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Supplementary Material

Confirmation of mesophyll signals controlling stomatal responses by a newly devised transplanting method

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Table S1. ANOVA summary of the experiments

Figure number	Source of variation	d.f.	*SS*	*F*	*P*
Fig. 4	buffer	2	475.3	110.98	$<2.2 \times 10^{-16}$
	day	2	539.3	125.92	$<2.2 \times 10^{-16}$
	residual	485	1038.5		
Fig. 6 *(a)*	pretreatment	1	0.8	0.89	0.35
	day	2	27.5	14.87	5.3×10^{-7}
	residual	506	467.6		
Fig. 6 *(b)*	pretreatment	1	291.3	91.54	$<2.2 \times 10^{-16}$
	day	2	291.3	45.78	$<2.2 \times 10^{-16}$
	residual	536	1705.1		
Fig. 6 *(c)*	pretreatment	1	345.0	60.14	4.5×10^{-14}
	day	2	135.0	11.77	1.0×10^{-5}
	residual	536	3074.7		
Fig. 6 *(d)*	pretreatment	1	174.7	75.46	$<2.2 \times 10^{-16}$
	day	2	87.4	18.87	1.3×10^{-8}
	residual	476	1102.1		
Fig. 6 *(e)*	pretreatment	1	259.1	76.62	$<2.2 \times 10^{-16}$
	day	3	766.3	75.60	$<2.2 \times 10^{-16}$
	residual	715	2717.7		
Fig. 6 *(f)*	pretreatment	1	34.1	11.40	7.8×10^{-4}
	day	2	370.0	61.93	$<2.2 \times 10^{-16}$
	residual	536	1601.4		
Fig. 6 *(g)*	pretreatment	1	91.9	35.81	3.2×10^{-9}
	day	4	1489.6	145.09	$<2.2 \times 10^{-16}$
	residual	894	2294.6		
Fig. S7	time	2	314.7	53.60	$<2.2 \times 10^{-16}$
	day	2	505.6	85.12	$<2.2 \times 10^{-16}$
	residual	515	1511.8		
Fig. S9	pretreatment	1	2.9	0.68	0.41
	day	2	11.0	1.31	0.27
	residual	536	2257.4		

Fig. 4: Experimental days were treated as the block factors.

Figs. 6 and S9: Experimental days (or pairs of the leaves) were treated as the block factors.

Fig. S7: Experimental days were treated as the block factors.

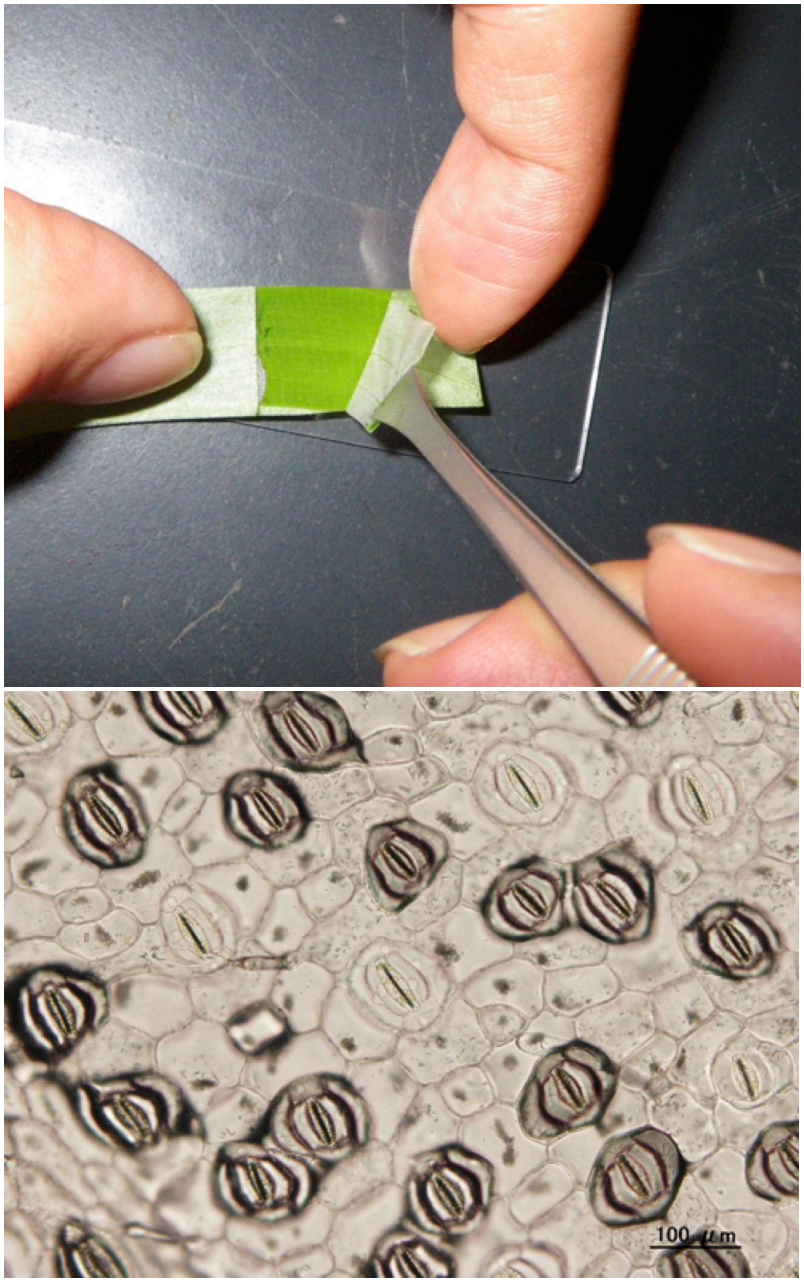

Fig. S1. Procedures to remove the abaxial epidermis from a leaf segment of *Commelina communis* (above) and the abaxial epidermal strip (below). The epidermis was cut with a razor blade, and peeled off with the forceps. Note that the epidermis was whitish and was free from mesophyll cells. Stomatal complexes with liquid-filled (transparent) and air-filled (refractive) substomatal cavities in an abaxial epidermal strip free from mesophyll cells.

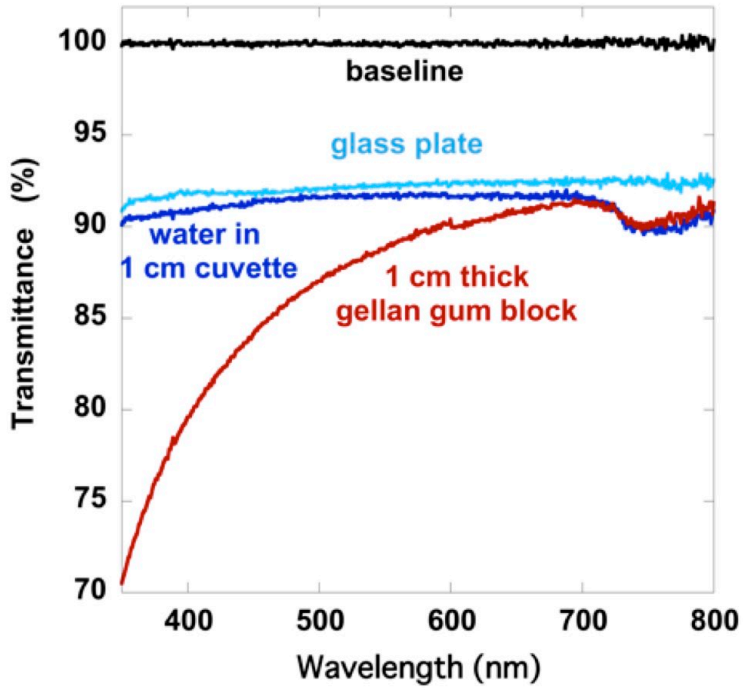

Fig. S2. Transmittance spectra of the glass plate used for chamber windows and a gellangum block. Transmittance spectra were measured with a spectrophotometer (Hitachi U3310, Hitachi, Tokyo) in a transmittance mode. Note that water showed some absorption at around 750 nm. The gellangum block contained 30 mM KCl, 0.1 mM $CaCl_2$, and 10 mM MES (pH 6.5). The 1 cm block absorbed some blue light in addition to far-red light at around 750 nm.

Although the glass plate hardly absorbed light, the transmittance was about 92%. This is attributed to reflection occurring at the interface between the air and glass. The reflectance (R) at the interface is expressed as

$$R= [(n_1-n_2)/(n_1+n_2)]^2,$$

where n_1 and n_2 are refractive indices of the air and the glass. If the internal multiple reflectance is considered, the transmittance (T) of the glass place can be expressed as,

$$T = (1–R)/(1+R).$$

When T = 0.92 and the refractive index of air is 1, the refractive index of the glass was calculated to be 1.51.

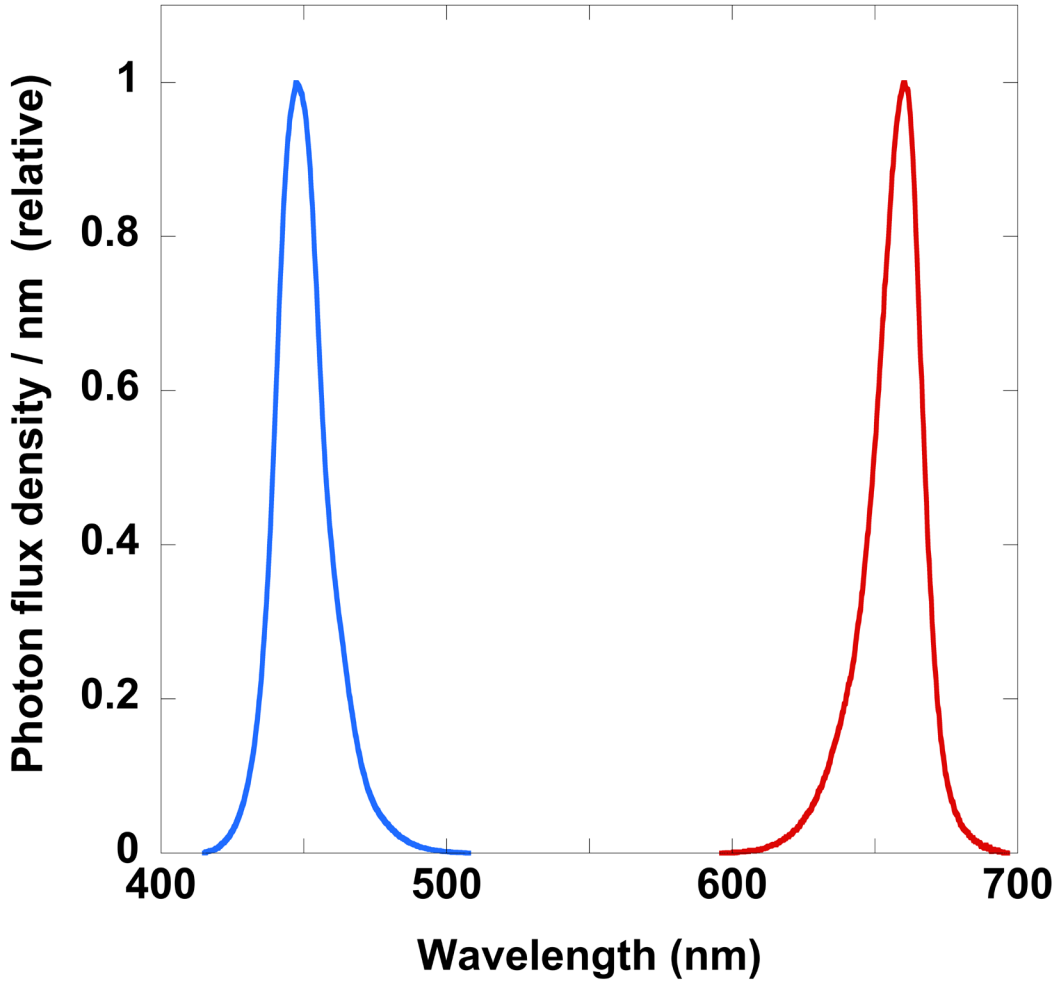

Fig. S3. Emission spectra of the blue (left) and red (right) LEDs used to illuminate the samples. The spectra were measured with a fiber optics spectrometer (USB2000+, Ocean Optics, Dunedin) with spectrometer operating software (SpectraSuite, Ocean Optics).

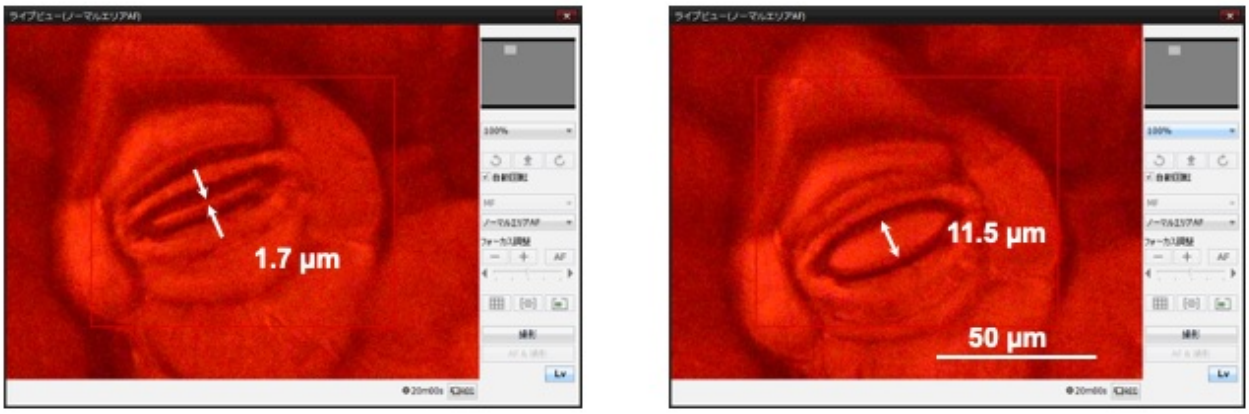

Fig. S4. Images used for aperture measurements in the red light. In the measurement, each stoma was focused. We moved the focal plane from the above and the aperture on the second focal plane, which corresponded the throat of the stomatal pore, was measured. The pictures of same stoma with apertures of 1.7 µm (left) and 11.5 µm (right) are shown. For detailed optical system see Fujita *et al*. (2013). Briefly, the sample was observed under a microscope (BH2; Olympus, Tokyo, Japan) with a long focal objective lens (SLIM x 20; working distance, 25 mm; Olympus). Digital images were obtained using a digital camera (D5100; Nikon, Tokyo, Japan) and analyzed using digital image analysis software (Macromax GOKO Measure; Goko Camera, Kawasaki, Japan).

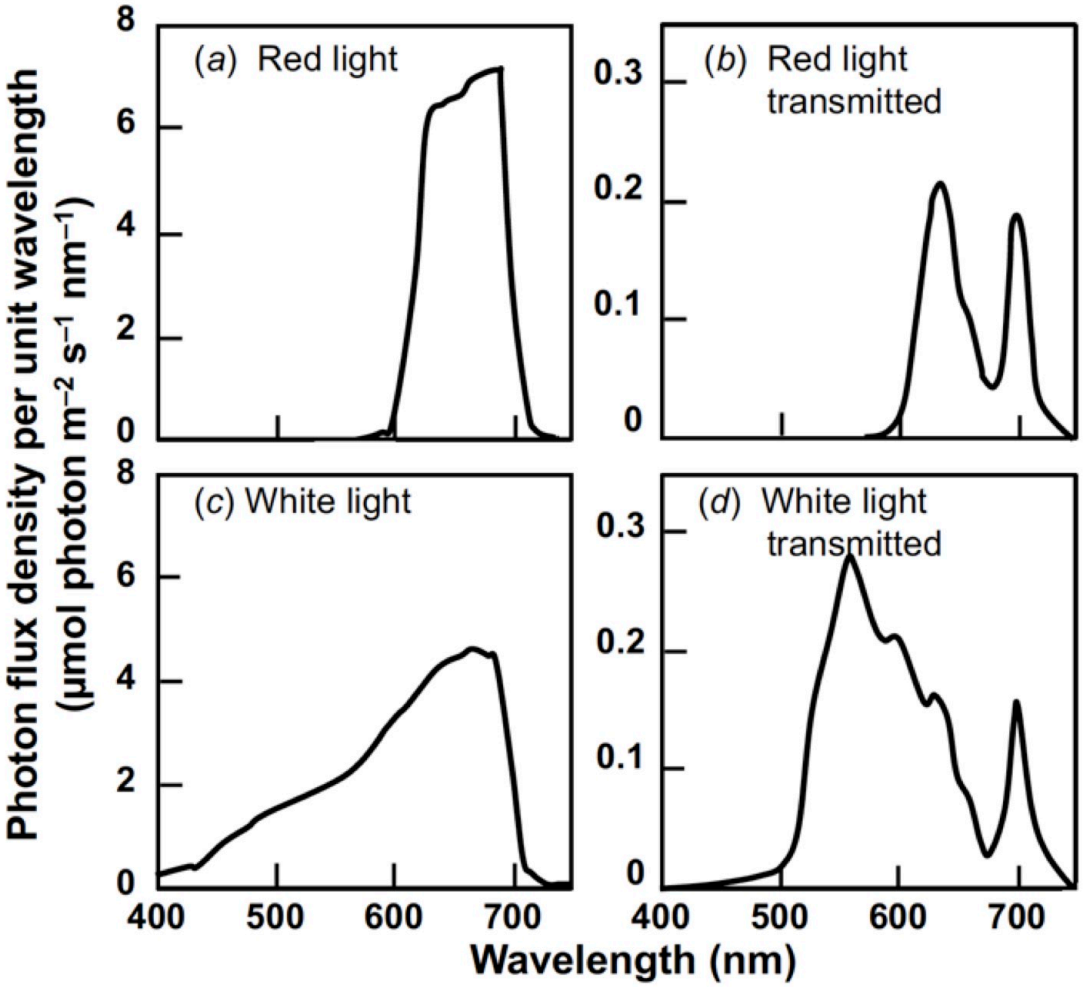

Fig. S5. Spectra of the red light (RL, (*a*)) and white light (WL, (*c*)) used to illuminate the samples. The spectra in (*b*) and (*d*) are RL and WL transmitted through a *Commelina communis* leaf. Redrawn from Fujita *et al*. (2013). The spectra were measured with a fiber optics spectrometer (USB2000+, Ocean Optics, Dunedin) with spectrometer operating software (SpectraSuite, Ocean Optics).

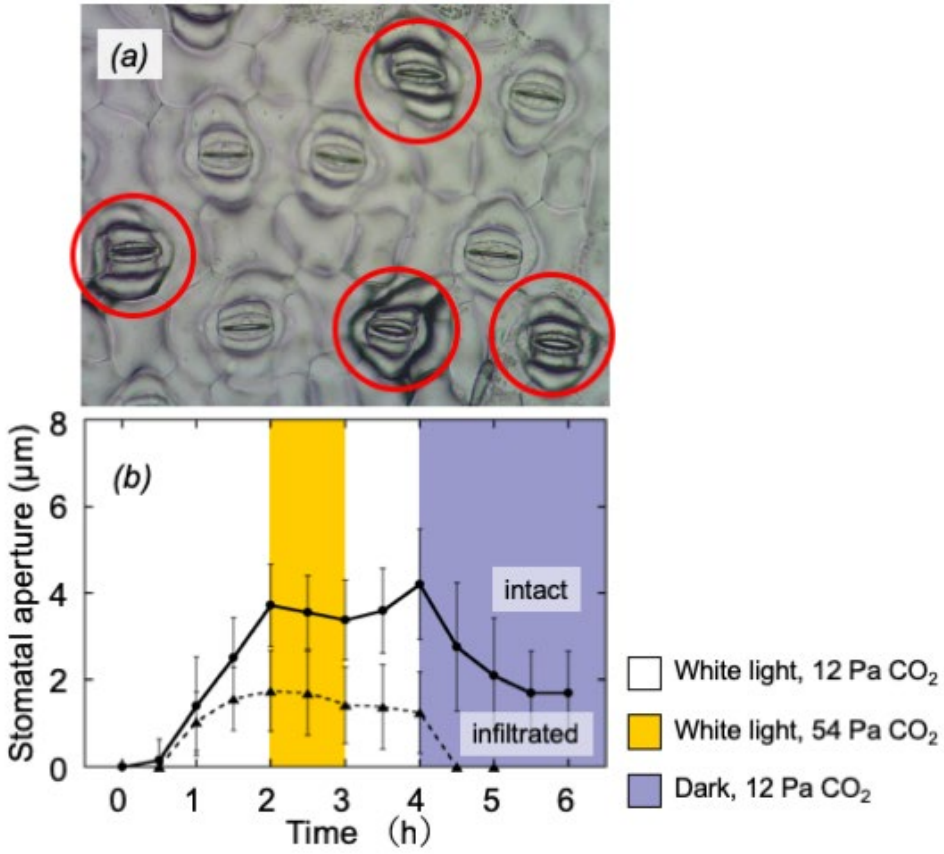

Fig. S6. Stomatal complexes with infiltrated and intact (enclosed in red circles) substomatal cavities *(a)* and responses of these stomata *(b)*. The epidermal strip was placed on a gellangum block containing 30 mM KCl, 0.1 mM $CaCl_2$, and 10 mM MES (pH 6.15) and subject to light/CO_2 treatments. The responses of the stomata with intact substomatal cavities were greater than those in the stomata with infiltrated substomatal cavity. The original data of Dr. Takashi Fujita in Lawson et al. (2018) were modified.

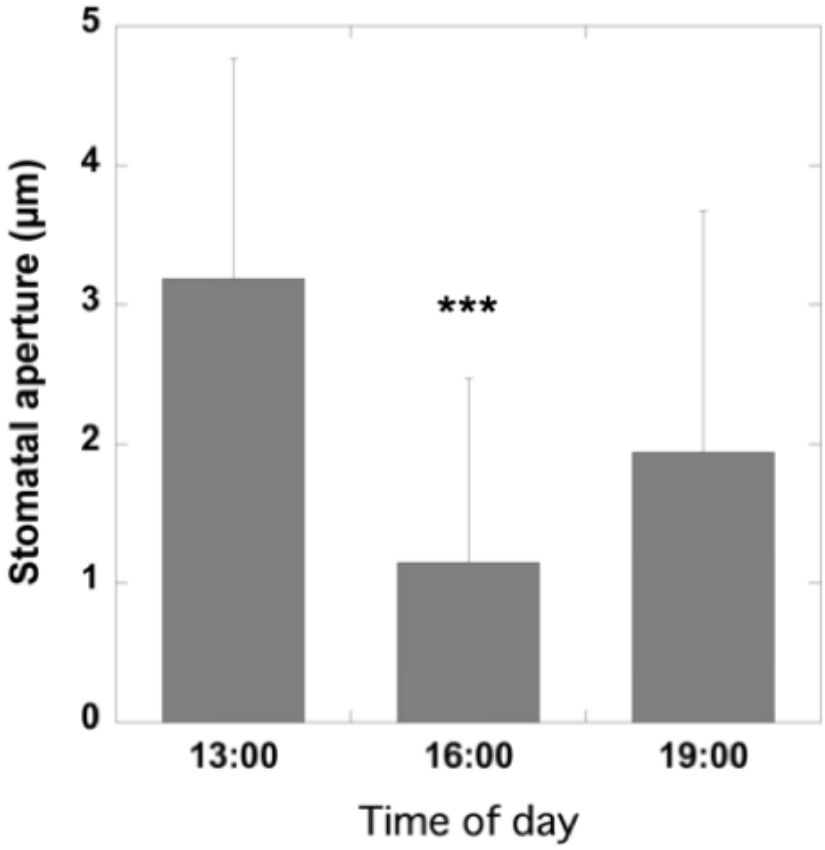

Fig. S7. Effects of experimental timing on stomatal responses to light.

Three experiments were conducted on three separate days. On each day, epidermal strips were prepared three times at 12:30, 15:30 and 18:30 from *Commelina communis* leaves from a plant that had been kept in the dark before the light period. The epidermal strips were placed on gels containing 30 mM KCl, 0.1 mM $CaCl_2$ and 0.1 mM MES (pH 6.15). Irradiation of the epidermal strips with red light (PPFD 550 μmol m^{-2} s^{-1}) + blue light (PPFD 50 μmol m^{-2} s^{-1}), R + B, was started at 13:00, 16:00 or 19:00 for 2 h. The epidermal strips on the gels were maintained at 23°C. The CO_2 partial pressure was maintained at 39 Pa. The data are shown as the weighted mean ± SD. The numbers of stomatal apertures measured on the respective days were 60, 60, and 40 for 13:00, 60, 60, and 60 for 16:00, and 60, 60 and 60 for 19:00. The effects of the time of day were significant at $P < 2.2 \times 10^{-16}$ (For ANOVA, see Supplementary Table S1).

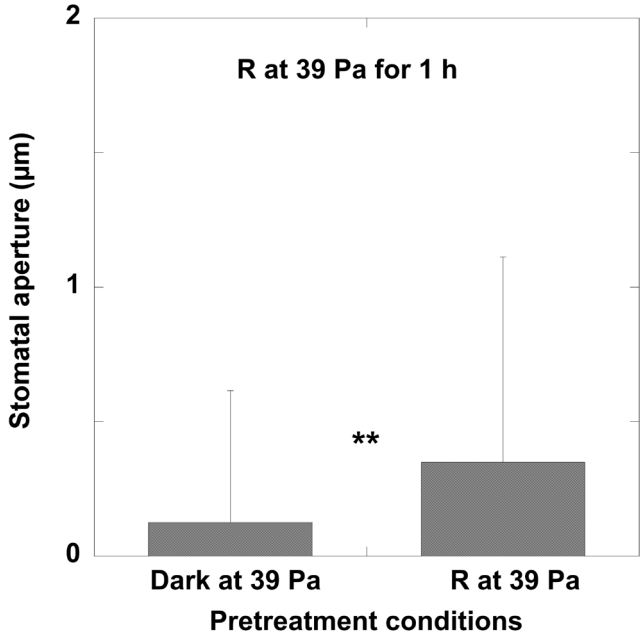

Fig. S8. Stomatal responses in epidermal strips from dark-treated plants transplanted onto mesophyll segments pretreated in the dark or in R for 1 h. Two plants were kept in the dark at 10:30. At 11:30, six leaf segments (10 × 10 mm^2) were prepared from a fully expanded mature leaf of one plant, and placed on gels containing 30 mM KCl, 1 mM $CaCl_2$ and 10 mM MES (pH 6.15) with their abaxial sides upwards. Each gel block with one leaf segment was placed in a petri dish (30 mm in diameter), and the dish was placed in one of two aluminium sample chambers (190 × 120 × 30 mm^3 W/D/H each) with glass windows. The pretreatments of the leaf segments in these chambers were started at 12:00. At 13:00, the adaxial epidermis was peeled off each leaf segment and an abaxial epidermal strip (15 × 15 mm^2), prepared from a leaf from another plant kept in the dark, was transplanted onto the mesophyll so that the inner sides of the epidermal strip and mesophyll segment were in contact. The preparation of the mesophyll segments and transplantation of the epidermal strips were conducted in dim light. Each transplant on the gel was placed in a petri dish and six dishes were placed in the aluminium chamber and illuminated with R from above from 13:30 for 1 h. The data are shown as the mean ± SD. Numbers of stomata measured for the transplants pretreated in the dark and in R, are 126 (34 + 48+ 47) and 149 (53 + 33 + 63), respectively. The difference between the two treatments was analysed by Welch's test because the equality of variances of the data set was not supported. Although the difference was statistically significant, apertures were small.

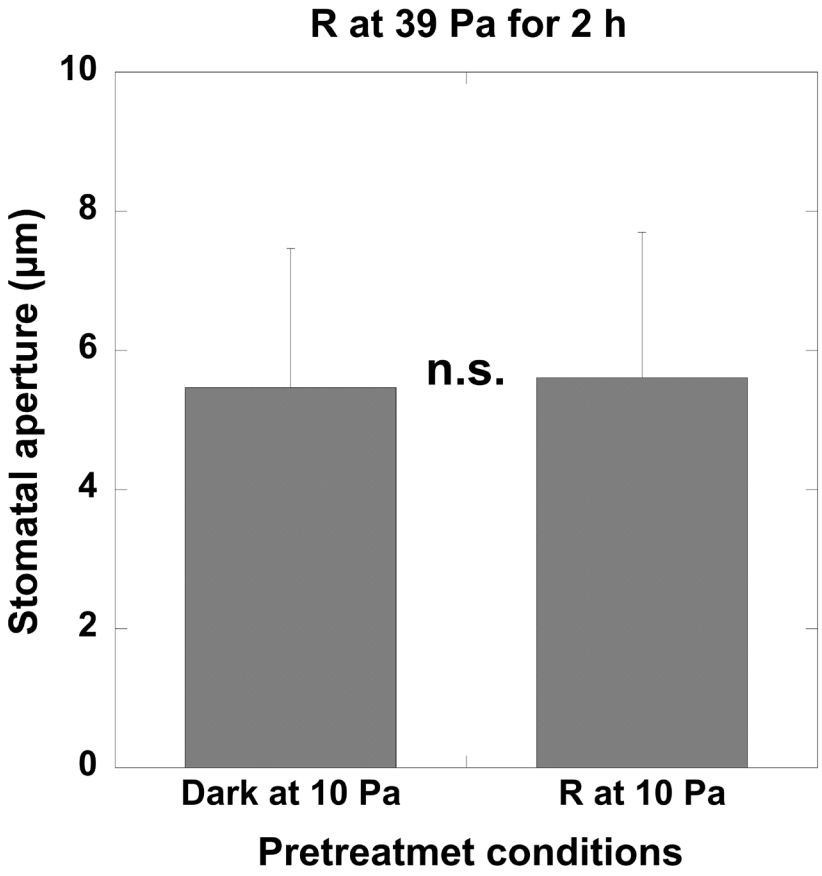

Fig. S9. Stomatal responses in epidermal strips from dark-treated plants transplanted onto mesophyll segments pretreated in the dark or in R at 10 Pa CO_2 and treated in R at 39 Pa for 2 h. For the pretreatment and treatment conditions and the number of stomata measured on each day for each pretreatment, see Table 1. The data are shown as the weighted mean ± SD. The difference between the two preparations was not significant (P = 0.42). Compare this figure with Fig. 6*c* and *e*. For ANOVA, see Supplementary Table s1

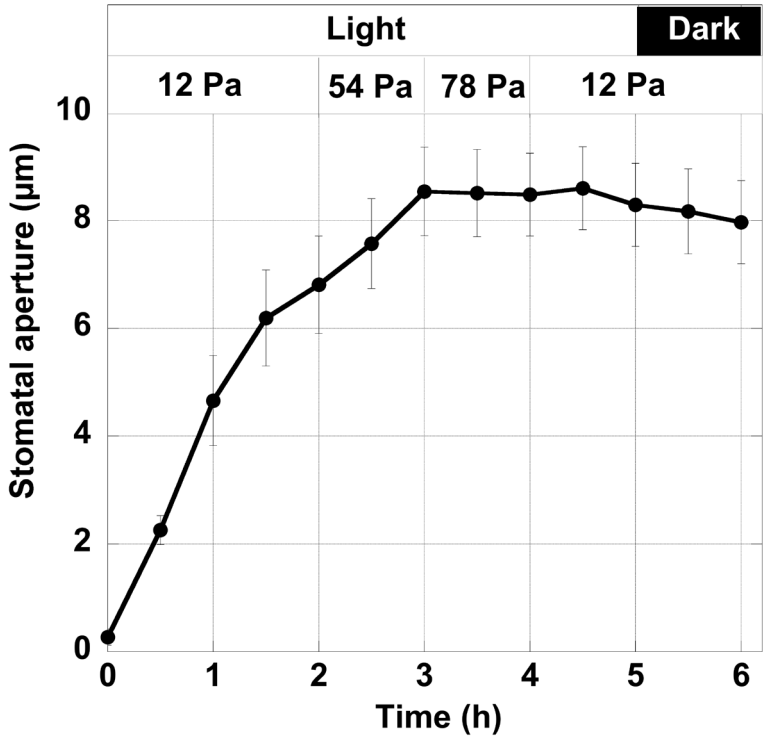

Fig. S10. Behaviour of stomata in an abaxial epidermal strip of *Commelina communis* placed over a hole (5 mm in diameter) made in a filter paper strip containing a buffer (50 mM KCl, 1 mM $CaCl_2$, and 10 mM MES at pH 6.15). The filter paper strip with the abaxial epidermis was sandwiched between the half-chambers with glass windows (45 × 55 × 10 mm^3 each) and one end of the filter paper strip was dipped in the buffer for perfusion. The system was kept in the dark for 1 h before the onset of illumination. White light at 150 μmol m^{-2} s^{-1} was provided by a halogen lamp attached to the microscope from the bottom. Thus, the light was given from the inner side of the epidermis through the filter paper. The CO_2 partial pressure was changed as indicated using mass flow controllers. The air was humidified by bubbling in water at 23°C. Data are the mean ± SD of 15 stomata.

Stomata continued to open for 3 h. Thereafter, the stomata responded neither to changes in the CO_2 partial pressure nor to darkness treatment. Because we used a buffer of 10 mM MES, the osmotic potential of the solution was lower than that of the solution used by Mott *et al*. (2008), which contained 50 mM KCl and 1 mM $CaCl_2$.