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### ► To cite this version:

Michele Faralli, Greg Mellers, Shellie Wall, Silvere Vialet-Chabrand, Guillaume Forget, et al.. Exploring natural genetic diversity in a bread wheat multi-founder population: dual imaging of photosynthesis and stomatal kinetics. *Journal of Experimental Botany*, 2024, 75 (21), pp.6733 - 6747. <10.1093/jxb/erae233>. <hal-04804050>

**HAL Id: hal-04804050**

**<https://hal.inrae.fr/hal-04804050v1>**

Submitted on 26 Nov 2024

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RESEARCH PAPER

# Exploring natural genetic diversity in a bread wheat multi-founder population: dual imaging of photosynthesis and stomatal kinetics

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Received 20 December 2023; Editorial decision 10 May 2024; Accepted 23 May 2024

Editor: Elizabete Carmo-Silva, Lancaster University, UK

## Abstract

Recent research has shown that optimizing photosynthetic and stomatal traits holds promise for improved crop performance. However, standard phenotyping tools such as gas exchange systems have limited throughput. In this work, a novel approach based on a bespoke gas exchange chamber allowing combined measurement of the quantum yield of PSII ( $F_q'/F_m'$ ), with an estimation of stomatal conductance via thermal imaging was used to phenotype a range of bread wheat (*Triticum aestivum* L.) genotypes. Using the dual-imaging methods and traditional approaches, we found broad and significant variation in key traits, including photosynthetic CO<sub>2</sub> uptake at saturating light and ambient CO<sub>2</sub> concentration ( $A_{sat}$ ), photosynthetic CO<sub>2</sub> uptake at saturating light and elevated CO<sub>2</sub> concentration ( $A_{max}$ ), the maximum velocity of Rubisco for carboxylation ( $V_{cmax}$ ), time for stomatal opening ( $K_i$ ), and leaf evaporative cooling. Anatomical analysis revealed significant variation in flag leaf adaxial stomatal density. Associations between traits highlighted significant relationships between leaf evaporative cooling, leaf stomatal conductance, and  $F_q'/F_m'$ , highlighting the importance of stomatal conductance and stomatal rapidity in maintaining optimal leaf temperature for photosynthesis in wheat. Additionally,  $g_{smin}$  and  $g_{smax}$  were positively associated, indicating that potential combinations of preferable traits (i.e. inherently high  $g_{smax}$ , low  $K_i$ , and maintained leaf evaporative cooling) are present in wheat. This work highlights the effectiveness of thermal imaging in screening dynamic  $g_s$  in a panel of wheat genotypes. The wide phenotypic variation observed suggested the presence of exploitable genetic variability in bread wheat for dynamic stomatal conductance traits and photosynthetic capacity for targeted optimization within future breeding programmes.

**Keywords:** Kinetics, MAGIC, photosynthesis, photosynthetic capacity, stomatal conductance, thermal images, water use efficiency, wheat.

## Introduction

Crop yield is the product of the cumulative rates of photosynthesis over the growing season (Zelitch, 1982). Indeed, several developmental processes occurring throughout the life cycle of a crop co-determine a series of yield components that are often limited by the availability of assimilates (Slafer, 2003). For instance, free-air concentration enrichment (FACE) experiments (Long *et al.*, 2006) and bioengineering approaches (Driever *et al.*, 2017) have provided evidence that increasing rates of photosynthesis can lead to yield gains. In many crops, while harvest index and light interception capacity are approaching their theoretical maximum ( $\sim 0.64$  and  $0.8\text{--}0.9$ , respectively, Long *et al.*, 2006), the efficiency of energy conversion into biomass (i.e. radiation use efficiency and thus photosynthesis) still has substantial room for improvement (Long *et al.*, 2006). Although it is well established that significant variation in photosynthesis exists between species (e.g. Wullschlegel, 1993; Lawson *et al.*, 2012), several more recent studies have reported significant variation between cultivars of the same species (Driever *et al.*, 2014; Carmo-Silva *et al.*, 2017; Faralli *et al.*, 2019a, b; Ferguson *et al.*, 2020; McAusland *et al.*, 2020; Wall *et al.*, 2023). Most of the intraspecific natural variation in photosynthesis for  $C_3$  plants has been attributed to differences in biochemical capacity, including electron transport rates and carboxylation efficiency (Driever *et al.*, 2014; Carmo-Silva *et al.*, 2017). In addition, under natural dynamic conditions, photosynthetic processes such as activation of Calvin cycle enzymes and/or stomatal dynamics can also be limiting (Lawson and Blatt, 2014; Taylor and Long, 2017; Faralli *et al.*, 2019a; Salter *et al.*, 2019).

Stomatal dynamics balance leaf  $\text{CO}_2$  uptake and water loss, and hence significantly influence the key components of crop productivity: the cumulative rate of photosynthesis, water use, and evaporative cooling. The opening and closing of stomata are driven by a series of environmental, hormonal, and hydraulic signals (Blatt, 2000), with significant variation observed in sensitivity and responsiveness among different species (Lawson *et al.*, 1998, 2003, 2012; Lawson, 2009) and genotypes driven by differences in morphology (Weyers and Lawson, 1997; Weyers *et al.*, 1997; Hetherington and Woodward, 2003; Drake *et al.*, 2013; McAusland *et al.*, 2015; Yoshiyama *et al.*, 2024). In general, stomata open in response to increasing light intensity, low  $\text{CO}_2$  concentration ( $[\text{CO}_2]$ ), high temperatures, and low vapour pressure deficit (VPD), while closure is driven by low light or darkness, high  $[\text{CO}_2]$ , and high VPD (Outlaw, 2003). In the natural environment, these factors can occur simultaneously and in a dynamic and fluctuating manner (Sharkey and Raschke, 1981; Zeiger and Zhu, 1998; Talbott *et al.*, 2003).

It has been extensively shown that the rapidity in stomatal movements under conditions such as shade or sun-flecks may be considered preferable traits for crop improvement due to reduced water loss when carbon gain is limited and a reduction in diffusional constraint on photosynthesis (Lawson

*et al.*, 2010; Lawson and Blatt, 2014). In wheat (*Triticum aestivum* L.), a major food crop accounting for up to 20% of the world's calorie consumption (Erenstein *et al.*, 2022), these traits have been sparsely explored (Faralli *et al.*, 2019a), although in other crops (e.g. rice) stomatal rapidity has been associated with an adaptation to dry conditions (Qu *et al.*, 2016; Faralli *et al.*, 2019a; Zhang *et al.*, 2019). Therefore, the main objective of this work was to phenotype, via a novel, non-invasive, and high-throughput method, stomatal rapidity in a wheat multi-parent advanced generation intercross (MAGIC) population along with steady-state gas exchange traits. A total of 192 greenhouse-grown wheat genotypes were analysed, including photosynthetic capacity and stomatal traits. Our work is the largest phenotyping study carried out so far to characterize wheat stomatal responses and photosynthetic diversity under dynamic light, and may provide methods and resources to open up new avenues to optimize wheat responses to natural fluctuating environmental conditions.

## Materials and methods

### Plant material

The MAGIC wheat population was used in this study (Mackay *et al.*, 2014). The population consists of recombinant inbred lines (RILs) generated from three cycles of intercrossing between eight elite European wheat cultivars (Alchemy, Brompton, Claire, Hereward, Rialto, Robigus, Soissons, and Xi-19) followed by five rounds of self-pollination to derive RILs as described by Mackay *et al.* (2014). A subset of the population comprising 192 lines and the eight parental lines was used for this work. These lines encompassed the important genetic variation present in the larger population.

### Plant growth, vernalization, and experimental design

Seeds were sown in plastic trays containing compost and germinated in a growth cabinet (Reftech BV, Sassenheim, the Netherlands) at the University of Essex at  $\sim 200 \mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetic photon flux density (PPFD), 14 h/10 h photoperiod (light/dark),  $\sim 15^\circ\text{C}$  on average, and  $\sim 60\%$  relative humidity. The compost (Levington F2S; Everris, Ipswich, UK) contained coir, sand, and fertilizer ( $144 \text{ mg l}^{-1} \text{ N}$ ,  $73 \text{ mg l}^{-1} \text{ P}$ ,  $239 \text{ mg l}^{-1} \text{ K}$ , adjusted to pH 5.3–6.0 with dolomitic lime). At BBCH (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) growth stage (GS) 12 (GS12, two seedling leaves unfolded; Lancashire *et al.*, 1991), seedlings were moved into a cold room for vernalization:  $4^\circ\text{C}$ ,  $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD at 10 h/14 h photoperiod (light/dark) for 8 weeks. After vernalization, seedlings (one per pot) were transplanted into 1.5 litre pots (15 cm diameter; 12 cm deep) containing F2S compost and transferred to a temperature-controlled glasshouse. To phenotype the population at flag leaf emergence (GS39–GS41), three batches of plants were grown from July 2017 to April 2018. Batch 1 consisted of 189 genotypes, with each line replicated twice. In Batch 2, 186 of these genotypes were grown again ( $n=2$ ), but the eight parental lines had a higher number of replications ( $n=5$ ). Batch 3 included an extra replicate of contrasting lines from Batch 1 (48 genotypes) and Batch 2 (47 genotypes), as well as 80 extra genotypes in  $n=2$ , along with 8-fold ( $n=8$ ) parental replication. After vernalization, plants from each batch were transferred to the glasshouse and spatially randomized with a two-block structure, and

genotypes exhibiting a non-uniform behaviour between replicates were discarded from the study (Supplementary Fig. S1).

### Phenotypic analysis

Plants were scored for the occurrence of flag leaf emergence (GS39) and flag leaf fully emerged (GS41). Plant height (soil surface to flag leaf tip) at GS41 was assessed with a ruler prior to stomatal conductance analysis. All measurements were made on flag leaves of plants that had reached GS41–GS45.

### High-throughput phenotyping of dynamic $g_s$ responses with thermal imaging

#### Thermal imaging

Dynamic responses of stomatal conductance to water vapour ( $g_s$ ) to a step change in irradiance were assessed by modifying an in-house system developed at the University of Essex (McAusland *et al.*, 2013). A FluorImager system (Technologica, Colchester, Essex, UK) was modified to allow thermal imaging and chlorophyll fluorescence imaging simultaneously. For thermal imaging, a thermal camera Optris 450i (Optris GmbH, Berlin, Germany) with a temperature resolution of 0.1 °C was configured with an emissivity ( $\epsilon$ ) of 0.96 and set to perform a non-uniform calibration every minute. The thermal camera was positioned at 0.45 m in the original location of the chlorophyll fluorescence camera, directly above the imaging port, while the chlorophyll fluorescence camera AVT manta (Allied Vision, Stadtroda, Germany) was positioned at a 90° angle. A silver-coated mirror (Thor-Optics, Dachau, Germany) was hinged on an axis directly above the original camera port and a servo motor connected to an Arduino was used to automatically move the mirror, switching between the two cameras while keeping the same field of view (Supplementary Fig. S1). Air temperature and relative humidity were collected using a sensor (HygroClip2, HC2A-S, Rotronic, Bassersdorf, Switzerland) connected to the same Arduino. A custom software was used to automatically collect images from both cameras, record environmental data, operate the servo motor, and process the data.

#### Imaging chamber and gas control

A modified open-top chamber based on that described by McAusland *et al.* (2013) was designed and constructed. The cuvette allowed the control of the concentration of gases (N, H<sub>2</sub>O, CO<sub>2</sub>, and O<sub>2</sub>), while keeping an open top for imaging. The chamber was built from Perspex and, due to the relatively high reflection inside the chamber at high light intensity, the interior surfaces were painted grey. With the exception of the base, the chamber consisted of an inner and outer wall separated by a 10 mm gap. The outer walls were connected on each of the four sides by 6 mm PTFE tubing connections that fed gas into the chamber wall cavity. The inner wall was perforated with 1 mm diameter holes at a density of 9 per 100 mm<sup>2</sup>, which was optimal for maintaining homogenous gas concentrations whilst minimizing leaf movement through turbulence. Within the chamber, target gas concentrations of nitrogen (N<sub>2</sub>) and CO<sub>2</sub> were individually maintained by mass flow controllers (EL Flow, Bronkhorst, Ruurlo, the Netherlands), connected to compressed gas cylinders containing 100% N<sub>2</sub> and CO<sub>2</sub>, respectively (British Oxygen Company-Industrial Gases, Ipswich, UK). To control water vapour concentration, a Controlled Evaporation and Mixing system (CEM Evaporator W-202A, Bronkhorst, Newmarket, UK) was used to precisely regulate the water vapour content of the air. Gas composition in the chamber was monitored at leaf height by sampling air with a diaphragm pump (Type 124, ADC Hoddesdon, Herts, UK) at 500 cm<sup>3</sup> min<sup>-1</sup>. Both CO<sub>2</sub> and H<sub>2</sub>O vapour concentrations were measured with an infrared gas analyser (IRGA) (Li-840, LI-COR, Lincoln, NE, USA). Throughout the phenotyping experiment and during each analysis, ambient CO<sub>2</sub> concentration ( $C_a$ )

was maintained at 400 μmol CO<sub>2</sub> mol<sup>-1</sup> air while relative humidity was maintained at 45–60% inside the cuvette.

### Estimating dynamic stomatal conductance from leaf temperature

Significant negative correlation exists between leaf conductance to water vapour and leaf temperature (Jones *et al.*, 2009). Due to the transition of water into water vapour during transpiration, energy (latent heat of vaporization) is taken from the leaf, leading to a reduction in surface leaf temperature. This evaporative cooling effect of transpiration can be used as an indirect method for the estimation of leaf  $g_s$  (Supplementary Fig. S1). For this reason,  $g_s$  was estimated following the equations proposed by Leinonen *et al.* (2006) and shown in Jones *et al.* (2009).

Mass leaf transpiration was estimated as follows:

$$E_m = \frac{\left[0.92g_b + \left(\frac{4\epsilon\sigma T_a^3}{\rho C_p}\right)\right] (\rho C_p) (T_{dry} - T_{leaf})}{\lambda}$$

Where  $g_b$  is the estimated boundary layer conductance to water vapour (see Supplementary Dataset S1 for an example of calculation), 0.92 indicates a proportional relationship between heat and water vapour transfer rates across the boundary layer under laminar flow,  $\epsilon$  represents sample emissivity,  $\sigma$  is the Stefan–Boltzmann constant,  $T_a$  is air temperature,  $\rho$  represents air density,  $C_p$  is the air specific heat capacity,  $T_{dry}$  is the temperature of the dry reference,  $T_{leaf}$  is the temperature of the leaf sample, and  $\lambda$  is the latent heat of vaporization.

Conversion of  $E_m$  to mol m<sup>-2</sup> s<sup>-1</sup> was carried out and total conductance to water vapour was estimated as:

$$g_w = \frac{E}{\frac{e_s - e_a}{P}}$$

where  $E$  is leaf transpiration,  $e_s$  is the saturated water vapour pressure in the leaf,  $e_a$  is the air vapour pressure, and  $P$  is the atmospheric pressure (i.e. leafVPD).

Leaf stomatal conductance to water vapour ( $g_s$ ) was then estimated as

$$g_s = \frac{1}{\frac{1}{g_w} - \frac{1}{g_b}}$$

A spreadsheet containing an example of calculating  $g_s$  from thermography and environmental conditions is provided in Supplementary Dataset S2.

### Protocol for estimating parameters in response to step changes in irradiance

Prior to each analysis, plants were moved from the greenhouse to a controlled-environment dark room (20 °C maintained with an air conditioner and ~60% relative humidity maintained with a humidifier). It was possible to run three plants simultaneously together with the dry reference. The dry reference was a flag leaf sampled with scissors from spare plants for each protocol, and petroleum jelly (Vaseline) was applied on both surfaces to prevent transpiration. Dark-adapted plants (~1 h per cycle, see below) were clamped onto the chamber and acclimated for 16 min at 100 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD followed by a step change in light to 1000 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD for 30 min (sufficient to reach steady state in wheat, Faralli *et al.*, 2019a), and thermal and fluorescence images were taken every 2 min. The thermal dataset generated was automatically

analysed with a bespoke system based on OpenCV that estimates leaf  $g_s$  in a pixel-based manner and averages over the leaf sample. Leaf segmentation was carried out using basal fluorescence in the dark and by applying Otsu thresholding. The time constant for the rapidity of  $g_s$  response to a step change in light intensity ( $K_{gs}$ , estimated from  $g_s$  kinetics; or  $K_t$ , estimated from  $T_{dry}-T_{leaf}$  kinetics) was estimated with the model proposed by [Viale-Chabrand \*et al.\* \(2013\)](#). The dynamic model predicts the temporal response of  $g_s$  at the leaf level using a sigmoid function for increasing  $g_s$ . It describes the temporal response of  $g_s$  using a time constant ( $k$ , min), an initial time lag ( $\lambda$ , min), and a steady-state  $g_s$  reached at a given PPFD. The model also allowed the estimation of  $g_s$  at 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD ( $g_{smin}$ ) and at 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD ( $g_{smax}$ ). In addition,  $\Delta T$  was estimated as the difference in temperature between the first point after the step change in light and the last point (i.e. cooling capacity). The light-adapted quantum yields of PSII ( $F_q'/F_m'$ ) at low and high light were also assessed as well as non-photochemical quenching (NPQ: high light) estimated following [Murchie and Lawson \(2013\)](#).

#### Photosynthetic CO<sub>2</sub> response curves ( $A/C_i$ )

The same leaf used for imaging was subjected to gas exchange measurements. Photosynthesis measurements ( $A/C_i$  curves) were performed between 09.00 h and 15.00 h on the fully emerged flag leaf at GS41–GS45 using a LI-6400. Measurements of the response of  $A$  to substomatal CO<sub>2</sub> concentrations ( $C_i$ ) were performed in the middle of the tagged leaf using an open infrared gas exchange system and a 2 cm<sup>2</sup> leaf cuvette with an integral blue–red LED light source (LI-6400-40; LI-COR). In the cuvette, PPFD was maintained at a saturating level of 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , a leaf temperature of  $20 \pm 0.1$  °C, a VPD between 0.9 kPa and 1.3 kPa, and a  $C_a$  of 400  $\mu\text{mol mol}^{-1}$ . When steady-state conditions were achieved,  $C_a$  was sequentially decreased to 300, 200, 100, and 75  $\mu\text{mol mol}^{-1}$  before returning to the initial concentration of 400  $\mu\text{mol mol}^{-1}$ . This was followed by a sequential increase to 550, 700, 1000, and 1200  $\mu\text{mol mol}^{-1}$ . Readings were recorded when  $A$  had stabilized to the new conditions. The maximum velocity of Rubisco for carboxylation ( $V_{cmax}$ ) and the maximum rate of electron transport demand for ribulose 1,5-bisphosphate (RuBP) regeneration ( $J_{max}$ ) were derived by curve fitting. We used the Plantecophys R package to determine  $V_{cmax}$  and  $J_{max}$  via non-linear least squares, while standard errors of the parameters were estimated with standard methods ([Supplementary Fig. S1](#)).

#### Validation of the estimated $g_s$ dataset with thermal imaging

An additional experiment was conducted to validate image-based estimations of  $g_s$  by comparison with  $g_s$  values estimated using standard gas exchange measurements. Lines with contrasting  $K_{gs}$  values were grown as for the phenotyping experiment. IRGAs (Li-Cor 6400XT) assessed the rapidity of stomatal responses to a step change in light (using the same protocols as described by [Faralli \*et al.\*, 2019a](#)). Briefly, prior to measurement, flag leaves of plants at GS41 were equilibrated to a PPFD of 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for ~60 min or until  $g_s$  reached ‘steady state’, defined as a  $\leq 2\%$  change in rate during a 10 min period. After equilibration, PPFD was increased to 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 50 min and subsequently returned to 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 1 h. Conditions inside the leaf cuvette were kept constant at  $20 \pm 0.1$ °C leaf temperature, a VPD of 1 kPa with a dew point generator (LI-610; LI-COR), and 400  $\mu\text{mol CO}_2 \text{ mol}^{-1}$  air (ambient CO<sub>2</sub> concentration,  $C_a$ ). The time constant for the rapidity of  $g_s$  responses to a step change in light intensity was estimated as previously described.

#### Statistical analysis

Data analysis was conducted using Rstudio. Due to significant variation in growing conditions in the glass house and spatial effects, the data were

analysed using a one-way analysis of covariance (ANCOVA), treating ‘greenhouse’ and ‘block’ as covariates. Normality checks were performed on all datasets. The associations between different traits were examined through correlation analysis, and the Pearson test was used to evaluate the strength of these associations.

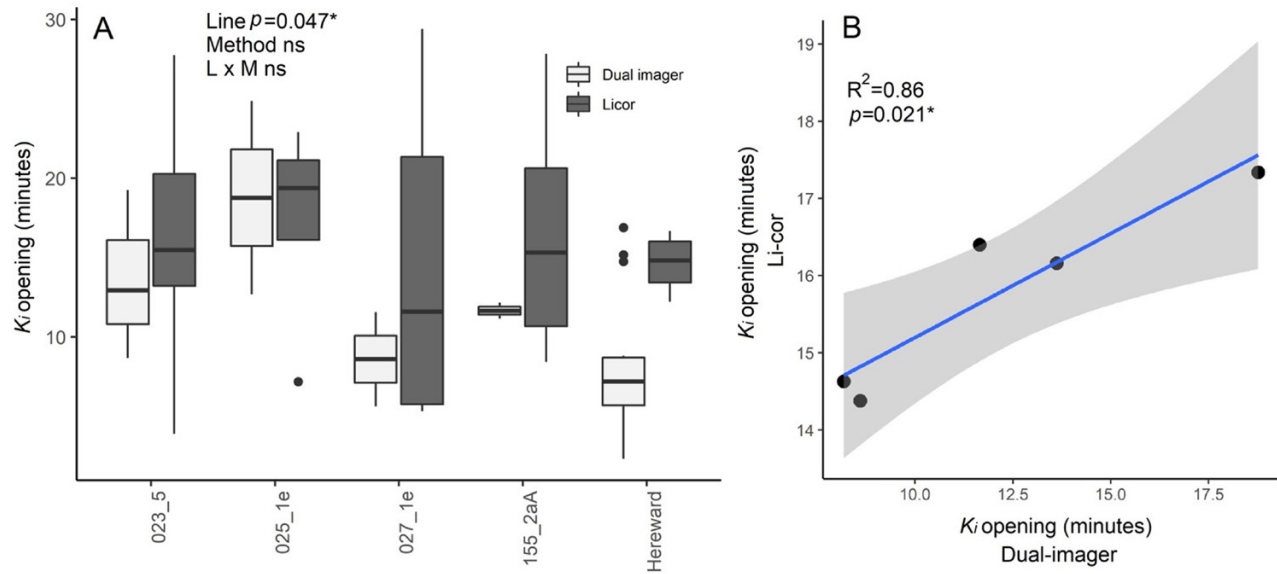
## Results

### Comparison of $g_s$ values obtained from imaging and standard approaches

To evaluate  $g_s$  values obtained from the dual imaging system compared with those from standard IRGA methods, a comparative analysis was performed on a subset of genotypes, selected based on their differential kinetic profiles ([Fig. 1](#)). Overall, the time constant for stomatal opening ( $K_i$ ) as determined by the dual imager method and standard gas exchange showed no significant difference between the two methods ([Fig. 1A](#)) ( $P > 0.05$ ). Additionally, a positive and significant association was observed for dual imager and IRGA estimates of  $K_i$  ([Fig. 1B](#)). Generally, the dual imager method showed higher variation between genotypes compared with the IRGA, yet the ranking among lines remained consistent.

### Photosynthetic capacity

The response of the photosynthetic assimilation rate to increasing CO<sub>2</sub> at saturating irradiance ( $A/C_i$  response curve) was analysed for all lines to assess variation in photosynthetic capacity.  $A/C_i$  curves for all genotypes measured followed the typical hyperbolic response. From these response curves, both the light-saturated rate of photosynthesis ( $A_{sat}$ ) and the light- and CO<sub>2</sub>-saturated rate of photosynthesis ( $A_{max}$ ) were determined. Significant differences ( $P < 0.05$ , [Fig. 2](#)) were observed between genotypes in both values, indicating variation in the maximum photosynthetic potential as well as operational rate across these genotypes.  $A_{sat}$  varied from a minimum of 18  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to a maximum of 34  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on average. For the eight parental lines, ‘Hereward’ and ‘Robigus’ showed the lowest (20–21.5  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) values, while the highest values (27–28  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) were observed in ‘Soissons’, ‘Claire’, and ‘Xi19’. For RILs of the population, the highest values were observed for MEL\_122\_1b and MEL\_036\_8 (up to 34  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), while the lowest values between 15  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 18  $\mu\text{mol m}^{-2} \text{s}^{-1}$  were observed in MEL\_139\_7 and MEL\_071\_1c.  $A_{max}$  varied significantly between genotypes, with values ranging from 25  $\mu\text{mol m}^{-2} \text{s}^{-1}$  to 45  $\mu\text{mol m}^{-2} \text{s}^{-1}$  on average, and a positive association between  $A_{sat}$  and  $A_{max}$  was observed ( $P < 0.001$ ). However, in some cases, the ranking was significantly altered; for example, Robigus had a relatively low  $A_{sat}$  while it was positioned in the middle of the distribution for  $A_{max}$ .  $A_{sat}$  and  $A_{max}$  for the 200 lines measured followed a normal distribution. Therefore, the differential ranking of lines between operational assimilation rates ( $A_{sat}$ ) and maximum capacity ( $A_{max}$ )



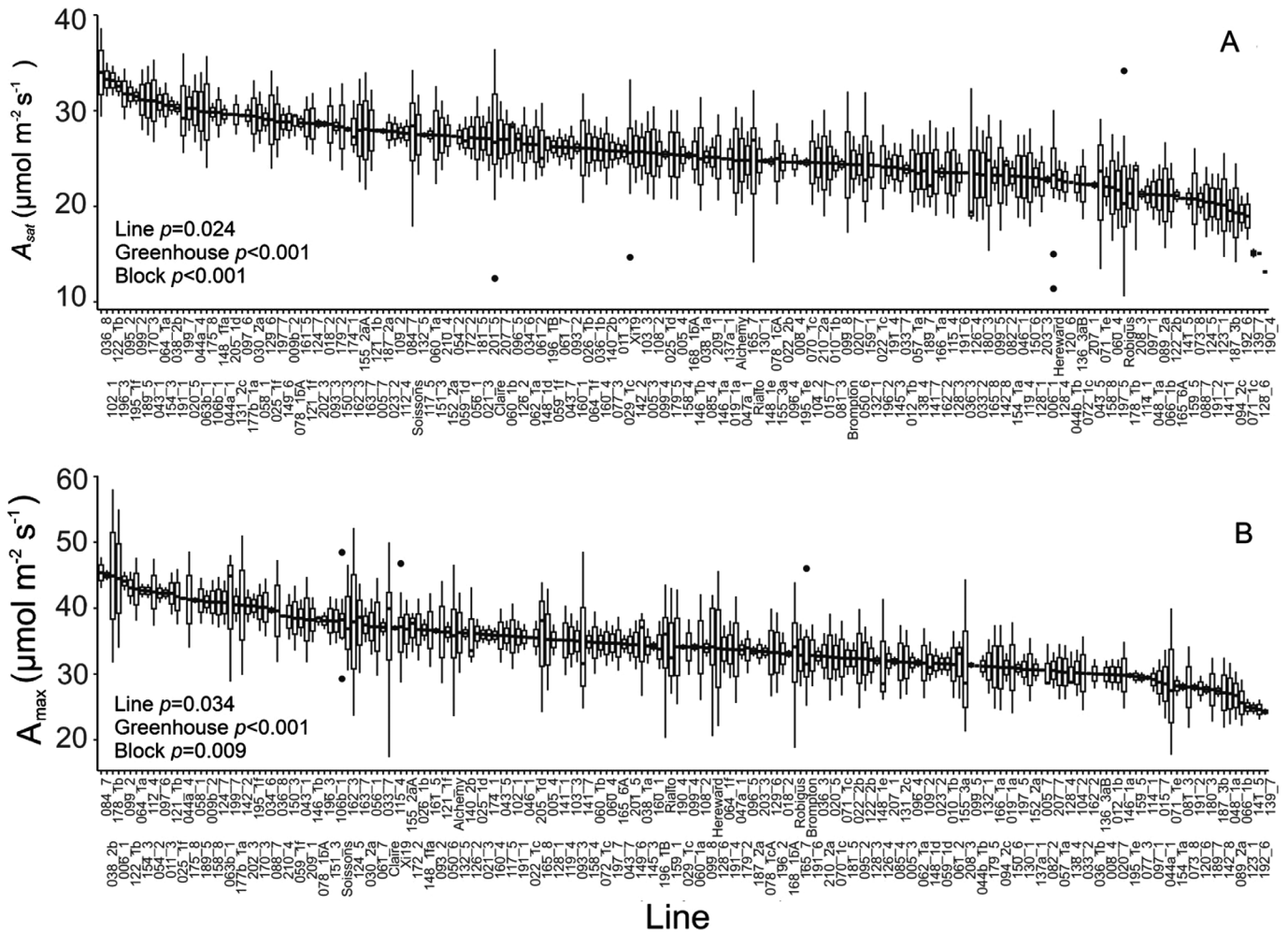
**Fig. 1.** Comparison of methods for the measurement of stomatal opening. (A) Time constant for stomatal opening analysed via an infrared gas analyser (dark grey) and the dual imager (light grey) ( $n=3-7$ ) in a subset of wheat genotypes. Data were analysed with two-way ANOVA and significant differences are indicated. (B) The linear association between the two methods. Data are means, and fitting was carried out via linear regression.

clearly indicates that these are influenced by differing factors and that a high photosynthetic potential does not always translate to a higher actual rate in the field.

Significant variation in  $V_{\text{cmax}}$  was observed between genotypes ( $P < 0.05$ , Fig. 3), with values varying  $>3$ -fold (from  $90 \mu\text{mol m}^{-2} \text{s}^{-1}$  to  $270 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). As stated above, on average for parental lines, ‘Hereward’ showed the lowest ( $140 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) values along with ‘Robigus’ ( $135 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), while higher values were observed for ‘Xi19’ and ‘Soissons’ ( $180 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). For RILs, MEL\_139\_7 and MEL\_073\_8 had the lowest values ( $\sim 90 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) while the highest values were observed for MEL\_062\_1a and MEL\_170\_3 ( $280-300 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The maximum electron transport rate capacity for RuBP regeneration ( $J_{\text{max}}$ ) also showed variation (although borderline significant,  $P=0.059$ ) between genotypes, and ranged between  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  and  $310 \mu\text{mol m}^{-2} \text{s}^{-1}$  on average.

In the field, the realized assimilation rate is influenced by  $g_s$  and the potential imposed diffusional constraints due to the dynamic behaviour of stomata. In order to assess stomatal dynamics in all 200 lines, a dual imager was utilized that adopted a high-resolution thermal camera to assess changes in leaf temperature as a proxy of  $g_s$  response (McAusland *et al.*, 2013; Vialet-Chabrand and Lawson, 2019, 2020) to a step increase in light intensity. Stomatal conductance values following 30 min at subsaturating light intensity ( $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) ( $g_{\text{smax}}$ ) showed trends of variation ( $P=0.064$ ) between lines, and an average value of  $0.7 \text{ mol m}^{-2} \text{ s}^{-1}$  (Fig. 4A). However, unsurprisingly, significant variation in individual measurements within the replicates was high (average SEM  $0.1 \text{ mol m}^{-2} \text{ s}^{-1}$ ). In the parental lines, the lowest  $g_{\text{smax}}$  values ( $0.6 \text{ mol m}^{-2} \text{ s}^{-1}$ )

were observed in Robigus, while Rialto displayed the highest value ( $0.85 \text{ mol m}^{-2} \text{ s}^{-1}$ ). Several RILs had low  $g_{\text{smax}}$ , ranging from  $0.3 \mu\text{mol m}^{-2} \text{ s}^{-1}$  to  $0.5 \text{ mol m}^{-2} \text{ s}^{-1}$  (e.g. MEL\_078\_1cA and MEL\_179\_2). The rapidity of changes in  $g_s$  after a 30 min sun-fleck, measured as the time constant to reach 63% of  $g_{\text{smax}}$  ( $K_i$ ), varied greatly between lines ( $P < 0.001$ ), and values ranged between 1.9 min and 19 min (Fig. 4B). For the parental material, Rialto, Brompton, and Hereward had the fastest responses (5–8 min on average) while Xi19 and Alchemy showed the slowest response (10 min). MEL\_146\_1b and MEL\_203\_3 had a very quick stomatal response ( $K_i$  between 1.9 min and 2.5 min), while MEL\_102\_1 and MEL\_071\_1c had the slowest stomatal response (16 min).  $K_i$  values followed a normal distribution, with the majority of speeds of responses between 5 min and 13 min, which agrees with previous reports on wheat (Faralli *et al.*, 2019a). Changes in stomatal conductance with differences in light intensity are critically important for evaporative cooling and maintaining optimal leaf temperature for photosynthesis and other metabolic processes. Differences between leaf temperature driven by the 30 min sun-fleck (i.e. cooling capacity,  $\delta T$ ) showed significant and wide variation with lines, and illustrate the ability of these lines to counteract the increase in irradiance via transpiration (i.e. positive evaporative cooling) (Fig. 4C). Values for  $\delta T$  ranged between  $-2 \text{ }^\circ\text{C}$  and  $4 \text{ }^\circ\text{C}$  changes in leaf temperatures. Values below zero indicate limited evaporative cooling with higher light intensity, whilst positive values show that changes in  $g_s$  result in increased cooling capacity. Most of the population (70%) showed positive values (i.e. cooling capacity) and all the parental material showed positive values, with Xi19, Robigus, and Alchemy showing the highest values ( $\delta T$   $1.5 \text{ }^\circ\text{C}$  on average). For the



**Fig. 2.** Steady-state photosynthetic traits estimated via  $A/C_i$  curves. (A) The variation between lines for photosynthetic  $\text{CO}_2$  uptake at saturating light and ambient  $\text{CO}_2$  concentration ( $A_{\text{sat}}$ ). (B) The variation between lines for photosynthetic  $\text{CO}_2$  uptake at saturating light and elevated  $\text{CO}_2$  concentration ( $A_{\text{max}}$ ). For graphs, horizontal lines within boxes indicate the median and boxes indicate the upper (75%) and lower (25%) quartiles. Whiskers indicate the ranges of the minimum and maximum values. Circles indicate outliers. Data were analysed with ANCOVA ( $n=2-10$ ), and  $P$ -values for the main effects are shown in the graph.

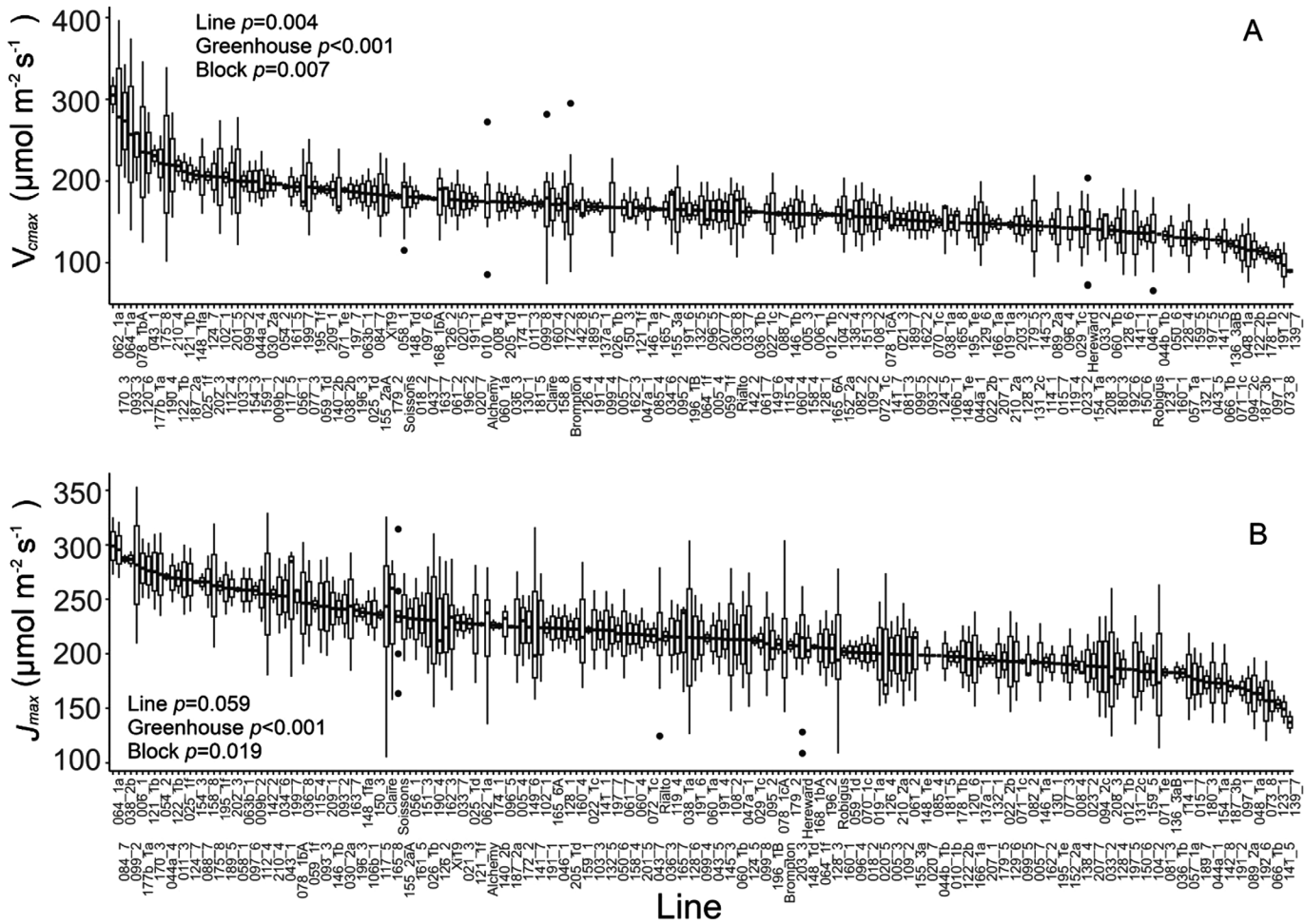
parental lines, the lowest values were observed for Hereward and Rialto ( $\delta T$  0.5 °C) while MEL\_081\_3 and MEL\_191\_2 had the most negative values ( $\delta T$  -2 °C). Significant differences were also observed for  $\delta g_s$ ; that is, the change in stomatal conductance after a step change in light (Fig. 4D). In general,  $\delta g_s$  ranged from 0.2 mol m<sup>-2</sup> s<sup>-1</sup> to up to 0.9 mol m<sup>-2</sup> s<sup>-1</sup>, with Alchemy showing the highest values between parental lines (0.7 mol m<sup>-2</sup> s<sup>-1</sup>) while Brompton and Rialto showed the lowest (0.5 mol m<sup>-2</sup> s<sup>-1</sup>). MEL\_201\_5 and MEL\_006\_1 showed the highest delta between  $g_{\text{smin}}$  and  $g_{\text{smax}}$  (0.9 mol m<sup>-2</sup> s<sup>-1</sup>) while MEL\_005\_3 and MEL\_036\_1b showed the lowest (0.2 mol m<sup>-2</sup> s<sup>-1</sup>).

The operating efficiency of PSII ( $F_q'/F_m'$ ) at low light levels showed significant variation between lines ( $P=0.048$ ), with an average value of 0.62 (Fig. 5A). In the parental lines, the lowest operating efficiency was observed in Soissons while Rialto

displayed the highest value (0.68). When plants were exposed to the subsaturating light intensity of 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , operating efficiency decreased and no significant differences were observed between lines. Similarly, although variation was present for NPQ, this was not significantly different between lines, potentially due to the subsaturating light conditions to which the plants were exposed.

### Stomatal density

Adaxial stomatal density was generally greater than abaxial density in all lines, which is typical for wheat although uncommon in most other species (Wall et al., 2022). Adaxial stomatal density showed significant ( $P<0.05$ , Fig. 6A) variation between lines analysed, with average stomatal density values ranging between 50 stomata mm<sup>-2</sup> and 80 stomata mm<sup>-2</sup>, while average



**Fig. 3.** Biochemical traits estimated via  $A/C_i$  curves. (A) The variation between genotypes for the maximum velocity for Rubisco carboxylation ( $V_{cmax}$ ). (B) The variation between genotypes for the maximum electron transport rate for RuBP regeneration ( $J_{max}$ ). For graphs, horizontal lines within boxes indicate the median and boxes indicate the upper (75%) and lower (25%) quartiles. Whiskers indicate the ranges of the minimum and maximum values, and dots indicate outliers. Data were analysed with ANCOVA ( $n=2-10$ );  $P$ -values are indicated.

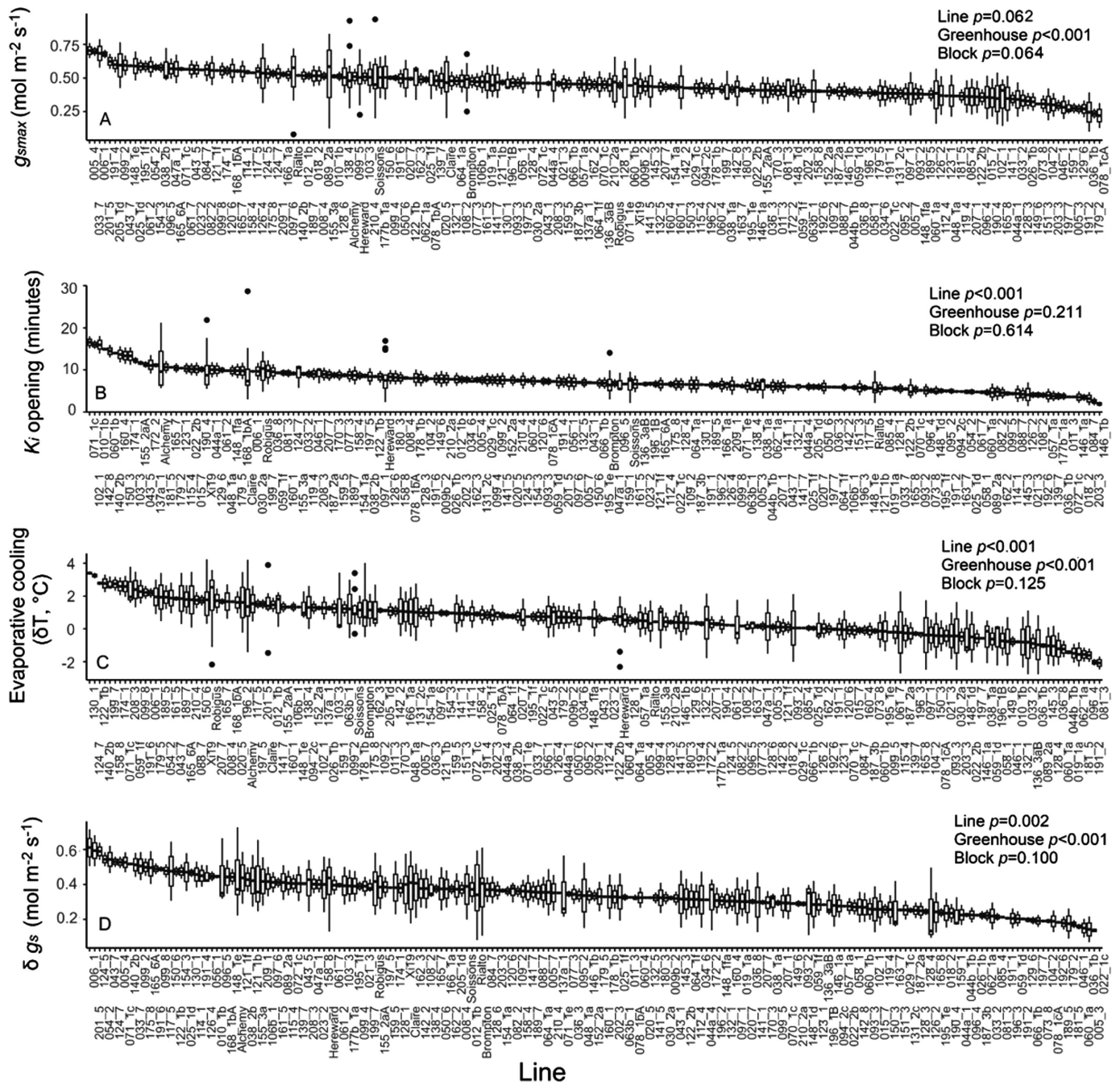
abaxial stomatal density values were between 40 stomata  $mm^{-2}$  and 65 stomata  $mm^2$ . Alchemy and Soissons had the highest average stomatal densities (57 stomata  $mm^{-2}$  and 61 stomata  $mm^{-2}$ , respectively) of the parental lines while Claire had the lowest (50 stomata  $mm^{-2}$ ). There was a strong positive correlation ( $P<0.001$ ) between adaxial and abaxial stomatal density (Fig. 7), suggesting a link between the two surfaces in terms of cell differentiation into guard cells.

Correlation analysis including all measurement parameters showed a number of significant associations (Fig. 7). Significant correlations ( $P<0.05$ ) were observed between  $A_{sat}$ ,  $A_{max}$ ,  $V_{cmax}$  and  $J_{max}$ . No significant associations were observed between stomatal anatomical features and estimated dynamic or steady-state  $g_s$  traits. However, a negative and significant correlation was observed between  $g_{smin}$  and  $K_i$  ( $P<0.001$ ). Similarly,  $\delta T$  significantly and positively correlated with  $K_i$  while a negative association existed between  $\delta T$  and  $g_{smin}$ .  $\delta g_s$  was correlated

with several traits ( $J_{max}$  and  $A_{max}$ ) and, in particular, with  $\delta T$  ( $r=0.47$ ).

### Discussion

The need to double food production in the next 50 years to feed a growing human population (Ray *et al.*, 2019; Asseng *et al.*, 2020; Furbank *et al.*, 2020; Billen *et al.*, 2024) and the requirement to do so in the face of predicted climate changes has led to increased research efforts to improve photosynthesis and other physiological processes in crops. Genetic modification (GM) of photosynthetic pathways has already proven successful (Raines, 2011; Evans, 2013; Kromdijk *et al.*, 2016; South *et al.*, 2019; Voss-Fels *et al.*, 2019); however, GM production is still met with resistance in many countries, and therefore exploiting the natural variation that exists in key crop characteristics represents an exciting and unexploited alternative.

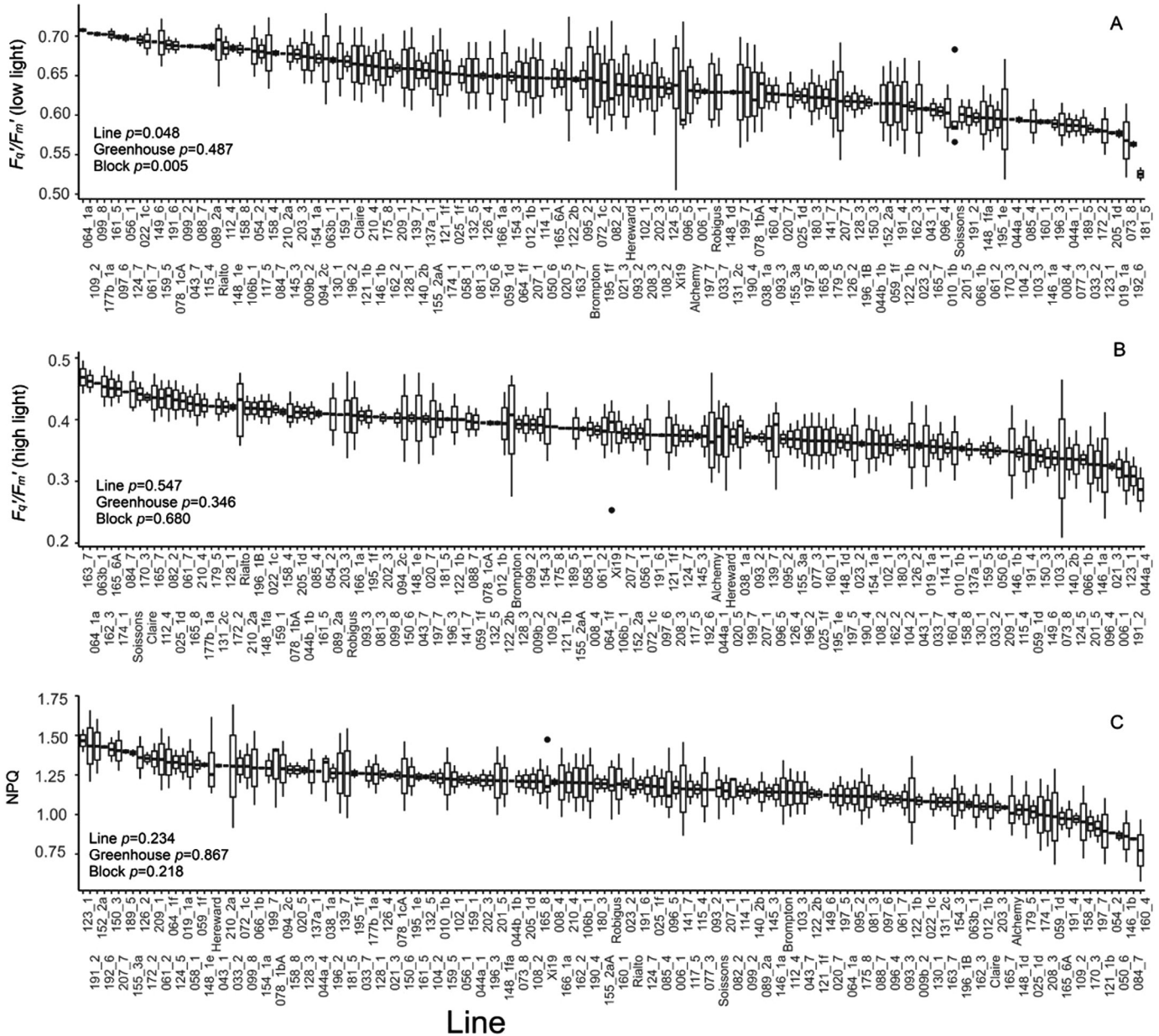


**Fig. 4.** Maximum stomatal conductance under near-saturating light ( $g_{smax}$ ), time for stomatal opening after a step change in light ( $K_i$ ), evaporative cooling capacity ( $\delta T$ ), and the difference between maximum stomatal conductance under near-saturating light and stomatal conductance before the step change in light ( $\delta g_s$ ) estimated with thermal imaging. For graphs, horizontal lines within boxes indicate the median and boxes indicate the upper (75%) and lower (25%) quartiles. Whiskers indicate the ranges of the minimum and maximum values. Data were analysed with ANCOVA ( $n=2-10$ ), and  $P$ -values are shown in the graph.

### Natural variation in photosynthetic capacity

Photosynthetic traits have been recognized previously as a potential source of natural variation that could be exploited for incorporation into breeding programmes to increase yield (Lawson et al., 2012; Driever et al., 2014; Faralli et al., 2019a).

Using standard IRGA techniques (i.e.  $A/C_i$ ) allowed us to take photosynthetic measurements in nearly 200 wheat genotypes from a germplasm that captures 80% of the single nucleotide polymorphism variation in North-West European bread wheat (Mackay et al., 2014), providing the largest screen to date of

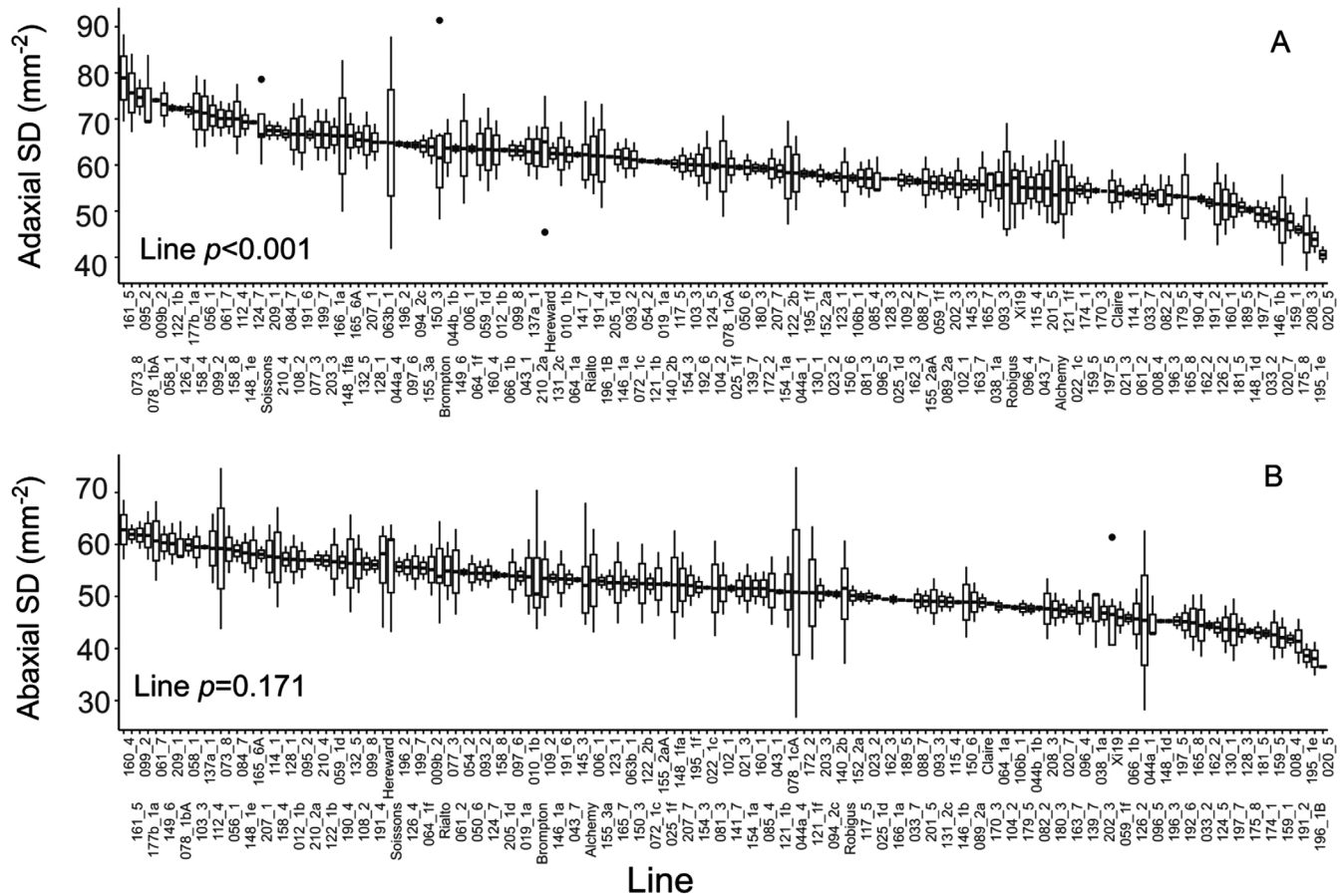


**Fig. 5.** PSII operating efficiency at 100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD ( $F_q/F_m'$  low light), PSII operating efficiency after 30 min at 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD ( $F_q/F_m'$  high light), and non-photochemical quenching after 30 min at 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD (NPQ). Data were estimated via chlorophyll fluorescence imaging. For graphs, horizontal lines within boxes indicate the median and boxes indicate the upper (75%) and lower (25%) quartiles. Whiskers indicate the ranges of the minimum and maximum values. Data were analysed with ANCOVA ( $n=2-10$ ), and  $P$ -values are shown in the graph.

several photosynthetic parameters of the flag leaf. We observed significant variation in both photosynthetic capacity ( $V_{\text{cmax}}$  and  $J_{\text{max}}$  Fig. 3, and  $A_{\text{max}}$  Fig. 2) and light-saturated rates of photosynthesis ( $A_{\text{sat}}$ ), highlighting the extent of intraspecific diversity that exists within UK bread wheat. The strong correlation we detected between  $V_{\text{cmax}}$  and  $J_{\text{max}}$  (Fig. 7) supports observations reported by others (Wullschlegler, 1993). Furthermore, a simulation analysis in rice suggested that genetic variation in both Rubisco-limited ( $V_{\text{cmax}}$ ) and electron transport-limited ( $J_{\text{max}}$ )

photosynthesis increased rice yields by 22–29% across distinct locations and years (Yin and Struick, 2017), potentially providing genetic targets for exploitation to improve wheat photosynthesis in a similar way.

Although questions have been raised regarding how closely linked photosynthetic rates per unit of leaf area are with yield (Driever et al., 2014; Zanella et al., 2023), ultimately photosynthesis is the primary determinant of all plant metabolic processes and therefore inevitably associated with reproductive

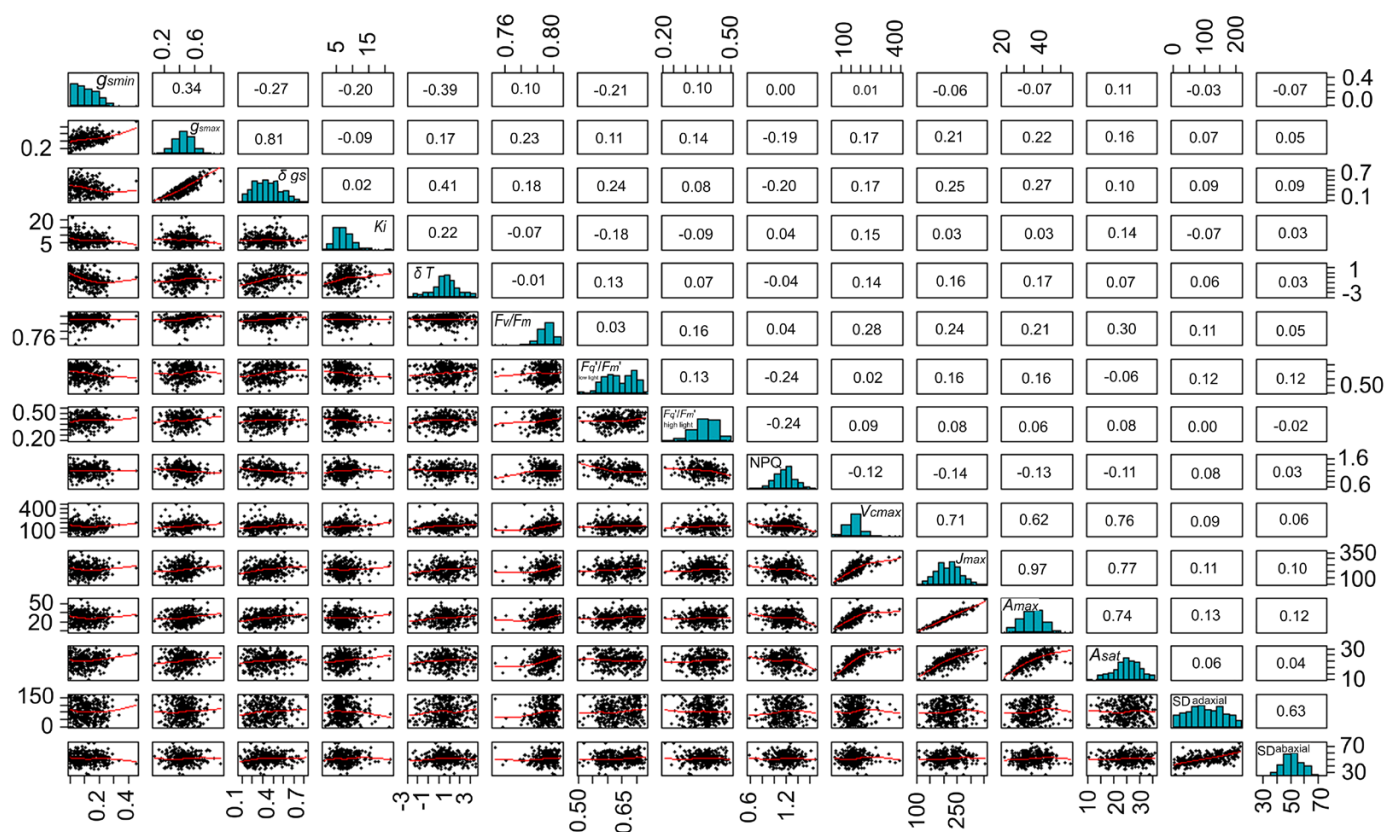


**Fig. 6.** Stomatal anatomical traits for a subset of the lines used for photosynthetic and dynamic screening. In (A), the adaxial stomatal density (SD) is shown while in (B) the abaxial SD is presented. For graphs, horizontal lines within boxes indicate the median and boxes indicate the upper (75%) and lower (25%) quartiles. Whiskers indicate the ranges of the minimum and maximum values. Data were analysed with one-way ANOVA ( $n=2-10$ ), and  $P$ -values are shown in the graph.

processes (Slafer and Araus, 2007) and yield (Long et al., 2006; Parry et al., 2011). Furthermore, many studies have reported increases in photosynthetic  $\text{CO}_2$  assimilation along with rises in yield when different  $\text{C}_3$  crops were grown in FACE facilities (Ainsworth and Long, 2005). Similarly, significant positive associations have been observed for different breeding lines in the field between  $\text{CO}_2$  uptake and  $g_s$  with grain yield components (Fischer et al., 1981, 1998; Blum, 1990; Reynolds, 2000; Carmo-Silva et al., 2017). However, contrary to this, many studies that have reported considerable variation in photosynthesis could not demonstrate that these differences translated into changes in yield (e.g. Chytky et al., 2011; Sadras et al., 2012; Driever et al., 2014). These contradictory findings may at least partially be due to the method used for photosynthetic assessment (Driever et al., 2014). Indeed, in most of the studies focusing on exploiting natural variation, photosynthesis is measured as maximum capacity which does not represent photosynthetic rates achieved in the field (Lawson et al., 2012). Although instantaneous measurements during the day and standardized for a specific leaf (Gaju et al., 2016)

provide a ‘real’ measure of photosynthesis at a specific moment in time, they fail to account for differences in microclimates, diurnal intracanopy variation, conditional effects prior to measurement, or circadian influences, all of which influence the measurements taken (Lawson et al., 2012; Driever et al., 2014; Faralli et al., 2019a). Here, we also determined the light-saturated rate of photosynthesis ( $A_{\text{sat}}$ ) (from the  $A/C_i$  analysis) which could be achieved in the field with sufficient light and no diffusional constraints from stomata (Lawson and Morison, 2004).

Significant associations were observed between  $A_{\text{sat}}$  and  $V_{\text{cmax}}/J_{\text{max}}$ , suggesting that measuring  $A_{\text{sat}}$  (Fig. 7) could provide an important step to further define potential methods for estimating the maximum carboxylation capacity of Rubisco and maximum electron transport for RuBP regeneration in wheat. Such strategies could be extremely beneficial for exploring and exploiting natural variation in photosynthesis as this is currently limited by the lengthy process for  $A/C_i$  measurements. While significant advances have been made recently in remote high-throughput measurements of photosynthetic



**Fig. 7.** Multiple scatter plots for traits assessed in this work. In each panel, the Pearson coefficient is shown for each correlation as well as the distribution. Significant associations are discussed in the text.

capacity through hyperspectral imaging (Meacham-Hensold *et al.*, 2020; Burnett *et al.*, 2021), further research is needed to enhance the predictive capabilities of these tools across various crops and environmental conditions.

Although there was strong correlation between  $A_{\text{sat}}$  and  $A_{\text{max}}$ , the differential rankings emphasize the influence of other factors on photosynthesis, for example limitation by low  $g_s$  which could impose a diffusional constraint on  $A_{\text{sat}}$  that would be removed by the high  $[\text{CO}_2]$  used to measure  $A_{\text{max}}$ .  $A_{\text{sat}}$  is very likely to represent realized carbon assimilation in the field, further emphasizing that a high photosynthetic potential does not always translate to a higher realized  $A$  as dynamic conditions and the impact on physiological processes need to be taken into consideration (see below).

The strong coordination between  $J_{\text{max}}$  and  $V_{\text{cmax}}$  both within and between species that has been demonstrated here and in other studies (Wullschleger, 1993), including those in which photosynthesis has been manipulated (Harrison *et al.*, 2001), suggests that plants employ a conservative strategy (Wullschleger, 1993). It has been suggested that this is to reduce the possibility of photoinhibition when carboxylation is limited; however, this could also limit the maximum photosynthetic rate under low light intensity (Walker *et al.*, 2014). In our work, the lack of any relationship between  $V_{\text{cmax}}$  and  $J_{\text{max}}$  with

stomatal anatomy and/or rapidity suggests that operational  $g_s$  may limit photosynthetic carbon gain in wheat, corroborating the hypothesis in which speedy stomata may be a preferable trait for maintaining or increasing carbon fixation under a natural environment (Lawson and Blatt, 2014).

#### Natural variation in dynamic stomatal responses

In order to function efficiently,  $\text{CO}_2$  uptake for photosynthesis must be balanced with water loss from the plant, to ensure sufficient substrate for photosynthesis, without compromise to plant water status. Under steady-state conditions, there is usually a strong correlation between  $g_s$  and  $A$  (Wong *et al.*, 1979); however, under dynamic conditions as experienced in the field, the slow stomatal response times to changing environmental conditions such as dynamic light patterns lead to a disconnect between  $g_s$  and  $A$  (Lawson and Vialet-Chabrand, 2018). The relatively slower change in stomata compared with changes in photosynthetic rate can lead to diffusion constraints limiting rates of carbon assimilation (Lawson and Blatt, 2014; McAusland *et al.*, 2016; Vialet-Chabrand, *et al.*, 2017; Lawson and Vialet-Chabrand, 2018). Therefore, the kinetics of  $g_s$  responses to changing light intensity have been identified as a potential novel trait

to increase photosynthesis (Long *et al.*, 2022) and plant water use efficiency (Lawson *et al.*, 2010; Lawson and Blatt, 2014; Matthews *et al.*, 2017). Previous work on natural variation for dynamic stomatal responses in wheat has been limited by the throughput, and therefore has been restricted to the evaluation of only a handful of genotypes. This is because such measurements usually rely on the use of IRGAs to measure stomatal responses during a step change in irradiance (e.g. McAusland *et al.*, 2016) which limits the throughput of this approach. Here, we used a novel thermal screening method to examine stomatal kinetics in ca. 200 wheat genotypes. Leaf temperature is ultimately determined by stomatal conductance (as well as boundary layer and other physical features), and therefore thermography can be used to determine differences in  $g_s$  (Jones *et al.*, 2009) and has been successfully employed as a screen for identifying stomatal mutants (Wang *et al.*, 2004). It has also been proven to provide a rapid screen for  $g_s$  kinetics in response to changing irradiance (Violet-Chabrand and Lawson, 2019, 2020; Kimura *et al.*, 2020; Yamori *et al.*, 2020), although this requires a range of references for energy balance calculations (see Violet-Chabrand and Lawson, 2019).

In order to use thermography to evaluate changes in  $g_s$  between different plant specimens and/or under changing conditions, temperature references (e.g. wet and dry reference) need to be included and maintained throughout the imaging process (see Violet-Chabrand and Lawson, 2019, 2020). Additionally, knowledge of the boundary layer conductance and considerable computation power are required. Here we used a simplified approach that employed only the dry reference (Leinonen *et al.*, 2006) along with a controlled-environmental chamber (similar to the one described by McAusland *et al.*, 2013 but modified for use in grasses) that controlled and maintained conditions around the leaves for image capture. The use of the chamber creating a constant boundary layer (see Supplementary Dataset S1 for the calculation of boundary layer) simplified the calculation of  $g_s$  from thermal signatures. To validate the use of thermal images to determine the rapidity of  $g_s$  responses ( $K_i$ ) to a step increase in light (Fig. 4), we demonstrated a strong and significant positive correlation between  $K_i$  using both methods, and no significant differences between individual genotypes were observed. However, a greater variation in  $K_i$  from IRGA measurements was evident, suggesting that  $K_i$  from thermography may if anything underestimate  $K_i$ . This can most likely be explained by the fact that thermal images take account of the spatial and temporal variation across the leaf surfaces that is not required with the smaller chambers of IRGAs, and therefore possibly provide a more realistic representation of whole-leaf responses.

As with the photosynthetic parameters, we demonstrated significant variation in both steady-state  $g_s$  at  $100 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD and the speed of  $g_s$  responses ( $K_i$ ).

In general, natural variation was previously detected in these traits, and this intraspecific variation was associated with stomatal anatomy in both wheat and barley (Faralli *et al.*, 2019a;

Stevens *et al.*, 2021) in a few genotypes. Although hypothesized and observed in studies focusing on interspecific diversity (e.g. Drake *et al.*, 2013), in the present work no link was found between stomatal anatomy and rapidity. Several anatomical, structural, and biochemical factors may affect stomatal rapidity to changing light intensity, and our data suggest that processes related to signalling, osmoregulation, or solute transport play a greater role than anatomical features alone (Lawson and Violet-Chabrand, 2018) in the variation observed. Significant associations were also observed between evaporative cooling and the magnitude of stomatal opening ( $\delta g_s$ ), suggesting, not surprisingly, that the degree of stomatal opening is a key component for maintaining optimal leaf temperature under dynamic irradiance. Indeed, previous studies have associated steady-state maximum stomatal conductance with yield and canopy temperature depression under water-limiting and high temperature conditions (Fischer and Stockman, 1986), suggesting that both stomatal dynamics and steady-state  $g_s$  represent a key target for maintaining optimal leaf temperature for photosynthesis. The fact that  $K_i$  and  $\delta g_s$  were correlated with  $g_{s\text{min}}$  suggests that maintaining high basal  $g_s$  under low light could represent an important trait that primes a faster stomatal responsiveness to changes in light intensity. In the genetic material studied here,  $g_{s\text{min}}$  and  $g_{s\text{max}}$  were positively associated, and this may indicate that potential combinations of preferable traits (e.g. inherently high  $g_{s\text{max}}$ , low  $K_i$ , and high evaporative cooling) are present in wheat and that this can be exploited for additional fine-tuning of gas exchange dynamics under fluctuating conditions. Wheat is amphistomatous and atypical, having greater stomatal numbers on the adaxial surface (Fig. 6), although this is often correlated with the abaxial density, as shown here, which suggests a common signal that determines cell differentiation between the two surfaces. Furthermore, previous studies have shown that  $g_s$  is generally higher on the adaxial surface, which could have implications for gaseous diffusion and evaporative cooling for maintaining photosynthesis (Wall *et al.*, 2022). This also highlights the potential of understanding the genetic targets that control stomatal development on the two surfaces to exploit for plants with enhanced diffusional capacity and/or cooling capacity and produce idiotypes for specific environments.

Interestingly, desirable stomatal traits were not necessarily observed in the same varieties as the desirable photosynthetic traits, suggesting that the presence of some traits may be at the expense of others. For example, the parental line Hereward had one of the fastest stomatal kinetic responses, but also one of the lowest  $A_{\text{sat}}$  and  $V_{\text{cmax}}$  values, whilst varieties such as Xi19 which had one of the highest photosynthetic capacities had one of the lowest  $K_i$  values. The rapid  $g_s$  responses in Hereward did not, however, result in a high  $\delta T$ , illustrating the importance of measuring actual  $g_s$  values and not just rapidity alone (Lawson and Blatt, 2014). However, as expected,  $g_s$  could explain some of the photosynthetic responses observed. The parental line Robigus had low  $g_{s\text{max}}$  values, which most probably

provides an explanation for the low  $A_{\text{sat}}$  observed, despite  $A_{\text{max}}$  being somewhere in the middle of the range. The low  $g_s$  values most probably created a diffusional constraint preventing high  $A_{\text{sat}}$  values from being achieved, which was overcome with the high  $\text{CO}_2$  for the  $A_{\text{max}}$  measurements.

The dual imaging system used here for the kinetic responses incorporated chlorophyll fluorescence imaging of photosynthetic efficiency ( $F_q'/F_m'$ ) (Fig. 5) alongside thermography. Cultivar differences in  $F_q'/F_m'$  were apparent at the lower light intensities, reflecting the variation observed using IRGA measurements; however no significant differences at the high light intensity were found. This is most probably explained by the fact that firstly,  $F_q'/F_m'$  decreases with illumination and therefore the values are greatly reduced, hence limiting the potential variation range; and secondly in  $C_3$  species, that the end-products of electron transport (ATP and NADPH) can also be utilized by sinks other than  $\text{CO}_2$  assimilation (such as photorespiration) (Baker, 2008). For this reason, the measurements of  $F_q'/F_m'$  are unable to distinguish differences in  $A$  that can be achieved through gas exchange, including those as a result of  $g_s$  diffusional constraints.

## Conclusion

This is the first study providing evidence of wide variation for steady-state and dynamic gas exchange traits in a bread wheat MAGIC population. This variation was detected using both standard eco-physiological approaches (IRGA) and novel methods (thermal imaging), allowing high throughput for stomatal dynamic phenotyping. Since natural variation in photosynthetic traits was identified in the wheat genotypes investigated, further work should focus on detecting the genetic loci controlling the traits employing larger numbers of RILs. Similarly, variation in dynamic stomatal responses was observed for the first time in the population as well as for  $g_{\text{smax}}$ ,  $\Delta g_s$ , and evaporative cooling using this novel high throughput method. This provides evidence of variability in bread wheat for dynamic  $g_s$  traits potentially providing unexploited targets for incorporation into ongoing breeding programmes. The strong relationships between our measured traits also provide proof of concept that taking the simpler measurement of  $A_{\text{sat}}$  and/or  $A_{\text{max}}$  could serve as a proxy for the more complex and time-consuming biochemical measurements of photosynthetic potential  $V_{\text{cmax}}$  and  $J_{\text{max}}$ . This is the first phenotyping study illustrating evidence of wide phenotypic variation in a wheat experimental population for several key traits important in yield determination, thus stressing the possibility to further exploit this variation for detecting the genetic control of stomatal and photosynthetic characters for crop improvement.

## Supplementary data

The following supplementary data are available at [JXB online](#).

Fig. S1. Visualization of the pipeline for plant growth including vernalization and subsequent greenhouse growth

Dataset S1. Spreadsheet for calculating boundary layer conductance.

Dataset S2. Spreadsheet for calculating  $g_s$  from thermal measurements.

## Acknowledgements

We would like to thank Dr Phil Davey for technical assistance.

## Author contributions

MF, TL, JC KAC, and EO: design; MF: performing all physiology experiments and data acquisition; MF, SVC, and GF: data analyses; SW: stomatal data collection; MF and TL: writing; all authors commented on the manuscript.

## Conflict of interest

The authors have no conflicts to declare.

## Funding

MF was supported via a BBSRC IPA funding with BASF (grant award to TL BB/N016831/1 and JC BB/N01698X/1). SW was funded by a (BBSRC) industrial studentship (1775930). BBSRC funding (BB/P027970/1 and BB/S005080/1) is also acknowledged.

## Data availability

Raw data can be accessed from the Dryad Digital Repository (Faralli *et al.*, 2024) (<https://doi.org/10.5061/dryad.79cnp5j4d>).

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