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Light, power, action! Interaction of respiratory energy- and blue light-induced stomatal movements

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Summary

- Although the signalling pathway of blue light (BL)-dependent stomatal opening is well characterized, little is known about the interspecific diversity, the role it plays in the regulation of gas exchange and the source of energy used to drive the commonly observed increase in pore aperture.
- Using a combination of red and BL under ambient and low [O_2] (to inhibit respiration), the interaction between BL, photosynthesis and respiration in determining stomatal conductance was investigated. These findings were used to develop a novel model to predict the feedback between photosynthesis and stomatal conductance under these conditions.
- Here we demonstrate that BL-induced stomatal responses are far from universal, and that significant species-specific differences exist in terms of both rapidity and magnitude. Increased stomatal conductance under BL reduced photosynthetic limitation, at the expense of water loss. Moreover, we stress the importance of the synergistic effect of BL and respiration in driving rapid stomatal movements, especially when photosynthesis is limited.
- These observations will help reshape our understanding of diurnal gas exchange in order to exploit the dynamic coordination between the rate of carbon assimilation (A) and stomatal conductance (g_s), as a target for enhancing crop performance and water use efficiency.

Introduction

Photosynthesis, the primary determinant of plant biomass, depends on light intensity (McCree, 1971) and CO_2 availability at the sites of carboxylation (Farquhar *et al.*, 1980). Gas exchange (CO_2 and H_2O) in and out of the leaf is controlled by stomata, microscopic pores surrounded by a pair of guard cells that open and close in response to environmental cues and internal signals. In addition to light, which is one of the main environmental cues driving variation of gas exchange, endogenous signals such as hormones and the circadian clock can further influence the diurnal behaviour of stomata (Gorton *et al.*, 1993). Stomatal conductance (g_s), a measure of the ease by which gas diffuses through stomata over the leaf surface, is closely correlated with the rate of carbon assimilation (A) under steady-state conditions, although the mechanisms coordinating the two are not clear (Wong *et al.*, 1979; Ball *et al.*, 1987). Variations in g_s balance CO_2 uptake and evaporative demands, which happen in opposite directions, resulting in a trade-off between biomass production and water loss at the plant level (Condon *et al.*, 2002; McAusland *et al.*, 2016). A temporal decoupling of A and g_s can appear under dynamic environmental conditions due to the stomatal response being an order of magnitude slower than A responses (McAusland *et al.*, 2016; Taylor & Long, 2017; Adachi *et al.*, 2019). Understanding and optimizing the mechanisms controlling the dynamic coordination between A and g_s is an unexploited avenue to increase plant productivity and contribute to achieving food security (Lawson *et al.*, 2010, 2012; Leakey *et al.*, 2019; Wu *et al.*, 2019).

Photosynthesis and stomatal movements are thought to be part of a positive feedback loop in which the products of photosynthesis are used to power changes in guard cell shape, which in turn alters pore dimensions and regulates CO_2 diffusion and A (Farquhar *et al.*, 1978; Buckley *et al.*, 2003). Alternatively, it has been proposed that these products or their intermediates could act as signalling molecules coordinating A and g_s (Lee & Bowling, 1993; Mott *et al.*, 2008; Fujita *et al.*, 2013; Mott & Peak, 2018), although the exact signal has yet to be identified. In a situation where stomata are closed (e.g. start of a dark to light transition) such a regulation loop could be expected to result in an initial slow increase in g_s due to the diffusive limitation of A, followed by an exponential phase triggered by the rise in A. However, several studies have reported rapid stomatal responses even in dark-acclimated plants (Flütsch *et al.*, 2020; Yamori *et al.*, 2020), which suggests that stored energy (in the guard cells or adjacent mesophyll) is used during the initial stomatal opening response (Outlaw & Manchester, 1979; Schnabl, 1980). The energy required for stomatal movements can originate from photosynthesis either from the guard cell chloroplasts or be imported from the surrounding mesophyll cells, although the exact contributions of each are not known and debated (Lawson *et al.*, 2002, 2003). The high ratio of mitochondria to chloroplast in guard cells

suggests a greater contribution from respiratory processes than photosynthesis (Shimazaki *et al.*, 2007) in maintaining energy supply for stomatal movements. In the guard cell, energy in the form of ATP is produced by electron transport within chloroplasts and has been reported to be 80% of that observed in the mesophyll (Lawson *et al.*, 2002, 2003). Functional chloroplasts are essential for guard cell energetics and turgor (Azoulay-Shemer *et al.*, 2016) with electron transport potentially providing energy to drive ion exchange and/or used to produce or transform organic compounds (e.g. sugars). These compounds can be subsequently utilized either as osmotica to drive changes in turgor (Horrer *et al.*, 2016) or as substrates to release energy by mitochondrial respiration (Medeiros *et al.*, 2018). It is important to note that previous work quantifying guard cell photosynthesis has reported limited Calvin cycle activity and suggested that sugars present in guard cells are mostly imported from the surrounding mesophyll (Outlaw, 2003).

Stomatal responses to irradiance depend not only on the intensity but also the wavelength, which triggers two distinct light transduction pathways: the red light (RL) and blue light (BL) responses (Shimazaki *et al.*, 2007; Matthews *et al.*, 2020). The RL-induced stomatal response is generally described as dependent on photosynthesis and is used to explain the close relationship between A and g_s. The BL-induced stomatal response occurs at low light intensities and is often considered independent of photosynthesis (because the low light levels are not enough to drive photosynthesis). Several studies have suggested that the intensity of the background RL influences the magnitude of the stomatal response to BL (Ogawa, 1981; Assmann, 1988; Shimazaki *et al.*, 2007). BL has been reported to be more effective at opening stomata than RL, which involves the release of stored energy and osmotica from starch degradation or lipid metabolism (Horrer *et al.*, 2016; McLachlan *et al.*, 2016). BL is sensed in guard cells by phototropins (Kinoshita *et al.*, 2001) that stimulate stomatal opening by an activation cascade of serine/threonine kinases such as BLUS1 and BHP leading ultimately to the activation of plasma membrane H^+-ATPases (Takemiya *et al.*, 2013; Takemiya & Shimazaki, 2016; Hayashi *et al.*, 2017). By activating the H^+-ATPase proton pumps on the plasmalemma, whilst simultaneously inhibiting S-type anion channels, BL stimulates membrane hyperpolarization, the activation of ion channels and K^+ uptake (Marten *et al.*, 2007; Inoue *et al.*, 2020). However, it is still not clear how BL-activated phototropins transmit the signal to the H^+-ATPases and inhibit plasma membrane anion channels (Marten *et al.*, 2007; Hiyama *et al.*, 2017; Hosotani *et al.*, 2021). The fact that BL-induced stomatal opening does not necessarily rely on photosynthesis (Karlsson, 1986; Roelfsema *et al.*, 2006) suggests that the guard cell mitochondria play a key role in powering the BL response. Little is known on the interaction between respiratory processes and the BL-induced stomatal response, and the impact on the A and g_s relationship during a diurnal period. BL could therefore play an important role in the regulatory feedback loop described above between A and g_s.

The rapidity and magnitude of changes in g_s in response to changing irradiance influence crop photosynthesis (McAusland *et al.*, 2016; Taylor & Long, 2017) and crop yield under natural environments (Adachi *et al.*, 2019; Yamori *et al.*, 2020). Despite having been identified in several species, the diversity of the BL-dependent stomatal response and its role during a diurnal period are still not well characterized. Recent studies have suggested that the stomatal BL response is present in seed plants, ferns from early diverged clades, and lycophytes (Doi *et al.*, 2015; Sussmilch *et al.*, 2019) and may have provided a competitive advantage (Doi *et al.*, 2015; Westbrook & McAdam, 2020), for example helping the diversification of modern ferns during the Cretaceous (Cai *et al.*, 2021). The nature of this advantage is unclear and we suggest that it could provide an advantage under dynamic light conditions favouring increased carbon fixation during the diurnal period. Comparing the temporal kinetics of g_s in response to changes in RL intensity with or without the addition of BL can reveal the contribution of this signalling pathway to leaf gas exchange in different species. Previous work has suggested that the BL stomatal response depends on the level of photosynthesis and/or respiration, although the majority of these studies have only considered short-term responses and/or have used epidermal peels and guard cell protoplasts to prevent mesophyll interactions (Mawson, 1993; Suetsugu *et al.*, 2014; Wang *et al.*, 2014). Here, we measured the impact of different light intensities and spectral quality (RL and BL) on gas exchange in intact leaves and used low $[O_2]$ to inhibit respiratory processes to determine the relative contributions of respiration and photosynthesis to the rapidity and magnitude of the stomatal response.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (A.T, Columbia, Col-0) seeds were germinated in 100 ml 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 ± 10 μmol m^{-1} s^{-1} for an 8 h photoperiod, whilst temperature and vapour pressure deficit (VPD) were 23°C and 1.1 kPa, respectively, day and night.

Phaseolus vulgaris (P.V, french bean), *Vicia faba* (V.F, broad bean), *Vigna unguiculata* (V.U, cowpea), *Glycine max* (G.M, soybean) and *Pisum sativum* (P.S, pea) were germinated in 650 ml pots containing peat-based compost (Levington F2S). *Solanum tuberosum* (S.T, potato), *Commelina communis* (C.C), *Dryopteris carthusiana* (D.C), *Pennisetum glaucum* (P.G, Millet), *Oryza sativa* (O.S, Rice), *Avena sativa* (A.S, oat), *Helianthus annuus* (H.A, sunflower), *Nicotiana tabacum* (N.T, tobacco), *Solanum lycopersicum* (S.L, tomato), *Sorghum bicolor* (S.B, sorghum), *Hordeum vulgare* (H.V, barley), *Triticum aestivum* (T.A, wheat) and *Zea mays* (Z.M, maize) were germinated in 5 litre pots containing peat-based compost (Levington F2S). Following germination, plants were grown under glasshouse conditions and well-watered during the experiment. Solar radiation was complemented with sodium vapour lamps (*c.* 200–400 μmol m^{-2} s^{-1},

Hortilux Schreder 600 W, Monster, the Netherlands) to maintain a 12 h photoperiod.

Leaf gas exchange measurements

Net CO_2 assimilation (A) and stomatal conductance to water vapour (g_{sw}) were measured every 10 s on the youngest fully expanded leaf using an infrared gas analyser (Li-Cor 6400 and 6800; Lincoln, NB, USA). Leaves were first equilibrated at a PPFD of 100 µmol m^{-2} s^{-1} until both A and g_{sw} reached a 'steady state'. Once a steady state was reached, PPFD was increased to 1000 µmol m^{-2} s^{-1} for 30 min before returning to 100 µmol m^{-2} s^{-1} for 30 min. The light spectrum was set initially to 'Red only' (RL, peak wavelength: 625 nm) or 'Red+Blue' (90% Red/10% Blue), and once complete, the same protocol was repeated with the light spectrum inversed, by adding or removing 10 µmol m^{-2} s^{-1} (at 100 PPFD) and 40 µmol m^{-2} s^{-1} (at 1000 PPFD) of BL (peak wavelength: 475 nm). The leaf cuvette was maintained at 400 µmol mol^{-1} CO_2 concentration (C_a), a leaf temperature of 22°C (±0.2°C) and a leaf VPD of 1.1 ± 0.1 kPa. All measurements were performed before 14:00 h to minimize any diurnal or circadian effects on gas exchange.

Measurements under low O_2 concentration (< 1%) were performed using an infrared gas analyser (Li-Cor 6800) with the inlet connected to an oxygen-free nitrogen cylinder (British Oxygen Company-Industrial Gases, Ipswich, UK). A T-fitting was used to avoid excess flow coming from the pressurized cylinder that could damage the pump. A flow meter monitored the incoming flow and made sure that the excess was vented off and no outside air was pumped in. CO_2 and H_2O were added to the mix by the Li-Cor 6800 and the infrared signal was corrected for a 1% [O_2]. The leaf cuvette was maintained at 400 µmol mol^{-1} CO_2 concentration (C_a), a leaf temperature of 22°C (±0.2°C) and a leaf VPD of 1.1 ± 0.1 kPa. It is important to note that using [O_2] > 1% in wheat led to different results from using [O_2] < 1%, as the inhibition of mitochondrial respiration is highly sensitive to [O_2] (Forrester *et al.*, 1966; Zabalza *et al.*, 2009) and values > 1% did not produce complete inhibition.

Over a diurnal period, gas exchange measurements were performed simultaneously on flag leaves of two different tillers. Dark-acclimated leaves were placed in the leaf cuvette and left to acclimate to the new conditions for 10 min in the dark. The Licor 6800 was programmed using a custom python script that recorded gas exchange every 2 min and changed the light intensity on average every 4 min to follow a predetermined pattern. The light pattern was described in a table (CSV file) containing the dwelling time and the intensity of the light (Supporting Information Fig. S1). A match of the two infrared gas analysers was automatically performed every 30 min to correct for any potential drift during the diurnal period. The leaf cuvette was maintained at 400 µmol mol^{-1} CO_2 concentration (C_a), a leaf temperature of 22°C (±0.4°C) and a leaf VPD of 1.1 ± 0.1 kPa. Each measurement was started at 08:00 h to avoid differences due to circadian effects.

Time-integrated leaf gas exchange

The responses of A and g_{sw} under 'Red' and 'Red+Blue' lights were integrated starting from the increase in light intensity and for the following 30 min. The spline function 'splinefun' was used to produce a continuous output from discrete observations and was used by the 'integrate' function from R to calculate the area under the curve. The percentage difference between values obtained for both light spectra were calculated and used to compare the BL-induced effects on gas exchange. To test if BL induced a significant increase in A and g_s, the percentage increase in A and g_s under BL was tested using a one-sample t-test comparing the percentage increase to 0 for each species.

Modelling the g_{sw} response to a step increase in light intensity

The temporal kinetics of g_{sw} in response to a step change in light intensity was modelled using two sets of equations describing the shape and the magnitude of the response. The shape was modelled using an exponential response:

$$\frac{ds}{dt} = \frac{S - s}{\tau}$$

where s is the current value and τ is a time constant. The steady-state target (S) was modelled as an exponential response changing between 0 and 1 and included a feedback loop (S is dependent on the current s) producing a slow initial increase until s reach a threshold value triggering an exponential response of s. This behaviour resulted in an equation capable of reproducing the exponential and sigmoidal response curve generally observed for g_{sw}:

$$S = 1 - e^{-s/\lambda}$$

where λ is the value corresponding to 63% of S. Increasing λ resulted in an increased initial lag time.

The results were then scaled using:

$$g_{sw} = s \cdot (g_f - g_i) + g_i$$

where g_i and g_f represent the initial and final steady-state values for g_{sw}.

Modelling circadian-driven g_{sw} response

The temporal kinetics of g_{sw} under weak light intensity can be modelled by two sinusoidal functions describing the variation of the steady-state target (S_g) through time (t):

$$\mathrm{Sin}_x(t) = P_x e^{\frac{-(t - T_{mx})^2}{2T_{sx}^2}}$$

$$S_g = \mathrm{Sin}_1(t) + \mathrm{Sin}_2(t)$$

where P_x is the magnitude of response, T_m is the time at which the maximum response is reached and T_s is the period.

The rapidity at which g_{sw} followed S_g was described by an exponential differential equation:

$$\frac{dg_{sw}}{dt} = \frac{S_g - g_{sw}}{\tau_s}$$

where τ_s represented a time constant (i.e. the time for g_{sw} to reach *c.* 63% of S_g). Different values of τ were used to describe an increase (τ_i) and a decrease in g_{sw} (τ_d).

Modelling the coupled g_{sw} and *A* response to step changes in light intensity and quality under different $[O_2]$

Modelling the dynamic of g_{sw} was performed using the assumption that the RL-induced stomatal response under a steady state (G_{red}) was linearly related to A and R_d (Wong *et al.*, 1979; Ball *et al.*, 1987) and was activated in the presence of light:

$$G_{red} = \begin{cases} \alpha(A + R_d), & \mathrm{PPFD} > 0 \\ 0, & \mathrm{PPFD} \le 0 \end{cases}$$

with α representing the slope of the relationship. In comparison to the Ball *et al.* (1987) model and its derivatives, atmospheric $[CO_2]$ (C_a) and leaf VPD were not included in the equation as both these variables were maintained relatively constant during the experiments.

The steady-state BL-induced stomatal response (G_{blue}) was modelled as an increase in g_{sw} activated by the presence of BL:

$$G_{blue} = \begin{cases} \beta, & \mathrm{PPFD} > 0 \\ 0, & \mathrm{PPFD} \le 0 \end{cases}$$

where β is the increase in g_{sw} induced by BL.

Both responses were added together to model the steady-state g_{sw} (G_{sw}) response to variations in light intensity and quality:

$$G_{sw} = G_{min} + G_{red} + G_{blue}$$

where G_{min} is the value of G_{sw} under darkness, representing incomplete stomatal closure.

The steady-state target G_{sw} was then used to model the temporal response of g_{sw} with an exponential response:

$$\frac{dg_{sw}}{dt} = \frac{G_{sw} - g_{sw}}{\tau}$$

where τ is the time constant representing the time to reach 63% of the total g_{sw} variation. Different values of τ were used to describe an increase (τ_i) and a decrease in g_{sw} (τ_d).

A modified version of the Farquhar, von Caemmerer and Berry model (FvCB, 1980) was used to predict A under a steady state (A_s) as a function of g_{sw}. The photosynthetic rate limited by Rubisco activity (A_c) was calculated as:

$$a = -\left(\frac{1}{g_{tc}} + \frac{1}{g_m}\right)$$

$$b = (Vc_{max} - R_d)\left(\frac{1}{g_{tc}} + \frac{1}{g_m}\right) + C_a + K_m$$

$$c = R_d(C_a + K_m) - Vc_{max}\left(C_a - \Gamma^*\right)$$

$$A_c = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

The photosynthetic rate limited by RuBP regeneration (A_j) was calculated as:

$$a = -\left(\frac{1}{g_{tc}} + \frac{1}{g_m}\right)$$

$$b = \left(\frac{J}{4} - R_d\right)\left(\frac{1}{g_{tc}} + \frac{1}{g_m}\right) + C_a + 2\Gamma^*$$

$$c = R_d\left(C_a + 2\Gamma^*\right) - \frac{J}{4}\left(C_a - \Gamma^*\right)$$

$$A_j = \frac{-b + \sqrt{b^2 - 4ac}}{2a}$$

where g_{tc} is the total conductance to CO_2 ($= 1/(1.6/g_{sw} + 1.37/g_{bw})$), g_{bw} the boundary layer conductance to water vapour, g_m the mesophyll conductance to CO_2, Vc_{max} the *in vivo* maximum rate of *RuBP* carboxylation, R_d the mitochondrial respiration, K_m the Michaelis–Menten constant ($= K_c\ (1 + O/K_o)$), with K_c and K_o the constants of Rubisco activity for CO_2 and O_2, O the $[O_2]$, J the electron transport rate and Γ^* the CO_2 compensation point in the absence of R_d. With $[O_2] \le 2\%$, the values of R_d and Γ^* were set to 0 to include the inhibition of respiration.

The resulting A_s was used as a target to model induction of photosynthesis due to enzyme activation such as Rubisco activase (Mott & Woodrow, 2000):

$$\frac{dA}{dt} = \frac{A_s - A}{\tau_A}$$

where τ_A is the time constant representing the time to reach 63% of the total variation in A.

Modelling the diurnal response of g_{sw} and *A* under fluctuating light intensity

The diurnal response of A and g_{sw} differs from the response to step changes in light intensity by the fact that circadian processes, such as those observed under weak light intensity, drive part of

the responses. Therefore, the diurnal model for A and g_{sw} combined the findings from the previously described model.

Bayesian inference

Parameter values from the previously described models were adjusted using CmdStan (https://mc-stan.org/users/interfaces/cmdstan) a program for statistical inference. Data were prepared in R and the models were written in the 'Stan' language. For each model, four Monte-Carlo Markov chains were produced that converged to the same optimum values. There was no divergent transition during the process and the effective sample sizes were all >100. The Bayesian inference results in the estimation of 95% credible intervals for each parameter are considered significantly different at $P < 0.05$ if they are not overlapping (or if the difference between two intervals does not contains 0).

Results

BL stomatal responses improve photosynthesis induction whilst reducing water use efficiency

Using RL (peak wavelength: 625 nm) with or without the addition of BL (peak wavelength: 475 nm), the rapidity and magnitude of g_{sw} responses were examined in response to a step change in light intensity (mimicking a sun-fleck) in species of scientific or agronomic importance (Fig. 1). The addition of BL to an RL background induced species-specific stomatal responses to the change in light intensity with increases in g_{sw} (when present) that were not constant over time. Therefore, to compare the effect of BL in different species, the differences between the RL- and RL+BL-induced responses of g_{sw} and A were expressed as a time-integrated difference in percentage relative to the RL treatment (Fig. 2a,b). The presence of weak BL resulted in most cases in an increase in both g_{sw} and A integrated over time, ranging from a few per cent to *c.* 100% for g_{sw} and *c.* 30% for A. The presence of weak BL significantly enhanced the magnitude of the g_{sw} response in most species except for *Solanum tuberosum* (S.T) and *Zea mays* (Z.M). In general, increases in g_{sw} with the addition of BL were accompanied by increases in A, although the differences were not always comparable or significant (Fig. 2b). Interestingly, major crops such as *Glycine max* (G.M), *Triticum aestivum* (T.A) and *Oriza sativa* (O.S) showed substantial increases in time-integrated A, suggesting that without the BL response, stomata strongly restricted CO_2 diffusion for A (Fig. 2b). Part of the observed diffusional limitation under RL was due to slow stomatal kinetics, with plants generally displaying a sigmoidal response for g_{sw} with an initial time lag characterized by a

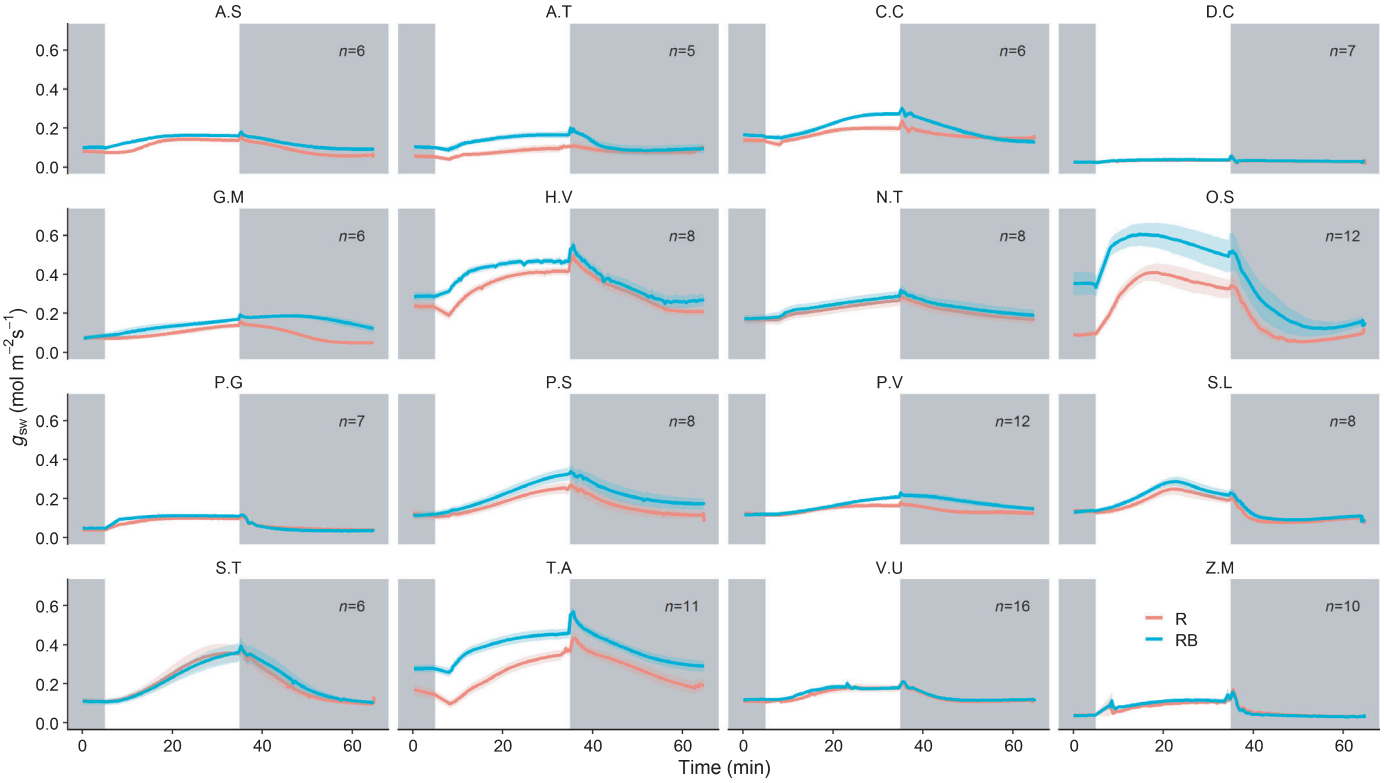

Fig. 1 Species-specific response of stomatal conductance (g_{sw}) to step changes in light intensity with different combinations of red and blue light. Dark shaded and white areas represent period where light intensity was 100 and 1000 μmol m^{-2} s^{-1}. Two light combinations were used: red or red+blue (960 + 40 μmol m^{-2} s^{-1}, RB) light. The shaded area around the curves represents the standard error of mean with n = 5–16 biologically independent samples. Species: *Avena sativa* (A.S, oat), *Arabidopsis thaliana* (A.T, Columbia, Col-0), *Commelina communis* (C.C), *Dryopteris carthusiana* (D.C), *Glycine max* (G.M, soybean), *Hordeum vulgare* (H.V, barley), *Nicotiana tabacum* (N.T, tobacco), *Oryza sativa* (O.S, rice), *Pennisetum glaucum* (P.G, millet), *Pisum sativum* (P.S, pea), *Phaseolus vulgaris* (P.V, french bean), *Solanum lycopersicum* (S.L, tomato), *Solanum tuberosum* (S.T, potato), *Triticum aestivum* (T.A, wheat), *Vigna unguiculata* (V.U, cowpea) and *Zea mays* (Z.M, maize).

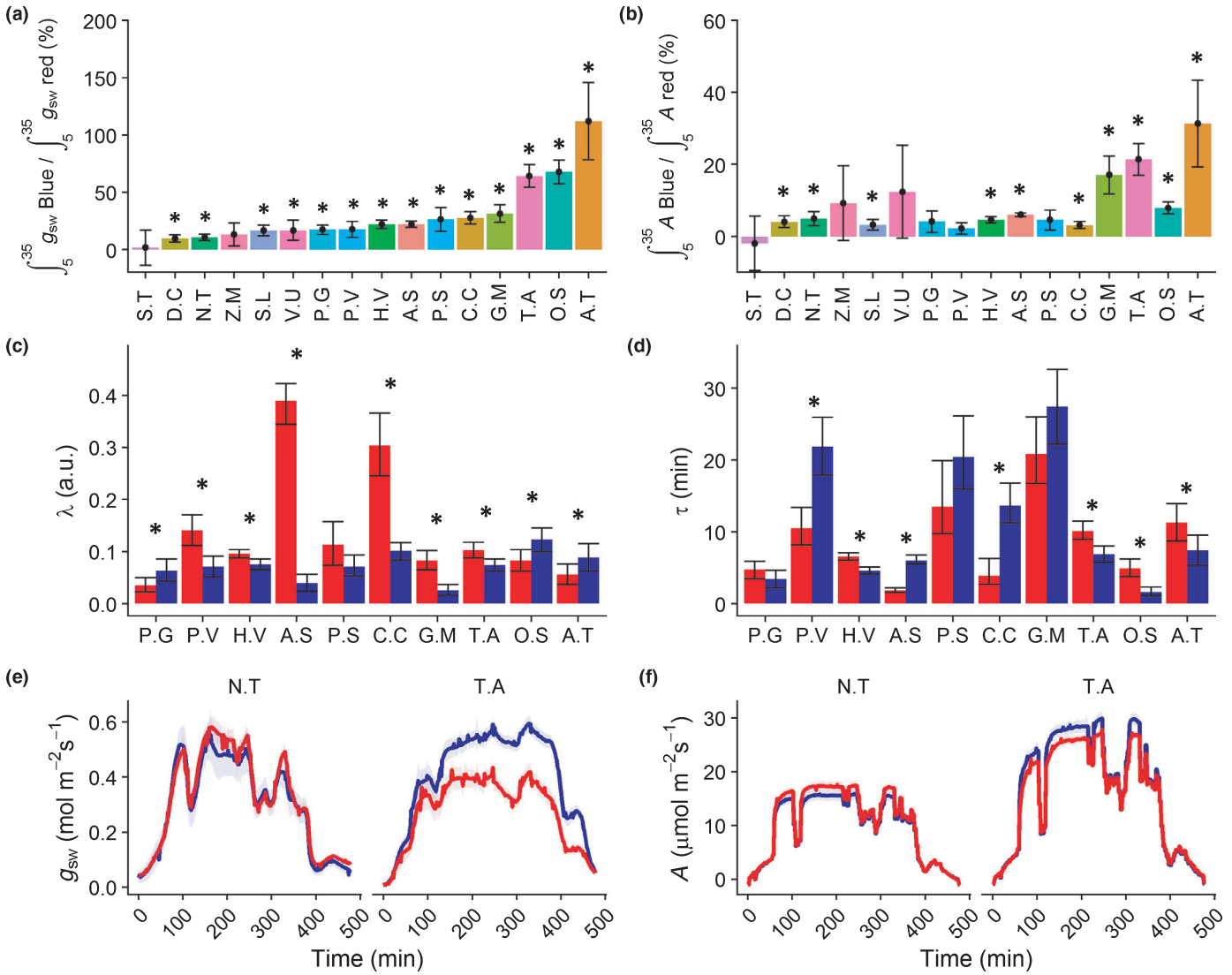

Fig. 2 Impact of light quality on the magnitude and rapidity of stomatal responses. (a) Relative difference of time-integrated stomatal conductance (g_{sw}) in response to a step increase from 100 to 1000 μmol m^{-2} s^{-1} of red (100%, R) or red+blue (90%+10%, RB) light. (b) Relative difference of time-integrated net CO_2 assimilation (A) under similar light conditions. (c) Comparison of the lag time observed before the exponential increase in g_{sw} in response to step increases in R or RB light. Error bars represent 95% confidence interval. (d) Comparison of the time constants representing the rapidity of the g_{sw} increase in response to step increases in R or RB light. Pairwise comparison of the parameter values is represented in Supporting Information Fig. S2. (e) Impact of the addition of blue light on diurnal kinetics of g_{sw} in *Nicotiana tabacum* (N.T) and *Triticum aestivum* (T.A), under fluctuating light intensity. Ribbons represent standard error around the mean. (f) Impact of the addition of blue light on diurnal kinetics of A under fluctuating light intensity. Error bars represent the standard error around the mean, n = 5–16 biologically independent samples; *, a significant difference between light treatments ($P < 0.05$).

quasi-absence of response followed by an exponential increase (Fig. 1). A model describing the kinetics of g_{sw} was used to quantify the importance of these two phases and how the addition of BL altered the kinetics (Fig. 2c,d). In most species, the initial time lag (λ) was significantly reduced with the addition of BL except for P.G, O.S and A.T, although the differences were relatively small (Figs 1c, S2A). The time constant (τ) describing the time required to reach 63% of the observed g_{sw} variation showed that the addition of BL did not necessarily result in faster stomatal responses (Figs 1d, S2B). In P.V, A.S and C.C, τ values were significantly higher with the addition of BL, and significantly lower in H.V, T.A, O.S and A.T (Fig. 2d). Overall, stomatal kinetics were significantly altered by the addition of BL, the main consequence being an increase in A and a reduction in intrinsic water use efficiency ($W_i = A/g_{sw}$), although the effects were species-specific.

The biological significance of BL on gas exchange was tested over a diurnal period in two species with low and high g_{sw} sensitivity to BL, *Nicotiana tobaccum* (N.T) and T.A, respectively (Fig. 2e,f). A diurnal light regime mimicking natural fluctuations in light intensity was used with or without the addition of BL to an RL background (Fig. S1) to assess the effect of BL on diurnal gas exchange. During the diurnal period, N.T did not display any significant differences in g_{sw} with the addition of weak BL on

an RL background (Fig. 2e). Under the same conditions, T.A showed a large increase in g_{sw} through the diurnal period, confirming the large effect of BL on stomatal behaviour described in Fig. 1. In T.A, BL induced a significant increase in A of *c.* 9% (2.3 μmol m^{-2} s^{-1}; $P < 0.05$), whilst no significant difference in A was observed for N.T (Fig. 2f). The increase in A in T.A was not sufficient to compensate for the significant decrease of *c.* 22% in W_i (Fig. S3). It is interesting to note that over the course of the diurnal period, the difference in g_{sw} with the addition of BL increased with time, possibly driven by an endogenous signal, with differences in g_{sw} values up to 0.2 mol m^{-2} s^{-1} in the later part of the diurnal period (Fig. 2e).

Stomatal responses to weak BL are driven by respiratory processes and depend on endogenous signals

The diurnal BL and RL stomatal responses observed in wheat leaves subjected to a constant weak RL or weak BL ($\leq$ 10 μmol m^{-2} s^{-1}) revealed variations in g_{sw} that can be interpreted as a response to an internal signal, here called 'endogenous' signal (Fig. 3a). After an initial increase in g_{sw}, a bimodal response was observed with peaks at *c.* 1 h 40 min and 5 h into the photoperiod that was not reliant on photosynthesis. Under RL, this endogenous response was shown to contribute up to *c.* 20% of the diurnal g_{sw} variation observed in Fig. 2(e).

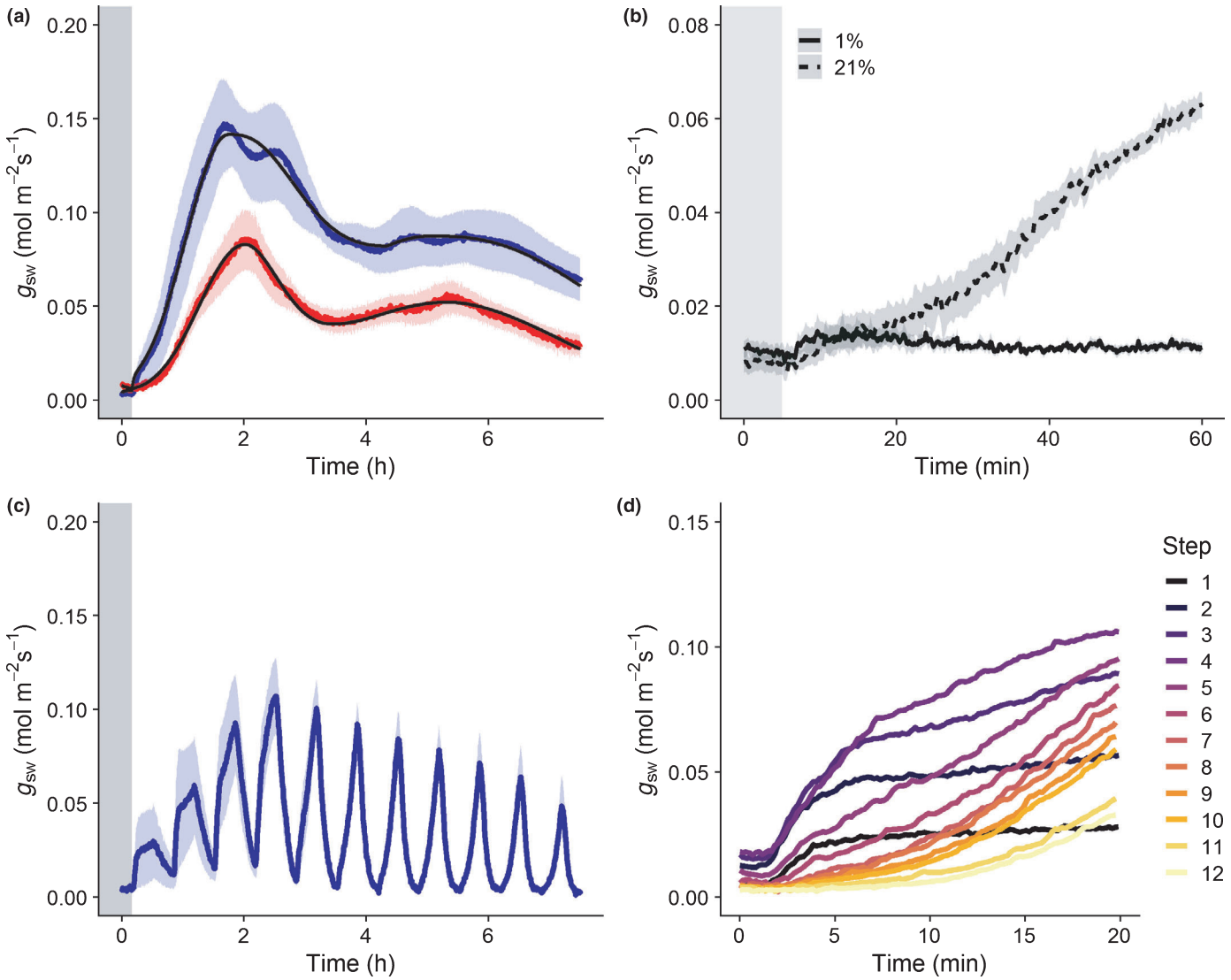

Fig. 3 Contribution of respiratory processes to light-induced stomatal responses in *Triticum aestivum*. (a) Observed and modelled (black line) g_{sw} response to constant 10 μmol m^{-2} s^{-1} of red or blue light. Shaded area represents the standard error around the mean, $n = 5$ biologically independent samples. The plants were maintained under dark conditions (grey area) before measurements. (b) Response of stomatal conductance (g_{sw}) to a step change from 0 (dark shaded area) to 5 μmol m^{-2} s^{-1} of blue light under 1% and 21% [O_2]. Shaded areas represent the standard error around the mean, $n = 5$ and 6 biologically independent samples. (c) Response of stomatal conductance (g_{sw}) to a cycle of step changes in light intensity from 0 to 10 μmol m^{-2} s^{-1} of blue light. The light intensity was maintained for 20 min between steps. (d) Successive g_{sw} kinetics in response to a step increase from darkness to weak blue light observed in (c). All responses were rescaled to start at $t = 0$, highlighting the change in the rapidity of the g_{sw} response over the diurnal period.

In comparison, BL resulted in both a faster initial g_{sw} increase and a 50% higher g_{sw} over the diurnal period. To evaluate the contribution of respiratory processes to BL-induced stomatal movements, mitochondrial and chloroplast respiration were inhibited by performing experiments under 1% [O_2] (Fig. 3b). Stomata failed to open under low [O_2] when weak BL (5 μmol m^{-2} s^{-1}) was applied to a dark-adapted wheat leaf, but stomata opened as expected under ambient [O_2]. When plants measured under low [O_2] were returned to ambient conditions, g_{sw} displayed a 'normal' response (Fig. S4) demonstrating that any lack of response in Fig. 3(b) was not due to damage or impaired function. Using a pattern of alternating dark and low light (without driving photosynthesis) to maintain a high energy demand for stomatal movement (opening and closing, Fig. 3c), we observed that the rapidity of the g_{sw} response decreased after each cycle and displayed a longer initial lag time (Fig. 3d). The highest g_{sw} achieved during each light period followed a similar trend to those observed in Fig. 3(a). Despite the low light intensity used, which was not sufficient to drive photosynthesis above the light compensation point, g_{sw} displayed rapid increases similar to those observed when high light intensity was used (Fig. 1).

BL enhances the rapidity of stomatal movements in interaction with respiratory processes

To evaluate the contribution of photosynthetic and respiratory processes on g_s responses, wheat leaves were subjected to a step change from dark to high red-light intensity at both ambient and low [O_2] (inhibiting respiratory processes) (Fig. 4a,b). Surprisingly, under low [O_2] g_{sw} increased to only 38 mmol m^{-2} s^{-1} after 60 min (Fig. 4a) and was still slowly increasing after 4 h (Fig. 4c). These findings demonstrate that the stomata could still open under low [O_2] albeit at a extremely slow speed and reduced magnitude. However, under ambient [O_2], g_{sw} reached 258 mmol m^{-2} s^{-1} after 60 min, showing that respiratory processes are essential for rapid stomatal movements under RL alone. The slow g_{sw} response under RL and low [O_2] displayed a strong linear relationship with A (Fig. 4c–e), which implies that when photosynthesis is the only source of energy and is itself limited by CO_2 diffusion (due to low g_{sw}) there is insufficient energy produced for rapid stomatal opening. When a similar step change from dark to high light was carried out using BL, g_{sw} under 21% and 1% [O_2] showed a fast initial response and greater magnitude of change compared with that observed under RL. In both cases this resulted in a faster induction of photosynthesis under BL compared with RL alone (Fig. 4b). At $t = 45$ min, g_s was significantly higher under BL than RL when subjected to 1% [O_2] and was further increased with 21% [O_2], with a significant interaction between the light colour and [O_2] conditions observed (Fig. S5). These differences in g_s under BL resulted in a release of the stomatal limitation of A, even under 1% [O_2]. These findings illustrate the importance of BL, in conjunction with the role of respiration, in the rapidity of stomatal responses and induction of photosynthesis. This is clear in Fig. 4(c,d); when weak BL was added to saturating RL under low O_2, g_{sw} doubled in < 3 min, releasing the diffusional constraint on A. The rapid increase in g_{sw} under BL was not directly dependent on the photosynthetic rate as g_{sw} increased before A (Fig. 4e) and may be due to the accumulation of solutes in guard cells under RL that were released or utilized to support rapid movements under BL.

A model describing A and g_s under different light quality and [O_2] was developed to test the validity of a photosynthesis feedback loop mechanism against these observations. The model described with high accuracy the observations (root mean square error (RMSE) for g_{sw}: ≤ 0.014 mol m^{-2} s^{-1} and for A: ≤ 0.46 μmol m^{-2} s^{-1} for all treatments; Fig. 4a,b) and therefore probably captures the key mechanistic responses. The g_{sw} response was 48% faster and g_{sw} increased by 29% under BL. Furthermore, when respiratory processes were inhibited, the model estimated a two-fold decrease in the parameter value controlling the coupling between A and g_{sw}, a doubling in the time required for a full induction of photosynthesis (time constant, K_{ai}) and a 64% lower maximum carboxylation of Rubisco (Vc_{max}) (Fig. S6). These results highlighted that BL impacts not only the kinetics of g_s but also directly influences the induction of photosynthesis and confirmed the role of respiratory processes in powering stomatal opening even under high irradiance.

Respiration is required for stomatal opening and closing

In light (RL+BL)-acclimated leaves, inhibiting respiration (switching from 21% to 1% [O_2]) resulted in a slow decrease in g_{sw}, which was independent of the light intensity (Fig. 5a) or the photosynthesis level (Fig. 5b). When [O_2] was restored to 21%, g_s slowly returned to its initial level. These data also revealed that the g_{sw} response to intercellular [CO_2] (C_i, Fig. 5b), which usually induces stomatal opening, was overridden under these conditions. Furthermore, stomata in T.A and N.T were unable to fully close for > 60 min when placed simultaneously under low [O_2] and darkness (Fig. 5b–d), stressing the importance of respiratory energy for both stomatal opening and closing at any time of the diurnal period.

Parallel contribution of respiratory and photosynthetic processes to diurnal gas exchange

Using a leaf gas exchange model including the previous findings, the contribution of respiratory and photosynthetic processes to diurnal gas exchange (Fig. 6a,b) was quantified in response to external and internal cues. The model was able to describe with high accuracy (RMSE for g_{sw}: 0.013 mol m^{-2} s^{-1} and for A: 0.6 μmol m^{-2} s^{-1}) the diurnal kinetics of g_{sw} under RL and RL+BL, which provided insights into the contribution of each process driving g_{sw}. Under RL, the feedback loop describing the coordination of A and g_{sw} explained *c.* 80% of the diurnal variation in g_{sw}, whilst the remaining *c.* 20% was due to endogenous signals mostly driven by respiratory processes. Adding weak BL during the diurnal period resulted in a faster increase (K_i) and slower decrease (K_d) in g_{sw} in response to variation in light intensity, promoting higher levels of g_{sw} and lower A limitation (Fig. 6c,d). The g_{sw} response to an endogenous signal(s) was doubled under BL (Fig. 6e,f) and showed a faster increase (Fig. 6g) and a

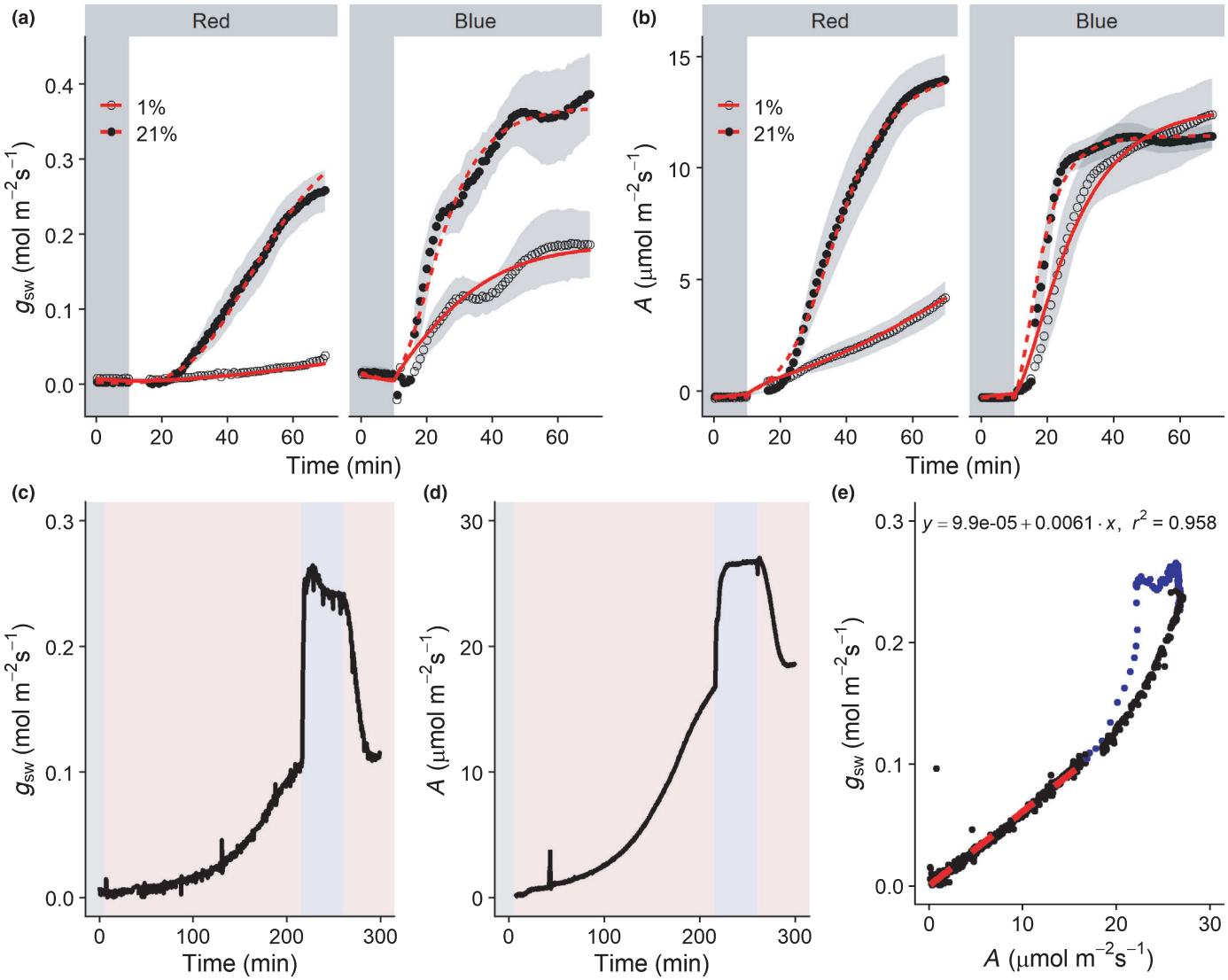

Fig. 4 Impact of respiratory processes on the rapidity and magnitude of stomatal responses in *Triticum aestivum*. (a) Response of g_{sw} to a step change from 0 (dark shaded area) to 1000 μmol m^{-2} s^{-1} of red or blue light under 1% (open circles) and 21% (closed circles) [O_2]. Shaded areas around the curves represent the standard error around the mean, $n = 5$ biologically independent samples. The red lines represent the modelled responses. (b) Response of net CO_2 assimilation (A) to a step change from 0 (dark shaded area) to 1000 μmol m^{-2} s^{-1} of red or blue light under 1% and 21% [O_2]. (c) Response of stomatal conductance (g_{sw}) to a step change in red light intensity from 0 to 1000 μmol m^{-2} s^{-1} that was maintained for 3.5 h, followed by the addition of blue light (red: 960, blue: 40 μmol m^{-2} s^{-1}) for 45 min and a final period of 1000 μmol m^{-2} s^{-1} of red light for 40 min. (d) Response of A under the same conditions described for (c). (e) Relationship between A and g_{sw} during the initial red-light period. The red dashed line represents the regression line between A and g_{sw}.

slower decrease (Fig. 6h), resulting in a higher g_{sw} through the diurnal period. Greater g_s resulted in a faster A induction and greater A reached during the diurnal period.

Simulations using the leaf gas exchange model used in Fig. 6 showed that the initial g_s value under darkness is an important determinant of the temporal kinetics of A and g_s in response to an increase in light intensity (Fig. 7). Under RL, a low g_s value induced a strong limitation of A, resulting in a slow increase in g_s. The presence of weak BL greatly improved the rapidity and magnitude of the g_s response, resulting in a faster A induction. Simulations showed that maintaining high g_{sw} values under dark conditions like those observed in N.T (Fig. 5d) can compensate for the lack of BL-induced stomatal opening during induction of A. The model also highlighted the interdependence of A and g_s by illustrating how the induction speed of A and the coupling with an increase in g_s influences their respective temporal responses. Overall, gas exchange simulations revealed that the coordination between A and g_s is not necessarily linear and is greatly improved in favour of A when the BL pathway is activated.

Discussion

Little is known about the role of BL in the regulation of diurnal gas exchange and the reason it has been evolutionarily conserved in many species (Doi *et al.*, 2015; Li *et al.*, 2015). Previous

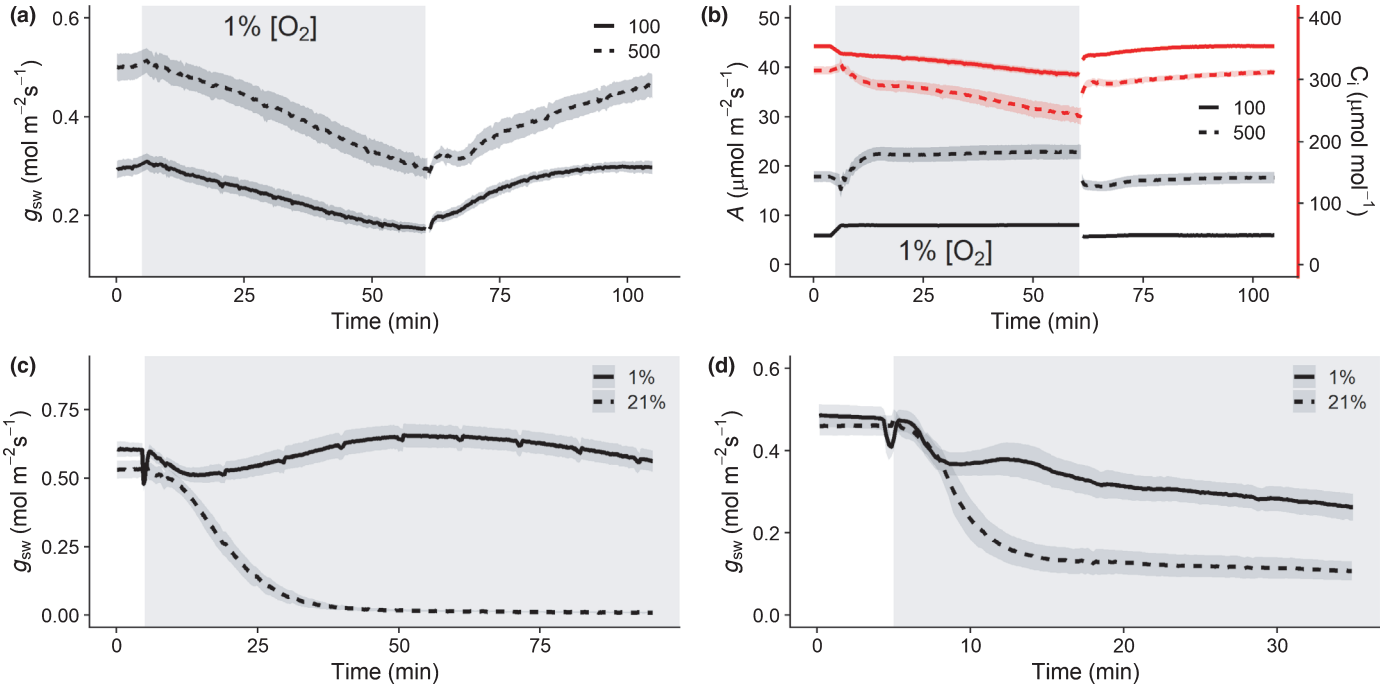

Fig. 5 Dependence of stomatal function on respiratory processes in *Triticum aestivum*. (a) Response of g_{sw} to a step change from 21% (non-shaded area) to 1% (shaded area) [O_2] at 100 and 500 μmol m^{-2} s^{-1} light intensity. Ribbon around the curves represent the standard error around the mean, n = 6–7 biologically independent samples. (b) Response of net CO_2 assimilation (A) and internal CO_2 concentration (C_i) to a step change from 21% to 1% [O_2] under 100 and 500 μmol m^{-2} s^{-1}. (c) Response of g_{sw} to the simultaneous decrease of light intensity (500 to 0 μmol m^{-2} s^{-1}) and [O_2] (21 to 1%). Shaded area represents the dark period. Ribbon around the curves represent the standard error around the mean, n = 5 biologically independent samples. (d) The same protocol described in (c) was applied to *Nicotiana tabacum*, n = 4 biologically independent samples.

studies hypothesized that BL removes stomatal limitation of photosynthesis early in the morning (Assmann & Shimazaki, 1999), which was supported here by the large and rapid increase in g_{sw} observed in dark-adapted plants subjected to weak BL. Our analysis went further and suggested that BL stomatal opening reduces diffusional limitations on A over the diurnal period in most species although at the cost of decreased W_i, and is species-specific. Traditionally, variations in g_s have been predicted from variations in A (Ball *et al.*, 1987), although our findings revealed that this relationship determines only a fraction of the g_s achieved during a diurnal period. This is due to the fact that both photosynthesis and respiration are required for rapid stomatal opening and that respiratory processes specifically contribute to BL-induced stomatal opening independent of A, which is overlooked in current views on stomatal function. These observations will help reshape our understanding of the diurnal dynamic coordination between A and g_s, an unexploited target for enhanced crop performance and water use efficiency.

BL reduces diffusive limitation and improves dynamic photosynthesis

In the species studied here, leaves subjected to BL showed diverse magnitude and rapidity of g_{sw} responses to BL that often resulted in a greater time-integrated A (due to a faster induction and increased magnitude of g_{sw}) and a reduction in W_i. In most species, the increase in g_s in response to the addition of BL was proportionally greater than the increase in A and is therefore a potential target to enhance W_i whilst maintaining A. Some of the major cereal grain and legume crop species used here (rice, wheat, barley, oat, soybean, pea) displayed large and significant g_s responses with the addition of BL, suggesting that breeding programmes may have already inadvertently selected for this trait to enhance A, at the detriment of W_i. By contrast, species of the family Solanaceae (tomato, potato, tobacco) displayed only a relatively small or no g_s increase with the addition of BL, suggesting a potential evolutionary aspect to the blue light signalling pathway. In wheat, higher g_s has been shown to be positively correlated with yield (Reynolds *et al.*, 1999; Fischer & Rebetzke, 2018), due to both reduced diffusional limitation as well as enhanced evaporative cooling for leaf temperature regulation. Despite the fact that most species seem to possess the major known genes involved in the BL signalling pathway (e.g. PHOT1, PHOT2 and BLUS1; Takemiya *et al.*, 2013; Li *et al.*, 2015), some species such as S.T (potato) and Z.M (maize) did not display a significant stomatal response to the addition of BL. The cause of this absence of response is currently unknown but could be a result of constant activation or inactivation of genes involved in the BL signal transduction pathway, and further research would be required to understand these interspecific differences in the BL-induced response.

To date, the majority of publications examining BL-induced stomatal responses have been performed using short-term protocols (seconds to minutes; Matthews *et al.*, 2020) and overlook the long-term variations in g_s with the addition of BL. Our results revealed that the increase in g_{sw} observed with the addition of BL

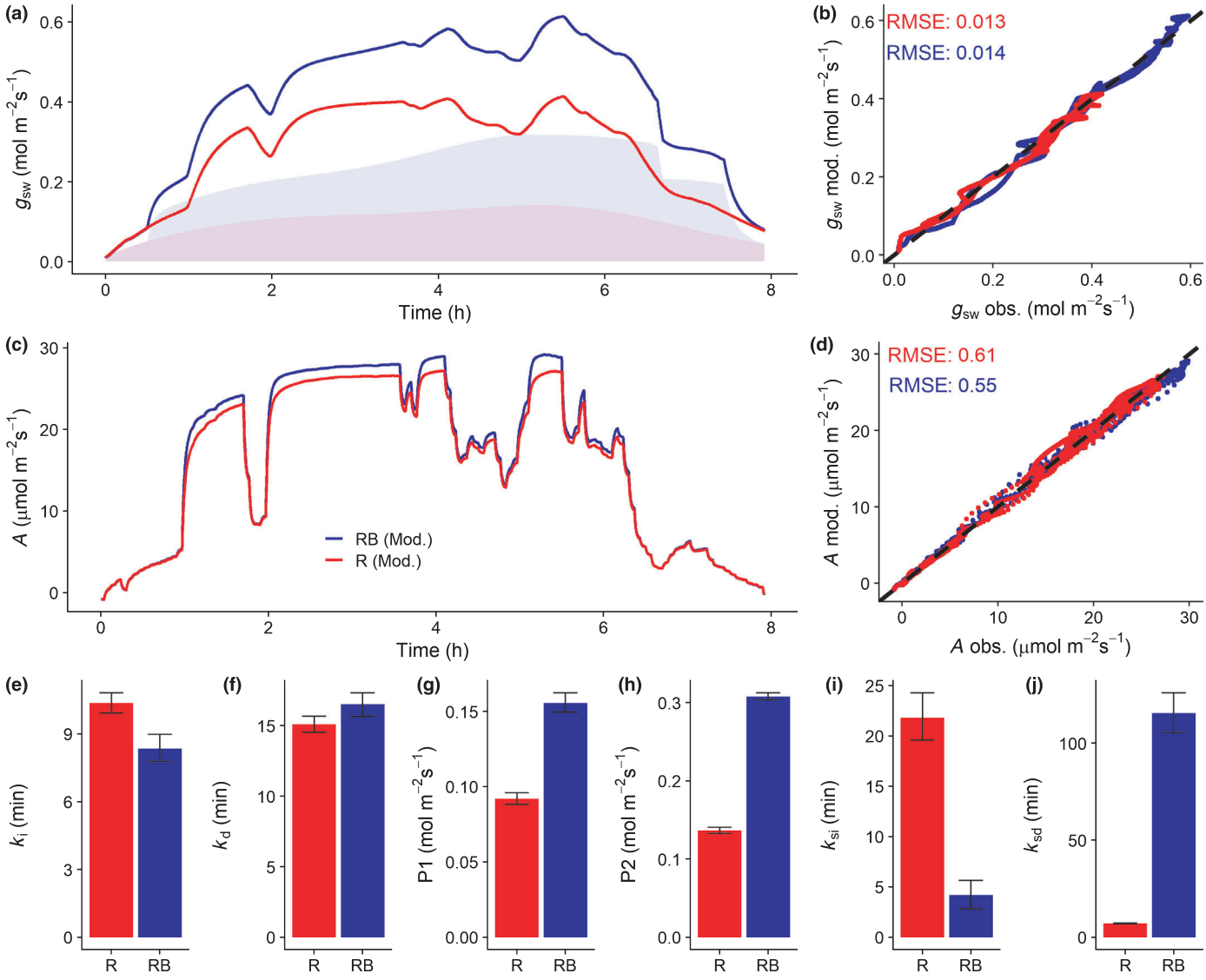

Fig. 6 Contribution of photosynthetic and respiratory processes to diurnal gas exchange under fluctuating R (red) and RB (blue) light in wheat. (a) Diurnal g_{sw} kinetics was modelled (lines) using a positive feedback loop coupling A and g_{sw} including the contribution of respiratory processes (shaded areas) modelled in Fig. 3(a). (b) Comparison of modelled and observed g_{sw}. (c) Diurnal net CO_2 assimilation (A) was predicted by the model. (d) Comparison of modelled and observed A. (e) Rapidity of stomatal opening (k_i). (f) Rapidity of stomatal closure (k_d). (g) Magnitude of the first diurnal sinusoidal response of g_s (P1). (h) Magnitude of the second diurnal sinusoidal response of g_s (P2). (i) Rapidity of the diurnal sinusoidal increase in g_s (k_{si}). (i, j) Rapidity of the diurnal sinusoidal decrease in g_s (k_{sd}). The dashed black lines in (b) and (d) represent the 1 : 1 line and the root mean square error (RMSE) was calculated for each light treatment. Error bars in (e–j) represent 95% credible intervals derived from Bayesian inference.

increased continuously during the diurnal period, independently of the external conditions. This was further supported by the observed impact of the endogenous signal on g_{sw}, which was 50% greater under BL and accounted for *c.* 25% of the maximum g_{sw} observed under fluctuating high light intensity. The self-entrained variation in g_{sw} was triggered by weak light intensity (below the compensation point) and resulted in a pattern similar to those reported for the circadian clock (Gorton *et al.*, 1993). These data agree with the concept of a diurnal endogenous signal or circadian system influencing stomatal behaviour over the course of the day, contributing to variations in W_i (Gorton *et al.*, 1993; Matthews *et al.*, 2018; Simon *et al.*, 2020) but also highlighting increased sensitivity to BL.

Respiratory processes are essential for rapid variations and maintenance of stomatal conductance

We hypothesized that the coordination observed between A and g_s (McAusland *et al.*, 2016) is due in part to the energy requirements of stomatal movements that are fulfilled at different times of the response by mitochondria and/or chloroplasts. Indeed, Mawson (1993) highlighted that the translocation of protons across the guard-cell plasmalemma, the first step in opening of the stomatal aperture, is an energy-requiring activity. The study suggested that both guard-cell chloroplasts and mitochondria contribute in synergy to supply energy for BL-induced proton pumping in guard-cell protoplasts, as both were inhibited by low

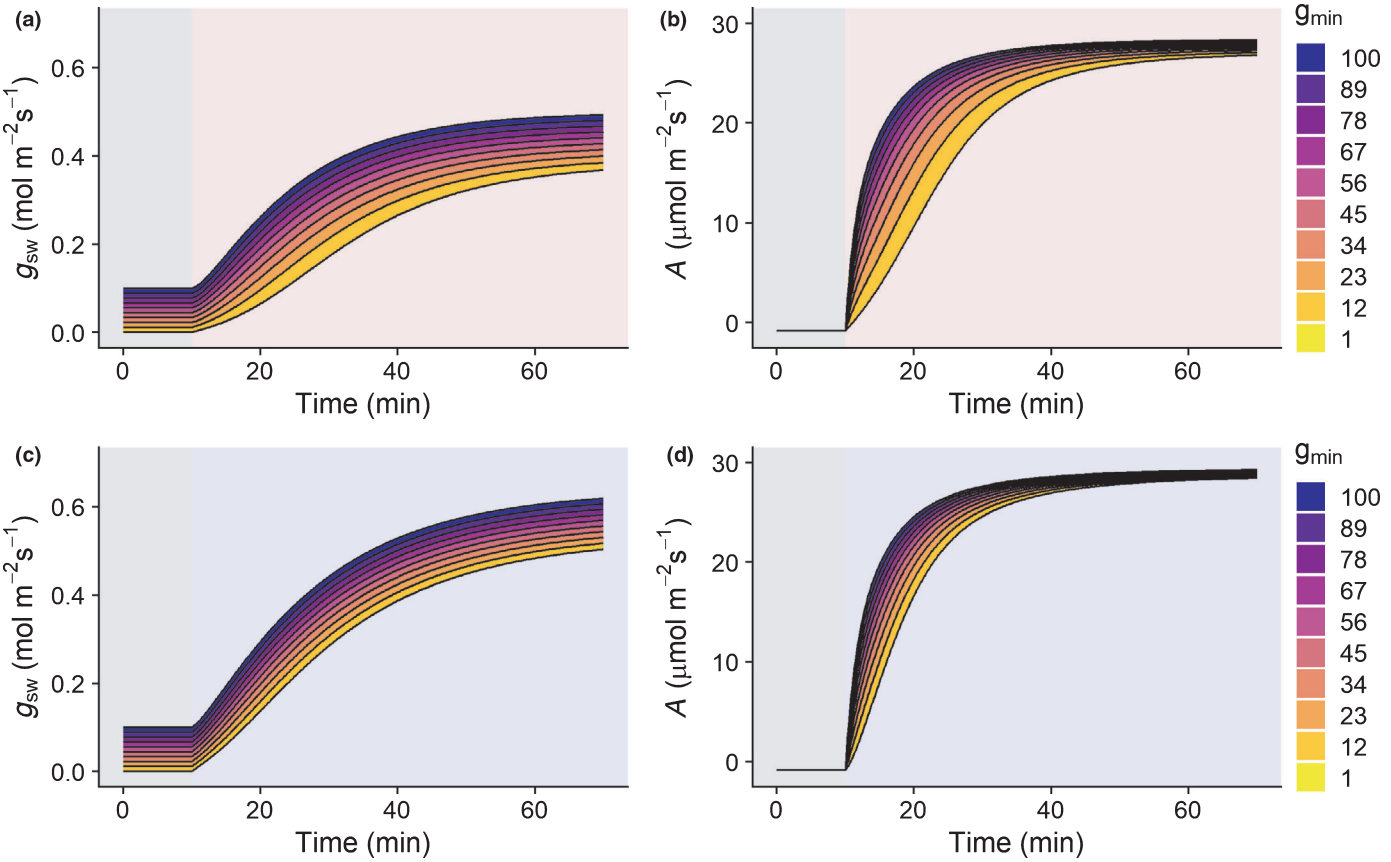

Fig. 7 Simulation of stomatal conductance (g_{sw}) and net CO_2 assimilation rate (A) responses to a step increase in light intensity. Gradation of the initial g_{sw} under dark conditions demonstrates the impact of the positive feedback loop driving g_{sw} (a, c) and A (b, d) variations under 1000 µmol m^{-2} s^{-1} of red light (red areas, a and b) or 960 µmol m^{-2} s^{-1} of red + 40 µmol m^{-2} s^{-1} of blue light (blue areas, c and d). An initially low g_{sw} results in a strong limitation of A, which in turn limits the energy produced to drive stomatal movements feeding back into the diffusion of CO_2.

[O_2]. Previous work using plants grown with the herbicide norflurazon or using the white areas of variegated plants showed that the stomata in such plants were still able to respond to BL but the RL response was greatly impaired, suggesting that alternative pathways to photosynthesis can provide the energy for stomatal responses (Karlsson *et al.*, 1983; Roelfsema *et al.*, 2006). The contribution of energy derived from both chloroplasts and mitochondria to drive the stomatal response was confirmed by our results in intact leaves under both red and BL. During the diurnal period, our results demonstrate the contribution of energy and osmotica originating from the mesophyll on stomatal behaviour, which is not possible to study in guard cell protoplasts. In addition to the activation of the plasma membrane H^+-ATPase, BL has been shown to influence sugar/lipid degradation pathways (Horrer *et al.*, 2016; McLachlan *et al.*, 2016) releasing the energy required (e.g. via mitochondria, Medeiros *et al.*, 2018) for the activation/deactivation of ion channels and pumps (Marten *et al.*, 2007; Inoue *et al.*, 2020), which promotes stomatal opening independently of *A*. By inhibiting respiratory processes during a transition from dark to high light intensity, stomata can rely only on photosynthetic processes (osmoregulation and energy) for opening, which are initially limited by CO_2 diffusion that induces the observed strong coupling between A and g_s. This supported the long-standing idea of a positive feedback loop controlling stomatal aperture based on mesophyll photosynthesis, with C_i potentially coordinating A and g_s (Farquhar *et al.*, 1978). In parallel with this feedback loop, our results suggest that respiratory processes and BL played a key role by initiating and promoting fast stomatal opening independently of *A*. Our model describing the temporal response of A and g_s using a feedback loop estimated a two-fold decrease in the rapidity of photosynthesis induction and maximum rate of carboxylation under low [O_2]. In the absence of respiratory energy, the initially low and slow g_s response limited CO_2 diffusion, prevented the CO_2 and energy (i.e. ATP) requirement of Rubisco activation to be met, and resulted in a lowered maximum carboxylation rate. Fig. 8 illustrates the theoretical framework for the interplay between photosynthesis and stomatal behaviour following a step increase in red or BL and highlights the importance of mitochondrial respiration in initiating and driving rapid stomatal movements. These findings provide a unifying theory for the observed 'RL-' (photosynthesis-dependent) and 'BL-' (photosynthesis-independent) induced stomatal opening that has been discussed extensively in the literature.

It is important to acknowledge that using low [O_2] to inhibit respiration can also inhibit other processes such as the production of reactive oxygen species (ROS), which are important signalling molecules in stomatal closure (Ehonen *et al.*, 2019). However,

the absence of ROS production under low [O_2] would be expected to increase or maintain stomatal aperture relative to ambient [O_2] conditions, which was not the case in our experiments (Fig. 4). When [O_2] was returned to ambient conditions g_{sw} was restored, suggesting no damage. Although these findings do not totally exclude ROS as a signal, a lack of ROS production could not explain the observed g_{sw} decrease under high light and low [O_2] (Fig. 5a). It has been suggested that stomatal closure under darkness (Fig. 5c,d) requires the accumulation of ROS (Desikan *et al.*, 2004; Ma *et al.*, 2018), which may explain why stomatal closure was impaired. In general, it is therefore unlikely that ROS production or lack of it can explain the observed differences in the rapidity of stomatal response shown here.

Over a diurnal period, our results suggest a significant role of BL along with the respiratory processes in powering and maintaining stomatal aperture independently of *A*. Under a repeated dark–light cycle applied over 8 h, the delay between the application of weak BL and the g_{sw} response increased after each cycle towards the end of the day, suggesting that stored energy used by respiratory processes had been exhausted. However, it is noteworthy that there was sufficient energy to power stomatal movements for the majority of the day. These results suggest that the rapidity and magnitude of the diurnal g_{sw} responses are determined in part by the status of the energy pool, and that photosynthates previously accumulated may influence stomatal behaviour.

Following a light to dark transition, stomatal closure in both wheat and tobacco subjected to low [O_2] was greatly impaired, as previously observed in wheat and barley (Akita & Moss, 1973) and in *Commelina communis* (Karlsson & Schwartz, 1988). In wheat, an unexpected small increase in g_s was observed, and in tobacco g_s showed a slow decrease. The g_s increase in wheat could be either due to an increase in guard cell turgor and/or a decrease

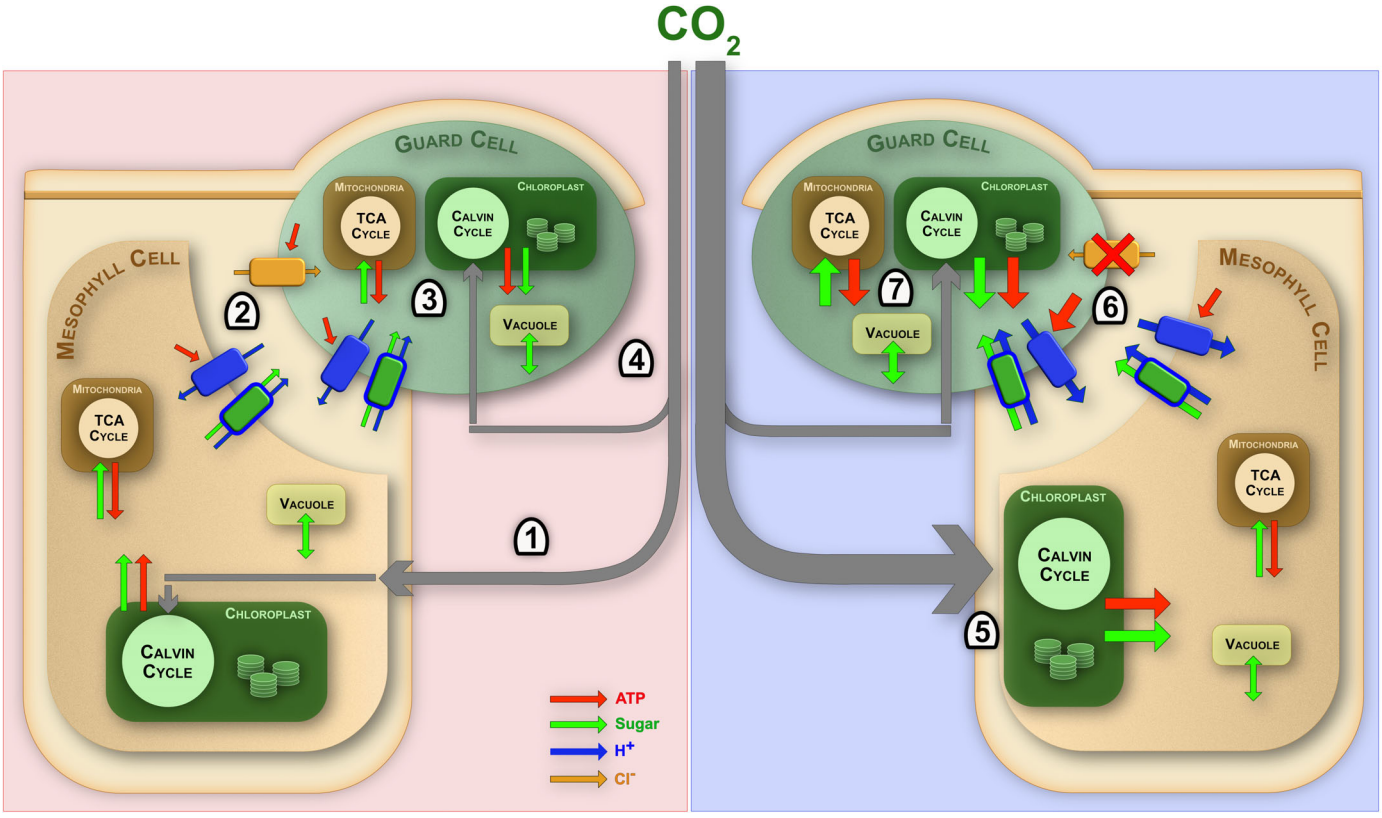

Fig. 8 Theoretical schematic illustrating potential energetics of light-induced stomatal opening. (1) Under red light (RL), reduced stomatal aperture limits CO_2 diffusion to the sites of carboxylation, limiting photosynthesis. Photosynthetic products such as sugars and chemical energy are available in limited quantities in mesophyll and guard cells. (2) Exchange of sugars between the mesophyll and guard cells is limited due to the lack of energy for active transport. (3) Initially, the main source of energy for guard cell movement is released by mitochondria using stored energy (e.g. starch). The proportion of mitochondrial and chloroplastic energy driving guard cell movement changes through time as the limitation on photosynthesis decreases, enabling increased stomatal aperture. (4) Changes in guard cell turgor are slow, depending strongly on the regulation loop where stomata both control and depend on CO_2 diffusion and the energy produced by photosynthesis. (5) Chloroplast migration in response to blue light (BL) reduces the path of CO_2 diffusion, helping to achieve higher photosynthesis rates. (6) BL initiates the release of stored energy and osmotica from starch degradation and/or lipid metabolism (Horrer *et al.*, 2016; McLachlan *et al.*, 2016). Additionally, BL stimulates stomatal opening by activating the H^+-ATPase proton pumps on the plasmalemma, simultaneously inhibiting S-type anion channels, resulting in a stronger membrane hyperpolarization, and the activation of ion channels and K^+ uptake (Marten *et al.*, 2007; Inoue *et al.*, 2020). Overall, BL stimulates energy release and exchange between the mesophyll and guard cells independently of photosynthesis. (7) The rapid removal of photosynthetic limitation and increased stimulation of the energetic mechanisms involved in changes in stomatal aperture induce rapid stomatal kinetics under BL and enable larger apertures compared to those observed under RL alone. Width of the arrows is proportional to flux rate.

in subsidiary cell turgor (Franks & Farquhar, 2007) in response to an imbalance of osmoticum. A previous study using detached leaves of *Solanum tuberosum* reported that stomata can close under low [O_2] (Hedrich *et al.*, 2001), which does not agree with our observations (Fig. 5c,d). Further investigations revealed that the g_s response is highly sensitive to [O_2], with values < 1% leading to an absence of decrease for > 1 h and values > 1% leading to a complete stomatal closure with a *c.* 10 min delay (Fig. S7). This sensitivity of the g_s response to [O_2] and the observed differences between species could explain why results differ between laboratories. Under darkness, the absence of ROS accumulation due to low [O_2] could have prevented stomatal closure, but this does not exclude the lack of energy as a possible cause. Indeed, blocking ATP formation, Karlsson & Schwartz (1988) obtained similar results to those observed here under low [O_2], suggesting that ROS alone cannot explain our findings. It is often overlooked that energy is required for stomatal closure (Karlsson & Schwartz, 1988; Willmer & Fricker, 1996) and our results stress the importance of respiratory processes in supplying energy for guard cell movements. Moreover, our results in wheat showed that stomata can maintain an aperture for > 1 h without energy and that energy is only consumed to drive changes in guard cell turgor. Surprisingly, light-acclimated leaves subjected to low [O_2] displayed a strong decrease in g_s independently of light intensity, highlighting that respiratory processes also play a key role in the maintenance of high g_{sw} throughout the diurnal period. A gas exchange model describing the role of respiratory processes in stomatal behaviour estimated up to 50% of the diurnal g_s under a 'natural' light regime was attributed to these processes. Overall, these results suggest that the large number of mitochondria in guard cells (Shimazaki *et al.*, 2007) are key for stomatal movements, especially when A is limited (e.g. early in the morning or during sun-flecks), as well as for the maintenance of high g_s and stomatal closure (e.g. end of the day or during shade-flecks).

BL acts as a dark/light switch optimizing stomatal behaviour for water saving

Thus far, our results have shown the influence of BL on diurnal stomatal behaviour and the resulting impact on photosynthesis. However, they do not explain why BL-induced stomatal opening during the diurnal period was essential for removing stomatal limitation of A in wheat but not necessary in tobacco. One major difference between these two species was the large nocturnal g_s observed in tobacco compared to the tight stomatal closure in wheat. The large nocturnal g_s in tobacco did not limit photosynthetic induction in the morning, whilst in wheat the rapid stomatal opening induced by BL was essential to remove the early morning diffusional limitation of A. This therefore suggests that a role for BL-induced stomatal opening is to enable a low nocturnal g_s in wheat without compromising photosynthetic induction, and to increase water savings by closing stomata during the night (Caird *et al.*, 2006). The interspecific differences observed in response to BL suggest that different strategies for the regulation of diurnal gas exchange exist, and this requires further research to determine species-specific advantages.

Conclusions

The BL-induced stomatal response is generally assumed to occur in the majority of species, although there is evidence that it is lacking in some ferns (Doi *et al.*, 2015; Westbrook & McAdam, 2020) and facultative CAM plants (when in CAM mode, Gotoh *et al.*, 2019). The findings presented here demonstrate that the BL response is far from universal, and that there is a significant interspecific diversity in stomatal response (both in rapidity and in magnitude). Our results give a broader context to the importance of BL for gas exchange and show how BL-induced stomatal responses interact with photosynthetic and respiratory processes over the course of the day. At the beginning of the day, BL was shown to uncouple g_{sw} variation from A and enables rapid stomatal opening, removing diffusional limitations and facilitating lower g_{sw} during the night. Respiratory and photosynthetic processes were both required for rapid stomatal movements and to maintain a high g_{sw}. We predict here that the energy produced by respiratory processes could drive up to 60% of the observed diurnal variations in g_{sw}, with photosynthesis providing the remaining 40% in wheat. Furthermore, we have shown that respiratory processes are essential for stomatal movements (opening and closing) and maintenance of the steady-state g_{sw} in the light. Surprisingly, in the absence of energy (both photosynthetic and respiratory), g_{sw} remained unchanged (for > 1 h) in wheat, suggesting there is minimal energy requirement to maintain g_{sw} at the current level. These results emphasize the unexploited potential of temporal optimization of A and g_{sw} over the diurnal period, based on tuning the sensitivity of the stomatal response to BL and the ratio of photosynthetic and respiratory energy driving the response.

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Author contributions

SV-C and TL conceptualized the research plan. SV-C designed and conducted the experiments and analysed the data. JSAM aided in collecting gas exchange data and constructed the schematic. SV-C and TL wrote the manuscript and JSAM reviewed and edited later versions of the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Diurnal light regime used for gas exchange measurements.

Fig. S2 Difference in rapidity of stomatal response to step changes in light intensity due to the addition of weak blue light.

Fig. S3 Impact of additional blue light on diurnal kinetics of intrinsic water use efficiency (W_i).

Fig. S4 Impact of low $[O_2]$ on temporal response of g_{sw} under weak light intensity.

Fig. S5 Effect of red and blue light on gas exchange under 1% and 21% $[O_2]$.

Fig. S6 Parameter values inferred using Bayesian inference and observations from Fig. 3(e,f).

Fig. S7 Effect of $[O_2]$ on stomatal conductance to a step change in light.

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