Variation in key leaf photosynthetic traits across wheat wild relatives is accession dependent not species dependent

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Introduction

Modern hexaploid bread wheat cultivars are the product of a genetic bottleneck – a reduction in genetic diversity brought about by domestication through polyploidization and the intensive selection for agronomically important traits over the past 10 000 yr (Charmet, 2011; Faris, 2014). However, despite breeding efforts in recent years, increases in global yields have slowed – averaging between 0 and 1.1% annually (Dixon et al., 2009). Potential yield gains have been circumvented by increasingly unpredictable environmental conditions, susceptibility to biotic stresses, and agronomic practices (Brisson et al., 2010). During the processes of domestication and selection, modern wheat may have lost key alleles required for adaptive robustness to abiotic and biotic stress – a negative side effect resulting from single trait selection. With pressure to raise yields between 1.6 and 2.4% per annum over the next 50 yr (Brisson et al., 2010; Ray et al., 2013), the emphasis falls to increasing the genetic diversity of modern wheat to maintain or improve yields under current environmental conditions (Evans & Lawson, 2020).

The wild, uncultivated relatives of modern wheat provide a global and mostly underutilized source of genetic and phenotypic diversity (King et al., 2017), with over 80 000 wheat accessions documented (Crop Wild Relative Diversity, 2019). The wild relatives represent adaptation to diverse habitats and climates, suggesting the existence of genes that are unavailable in the existing elite wheat germplasm. Exploration of the wild relatives may uncover previously untapped potential for wider and enhanced characteristics to face changing environmental conditions and disease resistance, ultimately aiming to improve the productivity and resilience of future modern varieties.

Wide crossing or hybridization events can be used as a means to generate plants with introgressions – fragments of wild relative DNA inserted into the genome of modern wheat. This approach can be used to markedly improve wheat genetic diversity (Chen et al., 2012; Molnár-Láng et al., 2014). Successful introduction has been noted in numerous species, including, but not limited to, Aegilops umbellulata (Sears, 1955, 1972), Secale cereale (Sebesta & Wood, 1978), Aegilops ventricosa (Doussinault et al., 1983; Burt & Nicholson, 2011), Aegilops speltoides (King et al., 2018), Triticum urartu (Grewal et al., 2018a), and Thinopyrum bessarabicum (King et al., 1997; Grewal et al., 2018b) (Supporting Information Table S1).

Despite the high probability of discovering novel traits and genes, the current number of species and accessions already investigated as potential candidates for crop improvement is relatively

Summary

- The wild relatives of modern wheat represent an underutilized source of genetic and phenotypic diversity and are of interest in breeding owing to their wide adaptation to diverse environments. Leaf photosynthetic traits underpin the rate of production of biomass and yield and have not been systematically explored in the wheat relatives.
- This paper identifies and quantifies the phenotypic variation in photosynthetic, stomatal, and morphological traits in up to 88 wheat wild relatives across five genera. Both steady-state measurements and dynamic responses to step changes in light intensity are assessed.
- A 2.3-fold variation for flag leaf light and CO₂-saturated rates of photosynthesis Amax was observed. Many accessions showing higher and more variable Amax, maximum rates of carboxylation, electron transport, and Rubisco activity when compared with modern genotypes. Variation in dynamic traits was also significant; with distinct genus-specific trends in rates of induction of nonphotochemical quenching and rate of stomatal opening.
- We conclude that utilization of wild relatives for improvement of photosynthesis is supported by the existence of a high degree of natural variation in key traits and should consider not only genus-level properties but variation between individual accessions.

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few. Though the majority of studies have generated lines that are resistant to biotic or abiotic stresses, a smaller proportion have reported the transfer of more complex polygenic traits, including yield stability, yield gains (Villarel et al., 1996), and increases in photosynthetic rates (Austin et al., 1982). Photosynthesis is a complex polygenic trait that fundamentally underpins the rate of production of biomass, and ultimately of yield. The close relationship demonstrated between enhanced photosynthetic CO₂ assimilation, biomass, and yield under elevated, ambient CO₂ supports the assumption that enhancing the capacity of individual leaves to fix carbon (C) will support higher yields though increased biomass (Long et al., 2006; Zhu et al., 2008, 2010, 2018; Murchie et al., 2009).

The relationship between photosynthesis and biomass accumulation is a ubiquitous and cumulative process involving numerous, interconnected processes. For example, at the canopy level, canopy architecture can drastically change both the amount of intercepted light and the efficiency with which it is converted into biomass and yield (Murchie et al., 2018; Wu et al., 2019). At the leaf level, under optimal environmental conditions, strong positive correlations are observed between stomatal conductance, carboxylation capacity, and the rate of leaf CO₂ uptake (Wong et al., 1979). Under saturating light, the Rubisco carboxylation efficiency and capacity are vital (Carmo-Silva et al., 2015). Under fluctuating environmental conditions, asynchronies arise between the changes in light intensity and the limitations imposed by the components of Rubisco, stomatal conductance (gₛ), and photoprotection (Lawson & Blatt, 2014; Kromdijk et al., 2016; McAusland et al., 2016; Taylor & Long, 2017; Lawson & Viallet-Chabrand, 2019; Murchie & Ruban, 2019), leading to restricted CO₂ assimilation and lowered water-use efficiency. Identifying species-specific rapid responses of stomata, photoprotection, and Rubisco is crucial in maximizing CO₂ uptake and minimizing water loss in response to a fluctuating field environment (Faralli et al., 2019b). In addition, the anatomy of the leaf also has a large impact on the diffusion pathway of CO₂ from traits such as stomatal density and distribution (Pearson et al., 1995; Faralli et al., 2019b), the size of the intracellular spaces (Lundgren et al., 2019), and the distance between veins (Richards, 2000; Terashima et al., 2010; Reynolds et al., 2012; Burgess et al., 2015; Driever et al., 2017).

The convergence of these traits provides a plethora of targets to improve photosynthesis, but natural genetic variation that can be used for breeding is unclear, especially in wheat (Driever et al., 2014; Salter et al., 2019). In some cases the underlying genes are known, along with the mechanism likely to underlie the improvement, and these currently rely on genetic modification to achieve both the proof of concept and the putative improved variety (e.g. Kromdijk et al., 2016). Clearly, the ability to combine discrete improvements will offer the greatest possibilities for yield improvement, and recent work has highlighted the need to improve photosynthesis in dynamic environments and across different climatic conditions (Tanaka et al., 2019; Wu et al., 2019; Faralli et al., 2019a,b).

As wild relatives are increasingly used to introduce genetic diversity into modern cultivars it is key to rapidly identify potential candidates with higher photosynthetic rates and efficiencies, to determine the basis for these improvements, and to link the physiological and/or anatomical traits with gene discovery. These steps are not linear, and one process may inform the discovery of another. One of the limiting factors has been the inability to rapidly genotype large numbers of lines, but this has recently been overcome (King et al., 2017; Devi et al., 2019; Grewal et al., 2020). Another significant bottleneck has been the availability of sophisticated high-throughput photosynthesis phenotyping tools. Recent advances in phenotyping techniques and pipelines provide the means to investigate variation on a larger scale – from screening populations of thousands to assessing the mechanistic basis behind that variation (McAusland et al., 2015, 2019; Murchie et al., 2018; Silva-Perez et al., 2018; Araus et al., 2018).

Here, we undertake a large-scale analysis of static and dynamic photosynthesis traits across accessions of wheat wild relatives. Our overall aim was to represent wide background adaptations, therefore we sourced these from a range of climates and continents across South America, western and eastern Europe, the Middle East, South and East Asia (Fig. 1). This work highlights for the first time the phenotypic and genomic diversity in the wild relatives as a source of variation for improving photosynthesis in modern wheat.

### Materials and Methods

Seed was obtained from the collection held by The Nottingham BBSRC Wheat Research Centre (University of Nottingham, Sutton Bonington, UK). A total of 88 accessions were investigated for variation in CO₂ and light-saturated photosynthesis, consisting of 41 species and five genera. A total of all plant material measured is presented in Table S2. Not all measurements were possible on all genotypes owing to the large variation in rates of growth and development. Accessions used were genotyped as a record that will be of use in future work to ensure that the same genotype was used across different/similar studies; these data are available at https://www.cerealsdb.uk.net/cerealgeneomics/CerealsDB/indexNEW.php. All sample sizes n stated in this paper are the number biological replicates.

### Assimilation rate–intercellular CO₂ analysis

Plants were grown and analysed at Sutton Bonington Campus, University of Nottingham. The wild relative accessions were germinated in compost (Levington M3; Everris, Ipswich, UK) and received 8 wk of vernalization at 5°C and a 16 h : 8 h, day : night cycle. The three modern *Triticum* cultivars received 4 wk of vernalization. After vernalization, all plants were moved to a glasshouse, potted into soil (John Innes No. 2; J. Arthur Bowers, Westland, Huntingdon, UK) and drip irrigated twice per day for 1 min with Hoagland’s solution. The wild relatives were grown and measured between September 2016 and August 2018. Owing to differences in the rate of development of the wild
deficit were maintained at 25 °C at an ambient relative humidity of 20%
and 2% oxygen (O2) was used to saturate the chamber to ensure consistent CO2 and O2 concentrations around the samples. The protocol consisted of three consecutive light steps of 15 min: 500, 100, and 1000 µmol m⁻² s⁻¹ PPFD (the latter is the capacity for the device). Saturating pulses were taken every minute throughout the protocol. The values of Fv/Fm and the responses of Fv'/Fm' (maximum efficiency of photosystem II (PSII) in the light), Fv'/Fm'' (operating efficiency of PSII in the light), photochemical quenching (qP), and nonphotochemical quenching (NPQ) were extracted from each protocol (for an in-depth description of these parameters see Maxwell & Johnson, 2000; Baker, 2008; Murchie & Lawson, 2013).

To determine the rate of NPQ relaxation (under 100 µmol m⁻² s⁻¹ PPFD) and induction (1000 µmol m⁻² s⁻¹ PPFD), data

Chl fluorescence imaging

Chl fluorescence imaging was performed on a subset of 25 wild relatives that represented good diversity in A–C1 responses and on three modern genotypes using a customized FluorCam imaging pulse-amplitude modulated fluorometer (Photon Systems Instruments, Brno, Czech Republic), as in McAusland et al. (2019). The same individual plants were used as those for the A–C1 analysis. Shutter time and sensitivity of the charge-coupled device were adjusted in accordance with the sample. The FluorCam was located in a temperature-controlled dark room maintained at 20 ± 2°C. Flag leaves were excised at 09:00 h and allowed to dark-adapt for 1 h in a custom imaging chamber. Fifteen minutes before the initial saturating pulse to determine Fv/Fm was taken, a pre-mixed gas of 400 µmol mol⁻¹ CO2 and 2% oxygen (O2) was used to saturate the chamber to ensure consistent CO2 and O2 concentrations around the samples. The protocol consisted of three consecutive light steps of 15 min: 500, 100, and 1000 µmol m⁻² s⁻¹ PPFD (the latter is the capacity for the device). Saturating pulses were taken every minute throughout the protocol. The values of Fv/Fm and the responses of Fv'/Fm' (maximum efficiency of photosystem II (PSII) in the light), Fv'/Fm'' (operating efficiency of PSII in the light), photochemical quenching (qP), and nonphotochemical quenching (NPQ) were extracted from each protocol (for an in-depth description of these parameters see Maxwell & Johnson, 2000; Baker, 2008; Murchie & Lawson, 2013).

To determine the rate of NPQ relaxation (under 100 µmol m⁻² s⁻¹ PPFD) and induction (1000 µmol m⁻² s⁻¹ PPFD), data
were fitted using a three-factor exponential function (Eqn 1) using the curve-fitting toolbox in MATLAB (R2018a; The MathWorks Inc., Natick, MA, USA):

\[ y = ae^{(-bx)} + c \]  
Eqn 1

where \( a \) determines the initial value, \( b \) is a constant representing the rate of exponential decay or growth and \( c \) is a constant describing the vertical shift in NPQ from start to end of the step.

\[ y = ae^{-bx} \]  
Eqn 2

(\( a \), initial value; \( b \), a constant representing the rate of exponential decay or growth). To determine the time \( t \) taken to achieve either 50% of the maximum NPQ values \( I_{50} \) or 50% of the maximum NPQ values \( R_{50} \), the equations were solved for \( b \) and the following calculations applied:

\[ t = \frac{1}{b} \]

\[ I_{50} \text{ or } R_{50} = t \log_{e}(2) \]  
(t, time constant; \( b \), obtained from the rearrangement of either Eqn 1 or Eqn 2).

Leaf properties

Flag leaf adaxial absorbance for the leaves used in the Chl fluorescence screen (25 wild relative accessions and three modern genotypes) was measured using an integrating sphere (LI1800-12; Li-Cor) and spectroradiometer (ASD HandHeld 2; Hand-held VNIR; Malvern Panalytical, Boulder, CO, USA). Multiple flag leaves from the same plant were aligned to cover the measurement window if a single flag leaf was < 2 cm². Absorbance was calculated in MATLAB (R2018a). Specific leaf area (SLA, m² kg⁻¹) was estimated following the protocol of Cornelissen et al. (2003); in brief, after photographing and measuring the fresh weight of the flag leaf, the leaves were placed in an oven at 70°C for 72 h. The leaves were reweighed to determine dry weight and the area calculated to produce SLA (IMAGE; Rashband, 1997–2018). Ear number per plant was also measured within this subset of plants.

Rubisco total activity and in vitro maximum carboxylation activity

Rubisco total activity was determined in flag leaves of glasshouse-grown plants (Sutton Bonington, University of Nottingham) for three modern Triticum cultivars (Triticum aestivum) and 19 wild relatives, from the same individual plants grown for \( A_{\text{C}} \) analysis in 2018 between the phenological Zadoks stages 4.2–5.5 (Zadoks et al., 1974). Leaf segments were snap-frozen in liquid nitrogen (N₂) and stored at −80°C. Rubisco was extracted as described by Carmo-Silva et al. (2017), and Rubisco total activity was measured by the incorporation of \(^{14}\)CO₂ into acid-stable products at 30°C (Party et al., 1997). The radioactivity was measured by liquid scintillation counting (Packard Tri-Carb; PerkinElmer, Waltham, MA, USA). Total soluble protein (TSP) was quantified by the Bradford assay (Bradford, 1976).

Rubisco was extracted from flag leaf tissue of three modern wheat cultivars and six wild relatives. The maximum \( \text{in vitro} \) carboxylation rate \( V_{\text{cmax}} \) of fully activated Rubisco was determined (Prins et al., 2016), incorporating the modifications described by Ort et al. (2016). Rubisco was quantified by the \(^{14}\)C-carboxyribulokinase bisphosphate binding method of Whitney et al. (1999).

Stomatal dynamics

Twenty-one accessions were grown in glasshouses at the University of Essex (Colchester, UK). Seedlings were vernalized as described earlier and potted into 650 cm³ pots containing peat-based compost (Levington F25). Solar radiation provided a PPFD of 500 μmol m⁻² s⁻¹, supplemented by sodium vapour lamps (600 W; Hortilux Schréder, Monster, the Netherlands) to 300 μmol m⁻² s⁻¹ PPFD when external PPFD dropped below 1200 μmol m⁻² s⁻¹ over a 10 h period. Air temperature was maintained at 25 ± 3°C during the day and 18 ± 3°C at night. The rapidity of the stomatal and photosynthetic responses was studied on fully expanded fourth leaves (growth stage Z1.4). Leaves were enclosed in a gas-exchange chamber (LCpro-SD; ADC BioScientific Ltd, Hoddesdon, UK) and left to equilibrate under dark conditions (< 30 min). Gas exchange was recorded every 1 min for 5 min under dark conditions, and light was set to 1200 μmol m⁻² s⁻¹ for another 1 h. Leaf temperature was set at 25°C, leaf vapour pressure deficit was maintained at c. 1.2 kPa, and [CO₂] was set at 400 ppm. In order to describe the temporal response of stomatal conductance to water vapour \( g_{s} \) to a single step-change in PPFD, an analytical model derived from the model by Vialet-Chabrand and co-workers (Vialet-Chabrand et al., 2013; McAusland et al., 2016) was used. In brief, this dynamic model predicts the temporal response of \( g_{s} \) to PPFD using an asymmetric sigmoid function parameterized by specific time constants to describe the opening response of stomata.

Statistical analyses

Statistical analyses were conducted in R (http://www.r-project.org/). A Shapiro–Wilk test was used to test for normality, and a Levene test of homogeneity was used to determine if samples had equal variance. Single factor differences were analysed using a one-way ANOVA with a Tukey–Kramer honest significant difference test where more than one group existed or using a Student’s \( t \)-test where only two groups were compared. For analysing more than two dependent variables, a MANOVA was used.

Results

Diversity of photosynthetic responses

\( A_{\text{C}} \) response curves were used to determine a 2.3-fold difference in light and CO₂-saturated photosynthetic assimilation \( A_{\text{max}} \)
between 88 accessions across five genera (Fig. 2). Overall, the modern varieties did not show a higher \( A_{\text{max}} \) than the wild relatives, and the variation was largely accession dependent, not species dependent. Of the 88 accessions, 11 wild relatives demonstrated significantly higher values of \( A_{\text{max}} (P < 0.05) \) than at least one of the three modern \( Triticum \) cultivars measured. Significant differences were observed between the individual accessions (\( P < 0.0001, F_{88,477} = 5.71 \)) and genera (\( P = 0.0016, F_{5,560} = 3.96 \); see Fig. S1a). \( Secale \) \( A_{\text{max}} \) values were significantly lower than all other wild relative genera (\( P < 0.03 \)), but not significantly different to the modern \( Triticum \). It is notable that \( A_{\text{max}} \) for many accessions exceeded that seen in elite lines (38.4–47.0 \( \mu \text{mol m}^{-2} \text{s}^{-1} \); Drier et al., 2014).

Accession and genus-specific significant differences in maximum rate of carboxylation \( V_{\text{cmax}} \) (\( P < 0.0001 \)) and electron transport \( J_{\text{max}} \) (\( P < 0.0001 \)) were also observed (Fig. 3). Contrary to expectations, modern wheat did not have a higher \( J_{\text{max}} \) or \( V_{\text{cmax}} \) than many of the wild relatives. \( Triticum dicoccon \) P95-98-3.2 (\#75) demonstrated the highest \( V_{\text{cmax}} \) (240.6 ± 26.3 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and \( J_{\text{max}} \) (313.5 ± 75.1 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), whereas the highest modern cultivar, \( T. aestivum \) ‘Paragon’ (#58), achieved 136.2 ± 25.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 198.6 ± 24.5 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), respectively. Again it is notable that \( J_{\text{max}} \) and \( V_{\text{cmax}} \) seen here for many accessions exceeded that seen in previously reported elite lines (233–280 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) and 124–161 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), respectively; Drier et al., 2014).

The \( Aegilops \) genus exhibited the greatest variation in \( V_{\text{cmax}} \) and \( J_{\text{max}} \) (5.8 and 4.4-fold, respectively; Fig. S1b,c), whereas the values of the modern \( Triticum \) cultivar were more conserved (2.7 and 2.4-fold, respectively). \( Secale \) accessions had significantly lower \( V_{\text{cmax}} \) and \( J_{\text{max}} \) (\( P < 0.05 \)) than the \( Aegilops \), \( Thinopyrum \), and wild and modern \( Triticum \) genera. Overall, the ratio \( V_{\text{cmax}} : J_{\text{max}} \) (Fig. 3) was significantly (\( P < 0.05 \)) lower in the modern \( Triticum \) cultivars than in \( Secale \), \( Thinopyrum \), \( Amblyopyrum \) and \( Aegilops \) genera, but not significantly different to the wild relative \( Triticum \) (\( P = 0.23 \)).

\( V_{\text{cmax}} : J_{\text{max}} \) has been suggested to indicate the limiting step of \( CO_2 \) assimilation, which is estimated by the transition point at which \( A \) shifts from being Rubisco to RuBP limited \( (G_{\text{transition}}) \). \( G_{\text{transition}} \) was significantly different between accessions (\( P < 0.0001 \)) and genera (\( P < 0.0001 \)) (Fig. S2a). Ranging between 197.73 and 455.36 \( \mu \text{mol mol}^{-1} \text{CO}_2 \), the modern \( Triticum \) genus demonstrated the lowest \( G_{\text{transition}} \) values (258 ± 65.16 \( \mu \text{mol mol}^{-1} \)), whereas members of the \( Secale \) (307.38 ± 117.74 \( \mu \text{mol mol}^{-1} \)) were the highest (Fig. S2b).

The amount of TSP and Rubisco total activity (\( RV_t \)) were used to quantify the investment of 19 accessions into leaf rubisco (Fig. 4). The ratio of \( RV_t \) to TSP was broadly genus specific, with modern \( Triticum \) showing considerably higher \( RV_t \) for similar values of TSP. This higher \( RV_t \) is likely to represent a greater investment of modern \( Triticum \) in Rubisco protein, since in vitro \( V_{\text{cmax}} \) was not significantly different between genera (Fig. S3).

Genus and accession-specific differences were determined for \( TSP (P < 0.02) \) and \( RV_t (P < 0.0001) \), although the modern \( Triticum \) cultivars were only found to have significantly higher \( TSP (P < 0.02) \) compared with the \( Amblyopyrum \) accessions. These modern cultivars had significantly higher \( RV_t (P < 0.003) \) when compared with the five genera studied. Cultivar \( T. aestivum \) ‘Paragon’ exhibited 3.2-fold greater \( RV_t \) than \( Aegilops muticum \) 2130004 did, which had the lowest activity of the accessions studied (31.9 ± 9.0 \( \mu \text{mol CO}_2 \text{m}^{-2} \text{s}^{-1} \)).

### Dynamic photosynthesis

The responses of 27 accessions in Chl fluorescence parameters to step changes in PPFD were analysed (Fig. S4; Table S3). This technique was utilized to uncover variation in dynamic photosynthetic traits; for example, in the speed of induction on transfer to high light or the kinetics of decay on transfer to low light (Fig. S4a). For a summary of the significant interactions for each light step, see Table S3. At steady state, the greatest number of significant differences were found under high light (1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{PPFD} \)). Maximum PSII efficiency in the light \( (F_v' / F_m') \) under 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \text{PPFD} \) was significantly higher in the \( Secale \) than in the wild relative \( Triticum \), \( Aegilops \) and \( Amblyopyrum \) genera (\( P < 0.05 \)), whereas no significant
differences were determined between the genera under 500 or 100 μmol m$^{-2}$ s$^{-1}$ PPFD. Similarly, no significant differences were determined under any light intensity between the modern Triticum cultivars and the five wild relative genera.

In general, accessions from the Amblyopyrum and Aegilops genera achieved the highest values of PSII operating efficiency ($F_{q0}/F_{m0}$; Fig. S4b) and electron transport rate (ETR) under 500 and 1000 μmol m$^{-2}$ s$^{-1}$ PPFD. These values were consistently, significantly higher ($P < 0.05$) than in the accessions from the wild and modern Triticum genera. Under low light (100 μmol m$^{-2}$ s$^{-1}$ PPFD), the Secale genus achieved significantly ($P < 0.05$) higher $F_{q0}/F_{m0}$ and ETR than the wild Triticum accessions did. By contrast, the wild and modern Triticum accessions achieved significantly higher values of NPQ under 500 and 1000 μmol m$^{-2}$ s$^{-1}$ PPFD intensities than the Secale and Amblyopyrum accessions did. The wild and modern Triticum also maintained the highest NPQ at 100 μmol m$^{-2}$ s$^{-1}$ PPFD when compared with Secale and Amblyopyrum. It was notable that those genera achieving the highest values of NPQ under 1000 μmol m$^{-2}$ s$^{-1}$ PPFD also demonstrated higher NPQ under low light but also obtained the greatest magnitude of change in NPQ from 100 to 1000 μmol m$^{-2}$ s$^{-1}$ PPFD (Fig. S5).

The kinetics of NPQ in response to changes in irradiance are indicative of the dynamic equilibrium between photoprotection and photochemistry in response to the fluctuating light environment experienced in the field. The analysis presented here indicates interesting and accession-dependent trends in the speed of NPQ induction and relaxation. For example, the time taken to induce 50% of the maximum NPQ under 1000 μmol m$^{-2}$ s$^{-1}$ PPFD ($I_{50}$; Fig. 5a) was consistently lower than the time taken to relax to 50% minimum NPQ under 100 μmol m$^{-2}$ s$^{-1}$ PPFD ($R_{50}$; Fig. 5b) for all accessions. Interestingly, there was no correlation observed between $I_{50}$ and $R_{50}$ ($R^2 = -0.06$, $P = 0.80$).

In general, although there were significant accession-specific differences in $I_{50}$ ($P < 0.0001$) and $R_{50}$ ($P < 0.0001$), the range of $I_{50}$ (1.9–7 s) was much narrower than that observed for $R_{50}$ (26–133 s). In general, the wild Triticum species took the least time to induce (2.13 ± 0.43 s), whereas the modern Triticum took the longest (5.34 ± 1.25 s). By contrast, the modern Triticum achieved the fastest $R_{50}$ time (79.32 ± 20.61 s),
whereas the Thinopyrum species took the longest to relax (111.02 ± 19.73 s). We conclude that modern wheat has faster NPQ relaxation than the wild relatives do with a slower induction. Some genus outliers were noted; for example, Aegilops biuncialis 550945 and Triticum monococcum took respectively 2.7 and 1.5-fold longer to induce NPQ than the other Aegilops and Triticum species did. On average, Triticum timopheevii took 245 s to relax to 50% minimum NPQ, compared with the Triticum average of 70 s. Interestingly, those plants with a faster NPQ induction might have a higher capacity for photosynthesis: a significant negative relationship was observed between \( A_{\text{max}} \) and \( I_{50} \) (Fig. 6a; \( R^2 = -0.46, P < 0.03 \)), whereas no correlation was observed between \( A_{\text{max}} \) and \( R_{50} \) (Fig. 6b; \( R^2 = 0.01, P = 0.96 \)).

Stomatal dynamics

Stomatal responses are an order of magnitude slower than metabolic processes. To investigate variation in stomatal responses, 24 accessions were subjected to a step increase in PPFD from 100 to 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PPFD and a model used to determine both magnitude of opening and rapidity of response (Viala-Chabrard et al., 2013). Substantial variation in dynamics of stomata was observed among the wild relatives, with some faster than the modern wheat. At steady state, no significant correlation (\( R^2 = -0.05, P = 0.81 \)) was determined between \( A \) and \( g_s \), under 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PPFD (\( A_{1000} \) and \( g_{s1000} \), respectively; Fig. 7a). In addition, no correlation was observed between \( g_{s1000} \) and \( g_s \) under 100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PPFD (\( R^2 = 0.14 \)) or between \( A_{1000} \) and \( A_{\text{max}} \) (\( R^2 = 0.02, P = 0.93 \)).

A significant positive relationship (\( R^2 = 0.87, P < 0.0001 \)) was observed for the time taken to achieve 95% \( A_{1000} \) and \( g_{s1000} \) (Fig. 7b); however, all species took longer to achieve 95% \( g_{s1000} \) compared with 95% \( A_{1000} \). Those species that took the longest to achieve 95% \( A_{1000} \) also achieved higher \( A_{1000} \) values (\( R^2 = 0.41, P < 0.05 \)), with the modern Triticum accessions achieving the highest \( A_{1000} \) values (19.9 ± 2.1 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)), on average 2.8 ± 0.6 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) greater than the other five genera measured. The species achieving the highest mean \( A_{1000} \) for the lowest \( g_{s1000} \) and – hence the highest intrinsic water-use efficiency (\( W_i \), \( \mu \text{mol CO}_2 \text{mmol}^{-1} \text{H}_2\text{O m}^{-2} \text{s}^{-1} \) – was T. monococcum TM01 (0.044 \( \mu \text{mol CO}_2 \text{mmol}^{-1} \text{H}_2\text{O m}^{-2} \text{s}^{-1} \)), whereas the lowest \( W_i \) was found for Aegilops caudata 2090001 (0.022 \( \mu \text{mol CO}_2 \text{mmol}^{-1} \text{H}_2\text{O m}^{-2} \text{s}^{-1} \)).

On average, the stomata from Thinopyrum accessions were the fastest (6.1 ± 1.3 min) to achieve 95% \( A_{1000} \), whereas the Aegilops accessions were the slowest (8.6 ± 1.5 min). To achieve 95% \( g_{s1000} \), all species opened for an average of 7.0 min (±2.3 min) longer than was required to achieve 95% \( A_{1000} \). The longest time was found in S. cereale 428373, which achieved 95% \( g_{s1000} \) c. 4 min later than the other genera (11.0 min). There was no correlation between the maximum rate of \( g_s \) increase to a doubling in PPFD from 100 to 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) \( (S_{\text{max}}) \) and the time constant used to describe the time taken to achieve steady-state \( g_s \) (\( k \)) and \( A_{1000} \) (\( R^2 = -0.14, P = 0.51 \) and \( R^2 = 0.19, P = 0.37 \), respectively). To relate speed with magnitude of opening, \( S_{\text{max}} \) and \( k \) were compared with \( g_{s1000} \) whereas a positive correlation was observed between \( S_{\text{max}} \) and \( g_{s1000} \) (\( R^2 = 0.60, P = 0.01 \)), no correlation was observed between \( k \) and \( g_{s1000} \) (\( R^2 = -0.04, P = 0.85 \)).

Finally, when comparing across experiments, a significant positive correlation was observed between \( S_{\text{max}} \) and NPQ under 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) PPFD (NPQ\( _{1000} \), \( R^2 = 0.52, P = 0.03 \)) and between \( g_{s1000} \) and \( I_{50} \) (\( R^2 = 0.48, P = 0.05 \)). Interestingly, a positive relationship was determined between \( A_{1000} \) and \( I_{50} \) (\( R^2 = 0.65, P = 0.005 \)), whereas a negative correlation was seen between \( A_{1000} \) and NPQ\( _{1000} \) (\( R^2 = -0.50, P = 0.04 \)).

Whole plant and leaf characteristics

Leaf morphological analysis was carried out on a selection of the accessions (25 wild relative accessions and three modern genotypes; Figs S6–S9). Though we do not expect yield components to be of value in the undomesticated species, we note some interesting relationships relevant to the theme of this paper. There was a genus-specific negative correlation between SLA and \( A_{\text{max}} \) (Fig. S6), suggesting a functional trade-off between leaf thickness, density of photosynthetic components, and photosynthetic capacity. We also noted negative correlations between SLA (Fig. S7a), leaf absorbance (Fig. S8, \( R^2 = -0.50, P = 0.03 \)), and total Rubisco activity (\( R^2 = -0.49, P = 0.04 \)). A positive correlation was also observed between SLA and ear number (Fig. S9; \( R^2 = 0.70, P = 0.0001 \)).

The Aegilops accessions were found to have the highest SLA (Fig. S7a; \( P < 0.003 \)), whereas Thinopyrum had the lowest (\( P <
Fig. 5 Time taken to (a) induce ($I_{50}$) and (b) relax ($R_{50}$) nonphotochemical quenching to 50% of maximum under 1000 μmol m$^{-2}$ s$^{-1}$ photosynthetic photon flux density (PPFD) and 100 μmol m$^{-2}$ s$^{-1}$ PPFD, respectively, in five genera. Outlying species are circled and annotated. The data are the means ($n = 3$–17 biological replicates). Boxplots show the median (horizontal line) and the quartiles (boxes). The whiskers represent 1.5-times the interquartile range above and below the 75th and 25th percentiles, respectively, with extreme values indicated as dots.

Fig. 6 The relationship between CO$_2$ and light-saturated rates of photosynthetic assimilation $A_{\text{max}}$ and (a) the time taken to achieve 50% maximum nonphotochemical quenching (NPQ) under 1000 μmol m$^{-2}$ s$^{-1}$ photosynthetic photon flux density (PPFD) and (b) the time taken to achieve 50% minimum NPQ under 100 μmol m$^{-2}$ s$^{-1}$ PPFD. Data are the mean for each species ($n = 3$–18), with genus indicated by shape: modern Triticum (black squares), Aegilops (black circles), Amblyopyrum (black triangles), Secale (black plus symbols), Triticum (black asterisks), and Thinopyrum (black enclosed crosses). The modern cultivar, Triticum aestivum ‘Paragon’, is highlighted (red squares). The larger the data point the greater the value of NPQ achieved under 1000 μmol m$^{-2}$ s$^{-1}$ PPFD. A linear regression is fitted to all data, with the shading indicating a 95% confidence interval on the fitted values. A Pearson’s correlation coefficient is shown in the bottom right corner of each plot.
0.03). With the exception of *T. timopheevii* P9599.11 (34.3 ± 6.4%), the wild *Triticum* accessions were found to have the highest percentage DW (Fig. S7b; 77.0 ± 14.0%), whereas modern *Triticum* and *Secale* exhibited the lowest (27.1 ± 1.4% and 30.5 ± 1.4%, respectively). Unsurprisingly, modern *Triticum* flag leaves absorbed the highest percentage of light (86.3 ± 1.9%; Fig. S8)—significantly (*P < 0.05*) greater than wild *Triticum* (83.2 ± 1.9%), *Aegilops* (81.1 ± 3.0%), and *Amblyopyrum* (80.7 ± 2.3%) genera. When illuminated under 2000 μmol m⁻² s⁻¹ PPFD, this variation in flag leaf absorbance accounted for between 280 and 400 μmol m⁻² s⁻¹ PPFD not utilized for C gain.

**Discussion**

Physiological and genotypic exploration of variation associated with the improvement of photosynthesis is crucial for the successful introduction and identification of interrelated traits that improve biomass acquisition in staple crops such as wheat and rice. Using wild relatives to introduce new genetic diversity into elite cultivars has gathered momentum in recent years (King *et al.*, 2017; Prohens *et al.*, 2017), generating a need for understanding the breadth of physiological variation available to breeding programmes, particularly traits that promote greater C acquisition. Here, we address this for the first time and encompass a wide selection of notable wild relatives, originating from >14 countries, covering 35 polyploid genomes, six genera, and 37 genetically distinct species (Fig. 1; Table S2). We have provided a database of photosynthetic traits, shown substantial variation for key photosynthetic traits, and discovered novel patterns among the wild relatives that partly explain the underlying causes of the differences observed.

Our results suggest that highly significant variation exists between the six genera, both for the capacity to fix CO₂ under saturating conditions (Figs 2–4) and for temporal processes that facilitate the achievement of high rates of CO₂ uptake under fluctuating conditions, such as those found in the field. High flag-leaf photosynthesis can be correlated to grain yield in modern wheat cultivars (Fischer *et al.*, 1998; Gaju *et al.*, 2016; Carmo-Silva *et al.*, 2017). This is often linked to Rubisco activity. Curiously, though Rubisco total activity was found to be highest in the modern cultivars, the high *Aₘₐₓ* values observed in the wild relative genera were underpinned by higher and wider ranges of maximum carboxylation *V_{cₘₐₓ}* and electron transport *I_{ₘₐₓ}*. In addition, the wild relative genera exhibited greater *I_{ₘₐₓ} : V_{cₘₐₓ}* ratios, driven by higher values of *I_{ₘₐₓ}* and hence the electron-transport-mediated rate of RuBP regeneration (Fig. 3). Whereas some accessions (such as *Aegilops juvenalis* 574463 (#1), *Thinopyrum ponticum* 547312 (#70) and *T. dicoccoides* P95983.2 (#76)) utilized this relationship for increased rates of C uptake, some accessions (e.g. *Aegilops biuncialis* 550940 (#29)) still achieved high *I_{ₘₐₓ} : V_{cₘₐₓ}* ratios without the accompanying increase in *Aₘₐₓ*. Therefore, there is broad lack of tightness between carboxylation...
capacity and electron transport capacity across the material analysed here. However, $J_{\text{max}}$ values estimated from $A$ vs $C_i$ analyses may not always accurately represent maximum electron transport rate (Buckley & Diaz-Espejo, 2015). Calculation of $C_{\text{transition}}$ indicates the likely point for limitation of CO$_2$ assimilation (i.e. RuBP carboxylation or regeneration) and also indicates $N$ partitioning among photosynthetic components (Yamori et al., 2011). Greater leaf $N$ content may decrease the $V_{\text{cmax}} : J_{\text{max}}$ (Yamori et al., 2011); therefore, it is possible that some variation could be explained by differences in $N$ investment between electron transport and Rubisco. To support this, modern lines in the current study had the lowest $C_{\text{transition}}$ (Figs 3, S2b). Higher Rubisco activity in modern varieties is not a surprise since it would be expected that these genotypes would take up and accumulate a higher leaf $N$ content for ready mobilization from leaf to the grain during senescence (Havé et al., 2017).

We speculate that the higher $V_{\text{cmax}}$ in some wild relatives may be related to possession of thick, narrow leaves (a tendency in some lines), which is supported by the negative relationship between $A_{\text{max}}$ and SLA (Fig. S6). Quantifying variation in $V_{\text{cmax}}$ and $J_{\text{max}}$ is vital in modelling C exchange at different scales (Rogers et al., 2017; Bloomfield et al., 2019) and is mediated by the balance of leaf $N$ and phosphate, often evidenced by changes in SLA (Reich et al., 1997; Evans & Poorter, 2001). Though changes in SLA can alter the $N$ content per unit leaf area, they can also change the light absorbance of the leaf, with decreases in $J_{\text{max}}$ being somewhat negated by increases in absorbance (Evans & Poorter, 2001). The modern cultivars in this study had some of the lowest electron transport rates but also achieved some of the highest absorbance values, accompanied by low SLA (Figs S1, S7a, S8).

It should also be noted that increases in $J_{\text{max}}$ (such as those observed for the wild relatives) are not always directly associated with improvements in CO$_2$ uptake by the Calvin–Benson cycle. With high electron transport rates increasing the reducing power for other essential pathways in the leaf, such as chloroplastic conversion of nitrate to ammonium (Anderson & Done, 1978; Searles & Bloom, 2003), driving production of isoprene (Morfopoulos et al., 2013), and driving alternative electron sinks, such as the Mehler reaction, reflecting changes in the apportionment of photosynthetic proteins (Yamori et al., 2005). Changes in the $V_{\text{cmax}}$ to $J_{\text{max}}$ ratio may also reflect the resource allocation bias of the plant to maintain high photosynthetic rates to set down biomass and remain competitive (Bryant et al., 1998), with leaves at the top of the canopy limited by $V_{\text{cmax}}$ rather than $J_{\text{max}}$ at ambient CO$_2$ (Quebbeman & Ramirez, 2016).

Walker et al. (2014) modelled the instantaneous relationship between $J_{\text{max}}$ and $V_{\text{cmax}}$ under fluctuating light, hypothesizing that increases in $J_{\text{max}}$ compensated for Rubisco carboxylation limitations under high light, thus increasing photoprotection and buffering against photoinhibition (Walker et al., 2014). By contrast, the work presented here showed NPQ$_{1000}$ was highest in modern Triticum and wild Triticum genera (Fig. S5), despite the lower $J_{\text{max}}$ values. Interestingly, the modern cultivars also demonstrated some of the slowest rates of NPQ induction ($I_{50}$; Fig. 5a). This suggests that higher rates of electron transport supported faster induction in a handful of wild relatives but not greater magnitudes of photoprotection under high light conditions (Fig. S5).

Manipulating photoprotection is another route for increasing yield, and through identifying and manipulating the magnitude and response timings of NPQ this has led to improvements in crop productivity (Hubbart et al., 2012; Kromdijk et al., 2016). Interestingly, 18 of the 24 wild relative accessions responded faster than the modern cultivar ‘Paragon’ to a step increase in PPFD, which suggests there may be some room to improve NPQ induction and relaxation in this modern cultivar. The significant negative correlation between $A_{\text{max}}$ and $I_{50}$ is a strong example of screening for a temporal trait that can be linked with improvements in photosynthetic capacity. In rice, fast induction of NPQ was linked to an inhibition of the rise in CO$_2$ assimilation (Hubbart et al., 2012), consistent with this trend. Furthermore, these data suggest that $I_{50}$ could be utilized as a proxy for the more time-intensive measurements of $A_{\text{max}}$ allowing greater numbers of plants to be screened more rapidly for variation in photosynthetic capacity (McAusland et al., 2019).

Decreasing the relaxation time could be advantageous for leaves constantly under rapid high light fluctuations, allowing efficient induction of photosynthesis to maximally utilize available PPFD (Murchie & Niyogi, 2011; Murchie & Ruban, 2019). A much greater range of $R_{50}$ values was observed between the accessions than with the $I_{50}$ values. Though there was no correlation between $A_{\text{max}}$ and $R_{50}$, this does not mean that the rate of relaxation is not important in C acquisition. Instead, it has been shown to be important during fluctuations in PPFD, and, if reduced, it could increase C fixation 7–30% during a diurnal time course (Long et al., 1994; Werner et al., 2001; Zhu et al., 2004; Kromdijk et al., 2016). In general, the modern cultivars took the shortest time to relax NPQ, but there were examples of specific wild relative accessions that relaxed more rapidly (Fig. 5b).

Stomatal behaviour is another temporal trait that has been the focus of recent work on optimizing the balance between C gained and water lost (Lawson & Vlaet-Chabrand, 2019). $I_{50}$ was found to positively correlate with the magnitude of stomatal opening $g_{1000}$ and CO$_2$ assimilation rates achieved under 1000 µmol m$^{-2}$ s$^{-1}$ PPFD ($A_{1000}$), highlighting that greater opening not only allows higher $A_{1000}$ but also facilitates more rapid induction of NPQ under high light. These data suggest that, under high light, the plant mediates a fine balance between maintaining high rates of carbon fixation, with subsequent induction of photoprotection, and minimizing loss of water. Interestingly, there was no correlation between $g_{1000}$ and $A_{1000}$ suggesting that the variation observed in $g_{1000}$ could be manipulated to reduce water loss without restricting C gain – exemplified by the two-fold difference in $W_i$ values between the accessions measured (Fig. 7b).

Though large variation in the rate of stomatal opening has been observed between different species (McAusland et al., 2016) and between cultivars of a single species (Faralli et al., 2019a), the total time taken to achieve steady-state $g_{i}$ ($\delta$) has been shown not to correlate with maximum opening under high light (Vlaet-
Chabrand et al., 2013; McAusland et al., 2016). Maximum rate of opening \( S_{\text{max}} \) is mathematically dependent on the magnitude of change in \( g_s \) (\( k \)) and maximum opening, and therefore it is not unsurprising that those species that achieved greater \( g_{s,1000} \) also achieved the highest \( S_{\text{max}} \). However, the variation in maximum opening is somewhat determined by the stomatal density : size relationship (Franks & Beerling, 2009). These data point to anatomical variability between the wild relative accessions; and though densities in modern cultivars are known to have increased through breeding (Fischer et al., 1998), there may still be an optimal density for minimizing water loss evidenced by a wild relative genus or accession. Maintaining high water-use efficiency is a particularly relevant target for modern cultivars, which typically exhibit a higher leaf water content than wild relative species (Fig. S7b). In addition, efficient water management will directly contribute to improved tolerance to drought and heat stress, therefore maintaining yields under increasing unpredictable climatic conditions (Bertolino et al., 2019).

As with stomatal density, internal leaf anatomy will also contribute to the photosynthetic variation discovered in this study; with traits such as airspace volume (Lehmeier et al., 2017), mesophyll size (Austin et al., 1982), porosity and conductance (Lundgren et al., 2019), and distance to veins (Brodribb et al., 2007) playing a vital role in efficient C acquisition. Though no anatomical measurements are presented in this study, the significant variation in SLA (Fig. S7a) and absorbance (Fig. S8) suggests that extensive variation occurs at the cellular level.

It is important that both static and temporal responses are measured so that the capacity, dynamic adaptability, and the interrelated nature of the processes that support and maintain high rates of C acquisition are identified (Murchie et al., 2018; Lawson & Viala-Chabrand, 2019; Salter et al., 2019). Methodologies such as the Chl fluorescence screen described here (McAusland et al., 2019), and other powerful platforms (e.g. Viala-Chabrand & Lawson, 2019, 2020), offer rapid, high detail measurements that can be taken during the lifetime of the plant rather than at single phenological stages. Here, we cultivated plants in glasshouse conditions but note the importance of further studies in variable field-like conditions (Poorter et al., 2016). However, \( A_{\text{max}} \) values for ‘Paragon’ are similar to those observed by Driever et al. (2014), a field-based study. Complex emerging data sets may require development of functional models to predict optimal combinations in realistic field environments (e.g. Zhu et al., 2012; Wu et al., 2019).

Though only briefly mentioned here, the wild relatives of modern wheat also demonstrate a wealth of variation in agronomically important traits, such as disease (Table S1) and biotic resistance (Peleg et al., 2005), phenology (e.g. flowering time, perennial or annual), pollen fecundity, and root physiology (de Dorlodot et al., 2007; Atkinson, 2016). Quantifying and understanding the breadth of diversity available, tempered by the ease of introducing the material into modern wheat backgrounds, will not only enable efficient strategic crosses in breeding programmes designed to improve photosynthetic C gain but also provide traits to screen for in the subsequent backcross generations. In turn, this will speed up the identification of direct and related phenotypes associated with increased C assimilation per unit area, leading to greater biomass and improved yield.

Concluding remarks

The wild relatives of crop species represent a way of targeted introduction of beneficial traits to improve yield. Until now, photosynthetic variation in the wild relatives of wheat has not been explored. Here, we analyse relevant features of static and dynamic traits across a broad range of wild accessions and genera. The widest variation was found across individual accessions, suggesting local adaptation to be important when selecting crosses and that genotyping of individual accessions is important. We find key differences between wild relatives and modern lines for dynamic traits, notably that photoprotection relaxation is fast in modern lines but induction is slow. These results highlight fundamental variation in wild species and those that may have indirectly been selected for in breeding. The phenotypic and genotypic data presented are a first step to inform follow-on gene discovery and pre-breeding programmes.

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Author contributions

EHM, KJE, JK, IPK, ECS and TL planned and designed the research. SHE and KP contributed to the planning of the research. LM, SVC, AB, MJF and IJ performed experiments and analysed data. LM and EHM wrote the manuscript with input from the other authors.

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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** Light saturated rates of CO2 assimilation, carboxylation and electron transport in accessions from four wild relative genera and modern wheat cultivars.

**Fig. S2** The internal CO2 concentration (Ci) transition point, Ci_transition, for 88 accessions across six genus groups.

**Fig. S3** The maximum rate of *in vitro* carboxylation by Rubisco (V_cmax) determined for the modern *Triticum* varieties and four wild relative genera.

**Fig. S4** Mean response of PSII operating efficiency (Fv'/Fm') to step changes in PPFD in *T. aestivum* and six genus groups.

**Fig. S5** Steady-state non-photochemical quenching (NPQ) in five genus groups at 1000 μmol m−2 s−1 PPFD.

**Fig. S6** The relationship between A_max and specific leaf area (SLA) in five wild relative genera and modern wheat.

**Fig. S7** Variation in specific leaf area (SLA) and percentage dry matter for five wild relative genera and modern wheat.

**Fig. S8** Flag leaf absorbance of five genera in the wavelength region of 400–700 nm.

**Fig. S9** Number of ears per plant for five wild relative genera and modern wheat.

**Table S1** A summary of the wild relatives and traits successfully identified and/or introduced into modern wheat cultivar populations.

**Table S2** Parameters from A vs Ci response curves including light- and CO2 saturated photosynthetic rate (A_max), maximum carboxylation (V_cmax) and maximum electron transport (J_max) for 88 accessions across 5 genera.

**Table S3** Significant comparisons between five genera for four chlorophyll fluorescence parameters measured under three PPFD intensities.

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