

# Systemic Signaling in the Regulation of Stomatal Conductance<sup>1</sup>[OPEN]

Dear Editor,

Plants use systemic signaling networks to spread information on locally perceived stimuli and coordinate responses in different plant parts. In natural environments, sun flecks and leaf-shading may lead to rapid and large changes in light conditions within the canopy and plants need to continually adjust stomatal conductance and photosynthesis in the fluctuating conditions (Lawson and Blatt, 2014). Exposure of mature leaves to low light levels or high intercellular CO<sub>2</sub> concentrations ( $c_i$ ) triggers long-distance signaling that controls stomatal development and causes a reduction in stomatal density in young developing leaves (Lake et al., 2001; Miyazawa et al., 2006). Over shorter time periods, low light and  $c_i$  also affect stomatal conductance. However, it is unclear whether local changes in light and intercellular CO<sub>2</sub> levels also regulate stomatal conductance and photosynthesis in distal leaves. To address this question, we developed a unique experimental setup that enabled the application of treatments on local and distal leaves separately while simultaneously monitoring rapid changes in leaf gas exchange. Here we show that systemic signaling is involved in rapid stomatal closure in response to changes in ambient light conditions and CO<sub>2</sub> concentration in two different tree species, hybrid aspen (*Populus tremula* × *tremuloides*) and silver birch (*Betula pendula*), but not in *Arabidopsis* (*Arabidopsis thaliana*).

In our experimental setup, two leaf gas exchange measuring devices (model no. GFS3000; Heinz Walz) were placed inside a FitoClima Growth Chamber (Aralab). This allowed us to manipulate ambient CO<sub>2</sub> concentrations and light levels on local and distal leaves separately while simultaneously monitoring rapid changes in stomatal conductance and photosynthesis (Fig. 1A). The treatments were applied in three different combinations: sudden increase in ambient CO<sub>2</sub> concentration (from 400 to 1,100  $\mu\text{L L}^{-1}$ ) or darkness

treatment on (1) the measured leaf (cuvette); (2) the rest of the measured seedling (chamber); or (3) the whole seedling (cuvette and chamber). During each treatment, the stomatal conductance, assimilation ( $A$ ), and  $c_i$  of the cuvette leaves was continuously measured. Stomatal conductance of the measured leaf remained unchanged for the untreated *P. tremula* × *tremuloides*, *B. pendula*, and *Arabidopsis* samples (Fig. 1; see also Supplemental Figs. S1 and S2; Supplemental Tables S1 and S2). When CO<sub>2</sub> and darkness treatments were applied in the growth chamber only, stomatal conductance was significantly decreased in the nontreated (cuvette) leaves of *P. tremula* × *tremuloides* and *B. pendula*, indicating the involvement of systemic signaling in the regulation of stomatal conductance in both species. While the effect of systemic signaling on stomatal conductance after the onset of the dark treatment was evident in every measured seedling, increasing the ambient CO<sub>2</sub> concentration around the distal leaves triggered stomatal closure in the nontreated leaves only in half of the measured *P. tremula* × *tremuloides* and *B. pendula* seedlings. However, as none of the control measurements showed similar behavior, the increase of  $c_i$  can also be considered to trigger systemic signals that affect stomatal conductance in distal leaves but the signal and response may depend on yet unknown physiological conditions.

The effect of systemic signaling on the regulation of stomatal conductance in the two tree species was also evident when comparing whole seedling and local treatments. Stomatal conductance of *B. pendula* decreased more when CO<sub>2</sub> and dark treatments were subjected to the whole seedling (cuvette and chamber) than when the treatments were applied only on the measured leaf (cuvette). Furthermore, visual comparison of the stomatal conductance response trends of *B. pendula* leaves to different combinations of CO<sub>2</sub> and darkness treatments (Fig. 1) suggested that the observed additive effect of systemic signaling may account for the difference between the local leaf and whole seedling treatments. In *P. tremula* × *tremuloides*, variation in the stomatal responses was large and thus the responses to the whole seedling and cuvette treatments were not statistically different from each other.

A similar systemic effect on stomatal conductance was not observed in *Arabidopsis*. The stomatal conductance of the measured *Arabidopsis* leaves did not change significantly in response to growth chamber treatments and no additive effect of systemic signaling was observed when comparing the whole rosette and cuvette treatments (Fig. 1; Supplemental Table S2). Previously, local applications of high light (Devireddy et al., 2018), wounding, heat-stress, and dark-to-light transition (Devireddy et al., 2019) have been shown to regulate stomatal aperture also in nontreated systemic

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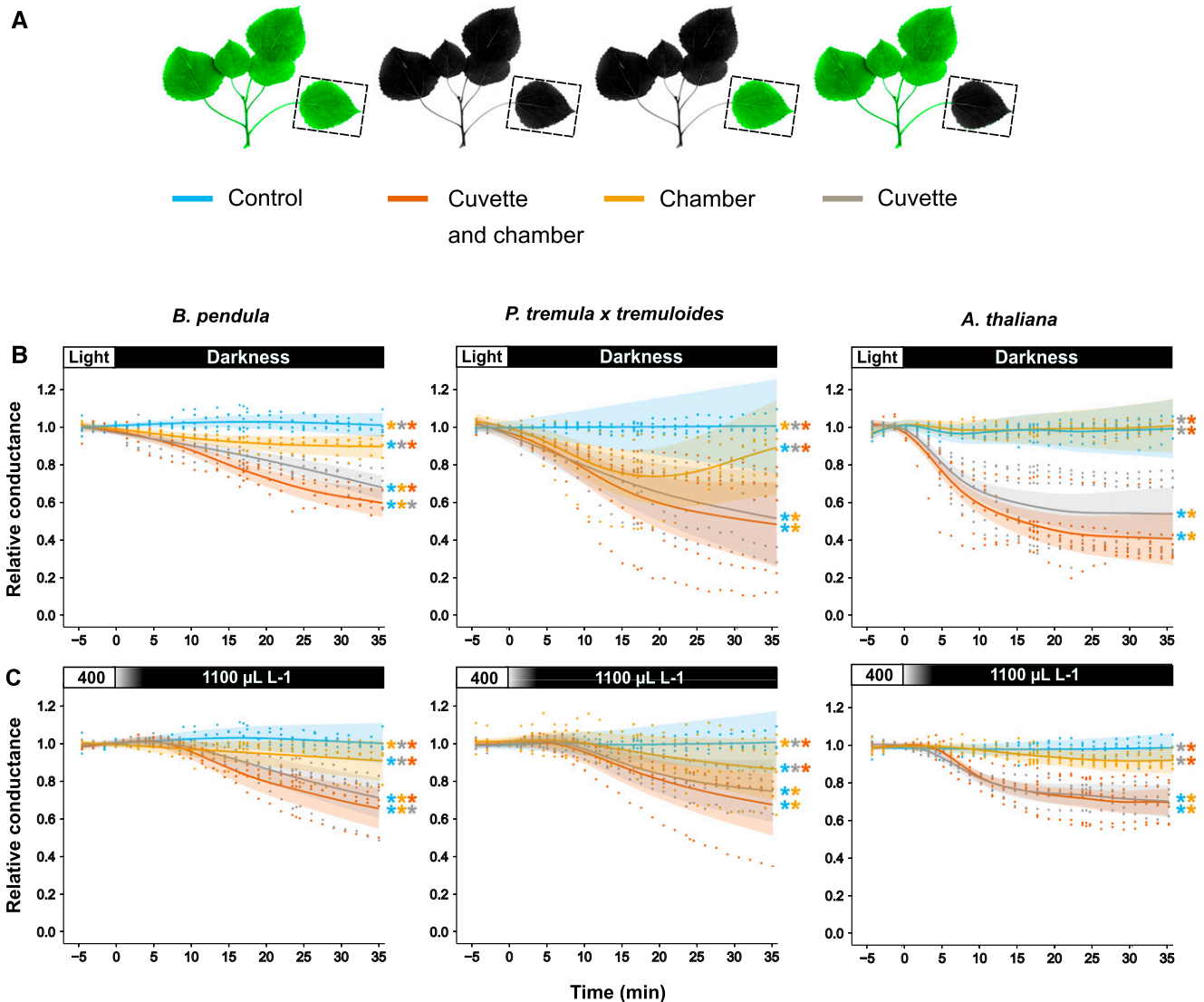
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S.E. designed and conducted the experiments and performed the data analysis; J.K. and T.H. supervised the study; S.E. and T.H. wrote the article; all authors commented, modified, and approved the article.

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**Figure 1.** Experimental setup and stomatal conductance measurements. A, Schematic representation of the experimental setup. Boxes with dashed borders represent the cuvette of the gas exchange measuring system and black indicates the treated tissues. A 1-h acclimation period in constant conditions (PAR 600/200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , relative humidity 60%, temperature 20°C/22°C, and  $\text{CO}_2$  concentration 400  $\mu\text{L L}^{-1}$ ) was followed by either of the two treatments: darkness after the light period or a rapid increase in  $\text{CO}_2$  concentration from 400 to 1,100  $\mu\text{L L}^{-1}$ . Treatments were conducted in three different combinations: treatment simultaneously in the cuvette and the chamber (orange), only in the chamber (yellow), or only in the cuvette (gray). Blue indicates the control treatment. Relative stomatal conductance of the measured (cuvette) leaf (normalized to pretreatment steady-state values) of *B. pendula* ( $n = 4$ ), *P. tremula*  $\times$  *tremuloides* ( $n = 4$ ), and *Arabidopsis* ( $n = 6$ ) in response to (B) sudden darkness (lights switched off) or (C) a rapid increase in  $\text{CO}_2$  concentration (from 400 to 1,100  $\mu\text{L L}^{-1}$  over  $\sim 4.5$  min). Black and white bars above the figures illustrate the onset and duration of the indicated treatment. Stomatal responses were statistically analyzed by fitting generalized additive mixed models (Wood, 2006) on the treatment responses as functions of time using the software package mgcv (v1.8-17) in R (v3.4.2; R Core Team; <http://www.r-project.org/>). Generalized additive mixed models are well suited for the analysis of gas exchange data as they do not assume linearity for the response and can take into account the nonindependence of the measuring points. Lines represent the model fits (predicted values) with 95% confidence intervals and dots are the observed relative stomatal conductance values. Asterisks indicate significant differences (adjusted  $P$  value  $< 0.004$ ) between the treatments. The color of the asterisk shows the subject of the pairwise comparison.

leaves of *Arabidopsis*. Although different signal elicitors, growth conditions, and study methods were used in the previous studies, it is rather surprising that we were unable to see the same systemic effect in response to  $\text{CO}_2$  and darkness treatments in *Arabidopsis*.

Therefore, further research is needed to evaluate the importance of systemic signaling on the regulation of leaf gas exchange in *Arabidopsis*.

The rapid systemic signaling seems to affect stomatal conductance in the opposite direction and at a different

time scale than would be expected for water potential- or sugar concentration-mediated stomatal regulation (see Supporting Materials and Methods for detailed explanation). We propose that the signals responsible for the systemic stomatal responses observed in our study are most likely transmitted by the rapid systemic signaling cascades mediated by ROS,  $\text{Ca}^{2+}$ , or electric waves. The involvement of systemic electrical signaling in the regulation of stomatal conductance in response to flaming of leaves has previously been demonstrated in several different species (Hlaváčková et al., 2006; Kaiser and Grams, 2006; Grams et al., 2007, 2009; Gallé et al., 2013). Recently, in *Arabidopsis*, extreme high light treatment (Devireddy et al., 2018), or wounding (Devireddy et al., 2019) have been shown to trigger a reactive oxygen species (ROS)/ $\text{Ca}^{2+}$  signal that leads to stomatal closure in distal untreated leaves.

In our study, the systemic stomatal response resulted in a subtle, but significant, decrease in stomatal conductance of the measured nontreated leaf within a few minutes of the treatment onset (Fig. 1; Supplemental Table S3). As the adjacent leaves were ~2–5 cm away from the measured leaf and the stomatal response could already be seen within 1.5 min after the onset of treatment, we estimated the maximum speed of the signal to be ~2 cm/min. ROS,  $\text{Ca}^{2+}$ , or electric signals have been shown to travel at a rate of 2.5 to several centimeters a minute (Huber and Bauerle, 2016). By contrast, changes in the sugar status are transmitted through the phloem on the order of tens of minutes, or even hours (Mencuccini and Hölttä, 2010). Changes in water potential can be transmitted in the xylem within a few minutes (Mencuccini and Hölttä, 2010), but changes in the water status of living cells in the mesophyll are on the order of tens of minutes (Nobel, 2009). Therefore, rapid systemically transmitted ROS,  $\text{Ca}^{2+}$ , and electric waves are the only known signaling mechanisms that correspond to the speed of the observed responses. However, as the evidence remains theoretical, further experimental studies are required to evaluate the role of ROS,  $\text{Ca}^{2+}$ , and electrical signaling in the systemic regulation of stomatal conductance in trees.

The kinetics of stomatal conductance and  $\text{CO}_2$  assimilation were also monitored and compared in the distal untreated leaf to examine how the systemic signal is perceived in the measured leaf. A decrease in stomatal conductance (Fig. 1) preceded the decrease in  $\text{CO}_2$  assimilation rate (Supplemental Fig. S3), while there was no change in the  $c_i$  levels (Supplemental Fig. S4) in the untreated leaf a few minutes after the initiation of darkness or  $\text{CO}_2$  treatment in the growth chamber. Moreover, the results suggest that the signal is likely perceived directly by the guard cells, leading to a reduction in stomatal conductance followed by a decrease in assimilation rate due to a decrease in the supply of  $\text{CO}_2$  through the stomata.

In conclusion, our data strongly suggest that trees are not only able to sense changing light conditions and  $c_i$ , but are also able to signal this information to distal parts of the plant. This work also highlights the fundamental

problems of single leaf measurements. The potential effect of systemic signaling on stomatal conductance and photosynthesis should always be taken into account when studying stomatal functions in both in vivo and in vitro conditions. A better understanding of different regulatory mechanisms at the local and systemic scales will contribute to the understanding of mechanisms by which changing environmental conditions modulate plant gas exchange.

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Statistical differences between the treatments, related to Figure 1.

**Supplemental Figure S2.** Statistical differences between the treatments, related to Figure 1.

**Supplemental Figure S3.** The kinetics of  $\text{CO}_2$  assimilation in response to different treatments, related to Figure 1.

**Supplemental Figure S4.** The kinetics of intercellular  $\text{CO}_2$  concentration ( $c_i$ ) in response to different treatments, related to Figure 1.

**Supplemental Table S1.** Statistical differences between the treatment responses in *B. pendula* and *P. tremula* × *tremuloides*, related to Figure 1.

**Supplemental Table S2.** Statistical differences between the treatment responses in *Arabidopsis*, related to Figure 1.

**Supplemental Table S3.** Absolute values of gas exchange parameters and response kinetics, related to Figure 1.

**Supplemental Materials and Methods.** Detailed description on growth conditions, experimental set up, statistical analyses, and water potential- or sugar concentration-mediated stomatal regulation.

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