The impact of slow stomatal kinetics on photosynthesis and water use efficiency under fluctuating light

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Abstract

Dynamic light conditions require continuous adjustments of stomatal aperture. The kinetics of stomatal conductance ($g_s$) is hypothesized to be key to plant productivity and water use efficiency (WUE). Using step-changes in light intensity, we studied the diversity of light-induced $g_s$ kinetics in relation to stomatal anatomy in five banana genotypes (Musa spp.) and modeled the impact of both diffusional and biochemical limitations on photosynthesis ($A$). The dominant $A$ limiting factor was the diffusional limitation associated with $g_s$ kinetics. All genotypes exhibited a strong limitation of $A$ by $g_s$, indicating a priority for water saving. Moreover, significant genotypic differences in $g_s$ kinetics and $g_s$ limitations of $A$ were observed.

For two contrasting genotypes, the impact of differential $g_s$ kinetics was further investigated under realistic diurnally fluctuating light conditions and at the whole-plant level. Genotype-specific stomatal kinetics observed at the leaf level was corroborated at whole-plant level by transpiration dynamics, validating that genotype-specific responses are still maintained despite differences in $g_s$ control at different locations in the leaf and across leaves. However, under diurnally fluctuating light conditions the impact of $g_s$ speediness on $A$ and intrinsic ($i$WUE) depended on time of day. During the afternoon there was a setback in kinetics: absolute $g_s$ and $g_s$ responses to light were damped, strongly limiting $A$ and impacting diurnal $i$WUE. We conclude the impact of differential $g_s$ kinetics depended on target light intensity, magnitude of change, $g_s$ prior to the change in light intensity, and particularly time of day.

Introduction

In order to survive, plants need to balance CO$_2$ uptake for photosynthesis ($A$) with water loss via transpiration. By adjusting their aperture, stomata control gaseous exchange between the leaf interior, and the external atmosphere. Stomatal aperture is adjusted by moving solutes into or out of the guard cells. These changes in osmotic potential elicit water movement in or out of the guard cells, altering turgor pressure and subsequently aperture. In general, stomatal opening in well-watered C3 and C4 species is triggered by high light intensity, low vapor pressure deficit (VPD), and low CO$_2$ concentrations. Opposite environmental conditions...
(low light, high VPD, and high CO₂) stimulate stomatal closure (Assmann and Shimazaki, 1999; Outlaw, 2003; Lawson and Morison, 2004). Therefore, in a dynamic field environment, stomata are continuously adjusting the aperture to achieve an appropriate balance between carbon gain and water loss (Pearcy, 1990; Lawson and Blatt, 2014). Most research has studied stomatal conductance (gₛ) and A under steady-state conditions. A high gₛ under steady-state conditions is associated with high A and consequently improved growth (Fischer et al., 1998; Franks, 2006). However, as gₛ kinetics are typically a magnitude slower than those of A, the speed at which these steady-state values are reached in a fluctuating environment have a great influence on the growth and water use efficiency (WUE; Lawson and Blatt, 2014; Kaiser et al., 2016; McAusland et al., 2016; Taylor and Long, 2017; De Souza et al., 2020; Yamori et al., 2020). In a fluctuating field environment, light intensity is one of the most variable environmental conditions as it changes continuously by moving cloud covers and shading from adjacent plants (Pearcy, 1990; Slattery et al., 2018; Morales and Kaiser, 2020). In this way, stomata frequently experience alternating light intensities, inducing stomatal responses that change A, gₛ, and the ratio of these, the intrinsic WUE (WUE). The balance between CO₂ gain and H₂O loss under changing light intensities is disturbed by delayed gₛ responses (Viale-Chabrand et al., 2017; Slattery et al., 2018). Limitations of A after an increase in light intensity are the combination of diffusional and biochemical limitations. Biochemical activation has been shown to majorly limit A during short light flecks (Soleh et al., 2017; Taylor and Long, 2017; Acevedo-Siaca et al., 2020). Under longer light periods, limitations have been mainly attributed to stomatal limitations, with biochemical activation only limiting for a short time (<10 min) because of rapid activation of RuBP regeneration and Rubisco (Mott and Woodrow, 2000; Kaiser et al., 2016; Deans et al., 2019a; De Souza et al., 2020). The slower gₛ increase to increased light intensity limits the CO₂ uptake for A, while the slower gₛ decrease to decreased light intensity results in unnecessary water loss. The limitation of A by the slower kinetics of gₛ has been shown to be significant in well-watered C₃ species (Farquhar and Sharkey, 1982; Jones, 1998; Lawson and Blatt, 2014; McAusland et al., 2016; Deans et al., 2019a). Rapid gₛ kinetics, therefore, have been hypothesized to maximize A and WUE, as steady-state values under the new conditions can be rapidly achieved (Lawson and Blatt, 2014; Papanatsiou et al., 2019; De Souza et al., 2020; Kimura et al., 2020). The gₛ kinetics are, together with the final steady-state gₛ the plant reaches, crucial to determine the plant performance (Franks and Farquhar, 2007; Vico et al., 2011; McAusland et al., 2016; Qu et al., 2016; Faralli et al., 2019b; Yamori et al., 2020). The importance of diversity in gₛ kinetics was highlighted by De Souza et al. (2020), who showed a three-fold higher variability in carbon assimilation between cassava genotypes under fluctuating light than under steady-state conditions, mainly caused by differences in stomatal limitation. However, to our knowledge, the diversity of gₛ kinetics across varieties has neither been investigated at whole-plant level nor under diurnally fluctuating light conditions.

Here our research aimed to explore biodiversity in light-induced stomatal dynamics across genotypes and evaluate for the first time the impact on whole-plant level. We studied the diversity of light-induced gₛ kinetics in relation to stomatal anatomy in five banana genotypes (Musa spp.) with distinct transpiration phenotypes (van Wesemael et al., 2019). We modeled the impact of diffusional and biochemical kinetics on A under single step-changes in light intensity and modeled the impact of differential gₛ kinetics on A and WUE under realistic diurnal fluctuating light conditions. By comparing the gₛ kinetics in response to step-changes with the gₛ responses under fluctuating light conditions, we gain insight into the importance of stomatal kinetics on diurnal carbon gain and WUE.

### Results

#### A and gₛ response to step changes

Increasing light intensity from 100 to 1,000 μmol m⁻² s⁻¹ induced a strong stomatal opening response (Figure 1). The gₛ response followed a sigmoidal pattern. A similar sigmoidal limiting pattern was observed for A in all genotypes, indicating a strong limitation of A by gₛ in banana (Figure 1). Between genotypes, there were significant differences in the speed of gₛ increase. Steady-state A and gₛ under high light intensity were reached in three out of five genotypes. In contrast, the genotypes Cachaco and Leite continued to increase gₛ and A slowly after 90 min of 1,000 μmol m⁻² s⁻¹. The subsequent decrease in light intensity from 1,000 to 100 μmol m⁻² s⁻¹ resulted in a rapid gₛ decrease, which also followed a sigmoidal pattern (Figure 1). Photosynthesis, on the other hand, as expected decreased instantly because light became the limiting factor (Figure 1).

#### Modeling steady-state and light-induced responses of gₛ

The steady-state gₛ at 100 μmol m⁻² s⁻¹ (gₛ,100) and 1,000 μmol m⁻² s⁻¹ (gₛ,1,000) did not differ significantly between genotypes (Figure 2A; Supplemental Table S1). gₛ,100 ranged from 0.023 to 0.040 mol m⁻² s⁻¹, while gₛ,1,000 ranged between 0.14 and 0.16 mol m⁻² s⁻¹ (Figure 2A; Supplemental Table S1). The speed of gₛ increase varied strongly between the banana genotypes and the modeled variables differed significantly (Figure 2, B and C; Supplemental Table S1). The genotype with the slowest gₛ increase, Cachaco, had an average time constant Kᵣ of 17 min, while the fastest genotype, Mbwazirume, had a Kᵣ of 6.4 min (Figure 2B; Supplemental Table S1). The speed of the decrease in gₛ (Kₜ) was also genotype-dependent (Figure 2C; Supplemental Table S1). Kₜ was about two-fold higher in Cachaco (9.5 min) than in Mbwazirume (4.4 min). Across all genotypes, Kᵣ was significantly correlated with Kₜ (R² = 0.41, P < 0.001; Figure 2D; Supplemental Figure S1). However, the decrease in gₛ was significantly faster than the increase (P < 0.001). Kᵣ was significantly correlated with the time to reach 95%, 90%, and
50% of steady-state gs under the high light intensity (R^2 = 0.27–0.57, P < 0.001; Supplemental Figure S1). Also the maximal slope of gs increase and decrease (Sl_{max,i} and Sl_{max,d}) were significantly correlated with the time constant K as the magnitude of gs change was similar across genotypes (R^2 = 0.52 and 0.49 for gs increase and decrease, respectively, P < 0.001; Supplemental Figure S1). During light-induced stomatal opening comparable differences across genotypes were present in Sl_{max,i} as in K_i. The lowest Sl_{max,i} values were observed for the genotype Cachaco and the highest values for Mbwazirume (Supplemental Figure S2 and Supplemental Table S1). Sl_{max,d} was highest for the genotype Klui Tiparot, while Leite showed the lowest Sl_{max,d} (Supplemental Figure S2 and Supplemental Table S1). Analogous to the opening and closing time constant, the absolute slope of closing was significantly higher than the opening slope (P < 0.001).

Impact of stomatal opening speed on A
The speed of the increase in gs, following a step-change in light intensity from 100 to 1,000 μmol m^{-2} s^{-1} strongly determined CO₂ uptake during this period. The speed of changes in gs, in all genotypes accounted for >89% of A limitation (Supplemental Figure S3A). The time to reach 95% of steady-state A at 1,000 μmol m^{-2} s^{-1} (A_{1,000}) was >30 min for almost all genotypes and differed significantly between Cachaco (51.9 min) and the genotypes Mbwazirume (30.3 min) and Banksii (29.5 min; Figure 3A; Supplemental Figure S4 and Supplemental Table S2). This timing of A limitation was significantly correlated with the time to reach 95%, 90%, and 50% of steady-state gs (P < 0.001, R^2 = 0.42–0.48), while there was no significant relation with the time to reach 95% or 90% of the maximum carboxylation rate of Rubisco (V_{cmax}, Supplemental Figures S1 and S3). The timing to reach 95% of steady-state V_{cmax} was <20 min in all genotypes, while the timing to reach 95% of steady-state gs was much longer and ranged between 41 and 69 min (Supplemental Figure S3 and Table S2). The durations of A limitation were also significantly correlated with the modeled time constant for gs increase (K_i; P < 0.001, R^2 = 0.67; Supplemental Figure S1). The percentage limitation of A was significantly higher in Cachaco (20.6%) compared to the genotypes Mbwazirume (10.2%), Leite (10.2%), and Banksii (8.5%; Figure 3B) and was significantly related to both K_i and the time to reach 90% and 50% of steady-state gs, confirming the impact of stomatal limitation on A (Supplemental Figure S1).

iWUE response to step-changes in light intensity
The step increase in light intensity induced an initial increase in A that was relatively larger than the increase in gs-
These responsiveness differences increased \( \text{iWUE} \), reaching the maximum \( \text{iWUE} \) during the light period in all cases within 7.5 min (Supplemental Figure S5). After reaching a maximal value, \( \text{iWUE} \) decreased as both \( g_s \) and \( A \) gradually increased (Supplemental Figure S5). \( \text{iWUE} \) only stabilized when both \( A \) and \( g_s \) reached steady-state. The genotype Cachaco had a significantly higher mean \( \text{iWUE} \) during the high light period compared to Mbwazirume (Supplemental Figure S6). The mean \( \text{iWUE} \) during the high light period was significantly correlated with the time constant \( K_i \) and \( S_{\text{max},i} \) with faster \( g_s \) responses resulting in higher \( \text{iWUE} \) \((R^2 = 0.36 \text{ and } 0.26, P < 0.001; \text{Supplemental Figure S1})\).

**Stomatal anatomy**
Banana has elliptical-shaped guard cells surrounded by four to six subsidiary cells (Rudall et al., 2017). Abaxial stomatal density, stomatal length, guard cell size, and subsidiary cell size were quantified from the leaf part enclosed in the gas exchange cuvette and significant differences between genotypes were observed (Supplemental Figure S7). Stomatal density and stomatal length were not correlated with any of the modeled light-induced \( g_s \) kinetics (Figure 4; Supplemental Figure S1). However, these correlations between anatomy and \( g_s \) kinetics were significant if the genotype Cachaco with the lowest \( g_s \) rapidity was not considered...
In this case, stomatal density was significantly correlated with the time constant \( K \) as well as the maximum slope of \( g_s \) response \( S_{l_{\text{max}}} \) during both stomatal opening and closing (\( P < 0.01; R^2 = 0.25–0.46 \)).

**Whole-plant transpiration response at dawn**

The significant differences in \( g_s \) speed at leaf level observed between the two extreme genotypes Cachaco and Mbwazirume were confirmed at the whole-plant level under a step increase in light intensity from darkness (Figure 5) and under a gradually increasing light intensity (Supplemental Figure S8). After the onset of light in the morning, the transpiration rate increased significantly faster in Mbwazirume compared to Cachaco (Figure 5, A and B; Supplemental Figure S8A). After a step increase in light intensity, a significant increase in transpiration rate was observed after c. 15 min in Mbwazirume, while in Cachaco this was only after 25 min (Figure 5, A and B). Similar faster increases in transpiration rate of Mbwazirume were observed under a gradually increasing light intensity (Supplemental Figure S8A). The temporal response of whole-plant transpiration rate to a step increase in light intensity was also modeled following the sigmoidal model (Eq. 1) and the time constants \( K_i \) differed significantly between genotypes (Supplemental Figure S9). Similar to the response at leaf level, Cachaco, had an average time constant \( K_i \) of 20 min, while Mbwazirume, had a \( K_i \) of 8.5 min (Supplemental Figure S9). The difference in transpiration responses was also reflected in the transpiration rate before and after dawn. The whole-plant transpiration rate did not differ significantly between both genotypes pre-dawn, but after the step change in light intensity, the transpiration rate was significantly higher in Mbwazirume for 90 min, whereafter both genotypes reached similar steady-state transpiration rates (Figure 5B). Likewise, the transpiration rate under gradually increasing light intensity did not differ pre-dawn, but was significantly higher in Mbwazirume after the onset of light (Supplemental Figure S8B).

**Impact of diurnal light fluctuations on \( g_s \), \( A \), and \( \phi \text{WUE} \)**

To evaluate the impact of \( g_s \) kinetics on diurnal \( A \) and \( \phi \text{WUE} \), plants were subjected to fluctuating light intensities.
and phenotyped over an entire diurnal period. Similar to the transpiration rate measured at the whole-plant level, the morning increase in \( g_s \) at leaf-level under gradually increasing light intensity was faster in Mbwazirume compared to Cachaco (Figure 6A). The time constant for the \( g_s \) increase (\( K_s \)) was significantly higher in Cachaco (\( P < 0.005; \text{Figure 6B} \)). However, the faster increase of \( g_s \) in Mbwazirume, did not result in increased \( A \) (Figure 6C). Maximum potential \( A \) values at specific light intensities were determined from light response curves and compared to those measured under the diurnal conditions. Under the gradual increasing light intensities experienced in the morning, maximum \( A \) values were achieved, indicating there was no \( g_s \) limitation under these light-limiting conditions (Figure 7). A similar \( A \) with lower \( g_s \) during the morning, led to a significantly higher mean \( \text{WUE} \) in Cachaco (\( P < 0.05; \text{Figure 6D} \)).

Throughout the day, \( g_s \) kinetics were in most cases significantly faster for the genotype Mbwazirume compared to Cachaco (Figure 8A), again confirming the previously observed kinetics (Figures 2 and 5). However, under fluctuating light conditions, \( g_s \) kinetics were dependent on the magnitude of light intensity change, \( g_s \) values prior to the light intensity change, and the time of the day (Figure 8A). During the afternoon, there was a setback in kinetics: the absolute \( g_s \) and the \( g_s \) responses to light were damped (Figures 7 and 8). Simultaneously, \( A \) decreased greatly in the afternoon, which could be mainly attributed to a reduction in \( g_s \). The limitation of \( A \) in the afternoon was 3 times higher in Cachaco (52.6%) compared to Mbwazirume (17.5%; Figures 7 and 9D). The reduction of \( g_s \) in the afternoon resulted in a significantly lower average diurnal \( g_s \) (Figure 9A) which translated into a greater diurnal \( \text{WUE} \) in Cachaco compared to Mbwazirume (Figures 8C and 9C).

**Discussion**

**Stomatal behavior greatly limits \( A \) in banana**

Step changes in light intensity have been shown to induce an uncoupling of \( A \) and \( g_s \) in many species (Barradas and Jones, 1996; Lawson and Blatt, 2014; McAusland et al., 2016; Faralli et al., 2019a). However, all banana genotypes maintain a tight coupling between \( A \) and \( g_s \) following a step increase in light intensity (Figure 1). This indicates a strong stomatal control of \( A \), which is demonstrated by diffusional limitations accounting for >89% of \( A \) limitation (Supplemental Figure S3A). This high stomatal limitation of \( A \) is explained by the slow \( g_s \) response (Figures 1 and 2) relative to the faster biochemical activation. The time required for biochemical activation was much lower and not correlated with the time for steady-state \( A \) and \( g_s \) (Supplemental Figure S3). Similar to Deans et al. (2019a) and De Souza et al. (2020), the speed of changes in \( g_s \) was the predominant limitation of \( A \). This behavior shows that banana strongly controls stomatal aperture, resulting in water conservation at the expense of potential carbon gain, which supports the early work of Aubert and Catsky (1970). This prioritizing of water conservation in banana can be
explained by its intrinsic need to maintain a high leaf water potential (Turner and Thomas, 1998).

**Diversity in light-induced stomatal responses**

Stomatal responses to changes in light intensity have been shown to vary at an inter- and intra-specific level (Vico et al., 2011; Drake et al., 2012; McAusland et al., 2016; Qu et al., 2016; De Souza et al., 2020; Durand et al., 2020). A higher steady-state $g_s$ has been linked with faster light-induced $g_s$ responses (Drake et al., 2012; Kaiser et al., 2016; McAusland et al., 2016; Wachendorf and Küppers, 2017; Sakoda et al., 2020). Although the differences observed in steady-state $g_s$ values between banana genotypes were not significant, their $g_s$ kinetics differed strongly (Figure 2). These results suggest that other factors such as stomatal anatomy, hydraulic conductance and membrane transporters are involved in determining the rapidity of changes in $g_s$.

The banana B genome is often related to drought tolerance because of its center of origin and its natural occurrence in drier habitats under full sunlight (Perrier et al., 2011; Janssens et al., 2016; Eyland et al., 2021). Within the investigated banana genotypes, we observed significant differences in the speed of increase and decrease in $g_s$ (Figure 2B and C). However, differences across genotypes were not explained by their genomic constitution (see “Materials and Methods” section), which is in agreement with the wide diversity of transpiration phenotypes observed irrespective of genomic constitution (van Wesemael et al., 2019).

Consistent with previous works in other species (Vico et al., 2011; McAusland et al., 2016; Faralli et al., 2019a), the speed of $g_s$ increase and decrease was significantly correlated (Figure 2D). Decreases in $g_s$ were faster than opening in all banana genotypes (Figure 2D), which is not the case for all crops (McAusland et al., 2016; Qu et al., 2016). The faster $g_s$ closure again indicates that banana prioritizes water conservation over maximization of carbon uptake.

The two most extreme genotypes Cachaco and Mbwazirume, with the slowest and fastest $g_s$ responses, respectively, also showed at the whole-plant level differences in the light-induced speed of transpiration rate increase (Figure 5; Supplemental Figures S8 and S9). This finding suggests that despite possible differences in $g_s$ control of water loss at different locations of the leaf (Matthews et al., 2017) and across leaves of different ages (Urban et al., 2008) genotype-specific responses are still maintained. Leaf-level measurements of light-induced $g_s$ kinetics are thus in line with whole-plant responses. To our knowledge, this is the first report confirming stomatal kinetics at the whole-plant level.
level. The genotype-specific difference in whole-plant transpiration responses at dawn was validated at the leaf level with \( g_s \) increasing faster in Mbwazirume under gradually increasing light intensity (Figure 6A). This faster \( g_s \) increase in Mbwazirume did not result in higher \( A \), indicating that at dawn, under gradually increasing low light intensities, \( g_s \) was not limiting \( A \) and was higher than necessary for maximal \( A \) (Figures 6 and 7). These results demonstrate that the impact of \( g_s \) kinetics on \( A \) and \( i_WUE \) depends on the time of the day and the light conditions. The uncoupling of \( g_s \) and \( A \) under increasing light conditions at dawn was not beneficial for carbon uptake. Gosa et al. (2019) called this period after dawn in tomato the golden hour because in dry climates it is the time of the day with the highest \( g_s \). Later in the day, VPDs become too high, restricting \( g_s \) (Gosa et al., 2019). Breeding for an even higher \( g_s \) during this golden hour was suggested to improve plant productivity. However, care must be taken to breed for an improved morning \( CO_2 \) uptake, rather than for a high \( g_s \) with associated uncoupling of \( A \) and \( g_s \). Although the absolute water loss resulting from excessive morning \( g_s \) might be relatively low because of low evaporative demands at dawn (Chaves et al., 2016), it may lead to a crucial decrease in overall plant water status.

Despite the confirmed genotypic differences in stomatal kinetics, the impact of \( g_s \) kinetics on \( A \) and \( i_WUE \) before noon hardly differed between the genotypes Cachaco and Mbwazirume under field-mimicking light conditions (Figures 7 and 8). This could be explained by lower amplitudes of light switches compared to a single step change in light intensity and/or \( g_s \) values not being at steady-state prior to changing light intensity. The genotype-specific speed of the \( g_s \) response observed under a single step change in light intensity did not explain the diurnal \( i_WUE \), indicating that \( g_s \) kinetics only partially affect diurnal WUE and carbon gain (Figure 9, B and C). The absolute \( g_s \) and the \( g_s \) responses to light decreased strongly in the afternoon, and this effect was more pronounced in the genotype Cachaco (Figures 7 and 8A). The 3 times higher
afternoon limitation of $A$ in the genotype Cachaco compared to Mbwazirume, resulted in a significantly higher diurnal $i\text{WUE}$ (Figure 9, C and D). The genotype Cachaco with the slowest $g_s$ kinetics thus achieved the highest $i\text{WUE}$, showing that not only $g_s$ speed but also the $g_s$ diurnal pattern determines the overall $i\text{WUE}$ and carbon gain. Although the mechanism behind the afternoon $g_s$ reduction remains largely unknown, it is commonly hypothesized to be related to circadian regulation of ABA sensitivity and associated endogenous signals regulating the clock, such as feedback loops from photosynthetic accumulation (Mencuccini et al., 2000; Haydon et al., 2013; Delorge et al., 2014; Resco de Dios and Gessler, 2018). We show that under fluctuating light conditions this intrinsic diurnal pattern of absolute $g_s$ decrease and $g_s$ light responsiveness reduction is decisive for diurnal $i\text{WUE}$ (Figure 9C).

**Impact of stomatal anatomy on responses**

Stomatal density, as well as the size, have been reported to affect $g_s$ kinetics (Hetherington and Woodward, 2003; Drake...
et al., 2012; Raven, 2014; Sakoda et al., 2020). However, McAusland et al. (2016) and Faralli et al. (2019a) reported no or only a weak inter- and intra-specific correlation between stomatal anatomy and light-induced \( g_s \) kinetics. We confirmed that stomatal density and size were not correlated with the \( g_s \) kinetics (Figure 4; Supplemental Figure S1). Remarkably, the genotype with the slowest increase in \( g_s \), Cachaco had the second highest density and the smallest stomata. Without this genotype a significant correlation between density and the speed of \( g_s \) increase and decrease was observed (Figure 4). This exception suggests that the surface-to-volumes ratios are not always directly related to stomatal speed as this assumes uniform ion transport activity per surface area (Lawson and Blatt, 2014).

**Conclusion**

Our findings show that there is diversity in \( g_s \) rapidity to light within closely related banana genotypes and that slow stomatal responses and not biochemical activation greatly limit \( A \). The priority of banana for water saving is shown by strong stomatal control of \( A \) and faster decrease in \( g_s \) than increase. The observed diversity in \( g_s \) rapidity was not related to stomatal anatomy and therefore suggests that variation is rather driven by functional components. We show here for the first time that the \( g_s \) rapidity observed at the leaf level can also be found at the whole-plant level. However, under fluctuating light conditions, \( g_s \) rapidity is only one of the many physiological factors determining overall plant WUE and carbon gain.

**Materials and methods**

**Experiment 1: Leaf gas exchange response to a step-change in light intensity**

**Plant material and growth conditions**

Banana plants (Musa spp.) were obtained through the International Musa Transit Center (ITC, Bioversity International), hosted at KU Leuven, Belgium. Plants of five genotypes from different subgroups were selected: Banksii (subgroup Banksii, AA genome, ITC0623), Cachaco (Bluggoe, ABB genome, ITC0643), Kluai Tiparot (Kluai Tiparot, ABB genome, ITC0652), Leite (Rio, AAA genome, ITC0277), and Mbwazirume (Mutika-Lujugira, AAA genome, ITC1356). Plants were grown in 800 mL containers filled with peat-based compost (Levingtons F2S, UK) under 350 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) photosynthetic photon flux density (PPFD) in a 12-h:12-h light:dark cycle with temperature and relative humidity at 26 ± 1°C and 70 ± 10%, respectively. Plants were well-watered and starting from Week 3 a Hoagland nutrient solution was added. Measurements were performed when plants were fully acclimated and 7 weeks old.

**Leaf gas exchange measurements**

\( A \) and \( g_s \) to water were measured every 30 s on the middle of the second youngest fully developed leaf using an LI-6400XT infrared gas analysis and dew-point generator model.
LI-610 (LI-COR, Lincoln, NE, USA). Light was applied by an integrated LED light source. The leaf cuvette maintained a CO$_2$ concentration of 400 μmol mol$^{-1}$, a leaf temperature of 25°C, and a VPD of 1 kPa. All measurements were performed before 14:00 h to avoid circadian influences.

### Stomatal response to a step change in light intensity

The light intensity was kept at 100 μmol m$^{-2}$ s$^{-1}$ until A and $g_s$ were stable for 10 min. Once steady-state was reached, light intensity was increased to 1,000 μmol m$^{-2}$ s$^{-1}$ for 90 min. Then, light intensity was lowered back to 100 μmol m$^{-2}$ s$^{-1}$ for 30 min.

The increase in $g_s$ after the increase in light intensity and the decrease in $g_s$ after the decrease in light intensity followed a sigmoidal pattern and was modeled using the nonlinear sigmoidal model described in Vialet-Chabrand et al. (2017):

$$g_s = (g_{s,1000} - g_{s,100}) e^{\frac{t}{\lambda}} + g_{s,1000} \quad \text{(Eq. 1)}$$

With $g_s$, the $g_s$ at time $t$, $\lambda$ the lag time of the sigmoidal curve (min), $g_{s,100}$ and $g_{s,1000}$ (mol m$^{-2}$ s$^{-1}$) the steady-state $g_s$ at 100 and 1,000 μmol m$^{-2}$ s$^{-1}$, respectively. Parameter values were estimated for each individual plant using nonlinear model optimization in R version 3.4.3. $K_i$ indicates the $g_s$ increase time constant, $K_d$ the $g_s$ decrease time constant. The maximum slope of $g_s$ during opening and closing was calculated and defined as $S_{max}$. WUE was calculated as $\text{WUE} = A/g_s$. Outlying values (0.5% quantile; WUE < 0 or > 400 μmol mol$^{-1}$) caused by low $g_s$ were discarded for plotting.

### Stomatal and biochemical limitation analysis

A was considered to be limited until 95% of steady-state $A$ at 1,000 μmol m$^{-2}$ s$^{-1}$ was reached (McAusland et al., 2016). The percentage of limitation of $A$ was calculated by comparing the measured $A$ with the maximal steady-state $A$ under 1,000 μmol m$^{-2}$ s$^{-1}$ according to McAusland et al. (2016):

$$\text{Limitation of } A (\%) = \frac{\int_0^t(A_{\text{max}} - A_{\text{measured}}) dt}{\int_0^tA_{\text{measured}} dt} \quad \text{(Eq. 2)}$$

With $A_{\text{max}}$ the value reached at 95% of steady-state $A$ under 1,000 μmol m$^{-2}$ s$^{-1}$, $A_{\text{measured}}$ the measured $A$ and $t$ the time where 95% of steady-state $A$ is reached.

The delay in obtaining maximum potential $A$ under 1,000 μmol m$^{-2}$ s$^{-1}$ is determined by the stomatal opening speed as well as the rate of biochemical activation. The activation rate of Rubisco is the main biochemical limiting component during step changes in light exceeding several minutes (Mott and Woodrow, 2000; Way and Pearcy, 2012). To quantify the relative contributions of biochemical and stomatal limitations a differential method was applied (Jones, 1985; Wilson et al., 2000; Grassi and Magnani, 2005; Deans et al., 2019b). As explained by Deans et al. (2019b), the forgone $A$ because of biochemical and stomatal limitation was calculated as:

$$dA_{\text{biochem}} = \frac{\partial A}{\partial V_{\text{max}}} dV_{\text{max}} \quad \text{(Eq. 3)}$$

and

$$dA_{\text{stom}} = \frac{\partial A}{\partial g_{sc}} dg_{sc} \quad \text{(Eq. 4)}$$

where $V_{\text{max}}$ is the maximum velocity of Rubisco for carboxylation and $g_{sc}$ the $g_s$ to CO$_2$. $V_{\text{max}}$ at every time point was calculated by solving the Rubisco-limited $A$ as described by Farquhar et al. (1980) for $V_{\text{max}}$:

$$V_{\text{max}} = \frac{(A+R_d)(C_i+K_m)}{(C_i-\Gamma^c)} \quad \text{(Eq. 5)}$$

where $C_i$ is the CO$_2$ concentration in the intercellular airspaces of the leaf, $R_d$ represents the mitochondrial respiration for which average dark respiration rates were used. $\Gamma^c$ is the photorespiratory compensation point and $K_m$ is the effective the Rubisco Michaelis–Menten constant for CO$_2$ under 21% O$_2$. Values for $\Gamma^c$ and $K_m$ were taken as the average for C3 species at 25°C as described by Hermida-Carrera et al. (2016), 41.2 and 529.4 μmol mol$^{-1}$, respectively. Mesophyll conductance to CO$_2$ was assumed to be infinite. $g_{sc}$ at every time point was calculated as:

$$g_{sc} = \frac{g_s}{1.6} \quad \text{(Eq. 6)}$$

The relative stomatal limitation ($\sigma_{\text{stom}}$) was then calculated as:

$$\sigma_{\text{stom}} = \frac{\int_0^t dA_{\text{stom}} dt}{\int_0^t dA_{\text{biochem}} dt \int_0^t dA_{\text{stom}} dt} \quad \text{(Eq. 7)}$$

where $t$ represents the time where 95% of steady-state $A$ under 1,000 μmol m$^{-2}$ s$^{-1}$ was reached. Timings representing the $g_s$ and $A$ increase were calculated at 95%, 90%, and 50% of steady-state values under 1,000 μmol m$^{-2}$ s$^{-1}$. Timings for $V_{\text{max}}$ were calculated at 95% and 90% of steady-state values.

### Stomatal anatomy measurements

Stomatal impressions of the abaxial surface of the leaf were made when stomata were completely closed using impression material. Impression was made by applying dental polymer according to the protocol of Weyers and Johansen (1985), followed by covering the polymer with nail varnish and placement on a microscope slide. Impressions were only taken from the abaxial side, because stomatal densities are generally 75% higher compared to the adaxial side in banana, therefore majorly determining gas exchange as shown.
by Brun (1961). Stomatal anatomy was quantified using an EVOS digital inverted microscope. Stomatal density was determined in three microscopic fields of views of 1.12 mm² captured with a 10× objective lens (54–117 stomata per field of view). Guard cell length (µm), guard cell size (mm²), and lateral subsidiary cell size (mm²) were determined in three microscopic fields of views of 0.07 mm² captured with a 40× magnification, respectively (four to seven stomata per field of view). Measurements were performed in ImageJ software (http://rsb.info.nih.gov/ij).

Experiment 2: Whole-plant transpiration response at dawn

**Plant material and growth conditions greenhouse experiment**

For the genotypes Cachaco and Mbwazirume, 12 plants were grown for 7 weeks in a greenhouse prior to the experiment. Plants were grown in 10 L containers filled with peat-based compost. At the start of the experiments, the six most homogenous plants per genotype were selected based on leaf area. Weight of each plant was followed by a multysimeter setup of high precision balances, registering the weight every 60 s (1 g accuracy, Phenospex, Heerlen, Netherlands). The soil was covered by plastic to avoid evaporation and ensure only waterloss through transpiration. The transpiration rate was calculated by differentiating the raw weight data over time. The soil water content was determined by subtracting the plastic pot weight, the dry soil weight, and the plant weight from the total weight measurement. Dry soil weight was calculated as a function of the soil volume (bulk density = 0.2267 g cm⁻³). Leaf area was calculated by weekly top view imaging and model over time by a power-law function (Paine et al., 2012):

\[
\text{leaf area} = k + a \times \text{days}^b
\]  

(Eq. 8)

The daily plant weight was estimated from the projected leaf area using genotype-specific correlations (n > 50; \( R^2 \geq 0.94 \)). Plants were watered with a nutrient solution during the night and kept at well-watered conditions. Radiation was collected every 5 min via a sensor (Skye instruments, Llandrindod Wells, UK) inside the greenhouse. Supplemental lighting of 14 W m⁻² at plant level was provided when solar radiation was < 250 W m⁻² during the daytime. Temperature and relative humidity data were collected using six data loggers (Trotec, Heinsberg, Germany) registering data every 5 min. The onset of light was defined as the moment when intensity increased > 2 W m⁻².

**Plant material and growth conditions controlled environment experiment**

For the genotypes Cachaco and Mbwazirume, three plants were grown in a growth chamber with relative humidity of 70% and temperature of 24°C. Plants were grown hydroponically in containers with 350 mL medium (see van Wensemael et al. (2019) for specific nutrient composition) and placed under adjustable LED panels (LuminiGrow 600R1; Lumini technology Co. Ltd., Zhejiang, China) providing 120 µmol m⁻² s⁻¹ in a 12/12-h light/dark cycle. Plants were 5 weeks old at the start of the experiment and weighted prior to the experiment to normalize for plant mass. Biomass was again measured after 8 d, at the end of the experiment. Water loss of each plant was followed by a multiysimeter setup of high precision balances (0.01 g accuracy; Kern, Balingen, Germany). Balances were connected to a computer registering the weight every 10 s.

Experiment 3: Impact of diurnal light fluctuations on \( g_s \), \( A \), and \( \psi \)WUE

**Plant material and growth conditions**

Four plants of the genotypes Cachaco and Mbwazirume were grown in a greenhouse. Plants were grown in 4 L containers filled with peat-based compost and maintained under well-watered conditions. After 8 weeks plants were moved to a growth chamber with relative humidity 70 ± 15% and temperature 28 ± 2°C.

**Leaf gas exchange measurements**

\( A \) and \( g_s \) were measured every minute on the middle of the second youngest fully developed leaf using an LI-6800 infrared gas analyzer (LI-COR, Lincoln, NE, USA). The leaf cuvette maintained a CO₂ concentration of 400 µmol mol⁻¹, a leaf temperature of 28°C and a VPD of 1 kPa. The light intensity was programmed to fluctuate throughout the day. Plants were placed under adjustable LED panels (LuminiGrow 600R1; Lumini technology Co. Ltd., Zhejiang, China) that mimicked light fluctuations inside the LI-6800 leaf cuvette. The \( g_s \) response was described using the nonlinear sigmoidal model of Vialet-Chabrand et al. (2013) where light or dark steps were sufficiently long for model optimization (Eq. 1). A light response curve with \( A \) in function of PPFD was modeled for each individual based on \( A \) values recorded during the first 6 h of the day that was not limited by \( g_s \). The non-rectangular hyperbola-based model of Proul and Chartier (1977) was optimized as described C. Lobo et al. (2013):

\[
A = \frac{\text{PPFD} \times \Phi_0 + A_{\text{max}} - \sqrt{\Phi_0 + \text{PPFD} + A_{\text{max}}^2 - 2 \Phi_0 \times \text{PPFD}}}{2 \Phi_0} \times \Phi_0 \times \text{PPFD} + A_{\text{max}} - R_n
\]

(Eq. 9)

With \( A \) the photosynthetic rate (µmol m⁻² s⁻¹), PPFD (µmol m⁻² s⁻¹), \( \Phi_0 \) the quantum yield at PPFD of 0 µmol m⁻² s⁻¹ (µmol µmol⁻¹), \( A_{\text{max}} \) the absolute maximum photosynthetic rate (µmol m⁻² s⁻¹), \( \theta \) the dimensionless convexity factor and \( R_n \) the dark respiration (µmol m⁻² s⁻¹). The percentage of limitation of \( A \) by \( g_s \) during the afternoon (> 6 h after light onset) was calculated by estimating the maximal potential \( A \) without \( g_s \) limitation and comparing it with the measured \( A \) (Eq. 2).
Statistical analysis and data processing

All data processing and statistical analysis were carried out in R version 3.4.3. Genotypic differences were tested by applying one-way analysis of variance with a post hoc Tukey HSD test. Segmented regression was performed on the whole-plant transpiration between –90 and 90 min relative to the onset of light. Data with no significant segmented regression (P-value Davies Test < 0.05, segmented R package, 7.5% of the data) and negative slopes (2.5% of the data) were removed. Transpiration rate was calculated as the mean water loss every 30 min. To use the sigmoidal model (1) on whole-plant transpiration data, 1 min weight measurements were smoothed according to the Savitzky and Golay (1964) method with a filtering window of 21 and a fourth-order polynomial. Each day of whole-plant transpiration responses was regarded as a new replicate by incorporating a plant-specific factor and a date-specific factor as a random effect in a linear mixed model.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Correlation matrix of gas exchange and stomatal anatomy variables.

Supplemental Figure S2. Maximum slope of gs response (SLmax) to an increase in light intensity from 100 μmol m⁻² s⁻¹ to 1,000 μmol m⁻² s⁻¹ and to a decrease in light intensity from 1,000 μmol m⁻² s⁻¹ to 100 μmol m⁻² s⁻¹.

Supplemental Figure S3. Stomatal limitation of A and timings until steady-state values of gs, A, and maximum velocity of Rubisco for carboxylation (Vcmax) were reached after an increase in light intensity from 100 μmol m⁻² s⁻¹ to 1,000 μmol m⁻² s⁻¹.

Supplemental Figure S4. Increase in A after increasing the light intensity from 100 μmol m⁻² s⁻¹ to 1,000 μmol m⁻² s⁻¹. A was considered limited until 95% of steady-state A was reached.

Supplemental Figure S5. Response of iWUE and the intracellular CO2 to a step increase and decrease in light intensity from 100 to 1,000 μmol m⁻² s⁻¹ and back.

Supplemental Figure S6. Mean iWUE after the increase in light intensity from 100 to 1,000 μmol m⁻² s⁻¹ and the decrease to 100 μmol m⁻² s⁻¹ afterward.

Supplemental Figure S7. Stomatal density, stomatal length, guard cell size, subsidiary cell size, and proportion of subsidiary cells of the five banana genotypes.

Supplemental Figure S8. Gravimetric transpiration rate analysis of genotypes Cachaco and Mbwazirume under gradual increasing light intensity.

Supplemental Figure S9. Modeled time constant (KI) for the whole-plant transpiration rate increase of genotypes Cachaco and Mbwazirume after a step increase in light intensity from 0 to 120 μmol m⁻² s⁻¹.

Supplemental Table S1. Modeled steady-state and light-induced variables of the g respond to a step increase and decrease in light intensity from 100 to 1,000 μmol m⁻² s⁻¹ for five different banana genotypes.

Supplemental Table S2. Time to reach 95%, 90%, and 50% of steady-state A, gs, and Vcmax after a step increase in light intensity from 100 to 1,000 μmol m⁻² s⁻¹ for five different banana genotypes.

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Conflict of interest statement. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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