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SYSTEMIC SIGNALING IN PLANT GAS EXCHANGE

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DOCTORAL DISSERTATION

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ABSTRACT

As plants are sessile organisms, the ability to adjust and withstand changing environmental conditions is essential for their survival. Systemic long-distance signaling is known to have an important part in plant responses to abiotic and biotic stresses. However, the role of long-distance systemic signaling in the regulation of plant gas exchange is not well understood. This thesis aims to shed light on how plants utilize systemic signaling mechanisms in the regulation of stomatal conductance and photosynthesis. To facilitate the research I developed a novel method, where the environmental conditions of a single leaf and the rest of seedling can be controlled separately while simultaneously monitoring the rapid changes in leaf gas exchange. The results indicate that in two tree species, *Populus tremula x tremuloides* and *Betula pendula*, systemic signals are involved in the fine-tuning of stomatal conductance and photosynthesis in response to step-changes in ambient light levels and CO₂ concentration. However, in *Arabidopsis thaliana*, leaf gas exchange responds to local changes but is not regulated systemically. I also investigated whether systemic signaling is involved in the stomatal and photosynthetic responses of *B. pendula* under naturally fluctuating environmental conditions. A comparative analysis of the gas exchange parameters of two separate leaves measured simultaneously under pre-set constant conditions and fluctuating ambient conditions revealed that leaves do not only respond to fluctuations in local light levels but also transmit and perceive information on light fluctuations across the canopy. This thesis also provides evidence that the systemic signals are likely perceived directly by the guard cells and that the concurrent systemic changes in photosynthesis are caused by changes in the supply of CO₂ through stomata. I suggest that the rapid systemic signals controlling stomatal conductance are transmitted by ROS, Ca²⁺ or electric waves as these are the only known signaling mechanisms that correspond to the speed and direction of the observed stomatal responses. Furthermore, the studies reported in this thesis indicate that systemic signaling helps plants to coordinate and fine-tune leaf gas exchange under naturally fluctuating light conditions.

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CONTENTS

ABSTRACT	5
ACKNOWLEDGEMENTS	6
CONTENTS	8
ORIGINAL PUBLICATIONS AND MANUSCRIPTS	9
AUTHOR’S CONTRIBUTIONS.....	10
ABBREVIATIONS	11
1 INTRODUCTION.....	12
2 LITERATURE REVIEW.....	13
2.1 Model species.....	13
2.2 Physiological properties of stomata	14
2.3 Plant response dynamics to environmental change	16
2.4 Natural fluctuations in irradiance and circadian rhythm affect plant gas exchange in space and time	17
2.4.1 Photosynthetic responses to fluctuating light	17
2.4.2 Stomatal responses to fluctuating light	18
2.4.3 Other factors affecting plant gas exchange	18
2.4.4 Acclimation to fluctuating light	19
2.5 Systemic signaling in the regulation of leaf gas exchange	20
3 AIMS OF THE STUDY	23
4 MATERIALS AND METHODS.....	24
4.1 Plant material	24
4.2 Methods	24
5 RESULTS AND DISCUSSION	26
5.1 Systemic signals are involved in the regulation stomatal conductance in trees	26
5.2 The signaling mechanism	27
5.3 Possible sources of error in systemic signaling studies.....	29
6 CONCLUSIONS AND FUTURE PERSPECTIVES	31
7 REFERENCES.....	33

ORIGINAL PUBLICATIONS AND MANUSCRIPTS

This thesis is based on the following articles:

- I **Ehonen S**, Yarmolinsky D, Kollist H, Kangasjärvi J (2019) Reactive oxygen species, photosynthesis, and environment in the regulation of stomata. *Antioxidants & Redox Signaling* 30: 1220-1237
- II **Ehonen S**, Hölttä T, Kangasjärvi J (2020) Systemic signaling in the regulation of stomatal conductance. *Plant Physiology* 182: 1829-1832
- III **Ehonen S**, Sierla M (2022) Gas exchange measurements in systemic signaling studies. *In* T Yoshida, eds, *Abscisic acid: methods and protocols*, Volume 2462: *Methods in molecular biology*, 2022. Springer, New York; NY, pp 111-123
- IV **Ehonen S**, Sierla M, Kangasjärvi J, Hölttä T (2022) Systemic signaling regulates stomatal responses to naturally fluctuating light. (Manuscript)

In the text, the articles and manuscript are referred to as their roman numbers.

AUTHOR'S CONTRIBUTIONS

- I I participated in conceiving the layout of the review article. I wrote approximately fifty per cent of the article with the advice from JK. I designed and prepared the figures together with YD.

- II I conceived the project, designed and conducted the experiments as well as performed the data analysis. JK and TH supervised the study. I wrote the manuscripts with contributions from TH and with the advice from JK.

- III I designed and conducted the measurements presented in the chapter. I wrote the chapter together with MS.

- IV I designed and conducted the experiments and performed the data analysis with the advice from TH. I wrote the manuscripts with the advice from TH, MS, and JK.

ABBREVIATIONS

Abbreviation	Full name
A	Photosynthetic CO ₂ assimilation
ABA	Abscisic acid
Ca ²⁺	Calcium
Ci	Intercellular CO ₂ concentration
CO ₂	Carbon dioxide
g _s	Stomatal conductance
Rubisco	ribulose-1,5-bisphosphate carboxylate-oxygenase
RuBP	ribulose-1,5-bisphosphate
ROS	Reactive oxygen species
SAA	Systemic acquired acclimation
SLAC1	SLOW ANION CHANNEL 1
VPD	Vapor pressure deficit

1 INTRODUCTION

Plants and algae capture light energy from sun and convert it into chemical energy by photosynthesis. The compounds essential for life, carbohydrates and oxygen, are produced in the process. In natural environments and in crop fields, plants grow under ever changing environmental conditions. Especially light intensities and spectral properties exhibit temporal and spatial variation due to changes in solar incident angle, cloud cover, and shading from overlapping leaves and neighboring plants. Therefore, transient light conditions have major impact on overall photosynthetic carbon assimilation (Percy, 1990; Percy and Way, 2012). As plants are sessile, they need to respond and acclimate to the prevailing light conditions in order to maintain performance and fitness.

At a given time, different parts of a plant may be subjected to different environmental conditions and information transmission between the different parts is thought to help in coordination of overall responses to biotic and abiotic stresses (Gilroy et al., 2016; Szechyńska-Hebda et al., 2017). Plants perception and response to changes in the surrounding environment involves a complex network of signaling pathways which transmit the information throughout organs, tissues or the whole plant by short- or long-distance signals such as the transport of mRNAs, proteins, metabolites, phytohormones, ions as well as ROS, Ca^{2+} , electrical and hydraulic waves. In addition, stomata functions as master regulator in the responses to environmental variables as they control the evaporation of water vapor and supply of CO_2 for photosynthesis. Stomatal responses to different environmental factors are well studied both at ecophysiological and molecular level. However, research on this topic is usually focused on studying the effect of local step-changes in different environmental variables on leaf gas exchange and little is known about the possible mechanisms that coordinate stomatal responses at the whole plant. Particularly little is known about the role of systemic signaling in the regulation of stomatal conductance.

Majority of the previous studies on systemic long-distance signaling have focused on plant stress reactions and acclimation to stress. It is likely that the same signaling pathways functioning in long-distance stress signaling and acclimation are also used in the systemic regulation and fine-tuning of plant gas exchange. The aim of this thesis is to fill the gap in knowledge and study the involvement of systemic signaling in the fast regulation of stomatal conductance. As stomatal conductance and photosynthesis are tightly coupled processes, I also studied fast photosynthetic regulation by systemic signaling. However, the experiments reported in this thesis were specifically designed to explore the involvement of systemic signaling in stomatal regulation and its effect on photosynthesis. Thus, further research is needed to determine the importance of systemic signaling in the regulation of photosynthetic metabolism.

2 LITERATURE REVIEW

2.1 Model species

The research reported in this thesis was conducted in three different species, thale cress (*Arabidopsis thaliana*), hybrid aspen (*Populus tremula x tremuloides*) and silver birch (*Betula pendula* Roth), that could all be considered as model species in their own terms.

A. thaliana is the most important model species in modern plant research, which has become increasingly focused on finding molecular mechanisms behind the complex ecophysiology of plants. *Arabidopsis* has many attributes that make it a suitable model for plant molecular genetic research, such as small and simple genome, reproduction by self-pollination, short generation time, genetically well characterized and easy to transform. Plant scientists are trying to combat the consequences of population growth, pollution, climate change and biodiversity loss by developing new crop varieties that would yield well even in suboptimal conditions (Stepanova, 2021). The ease with which research can be carried out in *Arabidopsis* has allowed an undisputed, fundamental progress in our knowledge on plant ecology, physiology, development, metabolism, genetics, and epigenetics. Yet, the biggest disadvantage of using *Arabidopsis* as a model species is its ecology or rather ignorance of its ecology by scientists. For research purposes, *Arabidopsis* is commonly grown in steady-state conditions, under very low light and in the absence of any major biotic and biotic stress factors. However, in nature, *Arabidopsis* is a pioneer species of disturbed, poor, shallow or sandy soils (Krämer, 2015) and can be found in open habitats with high irradiance. Its general strategy to stress is to escape by prioritizing reproduction followed by a fast completion of its life cycle, and unlike the majority of flowering plants, *Arabidopsis* does not have mycorrhizal or rhizobial symbionts (Krämer, 2015). The natural growth conditions and ecology of the species should always be taken into account when placing one's research within a larger context.

Forest are the largest terrestrial ecosystems in the Northern Hemisphere and their importance in mitigating climate change and maintaining biodiversity is huge. However, Boreal forests also have high commercial value as they produce wood for timber, plywood, pulp and paper. Therefore, understanding how forest trees may respond to the anthropogenic environmental changes and degradation in the future, is essential not only for forestry but also for conservation of habitats and species, including our own.

Hybrid aspen (*P. tremula x tremuloides*) was used in this thesis to study stomatal regulation. *Populus* species have been widely used as a model species in woody plant research. The genus consist of approximately 30 species that grow throughout the northern hemisphere and are considered as one of the most ecologically and economically important tree species (Taylor, 2002; Cronk, 2005; Nieminen et al., 2012). *Populus spp.* have several traits that make them good models for ecological and molecular studies, such as large genetic variation within the genus, rapid growth, relatively small genome size, ease of vegetative propagation and transformation, as well as the availability of genetic tools, and complete *P. trichocarpa* genome (Taylor, 2002; Nieminen et al., 2012; Tuskan et al., 2022). However, according to a phylogenetic analysis carried on 37 phylogenetically different plant species, the *Populus* genus appears to be the only known plant family that do not have the SLOW ANION CHANNEL 1 (SLAC1) protein (unpublished), an anion channel that plays a central role in fast stomatal closure (Negi et al., 2008; Vahisalu et al., 2008). In addition, *Populus* species have been found to have less sensitive stomata when compared to some other deciduous tree species (Aasamaa and Söber, 2011) and maintain relatively high night-time conductance even under drought (Cirelli et al., 2015). These findings imply that the mechanisms of stomatal regulation in *Populus* species may have fundamental differences when compared to other deciduous tree species.

Silver birch (*B. pendula*) and downy birch (*B. pubescens*) are the most common deciduous tree species in Fennoscandian forests (Oksanen, 2021). As a common pioneer species of Eurasian forests, *B. pendula* has a great economic value but is also a highly important species in forest ecosystems. Similar to *Populus* species, its advantages as a model species are high plasticity and genetic variation as well as a sequenced and annotated genome (Salojärvi et al., 2017). In addition, the possibility to artificially accelerate flowering makes silver birch more feasible for molecular genetic studies than other model tree species (Salojärvi et al., 2017). The effect of different abiotic and biotic stress factors on *B. pendula* ecology and physiology have been studied intensively (reviewed in Oksanen, 2021) and the high plasticity and genetic variation makes it a good model species for studying physiological responses and acclimation to changing environmental conditions.

2.2 Physiological properties of stomata

Stomata are microscopic pores in plant leaves and stems, surrounded by two guard cells and subsidiary cells. Cell turgor derived conformational changes in the pore control the exchange of gases between the leaf interior and atmosphere (Lawson and Matthews, 2020). The regulation of stomatal aperture is essential for plant survival as it balances the CO₂ supply for photosynthesis with water loss via transpiration.

Stomatal aperture is adjusted by transport of potassium and other solutes into or out of the guard cells. The resulting increase or decrease in osmotic concentration inside the guard cells causes movement of water to the direction of lower water potential. The subsequent change in guard cell turgor pressure leads to the alteration in stomatal aperture. Increased turgor forces the guard cells to bend outward (due to mechanistic properties of the cell wall) and opens a pore between the cells whereas loss of turgor leads to shrinkage of the guard cells and closure of stomatal pore (Figure 1; Kollist et al., 2014). The cellular mechanisms and signaling pathways leading to the changes in stomatal aperture have been intensively studied. They are known to involve membrane channels and pumps as well as downstream enzymes, which are all regulated by changes in cellular and intercellular levels of abscisic acid (ABA), other hormones, bicarbonate, reactive oxygen species (ROS), and calcium (Ca^{2+}) (reviewed in Sierla et al., 2016; Ehonen et al., 2019). In general, stomatal opening is triggered by high light intensities, low vapor pressure deficit (VPD) and low intercellular CO_2 concentration (C_i) while closure is induced by the opposite conditions (low light, high VPD and high CO_2) (Ehonen et al., 2019; Ferrando et al., 2020; Grossiord et al., 2020). In addition, stomata respond to several different abiotic and biotic stress factors, such as low water availability, pollution, high light, pathogen attack or herbivory. Moreover, stomata need to actively adjust their aperture in order to balance carbon gain and water loss under naturally fluctuating environmental conditions as well as during abiotic and biotic stresses (Percy, 1990; Lawson and Blatt, 2014).

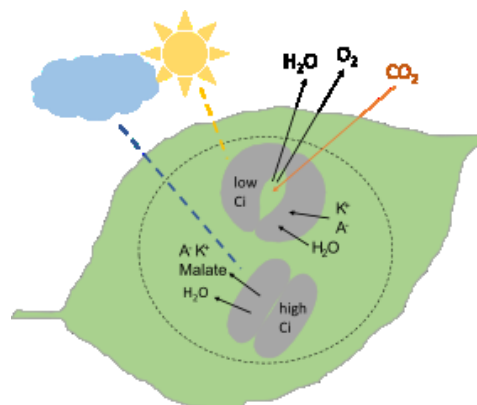


Figure 1. A simplified overview on the rapid regulation of stomatal aperture. Stomatal guard cells react to changes in environmental conditions on both sides of the leaf epidermis, ambient air and intercellular airspace in the mesophyll. Under increasing light and decreasing intercellular CO_2 concentration (C_i), guard cells accumulate osmotically active potassium (K^+) ions and anions (A^-). The consequent influx of water (H_2O) increases the guard cell volume and stomata open allowing the diffusion of CO_2 to the site of carboxylation. Conversely, in response to decreasing irradiation and increasing C_i , efflux of K^+ , A^- , and H_2O reduces guard cell volume and stomata close preventing unnecessary water loss.

Stomatal closure conserves water when the photosynthetic need for CO₂ is low but under conditions favorable for photosynthesis, stomata must open to enable diffusion of CO₂ to chloroplast. Thus, stomata must continuously monitor and coordinate the responses to external environmental changes as well as the internal changes in intercellular CO₂ concentration (C_i). Stomatal conductance and photosynthesis are usually well correlated and coordinated in steady-state conditions (Farquhar and Sharkey, 1982). At low light levels, the photosynthetic assimilation of CO₂ (A) is limited by chloroplast related processes. When irradiance increases, the stomatal and mesophyll related processes limiting diffusion of CO₂ to chloroplasts becomes limiting factors of A. When irradiance increases to levels at which the photoprotective mechanisms fail to protect the photosynthetic machinery or photosystem II function is downregulated, photoinhibition causes reduction in the efficiency on photosynthesis (Foyer et al., 2017). At gradually increasing light, also the kinetics of A and stomatal conductance (g_s) will limit the rate of photosynthetic induction (discussed later). Moreover, in order to maintain a high rate of photosynthesis but at the same time protect the plant from adverse environmental conditions, stomata need to actively monitor the fluctuations in intercellular CO₂ levels, plant water status and ambient environmental conditions and adjust their aperture accordingly (Figure 1; Ehonen et al., 2019).

2.3 Plant response dynamics to environmental change

Plant responses to changes in irradiance vary from short-term (from seconds to minutes) adjustments in cellular processes to long-term (days) physiological changes. Plants respond to a sustained change in environmental conditions by adjusting their physiology to suit the prevailing growth conditions through processes defined as acclimation. The physiological changes involved in acclimation may be developmental or dynamic. Developmental acclimation introduces morphological changes in developing tissues whereas dynamic acclimation causes changes in proteins or metabolites in fully developed organs or tissues (Walters, 2005; Athanasiou et al., 2010). Both processes usually take multiple days and involve notable changes in plant gene expression (Gjindali et al., 2021) and thus acclimation is usually a response to sustained changes in growth conditions, such as seasonal variation in temperature, light irradiance or water and nutrient availability. However, plants also need to respond to much faster, abrupt changes in their growth environment, such as sun flecks or pathogen attack. Plants respond to sudden changes by regulating the activity of cellular processes. Regulatory responses occur within seconds or minutes and involve activation of proteins rather than changing their abundance (Herrmann et al., 2019). It must be noted that these fast regulatory responses may trigger acclimation

and be affected by acclimation processes and thus it is difficult to draw a line between these processes.

2.4 Natural fluctuations in irradiance and circadian rhythm affect plant gas exchange in space and time

2.4.1 Photosynthetic responses to fluctuating light

The amount of solar radiation reaching Earth's surface varies during the day and season and is affected by solar angle, cloud cover, aerosols and water vapor (Slattery et al., 2018; Durand et al., 2021). These sources of variation in incident solar radiation have major impact on photosynthesis in the upper canopy. While the effect of clouds on sunlight depends on many factors, clouds can substantially reduce the irradiance perceived by plants (Slattery et al., 2018; Durand et al., 2021). In addition, leaves in lower canopy experience more frequent, short-term fluctuations in irradiance due to transmission and scattering of light by leaves and stems above them. The duration and amplitude of these sun- and shade-flecks are affected by wind speed, canopy structure, position in the canopy and leaf biomechanical properties (Tang et al., 1988; Pearcy, 1990; Kaiser et al., 2018; Morales and Kaiser, 2020) and in the lower canopy plants are thought to receive most of the light energy from sunflecks (Pearcy, 1990).

Plant responses to fluctuating light varies from seasonal scale to less than a second. Photosynthesis is a highly regulated process in which the light harvesting and electron transport reactions have to be tightly coupled with the downstream metabolic processes (Foyer et al., 2012). The dynamic change in leaf photosynthesis in response to light fluctuations at various time scales involve several mechanisms that help to avoid photoinhibition and maximize photosynthetic yield. The short-term responses include changes in thylakoid membranes (such as changes in light harvesting capacity and the amount of non-photochemical quenching (NPQ)), regulation of enzyme activity, metabolite buffering, chloroplast movements and regulation of CO₂ diffusion into the chloroplast (Morales and Kaiser, 2020; Gjindali et al., 2021). When irradiance increases rapidly from low light (shade) to high light (sun), the rate of photosynthetic carbon assimilation (A) does not increase to its full capacity promptly but is delayed by several minutes. The gradual increase in photosynthesis is known as photosynthetic induction (Walker, 1973) and it is limited by three main steps: 1) the activation of ribulose-1,5-bisphosphate (RuBP) regeneration 2) the activation of ribulose-1,5-bisphosphate carboxylate-oxygenase (Rubisco) (Mott and Woodrow, 2000) and 3) light induced stomatal opening (Kirschbaum and Pearcy, 1988). The first two steps limit photosynthesis only for short time (less than ten minutes) whereas stomatal limitations may continue up to an hour (Pearcy, 1990; Deans et al., 2019).

2.4.2 Stomatal responses to fluctuating light

In the natural environment, stomata function as major regulators of the availability of CO₂ for carbon fixation and the differential kinetics of g_s to A, limit photosynthesis. The maintenance of a high C_i level is essential for sustaining high rate of photosynthesis as the enzyme catalyzing the first step of CO₂ assimilation in the Calvin cycle, Rubisco, has a dual role as carboxylase and oxygenase (Bathellier et al., 2018). A decrease in C_i and the subsequent decrease in carbon fixation leads to reduction in the amount of chemical energy (ATP) and reducing power (NADPH) consumed by the Calvin cycle. This, in turn, leads to over-reduction of electron transport chain and ROS production. In addition, the decrease in CO₂ at the site of carboxylation leads to increased oxygenase activity of Rubisco and induction of photorespiration (Wingler et al., 2000; Huang et al., 2015). During sunflecks, biochemical activation (of the first two steps of photosynthetic induction) has been shown to be the major limiting factor for A (Soleh et al., 2016; Soleh et al., 2017; Taylor and Long, 2017; Acevedo-Siaca et al., 2020).

The biochemical limitation of light induction is estimated to last less than 10 minutes (Pearcy, 1990; Kimura et al., 2020). Therefore, under longer sunflecks, stomatal dynamic become the major limiting factor of A (Huang et al., 2015; McAusland et al., 2016; Qu et al., 2016; Kimura et al., 2020; De Souza et al., 2020; Yamori et al., 2020; Eyland et al., 2021). However, a recent study on *Arabidopsis thaliana* mutants suggests that stomatal processes may limit photosynthesis already as early as one minute after light induction but the mechanism for this is not known (Kimura et al., 2020). Stomatal responses to fluctuating light conditions are significantly slower than the photochemical and biochemical changes involved in photosynthesis. The slower increase in g_s during increasing irradiation limits the CO₂ uptake for A, while the slower kinetics in g_s in response to decreasing irradiation leads to unnecessary water loss (Farquhar and Sharkey, 1982; Lawson and Blatt, 2014). Two recent studies highlight the importance of diversity in g_s kinetics under fluctuating light. De Souza et al. (2002) showed that fluctuating light caused a three-fold higher variation in A between different cassava (*Manihot esculenta*) genotypes than measured under steady-state conditions and the variation was caused mainly by differences in stomatal kinetics. In another study on closely related banana genotypes (*Musa* spp.) diurnally fluctuating light revealed diversity in g_s kinetics between banana genotypes and that slow stomatal regulation was the main limitation to A (Eyland et al., 2021).

2.4.3 Other factors affecting plant gas exchange

Diurnal variations in plant gas exchange are driven by alterations in environmental conditions and circadian rhythms (Resco de Dios and Gessler, 2018). Stomatal kinetics in fluctuating light can be affected by several biotic and abiotic factors such as drought,

VPD, nutrient availability, as well as pests and pathogens (Sun et al., 2022) and climate change is thought to increase the importance of these factors on plant productivity. For example, changing precipitation patterns and increasing temperatures will increase atmospheric evaporative demand (VPD) and the risk of drought in the future (Grossiord et al., 2020). In addition to the external factors listed above, circadian clock is known to play an important, intrinsic, role in the diurnal rhythm of plant gas exchange (Resco de Dios et al., 2016; Resco de Dios and Gessler, 2018). Reduction in absolute g_s and in the magnitude of stomatal responses towards the afternoon have been reported (Allen and Percy, 2000; Matthews et al., 2018; Lawson and Matthews, 2020; Eyland et al., 2021) but the mechanisms behind the diurnal g_s behavior remain largely unknown. Common hypothesis involve increase in VPD typically towards afternoon, diurnal changes in ABA sensitivity (Mencuccini et al., 2000), feedback loops from photosynthate accumulation and calcium signaling (Resco de Dios et al., 2016; Matthews et al., 2017; Resco de Dios and Gessler, 2018).

2.4.4 Acclimation to fluctuating light

Photosynthetic and stomatal responses to step changes or short-term fluctuations in environmental conditions have been extensively studied but very little is known about the acclimation of photosynthetic and stomatal processes to sustained environmental fluctuations (Morales and Kaiser, 2020). To my knowledge, only two studies, focusing on the photosynthetic acclimation to fluctuating light, have been published to date. According to these studies, acclimation to fluctuating irradiance caused decrease in photosynthetic capacity, biomass, leaf thickness and the amount of thylakoid proteins (Violet-Chabrand et al., 2017) as well as changes in stomatal kinetics and diurnal pattern of gas exchange in *Arabidopsis thaliana* (Matthews et al., 2018). Moreover, these findings suggest that experiments performed with plants grown under typical square-wave growth conditions (i.e. lights are turned on in the morning and switched off in the evening) may fail to predict plant performance and productivity under natural conditions. This could explain the difficulty in developing plants with better performance by manipulating genes involved in stomatal regulation. Under steady state conditions, high g_s is associated with high A and thus with high growth (Fischer et al., 1998; Franks, 2006). Therefore, altering stomatal kinetics have been suggested as potential way to improve plant growth and crop yield (Lawson and Blatt, 2014; De Souza et al., 2020). However, to date, only two studies conducted with transgenic *Arabidopsis* plants possessing altered stomatal kinetics, have reported increased A and growth in plants with faster stomatal kinetics. Expression of a synthetic light-gated K^+ -channel, BLINK1, (Papanatsiou et al., 2019) and overexpression of an plasma membrane proton pump translocator, PATROL1, (Kimura et al., 2020) have been shown to increase photosynthesis and biomass production in under fluctuating light. However, both studies were conducted in *Arabidopsis* under in vitro conditions and

thus it is too early to say whether such mechanisms could be used to improve biomass production of crop plants in agricultural environment.

2.5 Systemic signaling in the regulation of leaf gas exchange

Changes in the environmental conditions rarely affect the whole plant exactly at the same time but often a certain part of the plant (e.g. a single leaf, branch or part of a canopy) senses the changing conditions before the rest of the plant will. The sensing tissue may not only respond to the stimulus but also initiate a transmission of the signal to other parts of the plant. This enables the tissues that have not yet sensed the change in conditions or stress to activate response, acclimation or defence mechanisms in advance (Huber and Bauerle, 2016; Kollist et al., 2019; Fichman and Mittler, 2020). The response of the tissue that is exposed to the environmental change is termed “local” and the response of the non-exposed tissues is termed “systemic”.

Plants are able to coordinate responses to abiotic stress at a whole plant level through systemic signaling. These responses are termed systemic acquired acclimation (SAA) (Karpinski et al., 1999; Suzuki et al., 2013). Characteristic to this response is a rapid spread of locally initiated signals to other tissues of the plant within a few minutes after the application of a treatment. Signals involved in SAA may travel from cell to cell at different tissues or spread through the vasculature. These signals include reactive oxygen species (ROS) waves (Miller et al., 2009), calcium (Ca^{2+}) waves (Choi et al., 2014), electrical signals (Szechyńska-Hebda et al., 2010) hydraulic waves and ABA (Mittler and Blumwald, 2015). The cell-to-cell ROS, calcium, and electrical waves are able to mediate signaling at a very high speed (Huber and Bauerle, 2016; Kollist et al., 2019) and are likely to be interconnected at cellular level and regulate each other while the timing and intensity of these different waves could express the signal specificity (Gilroy et al., 2016). In *Arabidopsis*, local application of light stress caused increase in a high range of metabolites and expression levels of genes in systemic tissues only a few minutes after the induction of the treatment (Choudhury et al., 2018; Zandalinas et al., 2019). In tomato (*Solanum lycopersicum*), longer application of high light on the local leaf was reported to reduce photoinhibition in systemic leaves by enhancing the mechanisms involved in photoprotection (Jiang et al., 2020). The slow systemic signal was transduced by a mobile, light induced transcription factor LONG HYPOCOTYL 5 (HY5) (Jiang et al., 2020). In addition, the simultaneous application of high light and heat on different leaves of the same *Arabidopsis* plant was able to rapidly modify the signal as well as transcriptomic, metabolic and hormonal responses (Zandalinas et al., 2020) giving indication that plants are able to integrate different simultaneous signals.

As already reviewed here, stomata play a key role in protecting plants against immediate or long-term damage caused by adverse environmental conditions or pathogen attack (Sierla et al., 2016; Melotto et al., 2017; Lawson and Vialet-Chabrand, 2019). Application of heat stress or wounding on a local leaf have previously been shown to elicit electrical signals that can cause stomatal closure in non-treated distal leaves (Koziolek et al., 2004; Hlaváčková et al., 2006; Kaiser and Grams, 2006; Grams et al., 2009; Gallé et al., 2013). More recently, high-light treatment of a single *A. thaliana* leaf was shown to trigger a ROS-dependent systemic signal that lead to the closure of stomata in almost all untreated systemic leaves (Devireddy et al., 2018). Further work by the same group implied that rapid systemic stomatal responses are also triggered in response to wounding, heat and dark-to-light transition (Devireddy et al., 2020). Interestingly, another study showed that when light irradiation was applied to the whole *Arabidopsis* rosette, stomatal opening and consequently photosynthetic induction were improved in comparison to individually irradiated leaf (Shimadzu et al., 2019). From the evidence that the faster opening of systemic stomata was compromised in the ABA transporter mutants, *abcg25* and *abcg40*, the authors suggested that the improvement of stomatal opening was caused by altered leaf water relations. More recently, the involvement of ROS wave was shown to be involved also in the rapid systemic opening of in Soybean stomata. Locally applied sudden increase in light irradiance (from 40 to 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) induced stomatal opening in the treated local leaf and untreated systemic leaves of soyabean (Zandalinas et al., 2020).

Exposure of mature leaves to low light levels or high CO₂ concentrations is known to trigger long-distance signaling that control stomatal development and cause reduction in stomatal density in young developing leaves (Lake et al., 2001; Miyazawa et al., 2006). Over shorter time periods, low light and high CO₂ are also known to affect stomatal conductance. High CO₂ and dark treatments are commonly used to elicit rapid stomatal closure and several *Arabidopsis thaliana* mutants having impaired stomatal response to these treatments have been characterized (II; Merilo et al., 2013; Hashimoto et al., 2006; Hřrak et al., 2016). Mutant plants deficient in guard cell ABA (Merilo et al., 2013) and CO₂ signaling (Hashimoto et al., 2006; Hřrak et al., 2016) pathways show impaired stomatal closure in response to darkness or elevated CO₂. However, it remains unknown whether the same cellular signaling pathways could be activated through long distance signaling leading to systemic regulation of stomatal conductance.

This review of literature reveals a gap in knowledge needed to connect studies that consider stomatal responses to fluctuating light and those that focus on the role of systemic signaling in stomatal regulation. Stomatal responses to light fluctuations are usually studied by applying treatments to or performing measurements on a single

leaf. However, in natural conditions, fluctuating light often results in uneven light distribution in the canopy. Some studies have reported the occurrence of stress signaling between adjacent leaves but to my knowledge, no other studies, than the ones reported in this thesis, have covered the importance of systemic signaling on stomatal regulation in response to light fluctuations.

3 AIMS OF THE STUDY

The original idea for the studies reported in this thesis was derived from personal observations that hybrid aspen stomata in a cuvette of a gas exchange measuring device responded to step changes in light conditions stronger and more consistently when the whole seedling was subjected to the treatment. Moreover, I speculated whether the vast variation in stomatal conductance results could be explained by an ability of stomata to receive information on the ambient conditions from other leaves. Following this, I outlined three specific aims for further studies.

The specific questions addressed in this thesis were:

1. Is systemic signaling involved in the regulation of plant gas exchange in response to changes light and intercellular CO₂ concentration?
2. Do natural light fluctuations affect stomatal regulation and photosynthesis systemically?
3. What are the possible signaling mechanisms involved in the systemic regulation of stomatal conductance?

4 MATERIALS AND METHODS

Detailed descriptions to the materials and methods used in this thesis are found in the corresponding papers (Papers II and IV). Step-by-step instructions as well as specific challenges and important notions related to the methods used in the Paper II are described in Paper III. Here, I present a brief overview on the methods used in the papers II and IV.

4.1 Plant material

Three different model plant species were used in the experiments: *Arabidopsis thaliana* (II), *Populus tremula x tremuloides* (II) and *Betula pendula* (II, IV). The experiments reported in the paper II were conducted with three-year-old silver birch (*B. pendula*) and hybrid aspen (*P. tremula x tremuloides*). Five-year-old *B. pendula* seedlings were used in the experiment reported in paper IV. In all experiments, the potted tree seedlings were grown and overwintered outside. An annual plant, *A. thaliana*, Columbia ecotype, was grown in a growth chamber.

4.2 Methods

In order to study the role of systemic signaling in stomatal responses to specific changes in ambient growth conditions, we developed an experimental set up where a leaf gas exchange measuring device (model GFS3000; Heinz Walz) was placed inside a growth chamber (FitoClima; Aralab) (Figure 2, II). One leaf of the measured seedling was enclosed in the cuvette of the gas exchange measuring system (Figure 2). This simple set up allowed us to apply treatments on local and distal leaves separately while simultaneously monitoring changes in leaf gas exchange (g_s and A). The treatments, sudden darkness or increase in ambient CO_2 concentration (from 400 to 1100 ppm), were applied in three different combinations: treatment applied to (1) the measured leaf; (2) the rest of the seedling, but not the measured leaf; or (3) the whole seedling.

The effect of systemic signaling on leaf gas exchange under naturally fluctuating environmental conditions was studied with a slightly different experimental set up. In the experiment reported in the paper IV, two leaves of the same *B. pendula* seedling were measured simultaneously with two different GFS3000 gas exchange measuring systems. Fully expanded leaves from two different branches were placed inside the cuvettes of the measuring systems (one leaf in each). The two leaves of the same seedling were exposed to different conditions. In one cuvette the growth conditions were maintained constant (PAR 1000 $\mu mol m^{-2} s^{-1}$, relative humidity 60 %, temperature

two degrees Celcius lower than the maximum temperature of the day, and CO₂ concentration of 400 ppm), and in the other cuvette the conditions (light, temperature and humidity) followed the ambient fluctuations.



Figure 2. Photographs of the experimental set-up in field conditions (IV).

Results from the gas exchange measurements conducted in paper II were statistically analyzed by fitting generalized additive mixed models (GAMMs) (Wood, 2006) on the stomatal and photosynthetic responses (g_s and A) of the measured leaf as functions of time. The analyses were done with mgcv package (version 1.8-17,) in R (version 3.4.2; R core team, 2017; <http://www.r-project.org/>). GAMMs are well suited for the analysis of gas exchange data as they do not assume linearity in the response or independence of the measuring points and analyze the whole time course.

In the paper IV, where gas exchange measurements were conducted under naturally fluctuating light, GAMMs could not be used as there were no technical replicates (ie. every treatment was different). Moreover, we conducted an in depth visual evaluation of the data. In addition, cross-correlation function (function `ccf()` in R) was used to assess correlation coefficients as well as possible lags and leads in response times between A and g_s of the measured leaves and the environmental variables, PAR and temperature. The time series of A , g_s and PAR were linearly de-trended prior to CCF in order to exclude long-term (diurnal) trends from the analysis.

5 RESULTS AND DISCUSSION

5.1 Systemic signals are involved in the regulation stomatal conductance in trees

Changes in intercellular CO₂ levels and light conditions are known to trigger rapid closure or opening of stomata (l) but could local changes in light and intercellular CO₂ levels regulate stomatal conductance and photosynthesis also in the distal leaves? To address this question, we developed an experimental set up where sudden darkness or rapid increase of ambient CO₂ concentration could be applied in three different combinations: in growth chamber, in the cuvette of the gas exchange measuring device or both. No treatments were applied in the control measurements. Whole leaf g_s and A measured from the leaf inside the cuvette of the gas exchange measuring system remained unchanged for the untreated (control) seedlings of *B. pendula*, *P.tremula x tremuloides*, and Arabidopsis. Application of either of the treatments only in the growth chamber triggered a significant reduction in stomatal conductance also in the non-treated (cuvette) leaves of the two tree species *B. pendula* and *P. tremula x tremuloides*, indicating the involvement of systemic signaling in the regulation of stomatal aperture. When the treatments were applied only in the cuvette, stomatal conductance was decreased as expected, confirming a local response. An even larger reduction in stomatal conductance was observed when the treatment was applied simultaneously in the chamber and in the cuvette, further highlighting the role of the systemic response and suggesting that the local and systemic responses have an additive effect on stomatal conductance. A similar response was observed for both silver birch and hybrid aspen in response to both treatments but in silver birch the results were more coherent and statistically significant. These results indicate that systemic signaling has a role in CO₂ and light mediated stomatal regulation in trees.

In order to study whether the involvement of systemic signaling in the regulation of stomatal conductance could be considered as a conserved mechanism within vascular plants and to study the possible signaling mechanisms in more detail, we tested four week old seedlings of the model plant, *A. thaliana*, in our experimental set up. Interestingly, in Arabidopsis systemic signaling did not seem to have an effect on stomatal conductance in response to rapid increase in CO₂ concentration or sudden darkness. This was contradictory to previous studies that had reported systemic signaling to regulate stomatal aperture in response to high light, wounding and ABA (Devireddy et al., 2018; Devireddy et al., 2020) in low light grown Arabidopsis. However, in another recent study a local application of ABA did not change leaf temperature of distal leaves indicating that the local treatment had no effect on stomatal conductance (Hörak et al., 2020). The contradictory results between the

different studies could be explained by the application of different techniques (leaf peels, thermal imaging and stomatal conductance measurements) but it is more likely that the contradictions arise from the different experimental conditions and study methods. Therefore, further research is needed to understand the possible role of systemic signaling on the regulation of leaf gas exchange in Arabidopsis.

To follow the findings presented in paper (II), I wanted to study whether systemic signaling is involved also in the stomatal and photosynthetic responses to naturally fluctuating light. To address this question, another experiment on seedlings of *B. pendula* was performed in the field under naturally fluctuating growth conditions. Gas exchange from two different leaves was measured simultaneously. The measured leaves were exposed to different treatments; one leaf was kept in pre-set constant conditions and the other was subjected to fluctuating ambient conditions. In this study the stomatal conductance of the leaf under steady conditions responded to changes in ambient light levels with a similar trend than the leaf in ambient conditions. However, the fluctuations in the g_s of the leaf in constant conditions were less pronounced than the stomatal responses of the leaf in ambient conditions. These results give strong indication that birch leaves that are not only able to respond and adjust their stomatal conductance to local light levels but also to transmit this information and to perceive signals conveying it. Moreover, the results were in line with the previous studies that have demonstrated the involvement of systemic signaling on stomatal regulation during photosynthetic induction in Arabidopsis (Shimadzu et al., 2019) and soybean (Zandalinas et al., 2020) as well as on stomatal closure in response to step changes in C_i and light in birch and hybrid aspen (II). Taken together, the results reported in the papers II and IV give a strong indication that systemic signaling has an important role in the regulation of plant gas exchange in trees under natural growth conditions.

5.2 The signaling mechanism

The regulation of stomatal conductance and the assimilation of CO_2 by chloroplast affect and are affected by the changes in C_i (Engineer et al., 2016; I). In order to study how systemic signals are perceived in the distal leaves the kinetics of stomatal conductance and CO_2 assimilation were analyzed. In both studies reported in this thesis (II and IV) the systemic changes in A (under constant conditions) were very subtle and hardly noticeable but still had similar trends than g_s measured from the same untreated leaves. In the paper II, the decrease in g_s was shown to occur prior to the decrease in A in the untreated leaves implying that sufficient change in stomatal conductance is a prerequisite for the change in assimilation. This was supported by the observations in the paper IV where C_i was shown to be regulated solely by g_s and the changes in A followed the changes in C_i . In addition, the subtle changes in A would not be sufficient to cause the observed changes in g_s in the systemic leaves in either of the

studies (II and IV). Moreover, the data suggests that the closure of stomata in response to a systemic signal is not caused by the decrease of photosynthesis and the consequent increase of the C_i . Instead, the signal is most likely perceived directly by the guard cells and the change in assimilation rate reflects the change in the supply of CO_2 through the stomata.

The signals responsible for the systemic stomatal responses observed in this thesis (II and IV) are likely transmitted by systemic ROS, Ca^{2+} or electric waves and not by changes in water potential or sugar concentration. No specific experiments on the possible systemic signaling mechanisms were conducted and thus our suggestion on the possible signaling pathways rely on existing scientific knowledge and theoretical reasoning. Previously, several studies have demonstrated the involvement of heat triggered electrical waves in the systemic regulation of stomatal aperture in *Mimosa pudica* (Koziolek et al., 2004; Kaiser and Grams, 2006), *Zea mays* (Grams et al., 2009), *Nicotiana tabacum* (Hlaváčková et al., 2006) and *Glycine max* (Gallé et al., 2013). More recently, high-light, wounding, heat and dark-to-light transition treatments of a single leaf was shown to trigger a ROS-dependent systemic signal that lead to the alteration of stomatal aperture in untreated systemic leaves of *A. thaliana* (Devireddy et al., 2018; Devireddy et al., 2018; Devireddy et al., 2020). In our studies (II and IV) the systemic stomatal response could be seen in non-treated leaves within a few minutes after the onset of treatment. ROS, Ca^{2+} and electric signals are estimated to travel several centimeters a minute (Huber and Bauerle, 2016), in other words, at velocities that could explain the fast responses observed here. By contrast, changes in the sugar status and water potential are transmitted through the vascular system and mesophyll in the order of tens of minutes, or even hours (Nobel, 2009; Mencuccini and Hölttä, 2010). In addition, water potential or sugar concentration mediated stomatal regulation would be expected to affect stomatal conductance in the opposite direction that was observed in this study (see papers II and IV for further discussion). Moreover, rapid systemically transmitted ROS, Ca^{2+} and electric waves are the only known signaling mechanisms that correspond to the speed and direction of the observed responses and are able to transmit the observed signals (Figure 3). These signaling pathways are interlinked through shared molecular components (Gilroy et al., 2016). Signal specificities could arise from the timing and integration the ROS, Ca^{2+} and electric waves and thus the same signaling mechanism could transmit information on different stress conditions as well as on natural environmental fluctuations.

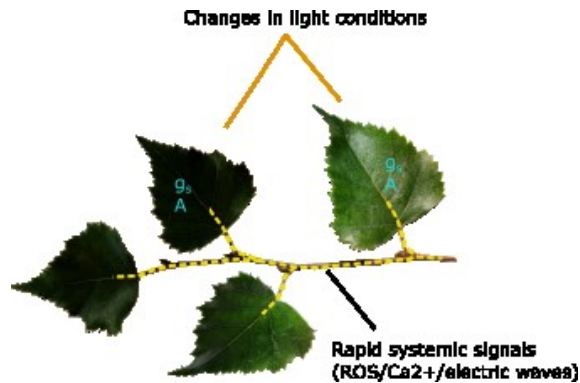


Figure 3. A proposed model for the rapid systemic stomatal responses to naturally fluctuating light in silver birch, *Betula pendula*. Local changes in irradiation cause changes in the photosynthetic assimilation of CO₂ (A) and consequently in stomatal conductance (g_s). The information on the changes in local light conditions is transmitted to distal leaves most likely through rapid systemic signaling pathways mediated by ROS, Ca²⁺ and electric waves (indicated by dashed yellow lines). In distal tissues, the signal is perceived directly by the guard cells, which adjust their aperture accordingly.

5.3 Possible sources of error in systemic signaling studies

Stomata respond to changes in various different environmental factors such as light irradiation, temperature, VPD, and chemical composition of the ambient air. This must always be taken into consideration when designing systemic signaling experiments. In laboratory conditions environmental variables may be changing, for example, due to changes in air conditioning or other activities performed in the same space. Even the presence of a researcher may alter the ambient CO₂ levels significantly (III). Unnoticed changes in ambient conditions may have significant impact on the results and if the changes occur simultaneously with the application of treatments they may even cause artefacts. The problems can be avoided by conducting the experiments in controlled conditions (e.g. inside a growth chamber) or including sufficient negative controls. Stomatal responses to different environmental variables are typically measured using several different techniques such as leaf gas exchange, thermal imaging and epidermal peels. These techniques are commonly used but each method has its limitations. In this thesis, I chose to use leaf gas exchange as a measure of stomatal regulation as it has several advantages compared to other methods: 1) a whole leaf is treated and measured at once, 2) the measured leaf can be kept in controlled conditions throughout the measurement, 3) noninvasive, high precision and time-resolved measurements on leaf gas exchange.

Time course data, where individual data points are from the same sample, should not be analyzed by commonly used simple statistical methods, such as ANOVA, as the data violates the basic assumption that each observation is independent. In this thesis we used Generalized additive mixed modelling (GAMM) and cross-correlation function (CCF) which are both appropriate and powerful statistical methods to analyze time course data and to test statistical differences between the treatment responses (Wood, 2006). In ecological research GAMMs (and other suitable methods) are already widely used but to my knowledge only in a few papers has such statistical modelling been used for leaf gas exchange data. GAMMs are often presented without original data as the model is considered as sufficient representation of the data. Gas exchange data is likewise almost always presented as mean values with standard deviation or error. However, in my opinion presenting the original data (in addition to the model estimate) is important as it visualizes the true variation in the data. Moreover, important biological information may be left unnoticed when presenting only the mean values or a statistical model.

Statistical significance does not always imply biological importance and correlation does not necessarily imply causation. Therefore, statistical analyses should always be considered together with the biological knowledge on the studied organisms. This should be taken into account already when designing experiments. Stomatal conductance data has usually a significant amount of variation. In the data presented in paper II, stomatal responses (measured as g_s) had large intraspecific variation. Although significant statistical differences were found between almost all the treatments, the variation caused uncertainties when interpreting the results. However, the same trends were seen between different treatment combinations and in different species. Moreover, the most convincing evidence for the involvement of systemic signaling on the regulation of stomatal conductance comes from the overall examination of the data. Whether or not there was a significant statistical difference (presented as p -values or model estimates) between individual treatments, the same trends was seen in the responses of both tree species (*B. pendula* and *P. tremula x tremuloides*) to both treatments (increase in ambient CO₂ and sudden darkness). This indicates that the statistical differences were biologically relevant and not caused by random artefacts.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The ability to perceive and react to changing environmental conditions is pivotal for the survival of plants. The results reported in this thesis give strong indication of the involvement of systemic signaling in the regulation of plant gas exchange in two different tree species, *B. pendula* and *P. tremula x tremuloides* (II) and that stomatal regulation is coordinated systemically under naturally fluctuating light (IV). I suggest that the systemic coordination of stomatal responses enables a leaf to prepare and adjust its responses to the upcoming change in ambient conditions and thus enhances plant productivity. In natural environment trees grow under constantly fluctuating light and different parts of a plant are receiving different amount of irradiation at a given time. Relatively slow stomatal kinetics limit the faster photosynthetic processes both in decreasing and increasing irradiation (Lawson and Vialet-Chabrand, 2019). Under increasing irradiation the limitations caused by stomatal processes have been estimated to delay reaching of the achievable maximum of photosynthetic rate for several minutes (Percy, 1990; Deans et al., 2019; Kimura et al., 2020). Similarly, under a decreasing irradiation, the decline in stomatal conductance takes several minutes longer than the almost instant decrease in assimilation (Lawson and Blatt, 2014; McAusland et al., 2016; Lawson and Vialet-Chabrand, 2019). Moreover, the relatively slow stomatal response to changes in irradiation may lead to reduction of photosynthetic efficiency or unnecessary water loss.

My motivation for the research presented in this thesis was to understand and explain the common variation in stomatal conductance measurements. I found that the possible reason for the variation are the systemic signals that affect stomatal conductance even if the measured leaf is not experiencing the concurrent changes in ambient conditions. Therefore, I suggest that the potential effect of systemic signaling on plant gas exchange should always be considered when studying stomatal processes both, *in vivo* and *in vitro* conditions. In addition, a suitable model plant must be carefully selected for every study. Ideally, the same phenomena should be studied in different species but, as scientists are only human and the passage of time is constant, conducting experiments with multiple species is usually difficult. In this thesis, I chose to use two different tree species as models to study the involvement of systemic signaling in the regulation of leaf gas exchange for two main reasons: the ecological importance and the ecophysiology of trees. Trees are long-lived perennial plants and thus they need to survive and thrive over centuries in set locations under constantly changing environmental conditions and biotic stress. Hence, they are good models for studying the regulation and acclimation of plant gas exchange in changing environment.

Previous studies have shown the role of systemic signals in the regulation of leaf gas exchange in response to extreme events or stress conditions. In this thesis, I argue that systemic signaling mechanisms are also involved in the continuous regulation of stomatal conductance under naturally fluctuating light. I have shown that rapid systemic signals are able to affect stomatal regulation but additional research is needed in order to understand the ecophysiological importance of the systemic coordination of plant gas exchange. This could be done, for example, by studying the phenomenon in full grown forest trees in natural growth environment. Further studies on the putative systemic signals in trees could be conducted by using different inhibitors of ROS and calcium signaling as well as by direct measurements of electric signals (Vian et al., 2015; De Vriese et al., 2018). In order to study the molecular mechanisms involved in the initiation, transduction and sensing of the systemic signals, other model plants should be considered. Molecular work would be easier with plants, such as barrel medic (*Medicago truncatula*) or tomato (*Solanum lycopersicum*) that have faster life cycle, widely used genomic tools and resources as well as fully sequenced and annotated genomes. However, the role of systemic signaling in stomatal regulation in these species should first be confirmed.

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