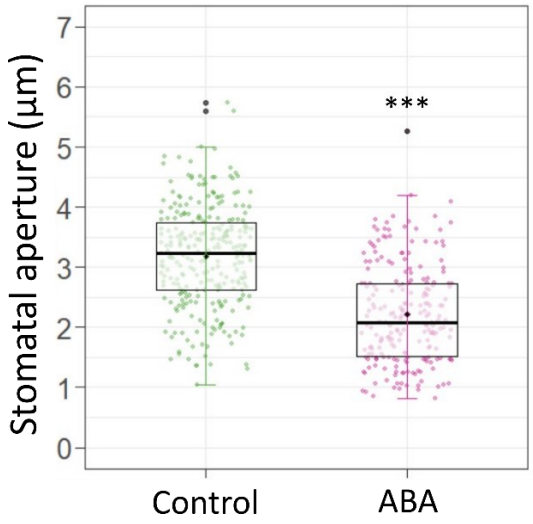

Figure 4. Stomatal closure response to abscisic acid (ABA). Epidermal peels were excised from the abaxial side of leaves from 5-week-old *Arabidopsis thaliana* wild-type ecotype Col-0 plants. Strips were subsequently floated in opening buffer (OB, 5 mM MES, pH 6.1, 50 mM KCl) for 3 h under light. Then, they were treated (ABA) or not (Control) for 90 min with 20 µM of abscisic acid (ABA). Stomatal aperture values are expressed as absolute values in micrometers and are represented as points in the boxplots, where the boxes are bound by the 25th to 75th percentile. The line in the middle is the median, the darker point is the mean, and the whiskers span from 10th to 90th percentile. The asterisk indicates statistical differences between treatments (*t*-test, $p < 0.05$).

B. Biosensors

1. Open files in Fiji (ImageJ) software. Both channels will appear.
2. Open the menu *Analyze* > *Set Measurements* and select *Mean grey value*.
3. Transform each channel to a 32-bit image.
4. Open Region of Interest (ROI) manager: *Analyze* > *Tools* > *ROI Manager*.
5. Select the *Freehand Line*, delimit the ROI to the cytosol of one guard cell avoiding the cytosol portion adjacent to the inner cell walls (Figure 2H), and press *Add* in the ROI Manager. Using a circle, draw a ROI in a region without stomata to obtain the background fluorescence value that is then going to be subtracted from guard cells' fluorescence.
6. Activate the window of one of both channels and press *Measure* in the ROI Manager to obtain the mean grey value as an expression of fluorescence intensity from all delimited regions. Repeat the procedure for the other channel.
7. Copy the values obtained in the *Results* window into a spreadsheet.
8. Subtract background values to guard cells' cytosolic fluorescence for each channel analyzed.
9. Calculate the 408/488 nm or the Venus/CFP ratio dividing the guard cell fluorescence intensity from each channel and represent results in a plot (Figure 5).

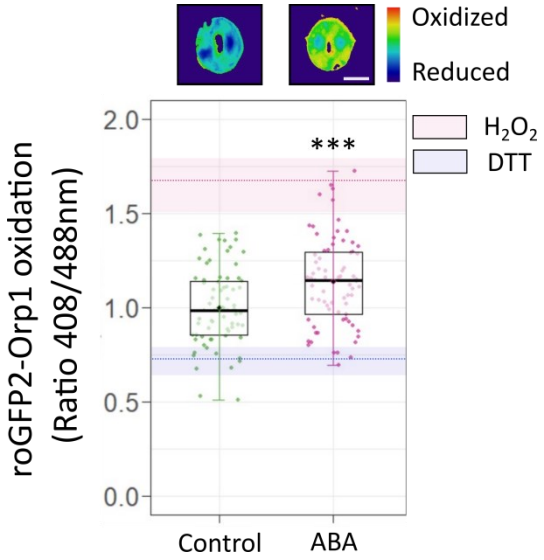

Figure 5. Cytosolic roGFP2-Orp1 oxidation in guard cells. Epidermal peels were prepared from *Arabidopsis thaliana* wild-type ecotype Col-0 leaves expressing the cytosolic biosensor roGFP2-Orp1, which detects H_2O_2. Peels were incubated in opening buffer (OB, 5 mM MES, pH 6.1, 50 mM KCl) for 7 h under light and then treated with 20 mM DTT or 1 mM H_2O_2 for 10 min, to fully reduce or oxidize the sensor, respectively, or with 20 µM of abscisic acid (ABA) for 10 min. Guard cells' images were obtained using an epifluorescence microscope. The ratio 405/488 nm was calculated for each guard cell, normalized to the control, and represented as individual points. Box plots are bound by the 25th to 75th percentile. The line in the middle is the median, the darker point is the mean, and the whiskers span from 10th to 90th percentile. Black points represent the outliers. Red and blue dotted lines and bands indicate the mean and the 25th to 75th percentile of maximum and minimum ratio values obtained or full oxidation (1 mM H_2O_2) and full reduction (20 mM DTT) of the sensor. Data was taken from at least three independent experiments for each treatment. The asterisk indicates statistical differences between treatments (*t*-test, $p < 0.05$). Ratiometric images are false-colored and represent the mean of different treatments. Scale bar: 10 µm.

Validation of protocol

This protocol or parts of it has been used and validated in the following research article (s):

A. Stomatal aperture

1. García-Mata, C. and Lamattina, L. (2010). Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. New Phytol.
2. Distéfano, A. M. et al. (2012). Phospholipase D δ is involved in nitric oxide-induced stomatal closure. Planta.
3. Scuffi, D. et al. (2014). Hydrogen Sulfide Generated by l-Cysteine Desulfhydrase Acts Upstream of Nitric Oxide to Modulate Abscisic Acid-Dependent Stomatal Closure. Plant Physiol.
4. Scuffi, D. et al. (2018). Hydrogen Sulfide Increases Production of NADPH Oxidase-Dependent Hydrogen Peroxide and Phospholipase D-Derived Phosphatidic Acid in Guard Cell Signaling. Plant Physiol.
5. Pantaleno, R. et al. (2023). Mitochondrial H2S donor AP39 induces stomatal closure by modulating guard cell mitochondrial activity. Plant Physiol.

B. Biosensors

1. Scuffi, D. et al. (2018). Hydrogen Sulfide Increases Production of NADPH Oxidase-Dependent Hydrogen Peroxide and Phospholipase D- Derived Phosphatidic Acid in Guard Cell Signaling. Plant Physiol.

2. Pantaleno, R. et al. (2023). Mitochondrial H2S donor AP39 induces stomatal closure by modulating guard cell mitochondrial activity. Plant Physiol.

C. GC-eRNA

1. Distéfano, A. M. et al. (2012). Phospholipase D δ is involved in nitric oxide-induced stomatal closure. Planta.
2. Scuffi, D. et al. (2014). Hydrogen Sulfide Generated by l-Cysteine Desulfhydrase Acts Upstream of Nitric Oxide to Modulate Abscisic Acid-Dependent Stomatal Closure. Plant Physiol.

General notes and troubleshooting

General notes

1. Double-blind testing for both image acquisition and analysis is highly recommended.
2. Use leaves with a similar stage of development to ensure that the stomata responses are comparable among them. For example, leaves 8–13 of *Arabidopsis* are usually of similar size and stage.
3. Take approximately three photos per peel and make sure to capture different sample fields.
4. Use at least four epidermal peels from different leaves per condition.
5. Keep the same objective and camera settings to take all the photos within an experiment.
6. Perform at least three or four independent assays for each type of experiment.

Competing interests

The authors declare no competing interests.

References

1. Lawson, T. and Matthews, J. (2020). Guard Cell Metabolism and Stomatal Function. *Annu Rev Plant Biol.* 71 (1): 273–302. https://doi.org/10.1146/annurev-arplant-050718-100251
2. Blatt, M.R. (2000). Cellular Signaling and Volume Control in Stomatal Movements in Plants. *Annu Rev Plant Biol.*16 (1): 221–241. https://doi.org/10.1146/annurev.cellbio.16.1.221
3. Schroeder, J. I., Allen, G. J., Hugouvieux, V., Kwak, J. M. and Waner, D. (2001). GUARD CELL SIGNAL TRANSDUCTION. *Annu Rev Plant Biol.* 52 (1): 627–658. https://doi.org/10.1146/annurev.arplant.52.1.627
4. WEYERS, J. D. B. and TRAVIS, A. J. (1981). Selection and Preparation of Leaf Epidermis for Experiments on Stomatal Physiology. *J Exp Bot.* 32 (4): 837–850. https://doi.org/10.1093/jxb/32.4.837
5. Raghavendra, A. S. (1981). Energy Supply for Stomatal Opening in Epidermal Strips of *Commelina benghalensis*. *Plant Physiol.* 67 (2): 385–387. https://doi.org/10.1104/pp.67.2.385
6. Raghavendra, A. and Vani, T. (1989). Respiration in Guard Cells, Pattern and Possible Role in Stomatal Function. *J Plant Physiol.* 135 (1): 3–8. https://doi.org/10.1016/s0176-1617(89)80215-9
7. Wille, A. C. and Lucas, W. J. (1984). Ultrastructural and histochemical studies on guard cells. *Planta*. 160 (2): 129–142. https://doi.org/10.1007/BF00392861
8. Montillet, J., Rondet, D., Brugière, S., Henri, P., Rumeau, D., Reichheld, J., Couté, Y., Leonhardt, N. and Rey, P. (2021). Plastidial and cytosolic thiol reductases participate in the control of stomatal functioning. *Plant Cell Environ.* 44 (5): 1417–1435. https://doi.org/10.1111/pce.14013
9. Thor, K., Jiang, S., Michard, E., George, J., Scherzer, S., Huang, S., Dindas, J., Derbyshire, P., Leitão, N., De Falco, T. A., et al. (2020). The calcium-permeable channel OSCA1.3 regulates plant stomatal immunity. *Nature.* 585 (7826): 569–573. https://doi.org/10.1038/s41586-020-2702-1

10. Yan, S., Mc Lamore, E. S., Dong, S., Gao, H., Taguchi, M., Wang, N., Zhang, T., Su, X. and Shen, Y. (2015). The role of plasma membrane H^+-ATPase in jasmonate-induced ion fluxes and stomatal closure in *Arabidopsis thaliana*. *Plant J*. 83 (4): 638–649. https://doi.org/10.1111/tpj.12915
11. Kostaki,K.-I., Coupel-Ledru, A., Bonnell, V. C., Gustavsson, M., Sun, P., McLaughlin, F. J., Fraser, D. P., McLachlan, D.H., Hetherington, A. M., Dodd, A. N. et. al. (2020). Guard Cells Integrate Light and Temperature Signals to Control Stomatal Aperture. *Plant Physiol.* 182 (3): 1404–1419. https://doi.org/10.1104/pp.19.01528
12. Merilo, E., Yarmolinsky, D., Jalakas, P., Parik, H., Tulva, I., Rasulov, B., Kilk, K. and Kollist, H. (2018). Stomatal VPD Response: There Is More to the Story Than ABA. *Plant Physiol.* 176 (1): 851–864. https://doi.org/10.1104/pp.17.00912
13. Tanaka, Y., Nose, T., Jikumaru, Y. and Kamiya, Y. (2013). ABA inhibits entry into stomatal-lineage development in Arabidopsis leaves. *Plant J*. 74 (3): 448–457. https://doi.org/10.1111/tpj.12136
14. Daloso, D. M., Antunes, W. C., Pinheiro, D. P., Waquim, J. P., AraÚJo, W. L., Loureiro, M. E., Fernie, A. R. and Williams, T. C. R. (2015). Tobacco guard cells fix CO_2 by both Rubisco and PEP case while sucrose acts as a substrate during light-induced stomatal opening: Isotope labeling kinetics in guard cells. *Plant Cell Environ*. 38 (11): 2353–2371. https://doi.org/10.1111/pce.12555
15. Kwak, J. M. (2003). NADPHoxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J.* 22 (11): 2623–2633. https://doi.org/10.1093/emboj/cdg277
16. Su, J., Zhang, M., Zhang, L., Sun, T., Liu, Y., Lukowitz, W., Xu, J. and Zhang, S. (2017). Regulation of Stomatal Immunity by Interdependent Functions of a Pathogen-Responsive MPK3/MPK6 Cascade and Abscisic Acid. *Plant Cell*. 29 (3): 526–542. https://doi.org/10.1105/tpc.16.00577
17. Thor, K. and Peiter, E. (2014). Cytosolic calcium signals elicited by the pathogen-associated molecular pattern flg22 in stomatal guard cells are of an oscillatory nature. *New Phytol*. 204 (4): 873–881. https://doi.org/10.1111/nph.13064
18. Ye, W., Munemasa, S., Shinya, T., Wu, W., Ma, T., Lu, J., Kinoshita, T., Kaku, H., Shibuya, N. and Murata, Y. (2020). Stomatal immunity against fungal invasion comprises not only chitin-induced stomatal closure but also chitosan-induced guard cell death. *Proc Natl Acad Sci U.S.A.* 117 (34): 20932–20942. https://doi.org/10.1073/pnas.1922319117
19. Zhang, J., Zhou, M., Ge, Z., Shen, J., Zhou, C., Gotor, C., Romero, L. C., Duan, X., Liu, X., Wu, D., et al. (2020). Abscisic acid-triggered guard cell L-cysteine desulfhydrase function and in situ hydrogen sulfide production contributes to hemeoxygenase-modulated stomatal closure. *Plant Cell Environ*. 43 (3): 624–636. https://doi.org/10.1111/pce.13685
20. García-Mata, C. and Lamattina, L. (2001). Nitric Oxide Induces Stomatal Closure and Enhances the Adaptive Plant Responses against Drought Stress. *Plant Physiol.* 126 (3): 1196–1204. https://doi.org/10.1104/pp.126.3.1196
21. Flütsch, S., Wang, Y., Takemiya, A., Vialet-Chabrand, S. R. M., Klejchová, M., Nigro, A., Hills, A., Lawson, T., Blatt, M. R. and Santelia, D. (2020). Guard Cell Starch Degradation Yields Glucose for Rapid Stomatal Opening in Arabidopsis. *Plant Cell*. 32 (7): 2325–2344. https://doi.org/10.1105/tpc.18.00802
22. Rodrigues, O., Reshetnyak, G., Grondin, A., Saijo, Y., Leonhardt, N., Maurel, C. and Verdoucq, L. (2017). Aquaporins facilitate hydrogen peroxide entry into guard cells to mediate ABA- and pathogen-triggered stomatal closure. *Proc Natl Acad Sci U.S.A.* 114 (34): 9200–9205. https://doi.org/10.1073/pnas.1704754114
23. Distéfano, A. M., Scuffi, D., García-Mata, C., Lamattina, L. and Laxalt, A.M. (2012). Phospholipase D δ is involved in nitric oxide-induced stomatal closure. *Planta*. 236 (6): 1899–1907. https://doi.org/10.1007/s00425-012-1745-4
24. García-Mata, C. and Lamattina, L. (2010). Hydrogen sulphide, a novel gasotransmitter involved in guard cell signalling. *New Phytologist*. 188 (4): 977–984. https://doi.org/10.1111/j.1469-8137.2010.03465.x
25. Pantaleno, R., Scuffi, D., Costa, A., Welchen, E., Torregrossa, R., Whiteman, M. and García-Mata, C. (2023). Mitochondrial H2S donor AP39 induces stomatal closure by modulating guard cell mitochondrial activity. *Plant Physiology*. 191 (3): 2001–2011. https://doi.org/10.1093/plphys/kiac591
26. Scuffi, D., Álvarez, C., Laspina, N., Gotor, C., Lamattina, L. and García-Mata, C. (2014). Hydrogen Sulfide Generated by L-Cysteine Desulfhydrase Acts Upstream of Nitric Oxide to Modulate Abscisic Acid-Dependent Stomatal Closure. *Plant Physiology.* 166 (4): 2065–2076. https://doi.org/10.1104/pp.114.245373
27. Scuffi, D., Nietzel, T., DiFino, L. M., Meyer, A. J., Lamattina, L., Schwarzländer, M., Laxalt, A. M. and García-

Mata, C. (2018). Hydrogen Sulfide Increases Production of NADPH Oxidase-Dependent Hydrogen Peroxide and Phospholipase D-Derived Phosphatidic Acid in Guard Cell Signaling. *Plant Physiol.* 176 (3): 2532–2542. https://doi.org/10.1104/pp.17.01636

28. Nietzel, T., Elsässer, M., Ruberti, C., Steinbeck, J., Ugalde, J. M., Fuchs, P., Wagner, S., Ostermann, L., Moseler, A., Lemke, P., et al. (2019). The fluorescent protein sensor roGFP2-Orp1 monitors in vivo H_2O_2 and thiol redox integration and elucidates intracellular H_2O_2 dynamics during elicitor-induced oxidative burst in Arabidopsis. *New Phytologist*. 221 (3): 1649–1664. https://doi.org/10.1111/nph.15550
29. Marty, L., Siala, W., Schwarzländer, M., Fricker, M. D., Wirtz, M.,S weetlove, L. J., Meyer, Y., Meyer, A. J., Reichheld, J. P. and Hell, R. (2009). The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in *Arabidopsis*. *Proc Natl Acad Sci U.S.A.* 106 (22): 9109–9114. https://doi.org/10.1073/pnas.0900206106
30. De Col, V., Fuchs, P., Nietzel, T., Elsässer, M., Voon, C. P., Candeo, A., Seeliger, I., Fricker, M. D., Grefen, C., Møller, I. M., et al. (2017). ATP sensing in living plant cells reveals tissue gradients and stress dynamics of energy physiology. *E life.* 6: e26770. https://doi.org/10.7554/elife.26770
31. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods.* 9 (7): 676–682. https://doi.org/10.1038/nmeth.2019
32. Voon, C. P., Guan, X., Sun, Y., Sahu, A., Chan, M. N., Gardeström, P., Wagner, S., Fuchs, P., Nietzel, T., Versaw, W. K., et al. (2018). ATP compartmentation in plastids and cytosol of *Arabidopsis thaliana* revealed by fluorescent protein sensing. *Proc Natl Acad Sci USA.* 115(45): e1711497115. https://doi.org/10.1073/pnas.1711497115