

# Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency

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## Summary

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- Both photosynthesis ( $A$ ) and stomatal conductance ( $g_s$ ) respond to changing irradiance, yet stomatal responses are an order of magnitude slower than photosynthesis, resulting in nonco-ordination between  $A$  and  $g_s$  in dynamic light environments.
- Infrared gas exchange analysis was used to examine the temporal responses and coordination of  $A$  and  $g_s$  to a step increase and decrease in light in a range of different species, and the impact on intrinsic water use efficiency was evaluated.
- The temporal responses revealed a large range of strategies to save water or maximize photosynthesis in the different species used in this study but also displayed an uncoupling of  $A$  and  $g_s$  in most of the species. The shape of the guard cells influenced the rapidity of response and the overall  $g_s$  values achieved, with different impacts on  $A$  and  $W_i$ . The rapidity of  $g_s$  in dumbbell-shaped guard cells could be attributed to size, whilst in elliptical-shaped guard cells features other than anatomy were more important for kinetics.
- Our findings suggest significant variation in the rapidity of stomatal responses amongst species, providing a novel target for improving photosynthesis and water use.

## Introduction

Stomata control the balance of gases between the internal leaf environment and the external atmosphere; regulating  $\text{CO}_2$  uptake for photosynthesis and water loss through transpiration ( $E$ ). Low stomatal conductance to water vapour ( $g_s$ ) can restrict  $\text{CO}_2$  uptake by limiting  $\text{CO}_2$  influx and thus net  $\text{CO}_2$  assimilation rate ( $A$ ), whereas high  $g_s$  facilitates high rates of  $A$ , but greater water loss is an inevitable consequence.

This balance between  $\text{CO}_2$  limitation and water loss is characterized by intrinsic water use efficiency ( $W_i$ ), which is the ratio between  $A$  and  $g_s$ . On an instantaneous timescale, maintaining a suitable and appropriate balance is impeded by the temporal stomatal responses, which are a magnitude slower than those of  $A$ . Therefore, in response to changing light, the kinetics of  $g_s$  can greatly impact  $\text{CO}_2$  uptake and water loss, which has significant implications water use efficiency (WUE). WUE can be defined as the ratio of net  $\text{CO}_2$  uptake relative to water loss through transpiration ( $E$ ) or as the ratio of biomass or yield accumulation to water use over the growing season. Consequently, WUE is often a target for improving crop performance; however, it should be noted that greater  $W_i$  is often at the expense of  $A$  (Blum, 2009; Lawson *et al.*, 2010; Lawson & Blatt, 2014). The rate of water transpired through the

stomata is an order of magnitude greater than the rate of  $\text{CO}_2$  uptake for  $A$  due to the greater water concentration gradient between the intercellular spaces within the leaf and the external atmosphere (as well as biochemical limitation on  $A$ ). In order to maintain an optimal balance between  $A$  and  $E$ , stomatal guard cells are continually adjusting to environmental and intracellular cues (Lawson & Blatt, 2014).

Many previous studies have reported a strong correlation between  $A$  and  $g_s$  (Wong *et al.*, 1979; Farquhar & Sharkey, 1982). This correlation is generally observed because steady-state values are often reported, yet under dynamic conditions  $g_s$  responses are not always coupled with  $A$  (Knapp & Smith, 1987, 1990). In natural environments, photosynthetic photon flux density (PPFD) fluctuates on timescales of seconds to days and seasons (Assmann & Wang, 2001) driven by changes in cloud cover, sun angle and shading from adjacent leaves in the canopy (Percy, 1990; Chazdon & Percy, 1991; Way & Percy, 2012). Plants therefore experience short and long term fluctuations in PPFD creating 'sun' and 'shade' flecks to which  $A$  and  $g_s$  respond. Slower stomatal opening when  $A$  responds rapidly to a PPFD increase can limit  $\text{CO}_2$  assimilation (Tinoco-Ojanguren & Percy, 1993), whilst delayed stomatal closing responses following a decrease in PPFD and photosynthesis result in unnecessary water loss when carbon gain is limited (Lawson *et al.*, 2010; Lawson & Blatt, 2014). Due to the difference in the rate of carbon gain to water loss, any disparity in the response of  $A$  and

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$g_s$  also increases the probability of water stress (Condon *et al.*, 2002). For example, slow closing of stomata when  $A$  has decreased will result in higher than necessary transpiration rates that will deplete the soil water more rapidly and thus potentially create a soil water deficit.

It has been estimated that stomata can limit  $A$  by up to 20%, which can impact substantially on crop yields (Farquhar & Sharkey, 1982; Jones, 1987, 1998; Fischer *et al.*, 1998; Lawson & Blatt, 2014). In order to maximize  $A$  and optimize  $W_i$ , species or cultivars with rapid stomatal responses would be intuitively desirable, as there would be greater synchrony with mesophyll demands for  $\text{CO}_2$ . Amplitude and rapidity of stomatal movements are therefore potential targets to improve  $A$  and  $W_i$ . The majority of studies reporting stomatal influences on photosynthesis describe steady-state values and explore the potential of increasing or decreasing  $g_s$  to enhance  $A$  or diminish water loss, however this often results in an overall reduction of  $A$  and thus productivity (Blum, 2009). We propose here to follow another approach for plant improvement, which exploits stomatal kinetics to facilitate synchronous  $g_s$  responses with mesophyll demands for  $\text{CO}_2$ , thus simultaneously reducing  $\text{CO}_2$  limitations as well as avoidable water losses, incidentally enhancing  $W_i$ .

Only a handful of investigations have focused on dynamic stomatal responses and even fewer of these have explored the effects on  $A$  and  $W_i$ . Most of these studies have examined forest understorey species and the impact of sun-fleck regimes on  $A$  and  $E$  (Pearcy, 1990; Tinoco-Ojanguren & Pearcy, 1993; Leakey *et al.*, 2005; Way & Pearcy, 2012). In addition, assessing the rapidity of stomatal movements is complicated by variation in both the sensitivity and responsiveness of stomata between different species (Ooba & Takahashi, 2003; Lawson *et al.*, 2010; Vico *et al.*, 2011) and between individuals of the same species grown in different habitats (Drake *et al.*, 2013). After a change in PPFD, the temporal response of  $g_s$  is usually composed of three steps: an initial lag where the value of  $g_s$  remains stable for several minutes, followed by an exponential phase during which rapid increases in  $g_s$  are observed before reaching final steady-state plateau (Naumburg *et al.*, 2001; Vialet-Chabrand *et al.*, 2013). Recently, a dynamic sigmoidal model has been developed by Vialet-Chabrand *et al.* (2013) to analyse the temporal response of  $g_s$  by estimating the initial lag time ( $\lambda$ ), a time constant ( $k$ ) and a steady-state target ( $G_{s\max}$ ; see Table 1 for a summary of parameters and units). The time constant was used to describe the rapidity of the exponential phase independently of the amplitude of the  $g_s$  response, facilitating species or cultivar comparisons as well as proposing a more accurate interpretation.

In order to determine the impact of stomatal responses to increasing and decreasing PPFD on limitation of  $A$ , water loss and intrinsic WUE, we have assessed and quantified the rapidity of stomatal movements in a range of plant types, including several major crops. We have selected species with kidney- and dumbbell-shaped guard cells to estimate the influence of anatomical features on rapidity of responses.

**Table 1** A summary of parameters referred to within the text with accompanying units

Parameter	Definition	Units
$A$	Net $\text{CO}_2$ assimilation rate	$\mu\text{mol m}^{-2} \text{s}^{-1}$
$A_{95}$	95% maximum $A$ under $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD	$\mu\text{mol m}^{-2} \text{s}^{-1}$
$C_a$	Atmospheric $\text{CO}_2$ concentration	$\mu\text{mol mol}^{-1}$
$C_i$	Intracellular $\text{CO}_2$ concentration	$\mu\text{mol mol}^{-1}$
$E$	Water loss via transpiration	$\text{mol m}^{-2} \text{s}^{-1}$
GCW	Guard cell width	$\mu\text{m}$
$g_s$	Stomatal conductance to water vapour	$\text{mmol m}^{-2} \text{s}^{-1}$
$G_{s\max}$	Predicted steady-state $g_s$ under $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD	$\text{mmol m}^{-2} \text{s}^{-1}$
$G_{s\min}$	Predicted steady-state $g_s$ under $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD	$\text{mmol m}^{-2} \text{s}^{-1}$
$k$	Time constant describing time taken to achieve steady-state $g_s$	min
$k_i$	Time constant for $g_s$ to increase to $G_{s\max}$ under $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD	min
$k_d$	Time constant for $g_s$ to decrease to steady-state under $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD	min
PL	Stomatal pore length	$\mu\text{m}$
PPFD	Photosynthetically active photon flux density	$\mu\text{mol m}^{-2} \text{s}^{-1}$
SD	Stomatal density	$\text{mm}^{-2}$
$S_{l\max}$	Maximum rate of $g_s$ opening to an increase in PPFD from 100 to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$	$\text{mmol m}^{-2} \text{s}^{-1}$
$r_0$	Minimum $g_s$ of the sigmoidal response of $g_s$ to a step increase in PPFD	$\text{mmol m}^{-2} \text{s}^{-1}$
VPD	Vapour pressure difference from leaf to air	kPa
$W_i$	Intrinsic water-use efficiency	$\mu\text{mol mol}^{-1}$
$W_{i95}$	$W_i$ at $A_{95}$	$\mu\text{mol mol}^{-1}$
$W_{i\max}$	Maximum $W_i$ under $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD	$\mu\text{mol mol}^{-1}$
$\lambda$	Initial lag in the response time of $g_s$ to a step increase in PPFD	min

## Materials and Methods

### Plant material and growth conditions

Thirteen important crop species (including three  $\text{C}_4$  species) were selected along with the model plant *Arabidopsis thaliana*, and the relict gymnosperm species *Ginkgo biloba*. Eight had kidney- or elliptical-shaped guard cells whilst four had dumbbell-shaped guard-cells that are typically found in grasses.

*Arabidopsis thaliana* (Columbia, Col-0) seed was germinated in  $100\text{-cm}^3$  pots containing peat-based compost (Levingtons F2S, Everris, Ipswich, UK) and grown in a controlled environment (Reftech BV, Sassenheim, the Netherlands). Photosynthetic photon flux density (PPFD) was maintained at  $155 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$  for an 8 h photoperiod, whilst temperature and vapour pressure deficit (VPD) were  $23^\circ\text{C}$  and 1.1 kPa, respectively, day and night.

Oat (*Avena sativa*), sunflower (*Helianthus annuus*), tobacco (*Nicotiana tabacum*), pea (*Pisum sativum*), tomato (*Solanum*

*lycopersicum*), Sorghum (*Sorghum bicolor*), Barly (*Hordeum vulgare*), wheat (*Triticum aestivum*), maize (*Zea mays*), French bean (*Phaseolus vulgaris*) and broad bean (*Vicia faba*) were germinated in 650-cm<sup>3</sup> pots containing peat-based compost (Levington F2S). Following germination, plants were grown in a temperature-controlled glasshouse for 4–8 wk before measuring. Established *Miscanthus* (*Miscanthus nepalensis*) were supplied in 1-l pots from a commercial nursery (Beth Chatto, Colchester, UK). Solar radiation provided a PPFD of *c.* 500 μmol m<sup>-2</sup> s<sup>-1</sup>, supplemented by sodium vapour lamps (600W; Hortilux Schrèder, Monster, the Netherlands) to 300 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD when external PPFD dropped below 1200 μmol m<sup>-2</sup> s<sup>-1</sup> over a 10 h period. Air temperature was maintained at 25°C ± 3°C during the day and 18°C ± 3°C at night. Plants were watered daily from below, with any excess water not absorbed by the pot within 2 h removed.

Rice (*Oryza sativa*) seeds were germinated and transferred to 650-cm<sup>3</sup> pots as described above and grown in a controlled environment with a photoperiod of 12 h : 12 h, light : dark at a PPFD of 500 ± 20 μmol m<sup>-2</sup> s<sup>-1</sup>, a day temperature of 25°C and VPD of 0.8 ± 0.2 kPa. Plants were measured after 12 wk.

### Leaf gas-exchange measurements

Photosynthetic carbon assimilation (*A*) and stomatal conductance to water (*g<sub>s</sub>*) were measured on the youngest fully expanded leaf using infrared gas analysis (Li-Cor 6400, Lincoln, NB, USA, and CIRAS-1, PP Systems, Amesbury, MA, USA). Light was provided by an integrated LED light source (Li-Cor, PP Systems). Leaves were first equilibrated at a PPFD of 100 μmol m<sup>-2</sup> s<sup>-1</sup> until both *A* and *g<sub>s</sub>* reached 'steady state', this being defined as a <2% change in rate during a 10-min period (*c.* 30–60 min). Once steady state was satisfied, PPFD was increased to 1000 μmol m<sup>-2</sup> s<sup>-1</sup> for 1 h before returning to 100 μmol m<sup>-2</sup> s<sup>-1</sup> for 30 min. The leaf cuvette was maintained at 400 μmol mol<sup>-1</sup> CO<sub>2</sub> concentration (*C<sub>a</sub>*), a leaf temperature of 20°C (±2°C) and a VPD of 1 ± 0.05 kPa. *A* and *g<sub>s</sub>* were recorded every 1 min. Intrinsic water use efficiency (WUE) was calculated as *W<sub>i</sub>* = *A/g<sub>s</sub>*. All measurements were completed before 14:00 h to avoid any unwanted diurnal or circadian effects on photosynthesis.

### Leaf anatomical measurements

Stomatal impressions of the ad- and abaxial leaf surfaces were taken of the same area, measured using gas exchange. A negative impression was made using a dental polymer (Xantoprene, Heraeus Kulzer Ltd, Hanau, Germany) following the methods of Weyers & Johansen (1985). Once the impression material had dried and was removed from the leaf, a positive impression was made from this by placing in nail varnish on a microscope slide. Stomatal density, guard cell length (*L*) and guard cell width were determined using IMAGEJ software (National Institute of Health, Bethesda, MD, USA) from twenty fields of view (size 1250 μm<sup>2</sup>) captured from each impression using a 5 MP eye-piece camera (MicroCAM 5 MP, Bresser Optics, Rhede, Germany).

### Modelling *g<sub>s</sub>*, *A* and *W<sub>i</sub>* responses to PPFD

In order to describe the temporal response of *g<sub>s</sub>* to a single step-change in PPFD, an analytical model derived from the model by (Violet-Chabrand *et al.*, 2013) was used (Fig. 1).

The model described the temporal response of *g<sub>s</sub>* using a time constant (*k*, min), an initial time lag (*λ*, min) and a steady-state *g<sub>s</sub>* (*G<sub>smax</sub>*, mmol m<sup>-2</sup> s<sup>-1</sup>) reached at given PPFD:

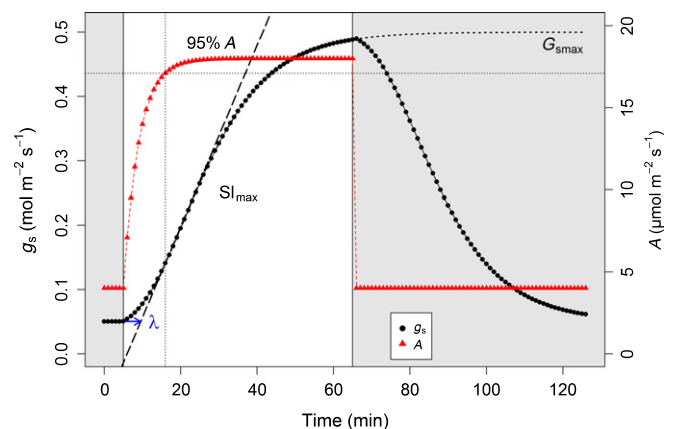
$$g_s = (G_{smax} - r_0)e^{-e^{\left(\frac{k-t}{k} + 1\right)}} + r_0 \quad \text{Eqn 1}$$

(*t*, time, where time 0 is the point at which PPFD was increased from 100 to 1000 μmol m<sup>-2</sup> s<sup>-1</sup>; *r<sub>0</sub>* (mmol m<sup>-2</sup> s<sup>-1</sup>), initial value of stomatal conductance before the change in PPFD). In this equation, the time constant *k* is a measure of the rapidity of response of *g<sub>s</sub>* independent of the amplitude of variation in *g<sub>s</sub>* (Eqn 2). To distinguish between the time taken for the stomata to open (increase) and to close (decrease), the abbreviations *k<sub>i</sub>* and *k<sub>d</sub>* are used.

A second parameter combining rapidity and amplitude of the response, the maximum slope (*S<sub>lmax</sub>*), was used to describe the maximal slope of the *g<sub>s</sub>* response to the step-change in PPFD:

$$S_{lmax} = \frac{k \cdot (G - r_0)}{e} \quad \text{Eqn 2}$$

Parameter values were estimated using a Metropolis Hasting algorithm and a Bayesian model. The priors (*a priori* probability of the parameter values) used were uniform covering a large range of possible values and the initial values were chosen randomly. The initial values were chosen from observed values (± 10%) of both *r<sub>0</sub>* and *G*. For *k*, the range of values were selected from between 10 and 60 min, whilst *λ* values were between 0.1 and



**Fig. 1** Theoretical temporal response of stomatal conductance (*g<sub>s</sub>*; black) and net CO<sub>2</sub> assimilation (*A*; red) to a step change in PPFD from 100 (shaded area) to 1000 (unshaded area) μmol m<sup>-2</sup> s<sup>-1</sup>. Where *S<sub>lmax</sub>* describes the maximum temporal response of *g<sub>s</sub>* (dashed line), *λ* describes the time-lag before *g<sub>s</sub>* starts to increase (blue arrow) and *G<sub>smax</sub>* describes the steady-state target of *g<sub>s</sub>* under 1000 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD. The dotted lines represented the time and the value were 95% *A* is reached.

5 min. After 100 000 iterations using a thinning factor of 15, the chains were checked for stability and convergence (see Table 1).

### Temporal responses in $g_s$ limits $A$

During a step increase in PPFD, photosynthesis was considered limited by stomatal conductance until 95%  $A$  ( $A_{95}$ ) was reached. Using this assumption, the percentage of limitation of  $A$  by  $g_s$  was estimated by:

$$\text{Limitation (\%)} = \frac{\int_0^t (A_{\max} - A)}{\int_0^{60} A_{\text{tot}}} \quad \text{Eqn 3}$$

( $\int_0^t (A_{\max} - A)$ , integral of the difference between the maximum potential  $A$  ( $A_{\max}$ ) and the observed limited  $A$  from the beginning of the observed curve to the time  $t$  where  $A$  reached 95% of the steady state;  $\int_0^{60} A_{\text{tot}}$ , maximum integral of  $A$  for 1 h period). Calculating the ratio using  $\int_0^{60} A_{\text{tot}}$  normalized  $g_s$  limitation over the 1-h measurement period (see Table 1 for a summary of parameters).

### The impact of different $g_s$ and $A$ responses on water loss

The nonsynchronous  $g_s$  and  $A$  response influences the temporal  $W_i$  response and the amount of water lost following a step increase in PPFD. To investigate the impact of  $g_s$  responses on water use efficiency, we predicted  $g_s$  from a simple model ( $g_s = A/W_i$ ) using a constant  $W_i$  during the transient response. The constant value of  $W_i$  was chosen close to the maximum  $A$  (95%), assuming that this would be close to an optimal  $W_i$  with no limitation of  $A$ . On the one hand, when observed values of  $g_s$  were greater than predicted by the constant  $W_i$  model, more water was 'lost' than required to maintain optimal  $A$ ; on the other, when observed values of  $g_s$  were lower than predicted, water was 'saved', illustrating

a close coupling of  $g_s$  with  $A$ . As an investigating tool, this approach allowed us to assess the percentage of water 'lost' and 'saved' by comparing the coupling between  $A$  and  $g_s$  in different species.

### Statistical analysis

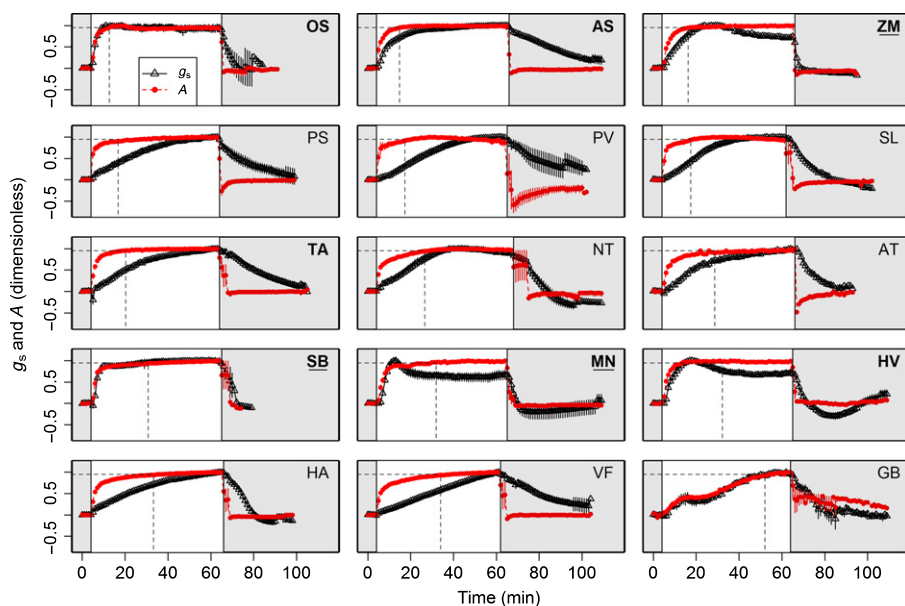
Statistics were conducted using SPSS (v.16; SPSS Inc., Chicago, IL, USA) and R (<http://www.r-project.org/>). A Shapiro–Wilk test was used to test for normality and a Levene's test of homogeneity was used to determine if samples had equal variance. Single factor differences were analysed using a one-way ANOVA with a Tukey–Kramer honest significant difference test where more than one group existed or a Student's  $t$ -test where only two groups were compared.

## Results

Most species measured achieved steady-state  $g_s$  after 60 min of high PPFD, except *Helianthus* and *Vicia*, which had not attained their maximum  $g_s$  values within this timeframe (Fig. 2), which might have led to an underestimation of their  $k_i$  values, which were already high (Fig. 3). Additionally, Ginkgo displayed atypical  $g_s$  and  $A$  behaviour (Fig. 2). The 30-min exposure to low light may not have been sufficient for complete, steady-state stomatal closure for some species; however, this does not greatly impact our estimations of additional water loss compared to instantaneous stomatal responses, because the major part of the water loss can be attributed to the initial rapid opening response of the stomata (Fig. 2).

### Quantifying $A$ and $g_s$ responses to step changes in PPFD

Steady-state  $g_s$  at the initial PPFD of  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  ( $G_{\text{min}}$ ) varied significantly among the species ( $F_{(14,49)} = 5.007$ ,  $P < 0.0001$ ), with the lowest values recorded for *G. biloba*



**Fig. 2** Normalized temporal response of net  $\text{CO}_2$  assimilation ( $A$ ; circles) and stomatal conductance to water vapour ( $g_s$ ; triangles) of 15 species to an increase in irradiance from 100 (shaded area) to  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  followed by a decrease to  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  (see Table 3 for abbreviations of species nomenclature). The dashed line indicates where 95% maximum  $A$  ( $A_{95}$ ) was achieved. Data are the mean  $\pm$  SE ( $n = 3\text{--}5$ ). Values were normalized to the initial values at  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD and maximum values at ( $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD).

(13.2 mmol m<sup>-2</sup> s<sup>-1</sup>) and highest values for *T. aestivum* (255.9 mmol m<sup>-2</sup> s<sup>-1</sup>) (Fig. 3a; Supporting Information Fig. S1), whereas *A* was below 9 μmol m<sup>-2</sup> s<sup>-1</sup> for all species (Figs 3b, S2). An increase in PPFD to 1000 μmol m<sup>-2</sup> s<sup>-1</sup> led to an immediate and rapid increase in *A* compared to *g<sub>s</sub>* for all species. After this initial period, the increase in *A* slowed to a magnitude similar to the concurrent increase in *g<sub>s</sub>*. For the majority of species, *A* reached steady state while *g<sub>s</sub>* continued to increase. These different periods of coupled and uncoupled responses of *A* and *g<sub>s</sub>* were species-dependent. Although the majority of species displayed a mainly uncoordinated *A* and *g<sub>s</sub>* temporal response (Fig. 2), final steady-state values of *A* and *G<sub>smax</sub>* were significantly correlated among species (*r*<sub>(51)</sub> = 0.78,

*P* < 0.001 for C<sub>3</sub> species and *r*<sub>(13)</sub> = 0.84, *P* < 0.001 for C<sub>4</sub> species – Fig. S3). In contrast to the majority of the species examined, *S. bicolor*, *O. sativa* and *G. biloba* all exhibited low *g<sub>s</sub>* and an unusually strong coupling between *A* and *g<sub>s</sub>*. The key difference between these three species was that *S. bicolor* and *O. sativa* exhibited a faster response of *A* and *g<sub>s</sub>*, whereas *G. biloba* showed rather slower responses.

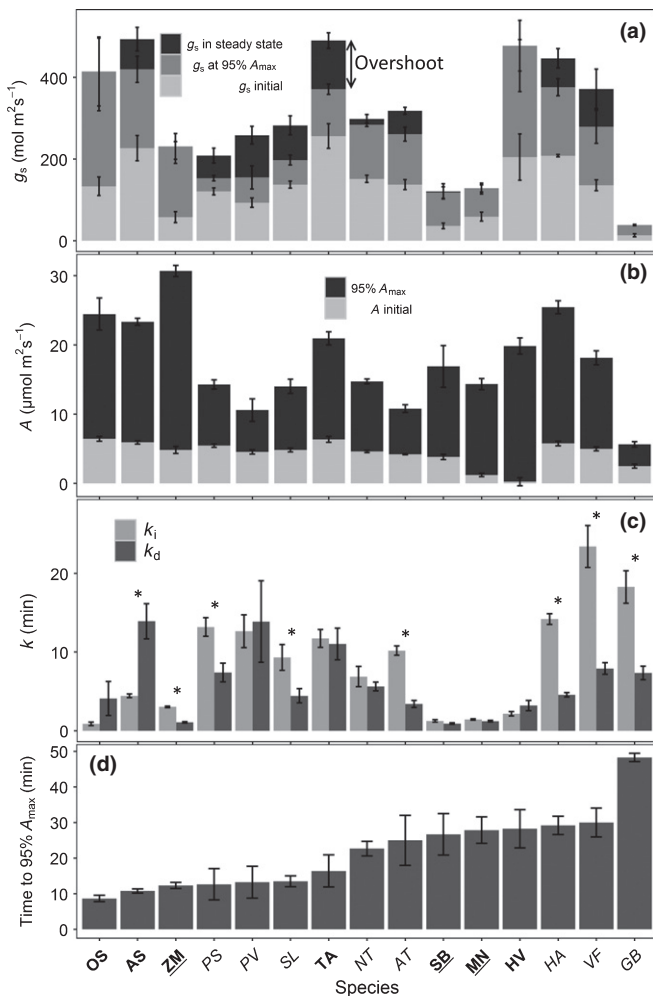
The steady-state values of *g<sub>s</sub>* (at 1000 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD) estimated by the model (*G<sub>smax</sub>*) were significantly different among species (*P* < 0.0001, *F*<sub>(14,49)</sub> = 14.469; Fig. 3a), with observed values 10-fold higher in *T. aestivum* (482.9 ± 18.7 mmol m<sup>-2</sup> s<sup>-1</sup>) compared with *G. biloba* (45.2 ± 1.9 mmol m<sup>-2</sup> s<sup>-1</sup>). Values of *G<sub>smax</sub>* were positively related to *S<sub>lmax</sub>* (*r* = 0.61, *P* < 0.01) in elliptical- and dumbbell-shaped guard cells (*r* = 0.41, *P* < 0.05), whereas *S<sub>lmax</sub>* was related to the total time taken to open to *G<sub>smax</sub>* (*k*) in elliptical- (*r* = -0.54; *P* < 0.01) and dumbbell-shaped (*r* = -0.68; *P* < 0.01) guard cells (Table 2). Six out of the seven species with dumbbell-shaped guard cells showed the highest values of *S<sub>lmax</sub>* (Table 3). Applying the Vialet–Chabrand model to the temporal response of *g<sub>s</sub>* to an increase from 100 to 1000 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD, showed that the initial lag time in the *g<sub>s</sub>* response (*λ*) was significantly different among species (*P* < 0.0001, *F*<sub>(14,49)</sub> = 5.819) and ranged from 12 s for *A. sativa* to 6 min for *G. biloba* (Table 3).

After PPFD was returned to 100 μmol m<sup>-2</sup> s<sup>-1</sup>, *A* decreased immediately whereas *g<sub>s</sub>* showed a slow, exponential and species-dependent decrease. Most species showed a temporal response of *g<sub>s</sub>* to decreasing light which was an order of magnitude lower than that of *A*, with some exceptions. *Sorghum bicolor* and *Z. mays* demonstrated significantly lower values of *k<sub>d</sub>* (< 1 min; Fig. 3c) and thus faster responses compared to other species, approaching the speed of assimilation rate response to light (Fig. 2). When PPFD was returned to 100 μmol m<sup>-2</sup> s<sup>-1</sup>, no significant differences in *λ* were observed (data not shown), although steady-state *g<sub>s</sub>* and *S<sub>lmax</sub>* varied significantly amongst species.

Significant differences in the opening (*k<sub>i</sub>*) and closing (*k<sub>d</sub>*) time constants were observed amongst species (Fig. 3c); *k<sub>i</sub>* ranged from 0.9 min in *O. sativa* to 23 min in *V. faba*, and *k<sub>d</sub>* ranged from 0.9 min in *S. bicolor* to 14 min in *P. vulgaris*. *k<sub>i</sub>* and *k<sub>d</sub>* were positively correlated in species with elliptical- (*R*<sup>2</sup> = 0.29, *P* < 0.01) and dumbbell-shaped (*R*<sup>2</sup> = 0.52; *P* < 0.001) guard cells. Although the majority of species showed tendencies for greater rapidity in stomatal closing than opening (Fig. 3c), this was significant in only six species (Fig. 3c). On average, the species with dumbbell-shaped guard cells were 10 min faster in opening than elliptical species, reaching *G<sub>smax</sub>* in significantly shorter periods of time (*t*<sub>(44)</sub> = -8.2, *P* > 0.0001). C<sub>4</sub> species increased *g<sub>s</sub>* more rapidly than C<sub>3</sub> species (*P* < 0.0001). Estimations from the closing response showed that dumbbell-shaped guard cells were also faster than elliptical-shaped guard cells (*P* < 0.04) and C<sub>4</sub> species closed faster than C<sub>3</sub> species (*P* < 0.0001) (Table 3).

### *g<sub>s</sub>* limitation of *A*

In order to assess the extent that *A* was limited by *g<sub>s</sub>* during the increase of PPFD, we determined the time taken to reach 95% of



**Fig. 3** Comparison between species of (a) steady-state *g<sub>s</sub>* under 100 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD (*G<sub>smin</sub>*), *g<sub>s</sub>* at 95% maximum net assimilation under 1000 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD (*A<sub>95</sub>*) and steady state (*G<sub>smax</sub>*) under 1000 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD for 15 species; (b) steady-state *A* under 100 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD (*A* initial) and *A<sub>95</sub>*; (c) time constants *k<sub>i</sub>* and *k<sub>d</sub>* for stomatal opening and closure, respectively; and (d) the time taken to reach *A<sub>95</sub>*. Data are the mean ± SE (*n* = 3–5). Asterisks represented a significant asymmetry of *k<sub>i</sub>*/*k<sub>d</sub>* (*P* < 0.05). Species in bold have dumbbell-shaped guard cells, underlined species have a C<sub>4</sub> metabolism and species in plain font have elliptical-shaped guard cells and C<sub>3</sub> metabolism (see Table 3 for species name abbreviations).

**Table 2** Correlation matrix between parameters (grey cells) describing the temporal response of  $g_s$  during opening and closing of elliptical- (upper triangle of the matrix) and dumbbell-shaped (lower triangle of the matrix) guard cells (see Table 3)

Elliptical								
Dumbbell	$G_{smax}$	ns	−0.48*	0.61**	0.47*	ns	ns	
	ns	$k_i$	ns	−0.54**	ns	ns	ns	
	ns	ns	$\lambda$	ns	ns	ns	ns	
	0.41*	−0.42*	ns	$SI_{max}$	ns	0.47*	0.46*	
	ns	ns	ns	ns	$SD$	ns	ns	
	0.47**	0.72***	ns	ns	ns	$PL$	0.91***	
	ns	ns	ns	ns	0.55**	−0.41*	$GCW$	
Elliptical								
Dumbbell	$G_{smax}$	ns	ns	ns	ns	ns	ns	
	0.44*	$K_d$	ns	0.68***	ns	ns	ns	
	ns	0.49**	$\lambda$	ns	ns	ns	ns	
	0.37*	0.62***	ns	$SI_{max}$	−0.68***	ns	ns	
	ns	ns	ns	ns	$SD$	ns	ns	
	0.6***	0.73***	ns	ns	ns	$PL$	0.91***	
	ns	ns	ns	ns	0.55**	−0.41**	$GCW$	

$G_{smax}$ , predicted steady-state  $g_s$  under 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD;  $k_i$ , time constant for  $g_s$  to increase to  $G_{smax}$  under 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD;  $K_d$ , decrease from  $G_{smax}$  to  $G_{smin}$  under 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ;  $\lambda$ , initial lag in the response time of  $g_s$  to a step increase in PPFD;  $SI_{max}$ , maximum rate of  $g_s$  opening to an increase in PPFD from 100 to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Anatomical parameters of stomatal density (SD), pore length (PL) and guard cell width (GCW) were also compared. Significance: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, not significant.

**Table 3** Parameters of the dynamic model of  $g_s$  as estimated from a step increase in irradiance from 100 to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 15 species

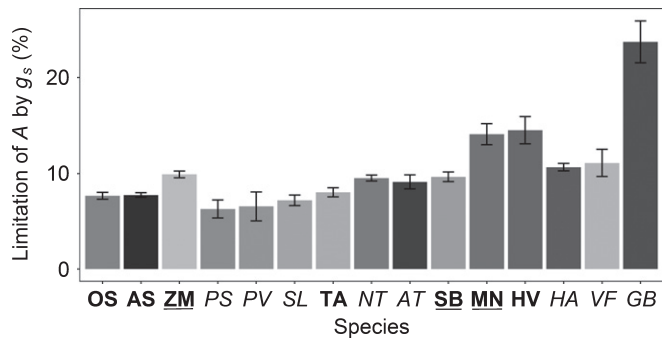
Species	Shape of guard cell/metabolism	Graph initials	$K_i$ (min)	$K_d$ (min)	$\lambda$ (min)	$SI_{max}$ ( $\text{mmol m}^{-2} \text{s}^{-2}$ )	G ( $\text{mmol m}^{-2} \text{s}^{-1}$ )
<b><i>Oryza sativa</i></b>	Dumbbell/C <sub>3</sub>	OS	0.9 ± 0.21 <sup>a</sup>	4.1 ± 2.16 <sup>abc</sup>	0.11 ± 0.02 <sup>a</sup>	1.91 ± 0.60 <sup>a</sup>	424.50 ± 89.99 <sup>abcd</sup>
<b><i>Sorghum bicolor</i></b>	Dumbbell/C <sub>4</sub>	SB	1.2 ± 0.16 <sup>a</sup>	0.9 ± 0.09 <sup>a</sup>	1.04 ± 0.17 <sup>a</sup>	0.46 ± 0.11 <sup>bc</sup>	118.32 ± 15.91 <sup>ef</sup>
<u><i>Miscanthus nepalensis</i></u>	Dumbbell/C <sub>4</sub>	MN	1.4 ± 0.11 <sup>a</sup>	1.2 ± 0.10 <sup>a</sup>	1.36 ± 0.16 <sup>ab</sup>	1.56 ± 0.11 <sup>bc</sup>	175.56 ± 18.53 <sup>ef</sup>
<b><i>Hordeum vulgare</i></b>	Dumbbell/C <sub>3</sub>	HV	2.2 ± 0.30 <sup>a</sup>	3.2 ± 0.70 <sup>ab</sup>	0.62 ± 0.37 <sup>a</sup>	1.01 ± 0.24 <sup>ab</sup>	529.07 ± 55.85 <sup>a</sup>
<b><i>Zea mays</i></b>	Dumbbell/C <sub>4</sub>	ZM	3.0 ± 0.10 <sup>ab</sup>	1.1 ± 0.08 <sup>a</sup>	1.37 ± 0.23 <sup>ab</sup>	0.37 ± 0.02 <sup>bc</sup>	244.31 ± 33.18 <sup>cdef</sup>
<b><i>Avena sativa</i></b>	Dumbbell/C <sub>3</sub>	AS	4.4 ± 0.23 <sup>ab</sup>	14.1 ± 2.37 <sup>d</sup>	0.20 ± 0.02 <sup>a</sup>	0.34 ± 0.03 <sup>c</sup>	478.95 ± 30.05 <sup>ab</sup>
<i>Nicotiana tabacum</i>	Elliptical/C <sub>3</sub>	NT	6.9 ± 1.28 <sup>abc</sup>	6.5 ± 0.57 <sup>abcd</sup>	5.91 ± 1.39 <sup>bc</sup>	0.13 ± 0.01 <sup>c</sup>	316.21 ± 10.92 <sup>bcdde</sup>
<i>Solanum lycopersicum</i>	Elliptical/C <sub>3</sub>	SL	9.3 ± 1.64 <sup>bcd</sup>	4.4 ± 0.90 <sup>abc</sup>	3.27 ± 0.22 <sup>abc</sup>	0.10 ± 0.01 <sup>c</sup>	286.12 ± 22.78 <sup>bcdde</sup>
<i>Arabidopsis thaliana</i>	Elliptical/C <sub>3</sub>	AT	9.9 ± 0.69 <sup>bcd</sup>	3.4 ± 0.61 <sup>abc</sup>	1.0 ± 0.56 <sup>ab</sup>	0.11 ± 0.01 <sup>c</sup>	307.91 ± 8.69 <sup>bcdde</sup>
<b><i>Triticum aestivum</i></b>	Dumb-bell/C <sub>3</sub>	TA	11.7 ± 1.13 <sup>cd</sup>	11.8 ± 2.54 <sup>cd</sup>	1.03 ± 0.69 <sup>a</sup>	0.13 ± 0.02 <sup>c</sup>	482.98 ± 18.66 <sup>ab</sup>
<i>Phaseolus vulgaris</i>	Elliptical/C <sub>3</sub>	PV	12.6 ± 2.08 <sup>cde</sup>	11.4 ± 6.10 <sup>abcd</sup>	3.75 ± 1.20 <sup>abc</sup>	0.10 ± 0.01 <sup>c</sup>	275.42 ± 23.58 <sup>cde</sup>
<i>Pisum sativum</i>	Elliptical/C <sub>3</sub>	PS	13.2 ± 1.18 <sup>cde</sup>	7.9 ± 1.04 <sup>abcd</sup>	0.26 ± 0.03 <sup>a</sup>	0.04 ± 0.00 <sup>c</sup>	209.78 ± 18.34 <sup>def</sup>
<i>Helianthus annuus</i>	Elliptical/C <sub>3</sub>	HA	14.2 ± 0.67 <sup>de</sup>	4.7 ± 0.35 <sup>abc</sup>	0.33 ± 0.03 <sup>a</sup>	0.09 ± 0.00 <sup>c</sup>	446.25 ± 25.52 <sup>abc</sup>
<i>Ginkgo biloba</i>	Elliptical/C <sub>3</sub>	GB	18.3 ± 2.07 <sup>ef</sup>	7.3 ± 0.85 <sup>abcd</sup>	6.13 ± 2.53 <sup>c</sup>	0.01 ± 0.00 <sup>c</sup>	45.20 ± 9.10 <sup>f</sup>
<i>Vicia faba</i>	Elliptical/C <sub>3</sub>	VF	23.4 ± 2.68 <sup>f</sup>	7.9 ± 0.75 <sup>abcd</sup>	0.29 ± 0.07 <sup>a</sup>	0.08 ± 0.02 <sup>c</sup>	430.14 ± 55.49 <sup>abcd</sup>

$k_i$  and  $K_d$ , time constants for stomatal opening and closing, respectively;  $\lambda$ , initial lag time in response to an increase in irradiance;  $SI_{max}$ , maximum slope of the temporal response of  $g_s$ ; G, steady-state target reached under 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. The data are means ± SE ( $n = 3-8$ ). Lowercase letters refer to significant differences ( $P < 0.05$ ) between species (Tukey–Kramer honest significant difference). Species in bold have dumbbell-shaped guard cells, underlined species have a C<sub>4</sub> metabolism and species in plain font have elliptical-shaped guard cells and C<sub>3</sub> metabolism.

maximum  $A$  ( $A_{95}$ ) at 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD (Fig. 3d). Further increases in  $g_s$  after  $A_{95}$  were substantially greater than the remaining 5% increase in  $A$ , suggesting that  $g_s$  was no longer limiting  $A$  (Fig. 2). Stomata opening did not increase significantly after  $A_{95}$  had been reached for the species with dumbbell-shaped guard cells (*Z. mays*, *S. bicolor*, *M. nepalensis*, *O. sativa*) and for *G. biloba* (Fig. 2). The majority of species achieved  $A_{95}$  within 30 min with values ranging between 5.7 and 30.7  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (Fig. 3b,d). *Zea mays* and *A. sativa* both attained the highest  $A$  and achieved  $A_{95}$  in the least time (12–11 min; Fig. 3d), whereas

*G. biloba* was the slowest, taking on average 48 min to achieve the lowest  $A_{95}$ . Species with elliptical-shaped guard cells achieve significantly lower  $A_{95}$  ( $P < 0.0001$ ) compared with species with dumbbell-shaped guard cells (Fig. 3b). The percentage of stomatal limitation of  $A$  during the opening response to  $A_{95}$  (Fig. 4) demonstrated significant variation among species ( $P < 0.0001$ ,  $F_{(14,49)} = 27.368$ ), with values ranging from 6.5% (*P. sativum*) to 24.3% (*G. biloba*). With the exception of *G. biloba*, which was statistically different from all other species, the limitation was 10–15%. Values of  $A_{95}$  also provided a set point from which to

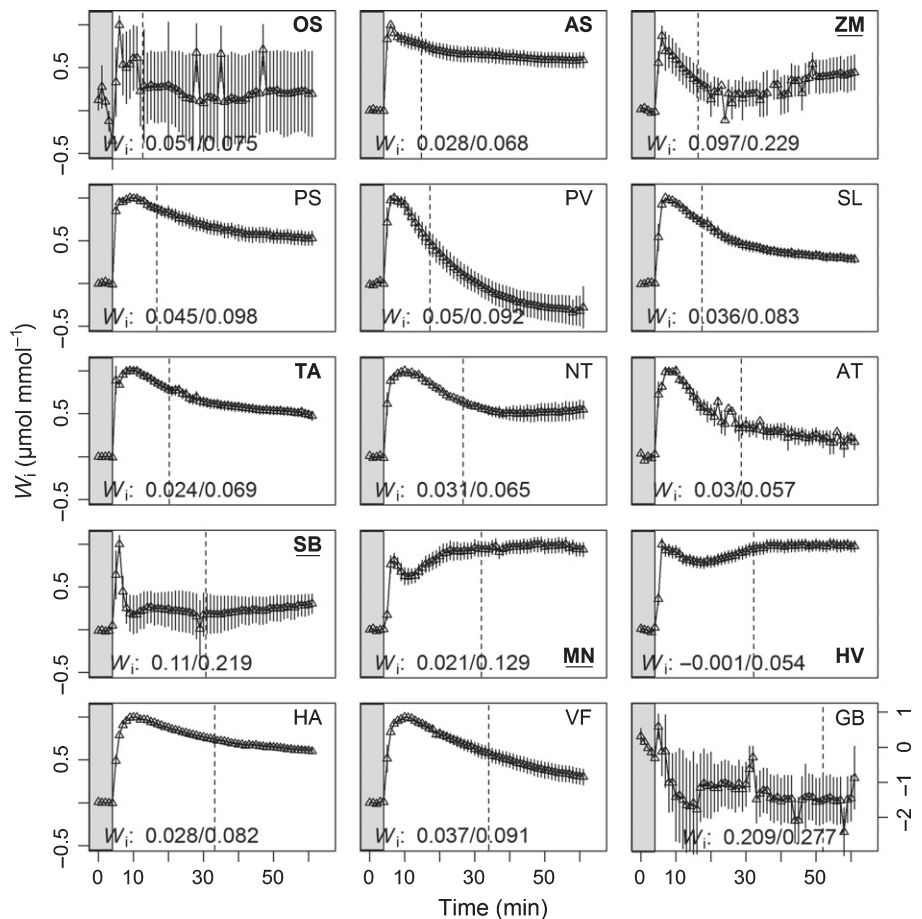
quantify the  $g_s$  response after  $A$  achieved near maximum steady state (Fig. 2, dotted line) with the majority of species with elliptical-shaped stomata showing an ‘overshooting’ in stomatal opening, demonstrated by a significant increase in  $g_s$  ( $P < 0.01$ ) between 10 and 125  $\text{mmol m}^{-2} \text{s}^{-1}$  (Fig. 3a). Species with dumbbell-shaped stomata (with the exception of *T. aestivum*; *A. sativa*) and *G. biloba* showed no or only a small ‘overshoot’ ( $< 3 \text{ mmol m}^{-2} \text{s}^{-1}$  (Fig. 3a)).



**Fig. 4** Percentage limitation of net CO<sub>2</sub> assimilation ( $A$ ) by stomatal conductance ( $g_s$ ) after the 60 min at 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD. Data are the mean  $\pm$  SE ( $n = 3-5$ ). Species in bold have dumbbell-shaped guard cells, species underlined have a C<sub>4</sub> metabolism and species in plain font have elliptical guard cells and C<sub>3</sub> metabolism (see Table 3 for species name abbreviations).

### Quantifying $W_i$ responses to a step change in PPFD

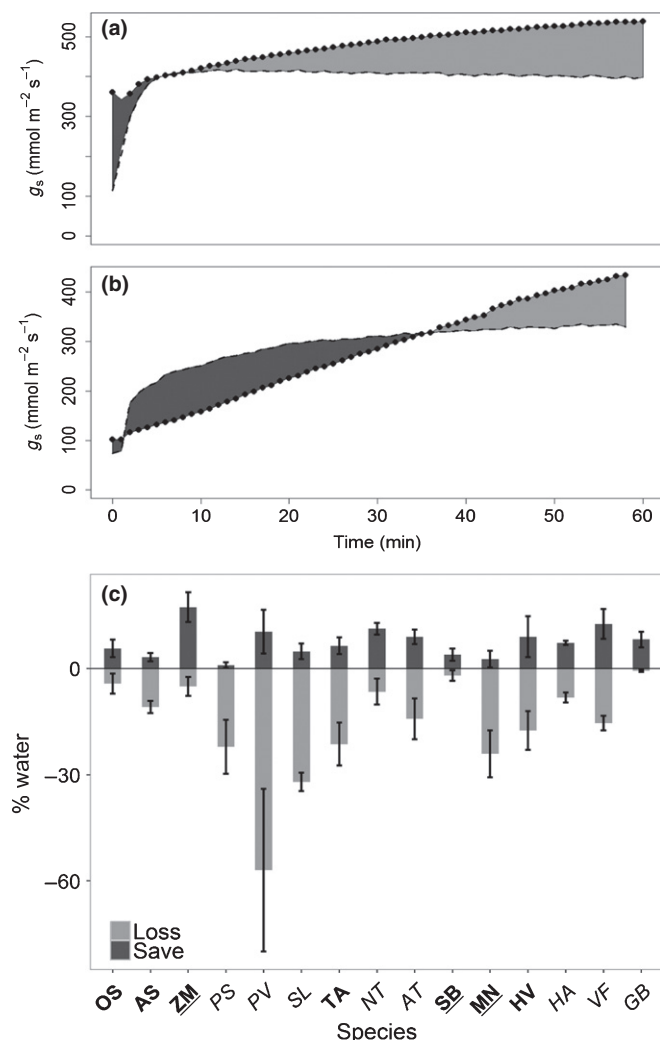
The consequence of the lack of synchrony between the responses of  $A$  and  $g_s$  to a step increase in PPFD can be illustrated by the temporal responses of  $W_i$  (Figs 5, S4). Following the step increase in PPFD,  $A$  rapidly increased compared to  $g_s$  (Fig. 2) and  $W_i$  reached a maximum value ( $W_{i\text{max}}$ ) well before  $A_{95}$  was achieved. The first 20 min of this response is shown in Fig. S4. The subsequent further increases in  $g_s$  drove a continuous decrease in  $W_i$  until both  $A$  and  $g_s$  reached steady state. In most of the C<sub>3</sub> species,  $W_i$  continued to decrease after  $A$  had reached a steady state due to the continued increase in  $g_s$ .  $W_{i\text{max}}$  represents the greatest CO<sub>2</sub> uptake for  $g_s$ ; however, it should be noted that this value occurs earlier in the transient response before  $A_{95}$  is reached and that  $W_{i\text{max}}$  is achieved only for an extremely brief period of time. The diversity in  $W_i$  between species was determined by  $G_{s\text{max}}$  rather than  $A_{\text{max}}$  as no correlation between  $A_{\text{max}}$  with  $W_{i\text{max}}$  was observed, whereas  $G_{s\text{max}}$  was negatively correlated with  $W_{i\text{max}}$  ( $P < 0.0001$ ,  $r_s = -0.67$ ) and with  $W_i$  before the decrease in PPFD ( $P < 0.0001$ ,  $r_s = -0.74$ ). For example, *G. biloba*, *S. bicolour*, *M. nepalensis* and *Z. mays* achieved and maintained the highest  $W_i$  ( $P < 0.001$ ) with vastly different values of  $A$  by maintaining a relatively low  $g_s$  compare to other species. The balance between CO<sub>2</sub> fixation and water loss was different between species, revealed by the four-fold difference in  $W_{i\text{max}}$  observed



**Fig. 5** Normalized temporal response of intrinsic water-use efficiency ( $W_i$ ) of 15 species to an increase in irradiance from 100 (shade area) to 1000 (white area)  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Data are the mean  $\pm$  SE ( $n = 3-5$ ). The initial and maximal average values of  $W_i$  are indicated above the x-axis for each species and a dashed line denotes net CO<sub>2</sub> assimilation rate at 95% of maximum ( $A_{95}$ ). Values were normalized to the initial values at 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD and maximum values at (1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD) (see Table 3 for species name abbreviations).

between the 15 species, with the lowest values observed in *H. vulgare* ( $0.054 \pm 0.006 \mu\text{mol mm}^{-1} \text{m}^{-2} \text{s}^{-1}$ ). On average, the percentage decrease between  $W_{i\text{max}}$  and  $W_i$  at the end of the response under  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD was significantly less in species with dumbbell-shaped guard cells ( $P < 0.0001$ ) than in species with elliptical-shaped guard cells.

The temporal response of  $W_i$  is driven by the temporal variation in  $g_s$  to increasing PPFD that is uncoordinated with  $A$ , resulting in unnecessary water loss (Fig. 2). To investigate the theoretical variation of  $g_s$  required to optimize  $W_i$ , if coordinated with  $A$ , a model with a constant  $W_i$  chosen at  $A_{95}$  ( $W_{i95}$ ) was



**Fig. 6** Examples of observed (dotted lines) and modelled (dashed lines) temporal response of stomatal conductance to water vapour ( $g_s$ ) for (a) wheat (*Triticum aestivum*) and (b) broad bean (*Vicia faba*). The modelled data represent  $g_s$  at constant water-use efficiency ( $W_i$ ) achieved at 95% net CO<sub>2</sub> assimilation ( $A_{95}$ ). The light grey shading represents water loss and the dark grey shading represents water conserved. (c) The percentage change in water loss (light grey) and water conserved (dark grey) for a 5% increase in  $A$  derived from the differences in observed and modelled for each species. Data are the mean  $\pm$  SE ( $n = 3-5$ ). Species in bold have dumbbell-shaped guard cells, underlined species have a C<sub>4</sub> metabolism and species in plain font have elliptical-shaped guard cells and C<sub>3</sub> metabolism (see Table 3 for species name abbreviations).

applied and the difference between observed and modelled  $g_s$  assessed (Fig. 6), with modelled values of  $g_s$  greater than observed signifying 'water saving' and values less than observed representing a 'water loss'. Figure 6(a,b) show examples for *T. aestivum* and *V. faba*. During the first part of the response, the amount of potential 'water saved' was not significantly different between species (0.98–17.3%) (Fig. 6c). However, after  $W_{i95}$  was achieved, a significant difference in 'water loss' between species, in terms of percentage change in  $g_s$  ( $P < 0.0001$ ,  $F_{(14,49)} = 3.454$ ) was observed. For example, in *P. vulgaris*,  $g_s$  increased by 57% ( $\pm 23\%$ ) for only a 5% gain in  $A$ , which illustrates the strong uncoupling of  $A$  and  $g_s$  in this species and the negative impact on  $W_i$  (Figs 3a, S5). By contrast, the observed response of  $g_s$  in *S. bicolor* was close to the modelled optimal  $g_s$  with minimal increases in  $g_s$  once  $A_{95}$  was reached (Figs 3a, 6c).

The results revealed that  $W_{i\text{max}}$  and  $A_{95}$  were not reached at the same point during the temporal response (Fig. 5). To reach  $A_{95}$  (denoted by the dotted line, Fig. 5), the species typically displayed a decrease in  $W_i$  from  $W_{i\text{max}}$ . The percentage increase in  $A$  from 100 to  $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD was significantly greater ( $P < 0.01$ ) than the percentage decrease in  $W_i$ . The highest gains in  $A$  were observed for *G. biloba* and the species with dumbbell-shaped guard cells (with the exception of *M. nepalensis*) which all achieved  $> 30\%$  increase in  $A$  (Fig. S5).

### Anatomical features

Stomatal density was significantly different between species with abaxial stomatal densities ranging from 68.5 to 376.3 mm<sup>-2</sup> and adaxial densities between 0 and 281.6 mm<sup>-2</sup> (Fig. S6). A positive correlation between ad- and abaxial density for species both with elliptical- ( $R^2 = 0.87$ ) and dumbbell-shaped ( $R^2 = 0.79$  excluding *M. nepalensis*) guard cells was observed. When considering both types of guard cells, a strong correlation between abaxial and adaxial values for stomatal density ( $R^2 = 0.76$  excluding *M. nepalensis*), pore length (PL;  $R^2 = 0.79$ ) and guard cell width (GCW;  $R^2 = 0.85$ ) was also observed, and therefore mean values were used to correlate with stomatal response traits. A strong correlation between PL and GCW was observed and hence only PL was used for further analyses.

With reference to opening responses of elliptical-shaped guard cells, no significant relationships were found between the anatomical features (PL and SD) and  $S_{i\text{max}}$ ,  $k$  or  $G$ , whereas in dumbbell-shaped guard cells, PL and SD correlated significantly with  $G_{s\text{max}}$ , and  $k_i$  was correlated with PL but not with SD. The same correlations were observed with reference to closing responses in both guard cell types; however, a significant relationship between SD and  $k_i$  was also observed in dumbbell-shaped guard cells (Table 2).

### Discussion

As light changes rapidly and is often considered the most dynamic and most important environmental variable influencing both stomatal behaviour and photosynthetic rate, we examined the kinetics of photosynthesis ( $A$ ) and stomatal conductance ( $g_s$ )



to a step increase followed by a decrease in photosynthetic photon flux density (PPFD), in a number of species; assessing the speeds of the  $g_s$  response, the amplitude of change,  $g_s$  limitation of  $A$  and the impact of these kinetics on intrinsic water use efficiency ( $W_i$ ). The temporal dynamics showed clear species-specific differences and noncoordination between  $A$  and  $g_s$ , with  $g_s$  exhibiting a slower and more varied response than  $A$ . Such uncoordinated  $A$  and  $g_s$  responses could have significant implications for cumulative carbon assimilation and transpirational water loss, especially in dynamic light environments. For example, Lawson & Blatt (2014) modelled synchronous  $g_s$  and  $A$  behaviour and calculated a theoretical 20% increase in water use efficiency if  $g_s$  responded instantaneously to the changes in PPFD and matched mesophyll demands for  $\text{CO}_2$ .

A combination of rapid responses and high steady-state values of  $g_s$  reduce  $\text{CO}_2$  diffusional limitations of  $A$ , but can also drastically reduce  $W_i$ , due to the nonlinear relationship between  $A$  and  $g_s$  (Wong *et al.*, 1979). We show for example that the high steady-state values and rapid responses observed in *Oryza sativa*, *Avena sativa* and *Triticum aestivum* facilitated high photosynthetic rates but ultimately resulted in low  $W_i$ , which may be indicative of traditional breeding and selection practices for high yield at the expense of water loss (Jones, 1987). Although high  $g_s$  reduces  $W_i$ , it is also possible that, under well-watered conditions, such stomatal behaviour would increase overall photosynthetic carbon gain by enabling plants to opportunistically use sun flecks in the canopy that can occur on a timescale of seconds to hours (Chazdon & Pearcy, 1991; Kirschbaum *et al.*, 1988; Pearcy, 1990; Way & Pearcy, 2012). Under the measurement conditions used here, when PPFD was raised  $A$  immediately (within 1 min) increased in all species, indicating that  $g_s$  at the lower light level was greater than required. However, a clear stomatal limitation of  $A$  was also apparent as all species took > 9 min to reach 95% final  $A$  ( $A_{95}$ ) (Fig. 3d).

A common feature of  $g_s$  dynamics was the noncoordination in  $A$  and  $g_s$  responses and the continued stomatal opening after  $A_{95}$  had been reached (or 'overshooting' of  $g_s$ ; Fig. 3a), resulting in decreases in  $W_i$ . The observed diversity in responses of  $A$  and  $g_s$  in the species measured questions the mechanisms that coordinate these parameters. Intercellular  $\text{CO}_2$  concentration ( $C_i$ ) was originally proposed as the mediator for the close correlation between  $g_s$  and  $A$  (Wong *et al.*, 1979; Farquhar & Wong, 1984; Mansfield *et al.*, 1990; Buckley *et al.*, 2003). It was assumed that stomata adjust to a steady-state aperture to maintain  $C_i$  at 2/3 atmospheric  $[\text{CO}_2]$  (Ehleringer & Pearcy, 1983) and therefore, when  $A$  is increasing the resulting decrease in  $C_i$  would cause stomata to open and *vice versa*. When  $A$  reaches steady state, further increases in  $g_s$  would result in a greater  $C_i$  that cannot increase  $A$  and therefore, following the  $C_i$  hypothesis, no further increases in  $g_s$  would be expected once steady-state  $A$  has been achieved. However, our results do not fully support this conclusion (e.g. *Vicia faba* in Fig. 2) and agree with findings from work on transgenic plants with reductions in photosynthesis, which showed increasing  $g_s$  with light despite high  $C_i$  (Von Caemmerer *et al.*, 2004; Baroli *et al.*, 2008; Lawson *et al.*, 2008). Many studies support  $C_i$ -driven stomatal responses (e.g. Roelfsema & Prins,

1995) and we do not argue against  $\text{CO}_2$  as a driver; however, our results show that  $C_i$  is clearly not of high priority in the hierarchy.

Stomata have been a key target for improving plant water use efficiency (WUE) and/or a plant's ability to cope with reductions in water availability. However, improvements of WUE in crop plants often come at the expense of photosynthetic rates (Yoo *et al.*, 2009, 2010) and are therefore of limited value, given that current global research efforts focus on increasing crop yield for sustainable food and fuel production (Long *et al.*, 2015). However, as we have illustrated here,  $W_{i\text{max}}$  does not correspond to maximum assimilation rate (Fig. 5) as maximum WUE can often only be achieved when  $g_s$  restricts  $A$ . Based on these observations of dynamic responses we could suggest a steady-state  $g_s$  target that would provide a compromise between  $A$  and  $W_i$  and propose that this target should be the lowest  $g_s$  value that enables  $A_{95}$  to be achieved. It should be noted that this is an optimal target and that fluctuations in the environment could result in different integrated values of  $A$ ,  $g_s$  and therefore  $W_i$ , highlighting the importance of appreciating the speed of stomatal responses and coordination between  $A$  and  $g_s$ .

It is well known that significant variation in photosynthetic capacity ( $A_{\text{max}}$ ) exists both within and amongst different species (Lawson *et al.*, 2012) and, as observed here, this is generally correlated with steady-state  $g_s$  (and  $G_{\text{smax}}$ ). As may be expected the  $C_4$  species measured in our study were able to achieve a greater  $A_{95}$  at a lower  $g_s$  (at  $A_{95}$ ) compared to  $C_3$  species (Fig. S3), and it is likely that the faster stomatal opening and closing responses observed in  $C_4$  species (Fig. 3c) facilitated this greater level of coordination between  $A$  and  $g_s$  (Fig. 2). This faster response was a common feature not just in  $C_4$  plants, but also  $C_3$  species with dumbbell-shaped guard cells. However, despite the close coupling in  $C_4$  species, the same stomatal limitation on  $A$  of c. 10% or greater was observed in both  $C_4$  and  $C_3$  plants (Fig. 4). This illustrates the importance of considering both  $\text{CO}_2$  uptake and water loss when evaluating steady-state or transient  $W_i$  (McAusland *et al.*, 2013), as maximum  $W_i$  is often not observed at maximum  $A$ . Here the decrease in  $W_i$  (between  $W_{i\text{max}}$  and  $A_{95}$ ) with increasing  $g_s$  was outweighed by a substantial gain in  $A$  in all species.

The temporal uncoupling between stomatal behaviour and carbon demand observed in many species can be evaluated by comparing measured  $g_s$  responses with those modelled assuming a stable  $W_i$  (at  $A_{95}$ , which represented a  $W_i$  value that is achieved without a limitation on  $A$ ), providing an estimate of the differences between variable and stable  $W_i$  in terms of water gain and expense for each species. Using this model over the period measured, stomatal behaviour in the majority of species resulted in water expense exceeding water conservation, illustrating that the latter was not the priority. As all the plants in these experiments were maintained under relatively well-watered conditions, this would have led to a higher stomatal conductance than would be observed in plants experiencing water limitation (Comstock & Ehleringer, 1993; Mott & Peak, 2012; Lawson & Blatt, 2014). In general,  $C_4$  species were an exception to this and either demonstrated a balanced water budget or greater gains than

losses, further exemplifying the more synchronous  $A$  and  $g_s$  responses observed in the three species measured. The most likely explanation for the greater loss of water in  $C_3$  species is the substantial overshoot in  $g_s$  after  $A_{95}$  had been achieved, which was not apparent in the two  $C_4$  species studied (Figs 3a, 6).

However,  $C_3$  species with rapid stomatal responses (e.g. *O. sativa*) also exhibited a positive water balance, whilst species with the slowest stomatal opening (with the exception of *G. biloba*) demonstrated the most negative water balances (Fig. 6c) hinting at the possible existence interspecific diversity of stomatal control.

The rapidity of response for stomata to open (increase;  $k_i$ ) and close (decrease;  $k_d$ ) was positively correlated for species with elliptical- as well as dumbbell-shaped guard cells, suggesting that similar mechanisms or pathways were involved in both opening and closing responses. Overall, significant asymmetry of the stomatal responses revealed a faster closing than opening, which has previously been associated with conserving water (Tinoco-Ojanguren & Percy, 1993; Ooba & Takahashi, 2003). In comparison, species with dumbbell-shaped guard cells displayed the fastest responses and most had greater similarity in the rapidity of opening and closing, which is consistent with the fact that these guard cells require fewer solutes and less water to achieve a given unit increase in aperture (Franks & Farquhar, 2007; Raven, 2014). The rapidity of increasing  $g_s$  impacts on  $A$ , with species with high  $k_i$  taking longer to achieve  $A_{95}$ , as low  $g_s$  restricts  $CO_2$  diffusion. Under field conditions with a dynamic light environment, slowly responding stomata could restrict  $CO_2$  uptake and thus have a compound effect on the cumulative  $A$  over the growing season and affect yield (Reynolds *et al.*, 1994; Fischer *et al.*, 1998). However, slow stomatal closure would negatively impact on  $W_i$  when environmental conditions reduce  $A$ . It should be noted that under field conditions, changing the light environment also results in changes in leaf temperature. A direct impact of increasing PPFD would be an increased leaf temperature, which would lead to higher leaf-to-air vapour pressure difference and thus exacerbate the transpirational losses of 'overshooting' stomata. However, higher transpirational losses would have a cooling effect. Therefore, concomitant temperature variation could have complex effects on the dynamic responses of  $A$ ,  $g_s$  and  $W_i$ , and should be studied in detail using appropriate experimental set-ups.

Variation between species was also observed in the maximum speed of  $g_s$  response ( $S'_{max}$ ) and the rapidity of opening ( $k_i$ ) to achieve steady-state conductance. Previous research has associated the speed of stomatal responses with the size of stomata, with smaller stomata facilitating rapid opening and closing (Hetherington & Woodward, 2003; Franks & Farquhar, 2007; Franks & Beerling, 2009; Drake *et al.*, 2013; Raven, 2014). The majority of these studies have used the maximum slope ( $S'_{max}$ ) as a measure of the maximum speed of response; however, this measurement is also dependent on the amplitude of the response (Eqn 2). Additionally, because  $g_s$  is determined by both stomatal aperture and density, small changes in aperture in plants with smaller, more numerous stomata will have a greater  $S'_{max}$  for the same change in aperture as species with fewer larger stomata.

Therefore, although  $S'_{max}$  may provide a useful comparative measure within species (in which anatomical features and the scales of stomatal responses are similar), it is not a useful parameter to compare speeds of response between species with different anatomical features and magnitudes of change. To address this issue, we used the time constant  $k$  to provide a measure of the rapidity of  $g_s$ , independently of the magnitude of response and the absolute  $g_s$  values observed. For elliptical-shaped guard cells, we did not detect any significant correlation between stomatal density and  $S'_{max}$  or  $k$ , suggesting that on an interspecific basis neither the speed nor the amplitude of the stomatal responses to PPFD were dependent on stomatal density. On the other hand, for dumbbell-shaped guard cells, variation among species in stomatal size impacted on the speed and amplitude of stomatal responses. This hints at the possibility that for elliptical-shaped guard cells attributes other than anatomical features are important contributors to the speed of stomatal responses (Hetherington & Woodward, 2003; Franks & Beerling, 2009) such as membrane permeability due to ion channels number or distribution (see discussion, Lawson & Blatt, 2014). By contrast, for dumbbell-shaped guard cells, anatomical variations seem to impact the rapidity of their response. Additionally, these species were also able to achieve a greater  $S'_{max}$  and tended to be faster. This may be due to the energetic requirements for stomatal movement of the often smaller dumbbell-shaped guard cells (Grantz & Assmann, 1991; Franks & Farquhar, 2007; Raven, 2014). The dumbbell-shaped design means that small changes in width can cause larger changes in stomatal aperture and maximize the potential of these stomata to track changes in environmental conditions (Hetherington & Woodward, 2003).

Although transients of leaf-level  $W_i$  provide insight into potentially optimizing stomatal behaviour, numerous other processes contribute to  $W_i$  in the field. Manipulation of the speed of  $g_s$  provides scope for improving carbon acquisition in fluctuating light environments but also enhances drought tolerance through improved conservation of water. Integration of these dynamic responses over daily or seasonal time periods is complex and would require a model that includes respiration (both from leaves and parts including stems and roots) and transpiration as a product of changes in diurnal saturation deficit (Cowan & Farquhar, 1977; Farquhar *et al.*, 1989; Jones, 2004). Identifying varieties or genotypes with more rapid stomatal responses could be used as an optimizing strategy for whole-plant water use over the growing period, potentially improving the ability of the plant to adapt to changing environments (Schulze & Hall, 1982; Campitelli *et al.*, 2016) which could feed forward to maintain or improve yields (Chaerle *et al.*, 2005; Lawson & Blatt, 2014).

## Conclusion

This is one of the few studies to investigate temporal responses in  $A$  and  $g_s$  in relation to carbon assimilation and  $W_i$ , and illustrates significant species-specific variation in the speed of stomatal responses and magnitude of change, as well as coordination with  $A$ . Slow stomatal responses can limit  $A$  by c. 10%, which could equate to substantial losses in photosynthetic rates, productivity

and reductions in yield. Previous research focusing on improving productivity has shown that by enhancing photosynthesis by only 2–3%, substantial increases in plant growth and biomass can be achieved over the season (Lefebvre *et al.*, 2005; Zhu *et al.*, 2007; Simkin *et al.*, 2015). The work presented here illustrates that similar short-term improvements in *A* could be gained by improving the rapidity of stomatal responses and coordination with *A*. Tighter coupling between stomata and *A* therefore has the potential to achieve a substantial improvement in WUE, as in the present study, overshooting of  $g_s$  by up to 80% was observed for only a 5% gain in *A* and fast closing responses resulted in substantial saving in water loss.

Our findings support faster responses in dumbbell- compared with elliptical-shaped guard cells and suggest that photosynthetic type ( $C_3/C_4$ ) also plays a role. The speed of stomatal responses might not be dependent on the same underlying processes when comparing elliptic- and dumbbell-shaped guard cells, with physiological processes being more important for the former and anatomical features for the latter. Improving the rapidity of stomatal responses could greatly improve productivity and  $W_i$  but achieving this will require greater knowledge of the physiological and molecular mechanisms that determine the speed of stomata and coordination with mesophyll demands for  $CO_2$ , and further field-based measurements that integrate the dynamics of *A*,  $g_s$  and  $W_i$  over seasons.

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## Author contributions

L.M., N.R.B. and T.L. planned and designed the research. L.M., S.V.-C., P.D. and T.L. performed experiments and analysed data. L.M., S.V.-C., P.D., N.R.B., O.B. and T.L. wrote the manuscript.

## References

- Assmann SM, Wang XQ. 2001. From milliseconds to millions of year: guard cells and environmental responses. *Current Opinion in Plant Biology* 4: 421–428.
- Baroli I, Price GD, Badger MR, Von Caemmerer S. 2008. The contribution of photosynthesis to the red light response of stomatal conductance. *Plant Physiology* 146: 737–747.
- Blum A. 2009. Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought stress. *Field Crops Research* 112: 119–123.
- Buckley T, Mott K, Farquhar G. 2003. A hydromechanical and biochemical model of stomatal conductance. *Plant, Cell & Environment* 26: 1767–1785.
- Campitelli BE, Des Marais DL, Juenger TE. 2016. Ecological interactions and the fitness effect of water-use efficiency: competition and drought alter the impact of natural MPK12 alleles in Arabidopsis. *Ecology Letters* 19: 424–434.
- Chaerle L, Saibo N, Van Der Straeten D. 2005. Tuning the pores: towards engineering plants for improved water use efficiency. *Trends in Biotechnology* 23: 308–315.
- Chazdon RL, Pearcy RW. 1991. The importance of sunflecks for forest understory plants. *BioScience* 41: 760–766.
- Comstock J, Ehleringer J. 1993. Stomatal response to humidity in common bean (*Phaseolus vulgaris*): implications for maximum transpiration rate, water-use efficiency and productivity. *Functional Plant Biology* 20: 669–691.
- Condon A, Rebetzke R, Farquhar G. 2002. Improving intrinsic water-use efficiency and crop yield. *Crop Science* 42: 122.
- Cowan I, Farquhar G. 1977. Stomatal function in relation to leaf metabolism and the environment. In: Jennings DH, ed. *Integration of activity in the higher plants; Symposia of the Society of Experimental Biology*. Cambridge, UK: Cambridge University Press, 471–505.
- Drake PL, Froend RH, Franks PJ. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany* 64: 495–505.
- Ehleringer J, Pearcy RW. 1983. Variation in quantum yield for  $CO_2$  uptake among  $C_3$  and  $C_4$  plants. *Plant Physiology* 73: 555–559.
- Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Biology* 40: 503–537.
- Farquhar GD, Sharkey TD. 1982. Stomatal conductance and photosynthesis. *Annual Review of Plant Biology* 33: 317–345.
- Farquhar G, Wong S. 1984. An empirical model of stomatal conductance. *Functional Plant Biology* 11: 191–210.
- Fischer R, Rees D, Sayre K, Lu Z-M, Condon A, Saavedra AL. 1998. Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. *Crop Science* 38: 1467–1475.
- Franks PJ, Beerling DJ. 2009. Maximum leaf conductance driven by  $CO_2$  effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences, USA* 106: 10 343–10 347.
- Franks PJ, Farquhar GD. 2007. The mechanical diversity of stomata and its significance in gas-exchange control. *Plant Physiology* 143: 78–87.
- Grantz D, Assmann S. 1991. Stomatal response to blue light: water use efficiency in sugarcane and soybean. *Plant, Cell & Environment* 14: 683–690.
- Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. *Nature* 424: 901–908.
- Jones HG. 1987. *Breeding for stomatal characters*. Stanford, CA, USA: Stanford University Press.
- Jones HG. 1998. Stomatal control of photosynthesis and transpiration. *Journal of Experimental Botany* 49: 387–398.
- Jones HG. 2004. *What is water use efficiency?* Oxford, UK: Blackwell Publishing.
- Knapp AK, Smith WK. 1987. Stomatal and photosynthetic responses during sun/shade transitions in subalpine plants: influence on water use efficiency. *Oecologia* 74: 62–67.
- Knapp AK, Smith WK. 1990. Stomatal and photosynthetic responses to variable sun light. *Physiologia Plantarum* 78: 160–165.
- Lawson T, Blatt M. 2014. Stomatal size, speed and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology* 164: 1556–1570.
- Lawson T, Caemmerer S, Baroli I. 2010. Photosynthesis and Stomatal Behaviour. *Progress in Botany* 72: 265–304.
- Lawson T, Kramer DM, Raines CA. 2012. Improving yield by exploiting mechanisms underlying natural variation of photosynthesis. *Current Opinion in Biotechnology* 23: 215–220.
- Lawson T, Lefebvre S, Baker NR, Morison JI, Raines CA. 2008. Reductions in mesophyll and guard cell photosynthesis impact on the control of stomatal responses to light and  $CO_2$ . *Journal of Experimental Botany* 59: 3609–3619.
- Leakey A, Scholes J, Press M. 2005. Physiological and ecological significance of sunflecks for dipterocarp seedlings. *Journal of Experimental Botany* 56: 469–482.
- Lefebvre S, Lawson T, Fryer M, Zakhleniuk OV, Lloyd JC, Raines CA. 2005. Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiology* 138: 451–460.
- Long SP, Marshall-Colon A, Zhu X-G. 2015. Meeting the global food demand of the future by engineering crop photosynthesis and yield potential. *Cell* 161: 56–66.
- Mansfield T, Hetherington A, Atkinson C. 1990. Some current aspects of stomatal physiology. *Annual Review of Plant Biology* 41: 55–75.

- McAusland L, Davey P, Kanwal N, Baker N, Lawson T. 2013. A novel system for spatial and temporal imaging of intrinsic plant water use efficiency. *Journal of Experimental Botany* **64**: 4993–5007.
- Mott KA, Peak D. 2012. Testing a vapour-phase model of stomatal responses to humidity. *Plant, Cell & Environment* **36**: 936–944.
- Naumburg E, Ellsworth DS, Katul GG. 2001. Modeling dynamic understory photosynthesis of contrasting species in ambient and elevated carbon dioxide. *Oecologia* **126**: 487–499.
- Ooba M, Takahashi H. 2003. Effect of asymmetric stomatal response on gas-exchange dynamics. *Ecological Modelling* **164**: 65–82.
- Pearcy RW. 1990. Sunflecks and photosynthesis in plant canopies. *Annual Review of Plant Biology* **41**: 421–453.
- Raven JA. 2014. Speedy small stomata? *Journal of Experimental Botany* **65**: 1415–1424.
- Reynolds M, Balota M, Delgado M, Amani I, Fischer R. 1994. Physiological and morphological traits associated with spring wheat yield under hot, irrigated conditions. *Functional Plant Biology* **21**: 717–730.
- Roelfsema MRG, Prins H. 1995. Effect of abscisic acid on stomatal opening in isolated epidermal strips of abi mutants of *Arabidopsis thaliana*. *Physiologia Plantarum* **95**: 373–378.
- Schulze E-D, Hall A. 1982. Stomatal responses, water loss and CO<sub>2</sub> assimilation rates of plants in contrasting environments. In: Lange OL, Nobel PS, Osmond CB, Ziegler H, eds. *Physiological plant ecology II*. Berlin: Springer, 181–230.
- Simkin AJ, McAusland L, Headland LR, Lawson T, Raines CA. 2015. Multigene manipulation of photosynthetic carbon assimilation increases CO<sub>2</sub> fixation and biomass yield in tobacco. *Journal of Experimental Botany* **66**: 4075–4090.
- Tinoco-Ojanguren C, Pearcy RW. 1993. Stomatal dynamics and its importance to carbon gain in two rainforest *Piper* species. *Oecologia* **94**: 395–402.
- Violet-Chabrand S, Dreyer E, Brendel O. 2013. Performance of a new dynamic model for predicting diurnal time courses of stomatal conductance at the leaf level. *Plant, Cell & Environment* **36**: 1529–1546.
- Vico G, Manzoni S, Palmroth S, Katul G. 2011. Effects of stomatal delays on the economics of leaf gas exchange under intermittent light regimes. *New Phytologist* **192**: 640–652.
- Von Caemmerer S, Lawson T, Oxborough K, Baker NR, Andrews TJ, Raines CA. 2004. Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. *Journal of Experimental Botany* **55**: 1157.
- Way DA, Pearcy RW. 2012. Sunflecks in trees and forests: from photosynthetic physiology to global change biology. *Tree Physiology* **32**: 1066–1081.
- Weyers JD, Johansen LG. 1985. Accurate estimations of stomatal aperture from silicon rubber impressions. *New Phytologist* **10**: 109–115.
- Wong S, Cowan I, Farquhar G. 1979. Stomatal conductance correlates with photosynthetic capacity. *Nature* **282**: 424–426.
- Yoo CY, Pence HE, Hasegawa PM, Mickelbart MV. 2009. Regulation of transpiration to improve crop water use. *Critical Reviews in Plant Science* **28**: 410–431.
- Yoo CY, Pence HE, Jin JB, Miura K, Gosney MJ, Hasegawa PM, Mickelbart MV. 2010. The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. *Plant Cell Online* **22**: 4128.
- Zhu X-G, de Sturler E, Long SP. 2007. Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. *Plant Physiology* **145**: 513–526.

## Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Response of stomatal conductance to water vapour ( $g_s$ ) of 15 species to an increase in irradiance from 100 to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD.

**Fig. S2** Response of net CO<sub>2</sub> assimilation ( $A$ ) of 15 species to an increase in irradiance from 100 to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD.

**Fig. S3** The relationship between 95% maximum net CO<sub>2</sub> assimilation ( $A_{95}$ ) and steady-state stomatal conductance under 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD ( $G_{\text{max}}$ ) for 15 species.

**Fig. S4** Normalized temporal response of intrinsic water-use efficiency ( $W_i$ ) of 15 species for the first 20 min after an increase in irradiance from 100 to 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

**Fig. S5** Determining the percentage decrease in intrinsic water-use efficiency ( $W_i$ ) for a percentage increase in CO<sub>2</sub> assimilation ( $A$ ) between maximum  $W_i$  max to 95% of the maximum  $A$  ( $A_{95}$ ) reached under 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD for 15 species.

**Fig. S6** Counts of stomatal density and measurements of guard cell length and width for 15 species from the adaxial and abaxial surfaces of the leaf.

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