Out of the blue; Phototropins of the leaf vascular bundle sheath mediate the blue light regulation of the leaf hydraulic conductance

Yael Grunwald [1*] Sanbon Chaka Gosa[1*] Nava Moran[1] Menachem Moshelion[1#]

[1]The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel

*Both authors contributed equally to this work

[#]Correspondence: menachem.moshelion@mail.huji.ac.il (M.Moshelion)

ABSTRACT

The leaf vascular bundle sheath cells (BSCs), which tightly envelop the leaf veins, are a selective dynamic barrier to water and solutes radially entering the mesophyll and play a major role in regulating the leaf radial hydraulic conductance (K_{leaf}). Blue light (BL) is known to increase K_{leaf}. Here we provide the mechanistic link for this phenomenon, based (a) on our recent demonstration that the BSCs' plasma membrane H^+-ATPase, AHA2, increases K_{leaf} by acidifying the xylem sap, and (b) on the presence of the BL receptor genes, *PHOT1*and *PHOT2*, in the BSCs.

The K_{leaf} of knockout mutant lines *phot1-5*, *phot2-1*, *phot1-5phot2-1* and *aha2-4* was lower than in WT and did not change under BL illumination. BSCs-directed (under a BSCs-specific promotor, SCR) respective complementation of *phot1-5* and *aha2-4* by *PHOT1* and *AHA2*, restored the normal K_{leaf}. BSC-directed knockdown of PHOT1 or PHOT2 expression sufficed to abolish the BL sensitivity of K_{leaf}. Xylem-fed tyrosine kinase inhibitor, tyrphostine 9, abolished the BL-induced K_{leaf} increase but not the BL-induced stomatal conductance increase. In parallel, in *phot1* mutants BL did not acidify the xylem sap, in contrast to WT and to leaves of *phot1* complemented with SCR: PHOT1. Our results link the blue light control of water fluxes from the xylem to the mesophyll via the BSCs: BL →BSCs' *PHOT*s activation →tyrosine phosphorylation→BSCs' H^{+-} ATPase activation →xylem acidification →K_{leaf} increase. Thus, a focus on the hydraulic valve in series with the stomata may provide new directions for crop manipulation for tolerance to the changing environment.

Introduction

Light is the energy source for photosynthesis. During evolution, plants have developed numerous mechanisms enabling them to compete over the light to optimize its absorption. In addition, light has also evolved as a signal that regulates growth and development (Kronenberg and Kendrick, 1986; Briggs and Huala, 1999) as well as physiological traits such as stomatal conductance (g_s) (Hsiao et al., 1973; Zeiger and Helper, 1977; Karlsson, 1986; Kinoshita et al., 2001; Talbott et al., 2003; van Ieperen et al., 2012) and leaf hydraulic conductance (K_{leaf}) (Voicu et al., 2008; Ben Baaziz et al., 2012; Aasamaa and Sõber, 2012; Prado and Maurel, 2013). One of the most conserved and well-studied light-sensing mechanisms is the blue light (BL, 390-550 nm) signal transduction pathway which invokes stomatal opening (Grondin et al., 2015). In this signal transduction pathway, BL is perceived by the guard cells (GC) light-activated protein kinases PHOT1 and PHOT2 (Kinoshita et al., 2001; Briggs and Christie, 2002); signals are eventually transmitted to the plasma membrane H^+-ATPases (Kinoshita and Shimazaki, 1999; Svennelid et al., 1999) which hyperpolarizes the GC plasma membrane and acidifies the GC apoplast (Kinoshita and Shimazaki, 1999; Ueno et al., 2005; Den Os et al., 2007; Elmore and Coaker, 2011). As a result, the hyperpolarization-activated inward-rectifying K^+ channels (K^+_{in}) are gated open enabling potassium ions (K^+) enter the GCs through the plasma-membrane by the direction of their electrochemical potential difference. Inward K^+ fluxes result in an osmolyte accumulation, which reduces the water potential inside the cell, thus eliciting the swelling of the GCs by an influx of water, enabling stomatal opening (Assmann, 1993; Roelfsema and Hedrich, 2005; Shimazaki et al., 2007; Oishi et al., 2010; Yamauchi et al., 2016). BL illumination has also been shown to increase the hydraulic conductance of the entire leaf in numerous plant species (Voicu et al., 2008 (bur oak); Voicu et al., 2009; Sellin et al., 2011 (silver birch); Aasamaa and Sber, 2012; Ben

Baaziz et al., 2012) . Interestingly, K_{leaf} has shown a faster response to light than g_s (Guyot et al., 2012). However, the molecular mechanism of K_{leaf} induced by BL is not yet fully understood.

In the past decade, it has been established that the bundle sheath cells (BSCs), a parenchymatous layer which tightly enwraps the entire leaf vasculature, can act as an active xylem-mesophyll selective barrier to water, and participate in K_{leaf} regulation (Shatil-Cohen and Moshelion, 2012; Sade et al., 2014; Grunwald et al., 2019). Moreover, we recently revealed that the AHA2, an H^+-ATPase proton pump, located in the BSCs participated in regulating $K_{leaf,}$ via changes in the xylem pH. There, K_{leaf} is positively correlated with BSCs-AHA2-driven xylem acidification which increases the osmotic water permeability of the BSC membranes (Grunwald et al., 2019). AHA2 was reported by (Wigoda et al., 2017) to have an over three-fold higher expression levels in the BSCs than in the neighboring mesophyll cells, explaining how such acidification is possible. In addition, the same BSC transcriptome analysis by (Wigoda et al., 2017) revealed that the BL receptors PHOT1 and PHOT2 were substantially expressed in the BSCs, as were also several other genes belonging to the BL signal transduction pathway (PHR2 (AT2G47590), CRY1 (AT4G08920), CRY2 (AT1G04400), HRB1 (AT5G49230)). These findings led us to hypothesize that a similar BL signal transduction pathway is active also in the BSCs and that it has a role in the regulation of K_{leaf}.

In confirmation of this hypothesis, we describe here some of the molecular and physiological details of the mechanism underlying the blue light-dependent K_{leaf} regulation by BSCs.

Materials and Methods

Plant material

Plant types. We used WT (wild type) *Arabidopsis thaliana* plants ecotype Colombia, Col-0 (aka Col) and T-DNA insertion AHA2 mutants *aha2-4* (Col) (SALK_ 082786) and SCR: AHA2 (line 56) as described in (Grunwald et al., 2019). *phot1-5 (nph1-5), phot 2-1(npl1-1 or cav1-1)* and the double mutant *phot1-5phot2-1 (npl1-5npl1-1)* as well as WT Arabidopsis *(Arabidopsis thaliana)* ecotype Glabrous (WT gl1), which were kindly provided by the Ken-Ichiro Shimazaki lab (Tokyo, Japan).

Construction of transgenic plant lines:

*SCR:mirPHOT Plants***:** The premiR-PHOT1or PHOT2 and synthetic genes were synthesized by Hylabs (Rehovot, Israel), based on a premiR164 backbone (Alvarez et al., 2006). We used the Web-based MicroRNA Designer (WMD, http://wmd3.weigelworld.org) to produce a premiRNA gene MIR319a as described in WMD. After sequence verification, the premiR-PHOT1or premiR-PHOT2 were cloned into the pDONR™ 221 and the SCR promoter into pDONRP4P1r (Invitrogen) vectors which are Gateway® compatible by BP reactions, and later cloned into the pB7M24GW (Invitrogen) two fragment binary vector by LR reaction according to the manufacturer's instructions. Each binary vector was transformed into Agrobacterium by electroporation and transformed to WT Col0 using the floral dip method (Clough and Bent, 1998). Transformants were selected based on their BASTA resistance, grown on plates with MS (Murashige and Skoog, Duchefa cat# M222.0050) Basal medium + 1 % sucrose and 20 µg/ml BASTA (Glufosinate Ammonium, Sigma cat # 45520). DNA insertion was verified in selected lines by PCR targeting the junction of the premiR-gene and the 35S terminator with forward primer about 1000bp from the 3' end of premiR-gene and reverse primer on

the 35S terminator (see primer list in supplemental Table S1), PCR fragments were then sequenced and verified.

SCR: PHOT1 plants: binary vectors were constructed with the PHOT1 gene as described above and then transformed into *phot1-5* (gl1) plants

Plant Growth Conditions: Plants were grown in soil (Klasmann686 Klasmann-Deilmann, Germany) + 4g/l Osmocote® 6M in a growth chamber at 22 °C and 70% relative humidity. During the 10-h light/14-h dark photoperiod, the illumination was provided by LED lights strips, of 150-200 µmol m^{-2} sec^{-1} light intensity at plant level (EnerLED 24 V-5630, 24 W/m, 3000 K (50%)/6000 K (50%)). The plants were irrigated twice a week.

Physiological characterization of the leaf (g_s and K_{leaf}).

Sample preparation: Fully expanded leaves from 7-8- week-old plants were excised at the petiole base using a sharp blade under a drop of "Artificial xylem sap" (AXS). Petioles were immediately perfused in 0.5 ml AXS filled Eppendorf tubes (AXS; 3 mM KNO3, 1 mM Ca(NO3)2, 1 mM MgSO4, 3 mM CaCl2, 0.25 mM NaH2PO4, 90 µM EDFC). The leaves were excised, shortly before "lights OFF" transition (around 5:00 pm) on the evening preceding the measurements and placed in gas-sealed transparent 25 cm x 20 cm x 15 cm plastic boxes with water-soaked tissue paper on the bottom to provide ~90% humidity. The transparent boxes were then inserted into a larger light-proof plastic box overnight and kept in total darkness until the start of light treatments in the next morning.

Light treatments

All gas exchange experiments were conducted in a dark room between 10:00 AM and 1:00 PM at a constant temperature of 22 °C. In all gas exchange experiments, excluding the K_{leaf} kinetics assay, the excised leaves were taken out of the light-sealed box, and

placed inside another sealed humid transparent box in one of two custom-made light chambers (either red light (RL) or combined red and blue light (R+BL), as specified further down). Boxes were left sealed for 7 minutes, and then opened for 3 minutes to expose the leaves to the ambient vapor pressure deficit (VPD) of 1.3-1.5 (10 minuts total ilumination in chamber) then, leaves were transfered to a Li-cor 6400xt for an additional 5 minutes, under the same illumination conditions. The total illumination intensity in both light chambers was set to roughly 215 µmol m^{-2} s^{-1}. In the RL chamber the leaves were illuminated only with red light (660 nm) and in the 'R+BL' chamber they were illuminated with approximetly 90% red light and approx. 10% blue light (450 nm). D-LED lighting with adjustable current source (DR-SD6/12) was used as light sources (http://www.d-led.net/). Light intensity was verified daily using a LI-250A light meter with a LI-190SA quantum sensor (LI-COR, Lincoln, NE, USA). In the K_{leaf} kinetics assay, light treatment durations varied: 0, 4, 6, 10, 15 or 30 minutes. For the 0 and 4 minutes durations, leaves were placed directly in the Li-cor 6400xt skipping the light chamber period.

Determination of gas exchange and hydraulic conductance, K_{leaf}. Immediately after the light treatments, the leaves were placed in a Li-cor 6400xt for the measurements of g_s and *E* (transpiration). The illumination in the cuvette was adjusted to match the preceding light treatments. Immediately thereafter, water potential (Ψ_{leaf}) was determined and K_{leaf} was calculated as described by (Grunwald et al., 2019), with the following changes: all experiments were conducted in a darkened room at 22 C^0.

Assays of inhibition of light transduction

Tyrphostin 9 (ENZO, cat. # BML-EI21, dissolved in DMSO (Sigma, cat # W387520) to a conc. of 100 mM and kept in aliquots at -18 ^{o}C), was added to the AXS to a final concentration of 10 μM for the overnight leaf perfusion.

Determination of xylem sap pH in detached leaves

Leaf perfusion: on the eve of the experiments, leaves from 6-7 week old plants, approximately 2.5 cm long and 1 cm wide, were excised with a sharp blade and perfused in AXS with the pH-sensitive dual excitation fluorescent probe, FITC-D (fluorescein isothiocyanate conjugated to 10 kD dextran, Sigma cat. #: FD10S), FITC-D was added to AXS from a 10 mM stock in water to a final concentration of 100 μM. The leaf samples were prepared on slides under low light conditions (<5 μmol m^{-2} sec^{-1}) and imaged on an inverted microscope (Olympus-IX8), as detailed in (Grunwald et al., 2019). Leaf samples were kept in the "double box" setup (i.e. sealed plastic box inside a dark box) in the dark until measurements in the next morning.

Dark-treated leaves: Leaves were taken out of the dark boxes and immediately laid on a microscope slide for vein imaging**.**

Light-treated leaves. Leaves were taken out of the dark boxes and placed in the growth chamber inside gas sealed boxes (boxes as in K_{leaf}) for 30 minutes of mixed illumination (see above), and were subsequently imaged.

Image capture and image analysis for intra-xylem pH determination were as described in (Grunwald et al., 2019).

2. RESULTS

Effect of blue light on K_{leaf} kinetics

In order to understand the hydraulic response of the leaf to light quality, we investigated the kinetics of K_{leaf} under two light regimes: red light alone (RL) and blue light superimposed on the background of red light (R+BL; see Materials and Methods). Under R+BL, K_{leaf} value reached its maximum within 10 minutes, and at the peak it was over seven-fold higher than K_{leaf} measured in dark treated leaves at time 0 (an increase from 13 to 91 mmol m^{-2} $sec^{-1}MPa^{-1}$; Fig. 1). RL treatment did not increase significantly the K_{leaf} values within the same time period.

The PHOT1 and PHOT2 light receptors involvement in K_{leaf} regulation

Based on the analysis of the Arabidopsis BSCs transcriptome (Wigoda et al., 2017), we revealed a substantial expression of the blue light receptor genes PHOT1 and PHOT2. To explore the role of these two light receptors in the regulation of K_{leaf}, we compared the K_{leaf} in knockout mutants lacking one or both light receptors to K_{leaf} of WT plants, under two light regimes (RL and R+BL). While K_{leaf} of WT leaves treated with R+BL was significantly higher than the K_{leaf} under RL treatment, R+BL did not seem to affect the K_{leaf} of the mutant lines lacking one or both light receptors, resulting in similar K_{leaf} values under both R+BL and RL treatments (Fig.2).

The kinase inhibitor, Tyrphostine 9, inhibits R+BL -induced K_{leaf} increase

The kinase inhibitor tyrphostine 9 was found to suppress the BHP (blue-light-dependent H^+-ATPase phosphorylation), a signaling mediator in blue light-dependent stomatal opening (Hayashi et al., 2017). To find out whether the vascular PHOT receptors activate a similar phosphorylating event in the light-dependent K_{leaf} regulation pathway, detached WT leaves were perfused with tyrphostin 9 and exposed to RL or R+BL treatments (Materials and Methods). The K_{leaf} values of R+BL -illuminated leaves pre-exposed to tyrphostin 9, were significantly lower than R+BL - illuminated leaves without tyrphostin,

and were no different than K_{leaf} in leaves illuminated with RL (whether or not exposed to tyrphostin 9; Fig.3A). Interestingly, while the R+BL treatment of the leaves increased their g_s, the xylem-fed tyrphostin 9 had no effect on g_s irrespective of the illumination regime and did not affect the increase of g_s under the R+BL treatment (Fig.3B). Perhaps the difference in our mode of tyrphostin application is the explanation: a) we used 10 µM tyrphostin instead of 50 µM (in Hayashi's work), b) while Hayashi et al applied tyrphostin in a solution smeared on the leaf, we applied it in a xylem perfusate via a petiole. We have already established the different effectiveness of ABA applied via these two routes (petiole-fed ABA lowered K_{leaf} while ABA smeared on the leaf did not; Shatil-Cohen et al., 2011)

BSCs-specific silencing of PHOT1 and PHOT2 receptors

Tyrphostin 9 reduction of K_{leaf} , resembling the tyrphostin 9 inhibition of the BL-PHOT pathway in GCs (Hayashi et al., 2017), strengthened our hypothesis that the PHOT receptors are involved also in a BL-pathway, which regulates K_{leaf}. To further focus our exploration on these light receptors in the BSCs, we silenced either the *PHOT*1 or the *PHOT*2 gene using amiRNA (artificial microRNA) under the BSCs-specific promotor, SCARECROW (SCR, see Materials and Methods). The K_{leaf} values of leaves from SCR: mirphot1 and SCR: mirphot2 were both significantly lower than the K_{leaf} values of WT leaves under R+BL treatment (Fig. 4).

BSCs-specific complementation of the *phot1* mutant with PHOT1 restored the normal K_{leaf}. *phot1-5* mutant (in gl background) plants were complemented with SCR: *PHOT1* to restore PHOT1 activity specifically in the BSCs (Materials and Methods). Fully expanded leaves of WT (gl ecotype) plants, *phot* 1-5 and SCR: *PHOT1* plants were illuminated and assayed as described in (Fig.2). Illumination of R+BL increased the K_{leaf} in the complemented plants to the values of WT (Fig. 5).

Light acidifies the xylem-sap and this is mediated by AHA2

During BL-induced stomatal opening, H^+-ATPases are activated extruding protons from the GCs and thus acidifying the apoplast around them. In order to find out whether light acidifies similarly the BSC apoplast, i.e., the xylem, we measured the intra-xylem pH in detached leaves comparing leaves without and with an exposure to 30 minutes of mixed illumination in the growth chamber following an overnight dark treatment (Materials and Methods). This comparison included leaves of WT and of *phot1-5* plants. Illumination of 30 minutes resulted in a significant acidification of the xylem sap in WT plants (by ~0.6 pH units, from 6.3 to 5.7), while the xylem pH of *phot1-5* remained unchanged. In contrast to the *phot1-5* plants, in the *phot1-5* mutants complemented with *PHOT1* directed specifically to the BSCs (i.e., under the SCR promoter), the 30 min exposure to light in the growth chamber decreased the xylem pH as in the WT (by ~0.7 pH units, from 6.4 to 5.7; Fig. 6). Recently we reported that AHA2 plays a major role in the K_{leaf} regulation by acidifying the xylem sap. To test whether AHA2 participates in the blue light-operated K_{leaf} regulation pathway, we examined the AHA2 knockout mutant *aha2-4*, and its BSCs-complemented transgenic plant line SCR:*AHA2* (i.e., the *aha2-4* mutant with AHA2 expressed only in its BSCs (Grunwald et al., 2019) under two light regimes (RL and R+BL). K_{leaf} of BL treated *aha2-4* leaves did not respond to the R+BL treatments and, unsurprisingly, was significantly lower than K_{leaf} of the R+BL treated WT leaves. In contrast, the complemented AHA2 (SCR:*AHA2*) plants treated with R+BL, revealed higher K_{leaf} than *aha2-4*, indicating that K_{leaf} regulation by R+BL was restored in these complemented plants depending on AHA2 (Fig. 7).

Discussion

Relative rate of response to R+BL illumination: K_{leaf} increase vs. stomata opening

Testing our BSCs autonomous BL signal transduction pathway hypothesis, we first studied the kinetics of K_{leaf} induction by R+BL and determined that this signal was effective already after 10 minutes of induction. In turn, stomata were reported to reach maximum aperture after approximately 30 minutes of exposure to light (Grondin et al., 2015), lagging behind K_{leaf} increase by approximately 20 minutes. Interestingly, K_{leaf} is known to respond faster than g_s also to other signals such as a decline of leaf water potential, ABA and drought (Martre et al., 2002; Shatil-Cohen et al., 2011; Sade et al., 2014; Scoffoni et al., 2014).

A simple consideration of all these comparisons shows they are invalid: their common denominator is the larger number of steps involved in gs increase (i.e., stomata opening) than in K_{leaf} increase. More suitably, one should compare how quickly H^+-ATPases are activated (phosphorylated) in these two cell types upon BL perception.

Beyond proton-pump activation – the paths of these two responses diverge and become different:

(a) The apoplast volume undergoing acidification relative to the activated proton-pumping membrane area in BSCs *vs.* GCs are likely to differ and hence – also the apoplastic pH attained; (b) The change in membrane permeability is different: in BSCs, the P_f is upregulated by external acidification which is reflected directly in K_{leaf} increase; in GCs, the Kin channel conductance is increased by hyperpolarization and acidification combined, and the P_f of GCs is probably also increased by apoplastic acidification; (c) And lastly, GCs swelling (i.e., accumulation of osmolytes and water till stomata opening) – constitutes an extra time-consuming step without a parallel in the process of BSCs K_{leaf} increase.

<u>BL signal transduction in the BSCs</u>

Stomatal opening as well as chloroplasts movement (Zeiger et al., 1983; Sakai et al., 2001) in response to R+BL are considered a "classical" short-term BL induction. In this study we confirmed that also in BSCs both PHOT1 and PHOT2 are required for the perception of the BL signal which increases the K_{leaf} (Figs. 2, 5). Furthermore, as found by (Hayashi et al., 2017) for stomata opening, also in BSCs, a tyrosine phosphorylation step is part of the BL signaling. We extended the parallel between the two signaling systems by showing that both the opening of stomata by BL and the increase of K_{leaf} by BL depend on the BL activation of the plasma membrane H^+-ATPase, present in the guard cells and in the BSCs (Figs. 6, 7).

The results from our genetic manipulations limited solely to the BSCs once again demonstrated the identification of K_{leaf} as reflecting the water permeability property of the BSCs and, moreover, localized the elements of the BL transduction pathway to the BSCs. Thus, the selective knockdown of the *PHOT1* or *PHOT2* in the BSCs (Fig. 4) which rendered the K_{leaf} insensitive to BL, or the complementation of *phot1* with BSCs-directed *PHOT1*, which restored this sensitivity (Fig. 5), combined with our demonstration that the light signal perceived by PHOTs in the BSCs is transduced onto the activation of the BSCs proton pump, AHA2 (Fig.7). The role of aquaporins in controlling K_{leaf} is a phenomenon already identified earlier (in Arabidopsis, Sade et al., 2014 and in walnut (Ben Baaziz et al., 2012).

Recently, we have already linked the acidification of the xylem sap by AHA2 to the increase of Kleaf (Grunwald et al., 2019). The fact that both are activated via the BSCs' PHOT receptors, serves as strong evidence for the following sequence of events:

BL →BSCs' *PHOT*s activation →tyrosin phosphorylation* →BSCs' H^+-ATPase activation →xylem acidification →K_{leaf} increase.

* Notably, the abovementioned tyrosine phosphorylation step – suggested to occur on the BHP protein in the guard cells, upstream of the H^+-ATPase (Hayashi et al., 2017) – is yet to be explored in the BSCs; currently, our guess is that it is similarly upstream of the BSCs' AHA2 (Fig. 8).

The physiological relevance of K_{leaf} enhancement by R+BL

Several studies reported an increase in K_{leaf} in the early morning hours (e.g., Brodribb and Holbrook, 2004; Domec et al., 2009). What is the advantage of such early K_{leaf} response to light? One possible advantage could be to prevent a hydraulic pathway failure which could occur in the morning when stomatal conductance is at its peak (Brodribb and Holbrook, 2004; Halperin et al., 2017), by increasing the K_{leaf} to ensure maximum water supply in this special "Golden hour" (Gosa et al., 2019). The fact that during this special time, the VPD (vapor pressure deficit, a measure of the driving force for leaf water evaporation) is still relatively low, weakens this assumption.

Another hypothetical explanation for this high K_{leaf} responsivity to R+BL may be related to CO_2 permeability via aquaporins. A few lines of evidence converge to support this hypothesis: (a) CO_2 can cross cell membranes through aquaporins (Uehlein et al., 2003; Tyerman et al., 2002; Kaldenhoff and Fischer, 2006; Heckwolf et al., 2011; Uehlein et al., 2012); (b) we demonstrated that aquaporins control K_{leaf} (Shatil-Cohen and Moshelion, 2012; Sade et al., 2014); (c) moreover, several studies suggested that xylem-transported CO_2 could be a source for CO_2 assimilated in the bundle sheath and mesophyll (Janacek et al., 2009; Hubeau et al., 2019) .

Thus, if the K_{leaf} increase with the first light of day parallels the increase of CO_2 permeability of aquaporins in the BSCs, the passage to the mesophyll of CO_2 from the xylem coming from respiration in the roots will be enhanced even before full stomatal opening. The increased CO_2 availability at this time, when the photosynthetic light is

already sufficient, would enhance CO_2 assimilation – a great advantage to the plant. Interestingly, photosynthetic CO_2 uptake reaches a maximum within 10 minutes of blue light ((Doi et al., 2015), similar to the rate of K_{leaf} increase we observed (Fig. 1), but over threefold faster than the full opening of stomata (Grondin et al., 2015). In conclusion, our data provide new evidence for the role of the BSCs' phototropins (*PHOT1* and *PHOT2*) in the *AHA2*-mediated increase of K_{leaf} by light. These findings provide new insights for better understanding the molecular basis of the leaf water influx regulation. Thus, a focus on the hydraulic valve in series with the stomata, may provide new directions for crop manipulation for tolerance to the changing climate.

References

Aasamaa, K. and Sõber, A. (2012). Light sensitivity of shoot hydraulic conductance in five temperate deciduous tree species. Funct. Plant Biol. **39**: 661.

Alvarez, J.P., Pekker, I., Goldshmidt, A., Blum, E., Amsellem, Z., and Eshed, Y. (2006). Endogenous and Synthetic MicroRNAs Stimulate Simultaneous, Efficient, and Localized Regulation of Multiple Targets in Diverse Species. Plant Cell **18**: 1134–1151.

Assmann, S. (1993). Signal Transduction in Guard Cells. Annu. Rev. Cell Dev. Biol. **9**: 345–375.

Ben Baaziz, K., Lopez, D., Rabot, A., Combes, D., Gousset, A., Bouzid, S., Cochard, H., Sakr, S., and Venisse, J.-S. (2012). Light-mediated Kleaf induction and contribution of both the PIP1s and PIP2s aquaporins in five tree species: walnut (Juglans regia) case study. Tree Physiol. **32**: 423–434.

Briggs, W.R. and Christie, J.M. (2002). Phototropins 1 and 2: Versatile plant blue-light receptors. Trends Plant Sci. **7**: 204–210.

Briggs, W.R. and Huala, E. (1999). Blue-Light Photoreceptors in Higher Plants. Annu.

Rev. Cell Dev. Biol. **15**: 33–62.

Brodribb, T.J. and Holbrook, N.M. (2004). Diurnal depression of leaf hydraulic conductance in a tropical tree species. Plant, Cell Environ. **27**: 820–827.

Clough, S.J. and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. **16**: 735–743.

Doi, M., Kitagawa, Y., and Shimazaki, K.-I. (2015). Stomatal Blue Light Response Is Present in Early Vascular Plants. Plant Physiol. **169**: 1205–1213.

Domec, J.C., Noormets, A., King, J.S., Sun, G., McNulty, S.G., Gavazzi, M.J., Boggs, J.L., and Treasure, E.A. (2009). Decoupling the influence of leaf and root hydraulic conductances on stomatal conductance and its sensitivity to vapour pressure deficit as soil dries in a drained loblolly pine plantation. Plant, Cell Environ. **32**: 980–991.

Elmore, J.M. and Coaker, G. (2011). The Role of the Plasma Membrane H+-ATPase in Plant–Microbe Interactions. Mol. Plant **4**: 416–427.

Gosa, S.C., Lupo, Y., and Moshelion, M. (2019). Quantitative and comparative analysis of whole-plant performance for functional physiological traits phenotyping: New tools to support pre-breeding and plant stress physiology studies. Plant Sci. **282**: 49–59.

Grondin, A., Rodrigues, O., Verdoucq, L., Merlot, S., Leonhardt, N., and Maurel, C. (2015). Aquaporins Contribute to ABA-Triggered Stomatal Closure through OST1-Mediated Phosphorylation. Plant Cell **27**: 1945–1954.

Grunwald, Y., Wigoda, N., Sade, N., Yaaran, A., Torne, T., Gosa, S., Moran, N., and Moshelion, M. (2019). Bundle-sheath cells are leaf “water valves” controlled via xylem acidification by H+-ATPase. bioRxiv Prepr.

Guyot, G., Scoffoni, C., and Sack, L. (2012). Combined impacts of irradiance and

dehydration on leaf hydraulic conductance: insights into vulnerability and stomatal control. Plant. Cell Environ. **35**: 857–871.

Halperin, O., Gebremedhin, A., Wallach, R., and Moshelion, M. (2017). High-throughput physiological phenotyping and screening system for the characterization of plant-environment interactions. Plant J. **89**: 839–850.

Hayashi, M., Inoue, S., Ueno, Y., and Kinoshita, T. (2017). A Raf-like protein kinase BHP mediates blue light-dependent stomatal opening. Sci. Rep. **7**: 45586.

Heckwolf, M., Pater, D., Hanson, D.T., and Kaldenhoff, R. (2011). The Arabidopsis thaliana aquaporin AtPIP1;2 is a physiologically relevant CO2 transport facilitator. Plant J. **67**: 795–804.

Hsiao, T.C., Allaway, W.G., and Evans, L.T. (1973). Action Spectra for Guard Cell Rb + Uptake and Stomatal Opening in Vivia faba. Plant Physiol. **51**: 82–88.

Hubeau, M., Thorpe, M.R., Mincke, J., Bloemen, J., Bauweraerts, I., Minchin, P.E.H., De Schepper, V., De Vos, F., Vanhove, C., Vandenberghe, S., and Steppe, K. (2019). High-Resolution in vivo Imaging of Xylem-Transported CO2 in Leaves Based on Real-Time 11C-Tracing. Front. For. Glob. Chang. **2**: 25.

van Ieperen, W., Savvides, A., and Fanourakis, D. (2012). Red and blue light effects during growth on hydraulic and stomatal conductance in leaves of young cucumber plants. Acta Hortic.: 223–230.

Janacek, S.H., Trenkamp, S., Palmer, B., Brown, N.J., Parsley, K., Stanley, S., Astley, H.M., Rolfe, S.A., Paul Quick, W., Fernie, A.R., and Hibberd, J.M. (2009). Photosynthesis in cells around veins of the C 3 plant Arabidopsis thaliana is important for both the shikimate pathway and leaf senescence as well as contributing to plant fitness. Plant J. **59**: 329–343.

Kaldenhoff, R. and Fischer, M. (2006). Aquaporins in plants. Acta Physiol. **187**: 169–

176.

Karlsson, P.E. (1986). Blue light regulation of stomata in wheat seedlings. II. Action spectrum and search for action dichroism. Physiol. Plant. **66**: 207–210.

Kinoshita, T., Doi, M., Suetsugu, N., Kagawa, T., Wada, M., and Shimazaki, K. (2001). Phot1 and Phot2 mediate blue light regulation of stomatal opening. Nature **414**: 656–660.

Kinoshita, T. and Shimazaki, K. (1999). Blue light activates the plasma membrane H+-ATPase by phosphorylation of the C-terminus in stomatal guard cells. EMBO J. **18**: 5548–5558.

Kronenberg, G.H.M. and Kendrick, R.E. (1986). The physiology of action. In Photomorphogenesis in plants (Springer Netherlands: Dordrecht), pp. 99–114.

Martre, P., Morillon, R., Barrieu, F., North, G.B., Nobel, P.S., and Chrispeels, M.J. (2002). Plasma Membrane Aquaporins Play a Significant Role during Recovery from Water Deficit. Plant Physiol. **130**: 2101–2110.

Oishi, A.C., Oren, R., Novick, K.A., Palmroth, S., and Katul, G.G. (2010). Interannual Invariability of Forest Evapotranspiration and Its Consequence to Water Flow Downstream. Ecosystems **13**: 421–436.

Den Os, D., Staal, M., and Elzenga, J.T.M. (2007). Signal integration by ABA in the blue light-induced acidification of leaf pavement cells in pea (Pisum sativum L. var. Argenteum). Plant Signal. Behav. **2**: 146–152.

Prado, K. and Maurel, C. (2013). Regulation of leaf hydraulics: from molecular to whole plant levels. Front. Plant Sci. **4**: 1–14.

Roelfsema, M.R.G. and Hedrich, R. (2005). In the light of stomatal opening: new insights into 'the Watergate.' New Phytol. **167**: 665–691.

Sade, N., Shatil-Cohen, A., Attia, Z., Maurel, C., Boursiac, Y., Kelly, G., Granot, D.,

Yaaran, A., Lerner, S., and Moshelion, M. (2014). The Role of Plasma Membrane Aquaporins in Regulating the Bundle Sheath-Mesophyll Continuum and Leaf Hydraulics. PLANT Physiol. **166**: 1609–1620.

Sakai, A.K. et al. (2001). The Population Biology of Invasive Species. Annu. Rev. Ecol. Syst.

Scoffoni, C., Vuong, C., Diep, S., Cochard, H., and Sack, L. (2014). Leaf Shrinkage with Dehydration: Coordination with Hydraulic Vulnerability and Drought Tolerance. Plant Physiol. **164**: 1772–1788.

Sellin, A., Sack, L., Õunapuu, E., and Karusion, A. (2011). Impact of light quality on leaf and shoot hydraulic properties: A case study in silver birch (Betula pendula). Plant, Cell Environ. **34**: 1079–1087.

Shatil-Cohen, A., Attia, Z., and Moshelion, M. (2011). Bundle-sheath cell regulation of xylem-mesophyll water transport via aquaporins under drought stress: a target of xylem-borne ABA? Plant J. **67**: 72–80.

Shatil-Cohen, A. and Moshelion, M. (2012). Smart pipes. Plant Signal. Behav. **7**: 1088–1091.

Shimazaki, K., Doi, M., Assmann, S.M., and Kinoshita, T. (2007). Light Regulation of Stomatal Movement. Annu. Rev. Plant Biol. **58**: 219–247.

Svennelid, F., Olsson, A., Piotrowski, M., Rosenquist, M., Ottman, C., Larsson, C., Oecking, C., and Sommarin, M. (1999). Phosphorylation of Thr-948 at the C Terminus of the Plasma Membrane H-ATPase Creates a Binding Site for the Regulatory 14-3-3 Protein.

Talbott, L.D., Shmayevich, I.J., Chung, Y., Hammad, J.W., and Zeiger, E. (2003). Blue Light and Phytochrome-Mediated Stomatal Opening in the npq1 and phot1 phot2 Mutants of Arabidopsis. Plant Physiol. **133**: 1522–1529.

Tyerman, S.D., Niemietz, C.M., and Bramley, H. (2002). Plant aquaporins: multifunctional water and solute channels with expanding roles. Plant, Cell Environ. **25**: 173–194.

Uehlein, N., Lovisolo, C., Siefritz, F., and Kaldenhoff, R. (2003). The tobacco aquaporin NtAQP1 is a membrane CO2 pore with physiological functions. Nature **425**: 734–737.

Uehlein, N., Sperling, H., Hekwolf, M., and Kaldenfoff, R. (2012). The Arabidopsis aquaporin PIP1;2 rules cellular CO2 uptake. Plant. Cell Environ. **35**: 1077–1083.

Ueno, K., Kinoshita, T., Inoue, S., Emi, T., and Shimazaki, K. (2005). Biochemical Characterization of Plasma Membrane H+-ATPase Activation in Guard Cell Protoplasts of Arabidopsis thaliana in Response to Blue Light. Plant Cell Physiol. **46**: 955–963.

Voicu, M.C., Cooke, J.E.K., and Zwiazek, J.J. (2009). Aquaporin gene expression and apoplastic water flow in bur oak (Quercus macrocarpa) leaves in relation to the light response of leaf hydraulic conductance. J. Exp. Bot. **60**: 4063–4075.

Voicu, M.C., Zwiazek, J.J., and Tyree, M.T. (2008). Light response of hydraulic conductance in bur oak (Quercus macrocarpa) leaves. Tree Physiol. **28**: 1007–1015.

Wigoda, N., Pasmanik-Chor, M., Yang, T., Yu, L., Moshelion, M., and Moran, N. (2017). Differential gene expression and transport functionality in the bundle sheath versus mesophyll – a potential role in leaf mineral homeostasis. J. Exp. Bot. **68**: 3179–3190.

Yamauchi, S., Takemiya, A., Sakamoto, T., Kurata, T., Tsutsumi, T., Kinoshita, T., and Shimazaki, K. (2016). Plasma membrane H+-ATPase1 (AHA1) plays a major role in Arabidopsis thaliana for stomatal opening in response to blue light. Plant Physiol.: pp.01581.2016.

Zeiger, E., Assmann, S.M., and Meioner, H. (1983). The Photobiology of Paphiopedilum stomata: opening under blue lightbut not red light. Photochem. Photobiol. **38**: 627–630.

Zeiger, E. and Helper, P.K. (1977). Light and Stomatal Function: Blue Light Stimulates Swelling of Guard Cell Protoplasts. Science (80-.). **196**: 887–889.

Figure legends

Figure 1: K_{leaf} response to R+BL illumination. Time course of the induction of leaf hydraulic conductance (K_{leaf}) in response to RL or R+BL illumination. Immediately after 16 h of dark, fully expanded excised leaves of WT Arabidopsis (ecotype Col0) were illuminated immediately after dark for different durations (0-30 min) with RL (215 µmol m^{-2} s^{-1}) or R+BL (200 µmol m^{-2} s^{-1} red + 20 µmol m^{-2} s^{-1} blue). Data are means (±SE) of 8-20 biological repeats determined in three independent experiments. Different letters indicate significant differences among the means (*Post hoc* Tukey's test, $P<0.05$). Note the dramatic effect, peaking in 10 min, of BL light addition.

Figure 2: **The effect of blue light on Kleaf of PHOT receptors mutants.** K_{leaf} of fully expanded excised leaves of WT (ecotype gl) and *PHOT* mutants (*phot1-5*, *phot2-1* and a double mutant, *phot1-5phot2-1*) after illumination for 15 min immediately after dark, with RL (215 µmol m^{-2} s^{-1}), or R+BL (215 µmol m^{-2} s^{-1} with 90% red light 10% blue light). Each column indicates mean values (±SE) of 8-25 biological repetitions measured on three independent experimental days. Different letters denote significantly different values (*Post hoc* Tukey's test $P< 0.05$). Note the lack of response to R+BL in the mutants.

Figure 3. **Xylem-fed kinase Inhibitor Tyrphostine 9 abolished the blue-light-induced K_{leaf} increase.** Fully expanded leaves of WT (gl) were pre-incubated (petiole deep)

overnight in darkness in AXS with or without Tryphostine A9 (10 μm) and then, immediately after dark, illuminated for 15 min with RL (215 µmolm^{-2} s^{-1} red) or R+BL (215 µmol m^{-2} s^{-1} with 90% red light 10% blue light). **A.** K_{leaf}. **B.** Stomatal conductance (gs). Columns are means ± SE (n = 20), determined in three independent experiments. Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05). Note that gs was not affected by tyrphostine.

Figure 4. The effect of R+BL on the leaf hydraulic conductance of *PHOT1*- and *PHOT2*- silenced (SCR:mir) plants. SCR:mir plants were generated in the WT (Col 0) background. Fully expanded leaves of WT and SCR: mirphot1 and phot2 plants were treated with light as described in Fig.2. Columns are means ± SE (n = 12-15), determined in three independent experiments. Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05). Note the lack of R+BL effect on the leaves of plants "amiR-mutated' solely in their BSCs

Figure 5. BSCs-directed *PHOT1* complementation of the *phot1-5* mutant restores the normal K_{leaf}. *Phot1-5* (in gl background) plants were complemented with SCR: *PHOT1* restoring PHOT1 activity specifically in the BSCs. Fully expanded leaves of WT (gl ecotype), *phot1-5* and SCR: *PHOT1* were treated with light as described in Fig.2. Columns are means ± SE (n = 12-15), obtained in three independent experiments. Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05).

Figure 6. White light treatment for 30 minutes acidifies the xylem pH of WT plants and SCR:*PHOT1*-complemented mutants, but not in the *phot1-5* mutant. The columns are means (±SE; n = 10-12) determined in three independent experiments (150-200 µmol m^{-2} sec^{-1} white light). Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05).

Figure 7. The BSCs H$^+$-ATPase, AHA2, mediates the blue-light-induced K_{leaf} increase. Fully expanded excised leaves of WT (ecotype Col0) plants, *aha2-4* mutant plants (in Col0 background) and *aha2-4* plants complemented in their BSCs with SCR:*AHA2* were illuminated for 15 min immediately after dark, with light as described in

Fig.2. The columns are means (±SE; n=8-10) determined in three independent experiments. Different letters denote significantly different values ($P < 0.05$; ANOVA). Note the marked restoration of K_{leaf} in the *aha2* mutant plants complemented solely in their BSCs.

Figure 8. Proposed pathway BSCs autonomous BL signal transduction pathway. A. Artist's rendering of a leaf radial water path, from xylem to mesophyll (K_{leaf}, blue hollow black arrow) via the BSCs, which tightly envelop the xylem. **B.** Blue-light (BL) signalling pathway (blue arrowheads) in a bundle sheath cell, from BL perception by the phototropin receptors (yellow circle), through an intermediate tyrphostin-sensitive tyrosine phosphorylation (TP), to the ultimate AHA2 (orange circle) activation resulting in proton extrusion via the pump and xylem sap acidification, presumably, at the expense of ATP breakdown to ADP with a transient phosphorylation (P) on the pump protein, as expected from a P-type H^+-ATPase.

Supplemental Table

Supplemental Table 1: List of Primers used in this work.

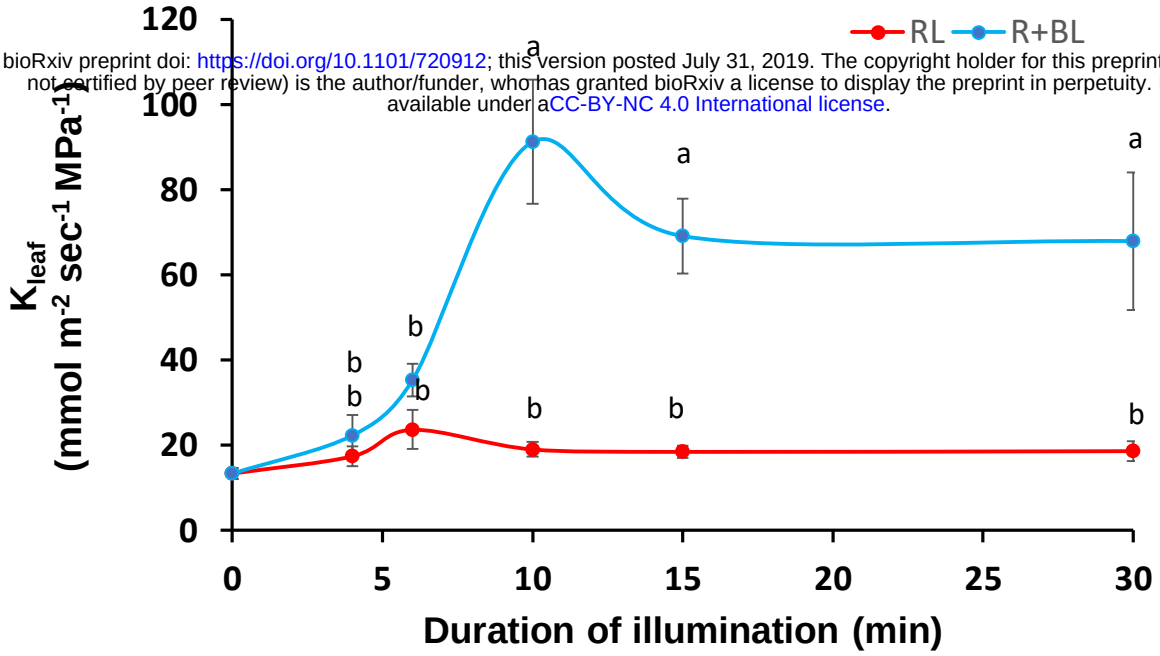

Figure 1: K_{leaf} response to R+BL illumination. Time course of the induction of leaf hydraulic conductance (K_{leaf}) in response to RL or R+BL illumination. Immediately after 16 h of dark, fully expanded excised leaves of WT Arabidopsis (ecotype Col0) were illuminated immediately after dark for different durations (0-30 min) with RL (215 µmol m^{-2} s^{-1}) or R+BL (215 µmol m^{-2} s^{-1} with 90% red light 10% blue light). Data are means (±SE) of 8-20 biological repeats determined in three independent experiments. Different letters indicate significant differences among the means (*Post hoc* Tukey's test, P<0.05). Note the dramatic effect, peaking in 10 min, of BL light addition.

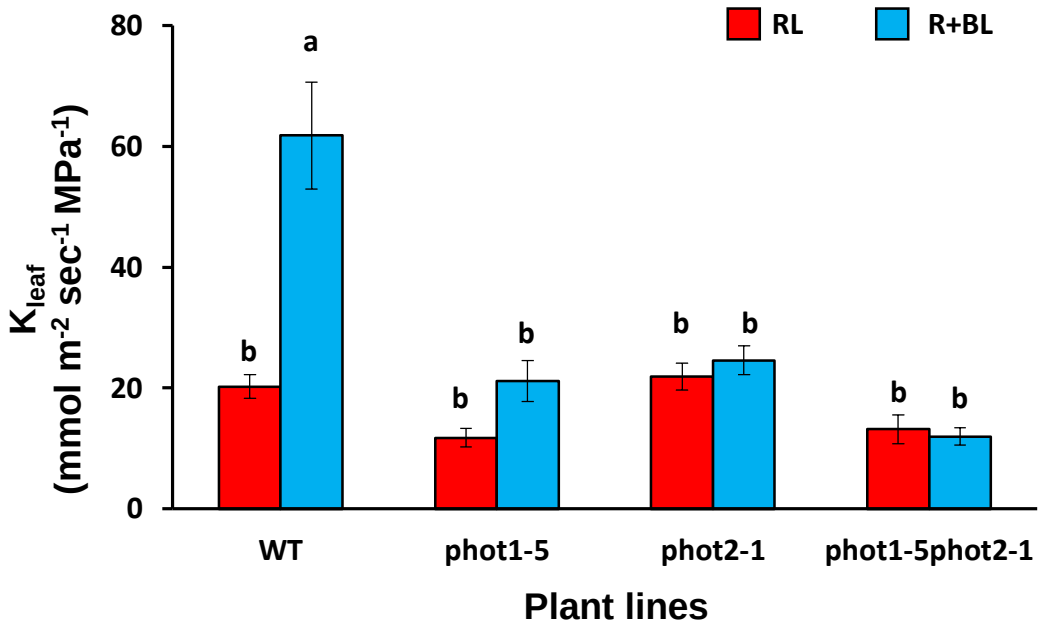

Figure 2: **The effect of blue light on K_{leaf} of PHOT receptors mutants.** K_{leaf} of fully expanded excised leaves of WT (ecotype gl) and *PHOT* mutants (*phot1-5*, *phot2-1* and a double mutant, *phot1-5phot2-1*) after illumination for 15 min immediately after dark, with RL (215 µmol m^{-2} s^{-1}), or R+BL (215 µmol m^{-2} s^{-1} with 90% red light 10% blue light). Each column indicates mean values (±SE) of 8-25 biological repetitions measured on three independent experimental days. Different letters denote significantly different values (*Post hoc* Tukey's test $P< 0.05$) . Note the lack of response to R+BL in the mutants.

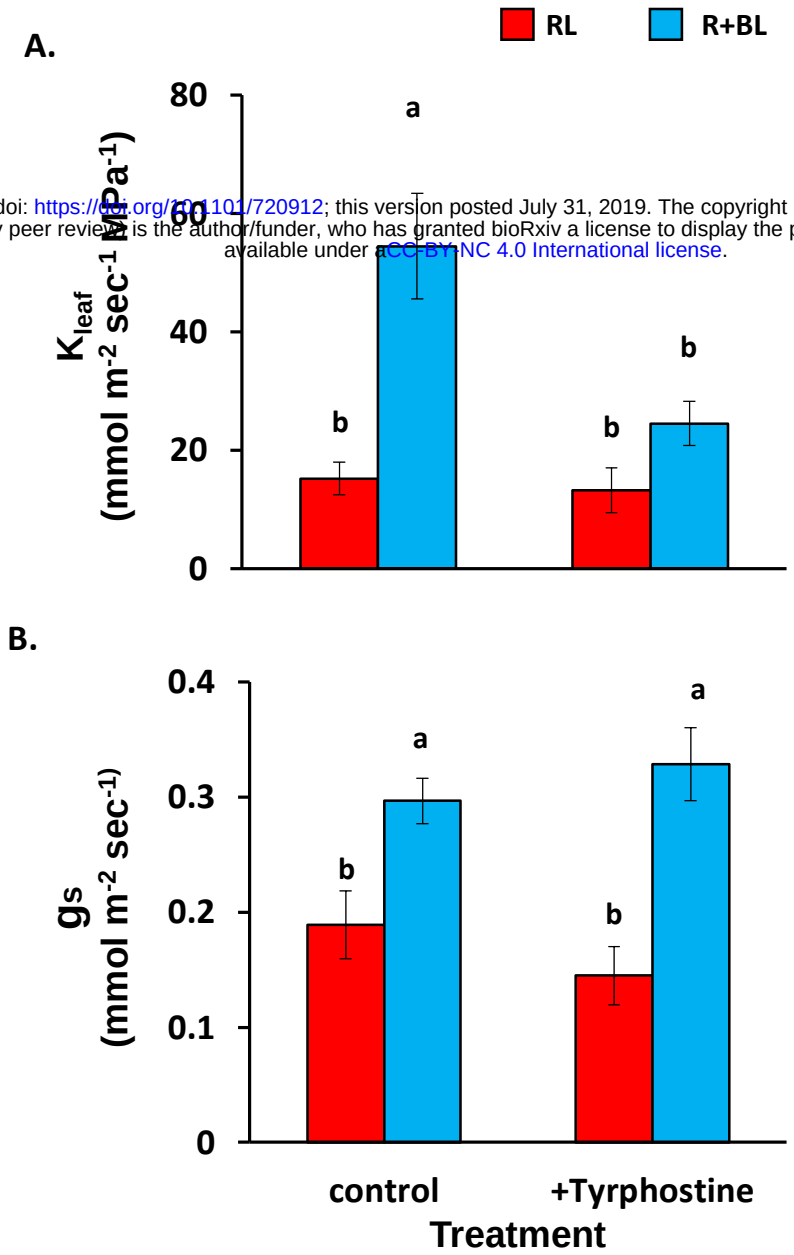

Figure 3. **Xylem-fed kinase Inhibitor Tyrphostine A9 abolished the blue-light-induced K_{leaf} increase.** Fully expanded leaves of WT (gl) were pre-incubated (petiole deep) overnight in darkness in AXS with or without Tryphostine A9 (10 μM) and, immediately after dark, illuminated for 15 min with RL (215 µmolm^{-2} s^{-1} red) or R+BL (215 µmol m^{-2} s^{-1} with 90% red light 10% blue light). **A.** K_{leaf}. **B.** Stomatal conductance (gs). Columns are means ± SE (n = 20), determined in three independent experiments. Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05). Note that gs was not affected by tyrphostine.

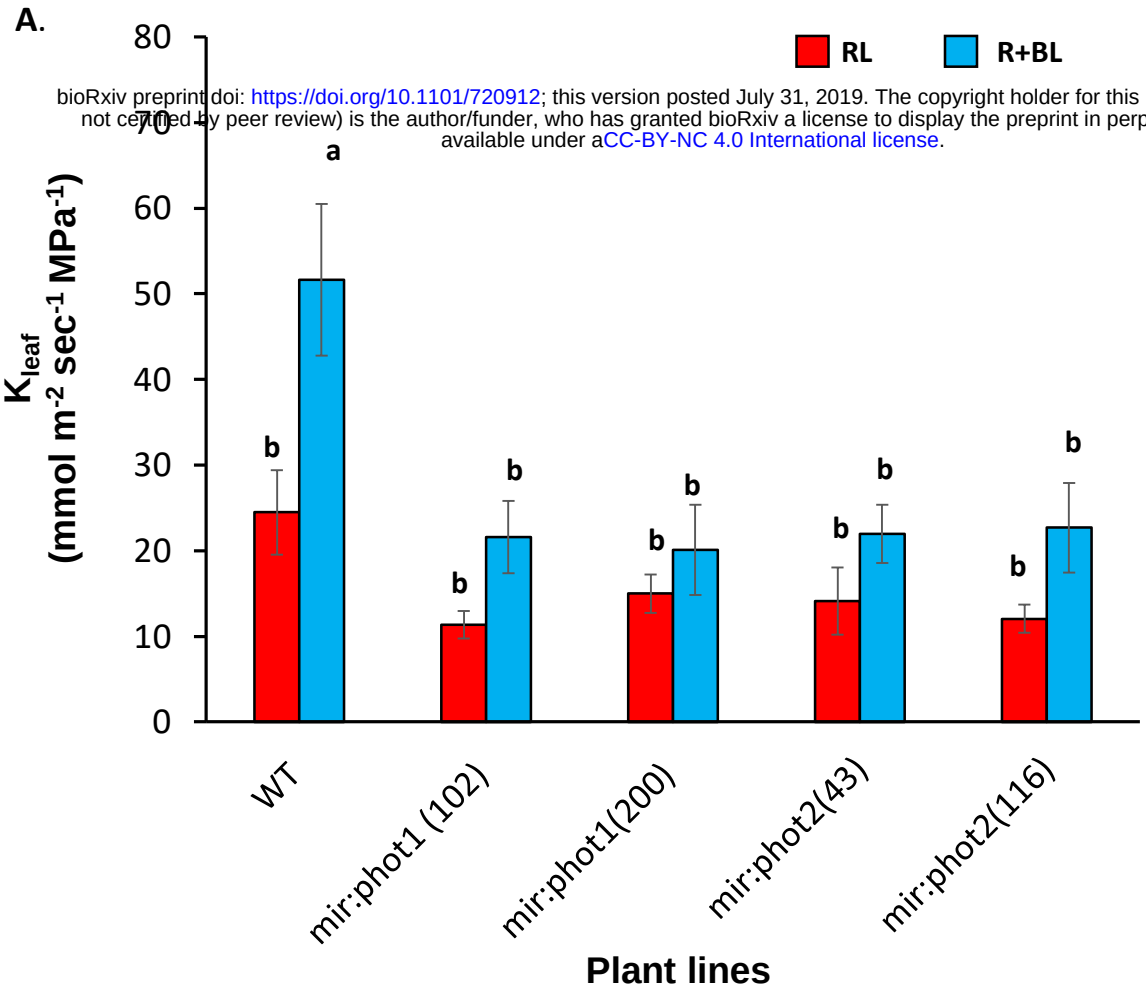

Figure 4. The effect of R+BL on the leaf hydraulic conductance of *PHOT1*- and *PHOT2*- silenced (SCR:mir) plants. SCR:mir plants were generated in the WT (Col 0) background. Fully expanded leaves of WT and SCR:mirphot1 and phot2 plants were treated with light as described in Fig.2. Columns are means ± SE (n = 12-15), determined in three independent experiments. Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05). Note the lack of R+BL effect on the leaves of plants "amiR-mutated' solely in their BSCs

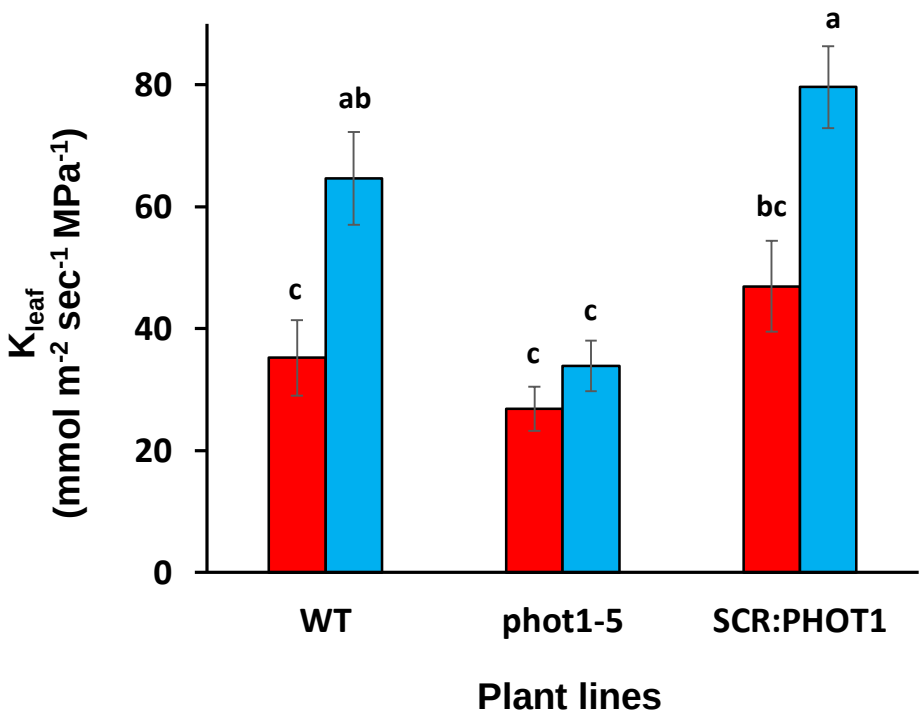

Figure 5. BSCs-directed *PHOT1* complementation of the *phot1-5* mutant restores the normal K_{leaf}. *phot1-5* (in gl background) plants were complemented with SCR:*PHOT1* restoring PHOT1 activity specifically in the BSCs. Fully expanded leaves of WT (gl ecotype), *phot1-5* and SCR:*PHOT1* were treated with light as described in Fig.2. Columns are means ± SE (n = 12-15), obtained in three independent experiments. Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05).

F5. Grunwald, Gosa et al., 2019

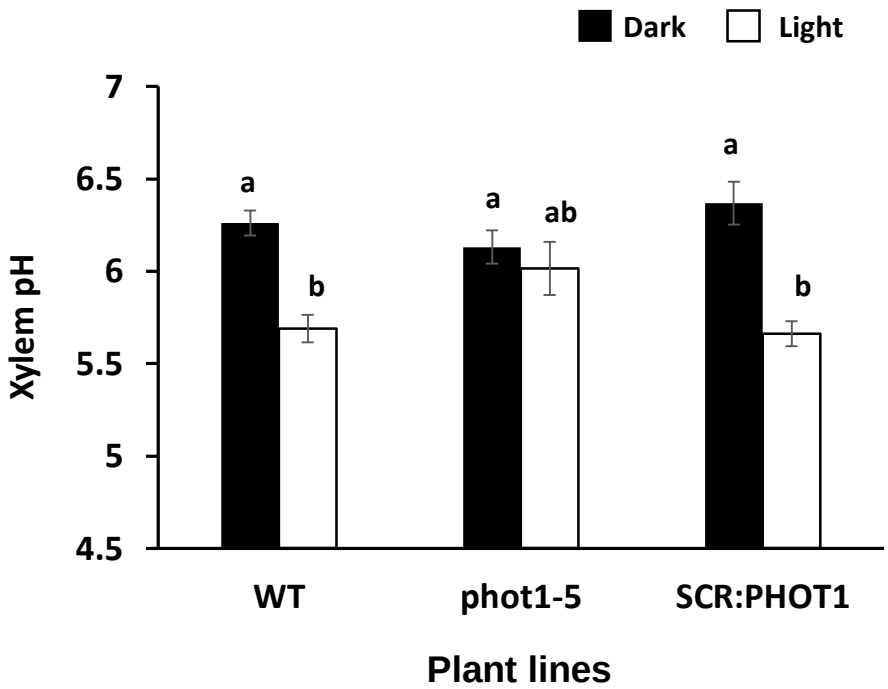

Figure 6. White light treatment for 30 minutes acidifies the xylem pH of WT plants and SCR:*PHOT1*-complemented mutants, but not in the *phot1-5* mutant. The columns are means (±SE; n = 10-12) determined in three independent experiments (150-200 µmol m^{-2} sec^{-1} white light). Different letters denote significantly different values (*Post hoc* Tukey's test P< 0.05).

.

F6. Grunwald, Gosa et al., 2019

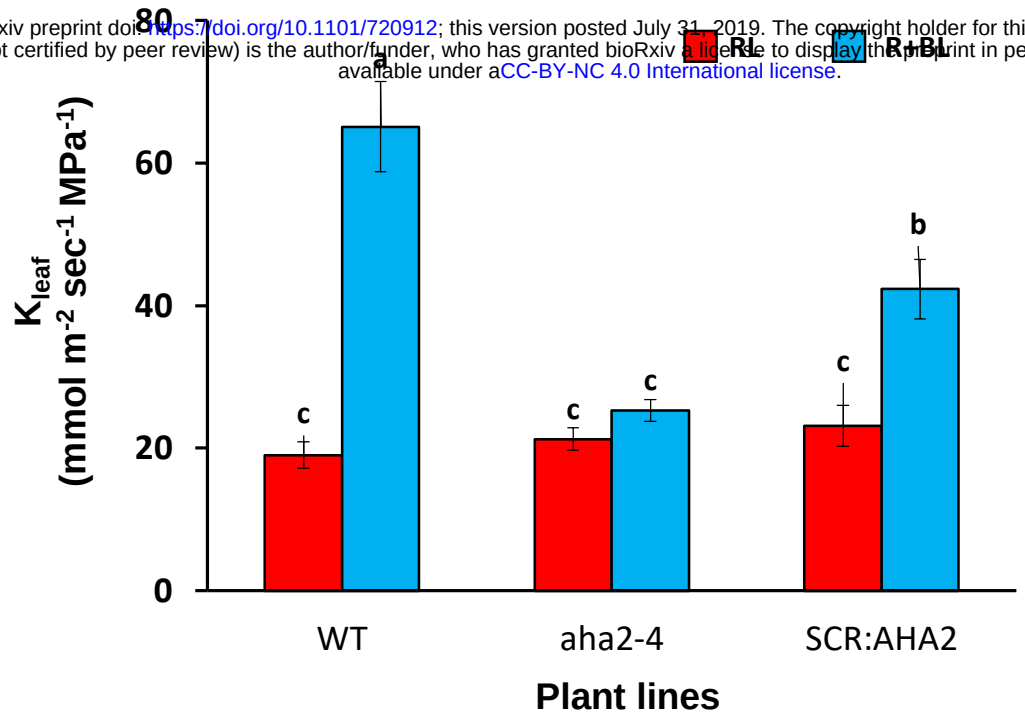

Figure 7. The BSCs H^+-ATPase, AHA2, mediates the blue-light-induced K_{leaf} increase. Fully expanded excised leaves of WT (ecotype Col0) plants, *aha2-4* mutant plants (in Col0 background) and *aha2-4* plants complemented in their BSCs with SCR:*AHA2* were illuminated for 15 min immediately after dark, with light as described in Fig.2. The columns are means (±SE; n=8-10) determined in three independent experiments. Different letters denote significantly different values ($P< 0.05$; ANOVA). Note the marked restoration of K_{leaf} in the *aha2* mutant plants complemented solely in their BSCs.

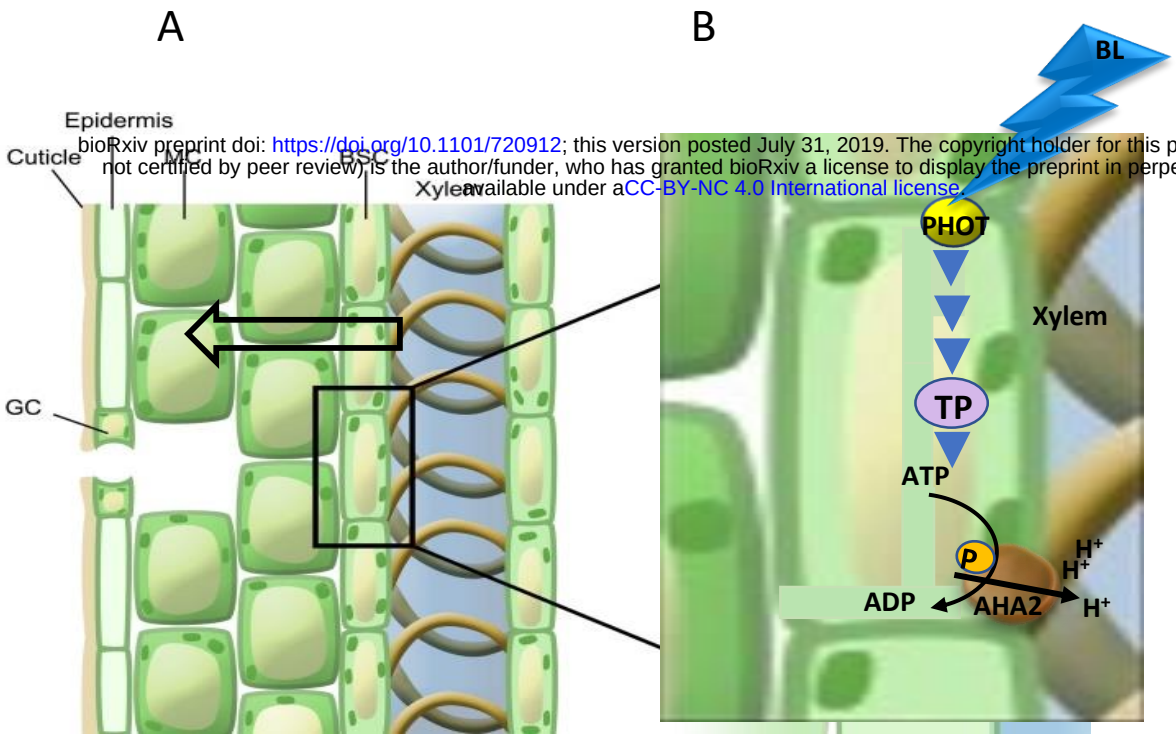

Figure 8. Proposed pathway BSCs autonomous BL signal transduction pathway. A. Artist's rendering of a leaf radial water path, from xylem to mesophyll (K_{leaf}, blue hollow black arrow) via the BSCs, which tightly envelop the xylem. **B.** Blue-light (BL) signalling pathway (blue arrowheads) in a bundle sheath cell, from BL perception by the phototropin receptors (yellow circle), through an intermediate tyrphostin-sensitive tyrosine phosphorylation (TP), to the ultimate AHA2 (orange circle) activation resulting in proton extrusion via the pump and xylem sap acidification, presumably, at the expense of ATP breakdown to ADP with a transient phosphorylation (P) on the pump protein, as expected from a P-type H^+-ATPase.

F8. Grunwald, Gosa et al., 2019