Use of Confocal Laser as Light Source Reveals Stomata-Autonomous Function

Roberto C. Cañamero1, Hernán Boccalandro1,2,3, Jorge Casal2,3, Laura Serna1*

1 Environmental Sciences Faculty, University of Castilla-La Mancha, Toledo, Spain, 2 Instituto de Investigaciones Fisiológicas y Ecológicas Vinculadas a la Agricultura (IFEVA), Faculty of Agronomy, University of Buenos Aires, Buenos Aires, Argentina, 3 Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

In most terrestrial plants, stomata open during the day to maximize the update of CO2 for photosynthesis, but they close at night to minimize water loss. Blue light, among several environmental factors, controls this process. Stomata response to diverse stimuli seems to be dictated by the behaviour of neighbour stomata creating leaf areas of coordinated response. Here individual stomata of Arabidopsis leaves were illuminated with a short blue-light pulse by focusing a confocal argon laser. Beautifully, the illuminated stomata open their pores, whereas their dark-adapted neighbours unexpectedly experience no change. This induction of individual stomata opening by low fluence rates of blue light was disrupted in the phototropin1 phototropin2 (phot1 phot2) double mutant, which exhibits insensitivity of stomatal movements in blue-illuminated epidermal strips. The irradiation of all epidermal cells making direct contact with a given stoma in both wild type and phot1 phot2 plants does not trigger its movement. These results unravel the stoma autonomous function in the blue light response and illuminate the implication of PHOT1 and/or PHOT2 in such response. The micro spatial heterogeneity that solar blue light suffers in partially shaded leaves under natural conditions highlights the physiological significance of the autonomous stomatal behaviour.

INTRODUCTION

Stomatal pores are located on the plant epidermis and regulate CO2 uptake for photosynthesis and water loss to drive transpiration. Stomatal opening is induced by several environmental factors, among them, blue light [1–3]. When either entire plants or epidermal strips adapted to darkness are exposed to low fluence rates of blue light, the stomata open their pores [4–6]. The blue-light receptors PHOTOTROPIN1 (PHOT1) and PHOTOTROPIN2 (PHOT2) control this response. In a redundant fashion, they mediate the stomatal opening, with both phot1 and phot2 single mutants being indistinguishable from wild-type plants when epidermal strips are illuminated with blue light, and phot1 phot2 double mutant exhibiting insensitivity of stomatal opening under these conditions [4]. Both, PHOT1 and PHOT2 are composed by a serine/threonine kinase domain located within the carboxy-terminus and repeated photosensory motifs referred LOV1 and LOV2 in the amino-terminus [7,8]. Blue-light-specific stomatal opening has an action spectrum typical of other blue-light responses of plants, showing a maximum at 450-nm and two minor peaks at 420-nm and 470-nm [9], which closely matches the absorption spectra of the LOV domains of PHOT1 and PHOT2 [9–12].

In dark-grown seedlings, both co-sedimentation experiments with plasma membrane enzymes [13,14] and aqueous two-phase partitioning [15–18] place PHOT1 at the plasma membrane. In addition, experiments with right-side-out plasma membrane vesicles show that it is associated only with the inner surface of the plasma membrane [16]. More recently, brief light treatments in PHOT1-GFP etiolated seedling have shown that a fraction of PHOT1 is released from the cell membrane to the cytoplasm in response to blue light [19]. Analysis of PHOT2-GFP has just shown that in the dark PHOT2 localizes, like PHOT1, mainly to the plasma membrane [20]. However, blue light illumination induces its association with the Golgi apparatus [20]. Stomata response to diverse stimuli seems to be dictated by the behaviour of neighbour stomata creating leaf areas of coordinated response [21,22]. For example, when a single stoma is exposed to a current of dry air, adjacent stomata also tend to close, despite being not exposed to the signal [21,23]. This coordinated behaviour is apparently due to hydraulic coupling among stomata [21,24]. Here, we experimentally address whether stomata might function autonomously in the blue light response by individuals cells irradiation with a laser. We show that stomata act independently regardless of the behaviour of their neighbours and highlight the implication of PHOT1 and/or PHOT2 in such response. The physiological advantage of the stomatal autonomous function is discussed.

RESULTS/DISCUSSION

We used simultaneously both a 458-nm line and a 476-nm line of an argon laser attached to a DMI8RB inverted Leica TCS SP2 confocal microscope, to investigate whether stomata might function autonomously in response to blue light. Individual stomata were exposed to 10 μmol m⁻² s⁻¹ of blue-light for 10 s,
and their neighbours were maintained in the dark (or illuminated with a 405-nm laser line of the diode laser, not shown). Of a total of 20 illuminated stomata, 17 (85%) increased the size of their pores (Figure 1A and 1C), whereas their dark-adapted neighbour exhibited no change (100%; n = 15; Figure 1B and 1C). This resulted in rejecting the null hypothesis of independence between the blue-light irradiation and the number of opened stomata (P < 0.0001). This result unravels the stomatal-autonomous opening in the blue light response, and also if demonstrates that the signal that triggers stomatal movements does not transmit across the epidermal tissue, at least from stoma to stoma. The 10 s-pulse illumination of 10 μmol m⁻² s⁻¹ resulted in an increase in aperture (Figure 1C), which does not differ from the data obtained when epidermal strips of Arabidopsis were illuminated with continuous 5 μmol m⁻² s⁻¹ blue light [5]. It should be noted that, like other authors discussed [12,25], the very low fluencies employed in our experiments ensure that the blue-light-induced stomatal movements are due to the photoreceptors rather than to photosynthesis.

In supporting the absence of a blue-light induced cell signalling across the epidermal tissue, irradiation of stoma neighbour epidermal cells with a short-pulse of blue light caused no effect on the opening of the adjacent stoma. The three neighbour epidermal cells that surround a given stoma were simultaneously illuminated, but the stoma making contact with such cells remained unaltered (Figure 2A). A total of 19 stomata (100%; n = 19) exhibited this behaviour (Figure 2B). Control stomata in these cells were illuminated, and as it might be expected they increased pore opening (n = 19; 90.5%; Figure 2B). This resulted in rejecting the null hypothesis of independence between the cell type that is irradiated and the number of opened stomata (P < 0.0001).

The induction of stomatal opening in wild type plants by blue light was disrupted in the phot1 phot2 double mutant (Figure 1A and 1C; P < 0.0001). Of a total of 15 illuminated stomata, every one of them showed insensitivity of stomatal opening to the blue-light-pulse. This result supports the previously established function of both PHOT1 and PHOT2 in the stomatal aperture in the blue-light response [4], and it demonstrates the cell-autonomous roles for these genes in controlling the stomatal movements. Like in the wild type, the dark-adapted neighbours of the illuminated stomata experienced no change (n = 20; 100%; Figure 1B and 1C). The three non-stomatal epidermal cells that make contact with every stoma were also illuminated and like in the wild type, the adjacent stomata did not increase the size of their pores (n = 15; 100%; Figure 2A and 2B). These results support the absence of a signalling response to blue light across the epidermal tissue. Stomata, in the peels of photosynthesis.

Figure 1. Stoma autonomy in its blue-light-induced opening

Individual stomata were irradiated with a short pulse of blue light by focusing an argon laser attached to a confocal microscope. (A) Irradiated stomata. (B) Nearest neighbour of the irradiated stoma. Confocal sections showing the stomatal opening in both wild type and phot1-5 phot2-1 double mutant, before (left) and two hours later (right) the blue-light treatment. (C) Differences between the stomatal opening before and two hours after the blue light treatment in both irradiated stomata and their nearest neighbours dark-adapted stomata. Wild-type irradiated stomata increased pore opening. In contrast, the irradiated stomata of the phot1-5 phot2-1 double mutant experienced no change. The nearest neighbours to the irradiated stomata remained unaltered in their movements. Bars indicate the mean of at least 15 measurements with standard deviations. Calcofluor staining (0.1%) produced a blue fluorescence in all cell walls when excited with a 405-nm laser line of a diode laser. White line shows the initial opening; red one represents the final aperture. Scale bar: 3 μm; all images are the same magnification.

doi:10.1371/journal.pone.0000036.g001
epidermal strips were exposed to prolonged blue plus red light [6] suggests that cryptochrome action might require a longer blue-light exposure, require a red background and/or function in a non-cell-autonomous manner.

In summary, we have demonstrated that stomata blue-light illumination is both sufficient and necessary to mediate stomatal opening, which, in addition, depends on PHOT1 and/or PHOT2 activity. This scenario is consistent with the observation that onion guard cells protoplasts swell when illuminated with blue light, but non-stomatal epidermal cell protoplasts do not swell under the same conditions [26]. The stomatal autonomy seems to extend in response to abscisic acid. Certainly, when single guard cells are injected with cyclic ADP-ribose, which mediates the abscisic acid-induced stomatal closure, its turgor decreases while those from the uninjected partner remains unchanged [22,27]. However, our finding that stomata act independently of the behaviour of those around them, contrasts with recent works suggesting that stomatal function is dictated by that of neighbour stomata [10,23,24], and opens the question on why the blue-light pulse has not a similar effect in the stomatal behaviour.

But, what advantage might stomata-autonomous function induced by blue-light confer on the plant? When a leaf is partially shaded by another leaf, incident blue-light irradiance is below the saturation value of phototropin action in the shaded region and above saturation in the lighted area (Figure 4). In addition, such change in blue-light irradiance occurs in a micrometric distance (Figure 4), similar to the average distance between two neighbour stomata [28]. In this context, the stomata-autonomous function would allow the opening of the lighted stoma, while maintaining the shaded neighbour one in a relatively closed state. This stomata-autonomous behaviour would optimise the balance between water loss and CO2 acquisition.

Light regulates many developmental and physiological processes in both plant and animal systems. Blue-light, for example, triggers de-etiolation, phototropic curvature, chloroplast movement, and stomatal opening [2,29,30]. The possibility of using the laser of a confocal microscope as a light source, opens an exciting and long way to investigate the cellular autonomy and/or cell-to-cell signalling in these and many others light-induced process.

MATERIALS AND METHODS

Plant materials and growth conditions

The blue-light dependent stomatal movements of the double mutant phot1-5 phot2-1 and its corresponding wild-type strain (Columbia) were previously described [4,6,31]. The phot1-5 mutant (originally nph1) is a null allele [7]. The phot2-1 mutant (originally npfl) results from a stop codon between the LOV2 and
The relative humidity in the growth chambers was maintained at 8 h of dark under fluorescent lamp (100 mól m⁻² s⁻¹). Seedlings were grown on soil at 20°C indicated [19]. Seedlings were grown on soil at 20°C and 16 h of light under fluorescent lamp (100 mól m⁻² s⁻¹). The relative humidity in the growth chambers was maintained at 60%.

Stomatal aperture measurements
Abaxial peels of mature, fully expanded leaves (3- to 6-week-old plants), were detached in the early morning and kept in the dark for 1 h under an incubation solution containing 0.1 mM CaCl₂ and 20 mM KCl. Dark-adapted peels were mounted on slides under a drop of the incubation solution. Apertures of randomly selected stomata were measured from transmission images taken at 10.000-fold magnification. They, or their three neighbour epidermal cells, were illuminated with blue light for 10 seconds. Two hours later, transmission images were again monitored to measure the final stomatal apertures. Controls were included in every set of laser irradiations. At least, 15 stomata, from a total of at least 8 leaves, were monitored for each treatment and genetic background. Results were evaluated by using χ²-test (99% confidence level).

Light transition measurements
Blue light (400–500 nm) was measured with a fibre optics touching the abaxial face of an Arabidopsis leaf. The fiber optics was connected to a spectroradiometer (Analytical Spectral Devices Field Spec Pro FR) and positioned at 100 m intervals with a calliper. The partial shade was produced by another Arabidopsis leaf placed 1 cm above the measured leaf. The leaf photograph was taken with a digital camera connected to an optic microscope. All data were collected at sunny middays.

ACKNOWLEDGMENTS
We thank W. R. Briggs for providing phot1-5 phot2-1 double mutant and PHOT1-GFP transgenic seeds; J. Doncel for technical advices about blue-light treatment; S. Verón, E. Goetz and M. Durante for helping in micro-scale light measurements; and S. Assmann for critically reading the manuscript.

Author Contributions
Conceived and designed the experiments: LS JC. Performed the experiments: RC HB. Analyzed the data: LS RC HB JC. Contributed reagents/materials/analysis tools: LS JC. Wrote the paper: LS.

REFERENCES
1. Schroeder JL, Allen GJ, Hugouvieux V, Kwak JM, Waner D (2001) Guard cell signal transduction. Annu Rev Plant Physiol Plant Mol Biol 52: 627–658.
2. Chen M, Chory J, Frankhauser C (2004) Light signal transduction in higher plants. Annu Rev Genet 38: 87–117.
3. Fan L-M, Zhao Z, Assmann SM (2004) Guard cells: a dynamic signalling model. Curr Opin Plant Biol 7: 537–546.
4. Kimokuta T, Dai M, Suetsugu N, Kagawa T, Wada M, et al. (2001) phot1 and phot2 mediate blue light regulation of stomatal opening. Nature 414: 656–660.
5. Talliot LD, Nikolova G, Ortiz A, Shamayevich I, Zeiger E (2002) Green light reversal of blue-light-stimulated stomatal opening is found in a diversity of plant species. Am J Bot 89: 366–368.
6. Mao J, Zhang Y-C, Sang Y, Li Q-H, Yang H-Q (2005) A role for Arabidopsis cryptochromes and COP1 in the regulation of stomatal opening. Proc Natl Acad Sci U S A 102: 12270–12275.
7. Huang E, Otter PW, Lucic E, Han JS, Larsen E, et al. (1997) Arabidopsis NPH1: A protein kinase with a putative redox-sensing domain. Science 278: 2121–2123.
8. Kagawa T, Sakai T, Suetsugu N, Ohkawa K, Ishiguro S, et al. (2001) Arabidopsis NPL1: A phototropin homolog controlling the chloroplast highlight avoidance response. Science 291: 2138–2141.
9. Karlsson PE (1986) Blue light regulation of stomata in wheat seedlings. II. Action spectrum and search for action dichromism. Physiol Plant 66: 207–210.
10. Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, et al. (2001) Arabidopsis nph1 and nph1: Blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci U S A 98: 6969–6974.

11. Christie JM, Salomon M, Nozue K, Wada M, Briggs WR (1999) LOV (light, oxygen, voltage) domains of the blue-light photoreceptor phototropin (nph1): Binding sites for the chromophore flavin mononucleotide. Proc Natl Acad Sci U S A 96: 8779–8783.

12. Eisiger W, Swartz TE, Bogomolni RA, Taiz L (2000) The ultraviolet action spectrum for stomatal opening in broad bean. Plant Physiol 122: 99–105.

13. Gallagher S, Short TW, Ray PM, Pratt LH, Briggs WR (1988) Light-mediated changes in two proteins found associated with plasma membrane fractions from pea stem sections. Proc Natl Acad Sci U S A 85: 8003–8007.

14. Hager A, Reich M (1993) Blue-light-induced phosphorylation of a plasma-membrane protein from phototropically sensitive tips of maize coleoptiles. Planta 189: 567–576.

15. Palmer JM, Short TW, Gallagher S, Briggs WR (1993) Blue-light-induced phosphorylation of a plasma membrane-associated protein in Zea mays L. Plant Physiol 102: 1211–1218.

16. Short TW, Reymond P, Briggs WR (1993) A pea plasma membrane protein exhibiting blue-light-induced phosphorylation retains photo-sensitivity following Triton solubilization. Plant Physiol 101: 647–655.

17. Salomon M, Zacherl M, Rüdiger W (1996) Changes in blue-light-dependent protein phosphorylation during the early development of etiolated seedlings. Planta 199: 336–342.

18. Sharma VK, Jain PK, Maheshwari S, Khurana JP (1997) Rapid blue-light-induced phosphorylation of plasma-membrane-associated proteins in wheat. Phytochemistry 44: 775–780.

19. Sakamoto K, Briggs WR (2002) Cellular and subcellular localization of phototropin I. Plant Cell 14: 1723–1735.

20. Kong SG, Suzuki T, Tamura K, Mochizuki N, Hara-Nishimura I, et al. (2006) Blue light-induced association of phototropin 2 with the Golgi apparatus. Plant J 45: 994–1005.

21. Mott KA, Buckley TN (2000) Patchy stomatal conductance: emergent collective behaviour of stomata. Trends Plant Sci 5: 258–262.

22. Hetherington AM, Woodward I (2003) The role of stomata in sensing and driving environmental change. Nature 424: 901–908.

23. Mott KA, Denne F, Powell J (1997) Interactions among stomata in response to perturbations in humidity. Plant Cell Environ 20: 1090–1107.

24. Mott KA, Franks PJ (2001) The role of epidermal turgor in stomatal interactions following a local perturbation in humidity. Plant Cell Environ 24: 657–662.

25. Zeiger E (1983) Biology of stomatal guard cells. Annu Rev Plant Physiol 34: 441–475.

26. Zeiger E, Hepler PK (1977) Light and stomatal function: Blue light stimulates swelling of guard cell protoplasts. Nature 196: 887–889.

27. Leckie CP, McArthur MR, Allen GJ, Sanders D, Hetherington AM (1998) Abscisic acid-induced stomatal closure mediated by cyclic ADP-ribose. Proc Natl Acad Sci U S A 95: 15037–15042.

28. Serna I, Fendoll C (2000) Stomatal development and patterning in Arabidopsis leaves. Physiol Plant 109: 351–358.

29. Briggs WR, Huala E (1999) Blue-light receptors in higher plants. Annu. Rev. Cell Dev Biol 15: 53–62.

30. Briggs WR, Christie JM (2002) Phototropins 1 and 2: versatile plant blue-light receptors. Trends Plant Sci 7: 204–210.

31. Talbott LD, Shayeovich IJ, Chung Y, Hammond JW, Zeiger E (2003) Blue light and phytochrome-mediated stomatal opening in the npq1 and phot1 pho2 mutants of Arabidopsis. Plant Physiol 133: 1522–1529.