Identification of QTLs for dynamic and steady state photosynthetic traits in a barley mapping population

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Highlight

Significant variation exists in the photosynthetic induction response after a switch from moderate to saturating light across a barley doubled haploid population. A QTL for rubisco activation rate was identified on chromosome 7H, as well as overlapping QTLs for steady state photosynthesis and stomatal conductance.

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Abstract

Enhancing the photosynthetic induction response to fluctuating light has been suggested as a key target for improvement in crop breeding programs, with the potential to substantially increase whole canopy carbon assimilation and contribute to crop yield potential. Rubisco activation may be the main physiological process that will allow us to achieve such a goal. In this study, we assessed the phenotype of Rubisco activation rate in a doubled haploid (DH) barley mapping population [131 lines from a Yerong/Franklin (Y/F) cross] after a switch from moderate to saturating light. Rates of Rubisco activation were found to be highly variable across the mapping population, with a median activation rate of 0.1 min\(^{-1}\) in the slowest genotype and 0.74 min\(^{-1}\) in the fastest genotype. A unique QTL for Rubisco activation rate was identified on chromosome 7H. This is the first report on the identification of a QTL for Rubisco activation rate in planta and the discovery opens the door to marker assisted breeding to improve whole canopy photosynthesis of barley. This also suggests that genetic factors other than the previously characterised RCA isoforms on chromosome 4H control Rubisco activity. Further strength is given to this finding as this QTL colocalised with QTLs identified for steady state photosynthesis and stomatal conductance. Several other distinct QTLs were identified for these steady state traits, with a common overlapping QTL on chromosome 2H, and distinct QTLs for photosynthesis and stomatal conductance identified on chromosomes 4H and 5H respectively. Future work should aim to validate these QTLs under field conditions so that they can be used to aid plant breeding efforts.

Keywords: barley, dynamic photosynthesis, genotyping, mapping, phenotyping, Rubisco activation, sunfleck.
Introduction

By 2050, the global population is expected to rise to 9 billion and to meet future food demand we will need to increase crop production worldwide by 70% (Paul et al., 2019). Recent progress has been hindered by stagnating rates of annual yield increase, therefore novel breeding targets to improve crop yield potential are urgently needed. Improving the photosynthetic efficiency of crop species has now been shown to boost plant growth under field conditions (Kromdijk et al., 2016; South et al., 2019). Whilst these studies used genetic transformation to achieve such gains, they have proven that significant photosynthetic gains are possible in the field and that these can contribute to plant growth and crop yield. It is now imperative that we identify natural variation in photosynthetic traits in diverse populations and harness this variation through marker-assisted plant breeding techniques (Furbank et al., 2020).

Improving photosynthetic efficiency in dynamic environments has recently been highlighted as a key target to increase whole canopy carbon assimilation (Murchie et al., 2018). The light environment of the lower canopy is subject to continuous and dynamic change across the course of a day, caused by movement of the sun across the sky, sporadic cloud cover and/or movement of upper elements in the canopy caused by wind (Slattery et al., 2018). These processes can cause a leaf in low/moderate light one moment to suddenly be exposed to saturating light conditions the next. Often these short periods of direct sunlit illumination (referred to herein as ‘sunflecks’) only last a short period of time, in the order of minutes, yet they can account for as much as 90% of the daily accumulated light of lower canopy leaves (Pearcy, 1990). Photosynthesis under these dynamic light conditions is highly inefficient. Specifically, the rates of stomatal opening and activation of Rubisco upon transition from low to high light significantly limit carbon assimilation. Interactive effects of several environmental factors on stomatal aperture are common in the field (Zeiger & Zhu, 1998; Talbott et al., 2003; Wang et al., 2008), meaning that stomata are operating in an integrated and hierarchical manner in response to multiple environmental stimuli (Lawson & Blatt, 2014). Stomatal responses to fluctuating light are therefore considered to be a much more challenging target for improvement than the biochemical limitations to photosynthesis (for a comprehensive review of limitations to dynamic photosynthesis see Kaiser et al., 2019).

Improving Rubisco activation rate could be the low hanging fruit that allows plant breeders to boost whole canopy photosynthesis with few associated costs, specifically in terms of water and nutrient
use. This is critical for a future where global environmental change is predicted to leave agricultural systems exposed to more frequent and more extreme drought and heat events. It has been estimated that we could increase daily carbon gain by as much as 21% in wheat if Rubisco activation was instantaneous (Taylor & Long, 2017). Variation in Rubisco activation kinetics has now been observed in crop species including soybean (Soleh et al., 2017), rice (Acevedo-Siaca et al., 2020) and wheat (Salter et al., 2019), and work with other species has indicated specific molecular targets and pathways that could accelerate Rubisco activation speed, with a particular focus on Rubisco’s catalytic chaperone Rubisco activase (Rca) (in Arabidopsis thaliana, Mott et al., 1997; in Nicotiana tabacum, Hammond et al., 1998; and in Oryza sativa, Yamori et al., 2012). In our recent work with wheat, we found that by increasing the rate of Rubisco activation of the slowest genotype included in our study to that of the fastest, daily carbon assimilation could be increased by 3.4% (Salter et al., 2019). However, in this work only ten genotypes of wheat were studied, the potential for improvement would likely be far more substantial if we were to investigate variation in this trait across a whole breeding population, and greater still if we were to investigate diversity within cultivars from diverse geographic locations or landraces.

Most recent studies of photosynthetic induction have tended to adopt the so-called ‘dynamic A/ci’ method (Taylor & Long, 2017; Salter et al., 2019), in which photosynthetic induction curves are measured at a number of different CO₂ concentrations, allowing for the reconstruction of A/ci curves throughout the induction response. Whilst this technique yields important fundamental data on the biochemical limitations during photosynthetic induction (most importantly Rubisco carboxylation capacity, V_{cmax}; and potential electron transport rate, J), it takes a very long time (> 6 hours per plant) and thus limits its use in large scale screenings for photosynthetic induction traits. Conversely, other techniques that yield less detailed information about the underlying physiology (such as that used by Soleh et al., 2016) take far less time (< 1 hour) and may be much more suitable for large-scale screenings of diverse plant material. The method of Soleh et al. (2016) involves measuring a single photosynthetic induction curve at a low intracellular CO₂ concentration (i.e. < 300 μmol mol), at which it can be assumed that photosynthetic biochemistry is limited by Rubisco rather than by electron transport. This allows for the reliable estimation of Rubisco activation rate, with data comparable to those obtained using the ‘dynamic A/ci’ method (Taylor & Long, 2017).
We hypothesize that variation in photosynthetic induction kinetics may be inadvertently confounding efforts to improve steady state properties of photosynthesis. Steady state measurement techniques [such as spot measurements of photosynthesis (A) and stomatal conductance (gs), CO₂ response curves and light response curves] all rely on the assumption that the leaf is fully acclimated to saturating light, and other environmental conditions inside the leaf chamber of the system, prior to measurement. Thus, these methods require a delay for the leaf to become equilibrated to the conditions inside the leaf chamber of the gas exchange system (referred to herein as the equilibration time). Although it is quite well established that adequate equilibration time is required for accuracy of steady state gas exchange measurements, an increasing demand for faster, higher throughput measurement techniques (Furbank & Tester, 2011) may make researchers complacent. Yet, few studies have quantitatively assessed the potential implications that may result from premature assumptions of steady state conditions, for instance, the identification of false quantitative trait loci (QTLs).

There is now compelling evidence that suggests whole canopy photosynthesis could be improved by harnessing natural variation in Rubisco activation rate that exists across genotypes of crop species. However, no study to date has investigated or performed trait dissection for Rubisco activation in a segregating mapping population. We sought to identify and characterise genetic variation in Rubisco activation rate across a barley (Hordeum vulgare L.) doubled haploid (DH) mapping population in planta using gas exchange techniques. We then used chromosome interval mapping to identify QTLs and closely associated molecular markers. We were also interested in assessing whether the strength of QTLs would be weakened for “steady-state” photosynthetic properties (A and gs) if equilibration times were not long enough for steady state conditions to be reached.

**Methods**

**Plant material and growth conditions**

A DH barley (H. vulgare L.) population was obtained from a cross between the Australian barley cultivars Yerong and Franklin (Y/F). This population contained 177 DH lines and was maintained at the Plant Breeding Institute at The University of Sydney. The Y/F mapping population has been extensively used for QTL mapping for both morphological (Xue et al. 2008) and physiological (Zhang et al. 2016) traits, as well as disease resistance (Singh et al. 2014; Dracatos et al. 2016). In this study 131 lines from this population were phenotypically assessed for steady state and dynamic photosynthetic traits. Due to the availability of seed and genotypic data, only 127 DH lines were
used for QTL analyses. A second DH barley population (from a cross between VB9104 and Dash) was also phenotyped for photosynthetic traits however due to the low number of lines with available genotypic data this population was not included in further analyses (phenotyping results are however presented in Figures S4 and S5).

Plants were grown in a controlled environment room for approximately five weeks prior to measurement and were measured at the advanced tillering stage. Day temperature was 25°C during a 14 h light period and night temperature was 17°C during a 10 h dark period. Relative humidity was maintained at 70% while daytime PPFD was approximately 600 μmol m$^{-2}$s$^{-1}$ at the top of the plants. Seeds were planted in potting mix enriched with slow-release fertilizer (Osmocote Exact, Scotts, Sydney, NSW, Australia). Six seeds per genotype were sown in 6 L pots and grown for three weeks before being thinned to three plants per pot. Seed was sown sequentially in time to make sure that all measurements were conducted at the same growth stage. Plants were watered daily to field capacity.

**Photosynthetic measurements**

Plants were moved from the controlled environment room to an adjacent temperature-controlled growth cabinet (TPG-2900-TH-3218; Thermoline Scientific, Wetherill Park, NSW, Australia) under the same environmental conditions [temperature 25°C; relative humidity 70%]. Care was taken to minimise the length of time that the plants were in transit between the controlled environment room and the growth cabinet however we note that this transition was not instantaneous. Two or three of the youngest fully expanded leaves of a single plant were sealed in a 2x6 cm leaf cuvette (6400-11; LI-COR, Lincoln, NE, USA) fitted to a LI-COR LI-6400XT gas exchange system to fill the cuvette without overlapping. This simulated a shift in light intensity from 600 μmol m$^{-2}$s$^{-1}$ to 1300 μmol m$^{-2}$s$^{-1}$. This moderate-to-high light transition allowed us to assess photosynthetic induction whilst overcoming complications often caused by low stomatal conductance of traditional dark-to-light transitions. Leaf chamber conditions were set to closely match those of the controlled environment room [leaf temperature 25°C; cuvette CO$_2$ [CO2S] 400 μmol mol$^{-1}$; relative humidity 70%], with the exception of PPFD which was set to 1300 μmol m$^{-2}$ s$^{-1}$ using a red-green-blue light source (6400-18A; LI-COR) set to 10% blue and 90% red light (set to replicate the commonly used settings of the standard 2 x 3 cm light source). Measurements of photosynthetic gas exchange rates ($A$ and $g_s$) were recorded once per minute immediately after the leaf was inserted into the chamber.
until photosynthesis had reached steady state. Logging was conducted at this frequency as the LI-COR system was also connected to a tuneable diode laser that required a low logging frequency, however if this study were to be repeated we would encourage logging more frequently. Preliminary photosynthetic light response curves were measured with plants grown under the same conditions to ensure that 1300 μmol m$^{-2}$ s$^{-1}$ was saturating and that 600 μmol m$^{-2}$ s$^{-1}$ was non-saturating (results shown in Figure S1).

Rubisco activation rate was calculated using a modified method of Woodrow and Mott (1989) and Soleh et al. (2016). Photosynthetic data was first normalised to an intercellular CO$_2$ concentration ($c_i$) of 300 μmol mol$^{-1}$, assuming that the relationship between $A$ and $c_i$ at any point in induction was described by a straight line through zero, using the following equation:

$$A^* = A \times \frac{300}{c_i}$$

where $A^*$ is the normalised photosynthetic rate, $A$ is the measured photosynthetic rate and $c_i$ is the measured intercellular CO$_2$ concentration. This effectively removed the influence of stomatal opening/closure for the induction phase. The choice to correct to a $c_i$ of 300 μmol mol$^{-1}$ followed that of Soleh et al. but was also found to be close to the steady state $c_i$ of our plants (as shown in Figure 2). We note that this choice did not affect subsequent calculation of the kinetics of Rubisco activation. The apparent Rubisco activation rate (1/$\tau$) was modelled from the plot of the logarithmic difference between $A^*$ and its maximum value after induction ($A^*_{\text{max}}$) against the time taken for induction (representative data shown in Figure 1). From this plot, the value of 1/$\tau$ was determined from the slope of the linear regression on data points in the range of 2 to 5 mins after induction, and points after this that maintained a regression with $R^2 > 0.9$. The first phase of induction (< 2 min after transition to saturating light) was not included in the regression as the slope is not related to Rubisco activation before this point, with other processes such as increases in stromal pH having a strong influence on this early induction phase (Woodrow and Mott, 1989). We refer to 1/$\tau$ as the apparent Rubisco activation rate here as we note that it could also be influenced by other photosynthetic processes, including dynamic responses of mesophyll conductance and non-photochemical quenching, however in the remainder of the text we refer to it simply as Rubisco activation rate.
Genetic analysis and QTL mapping

The genotypic data and genetic linkage map for the Yerong/Franklin DH population used for QTL analysis for Rubisco activity and steady state photosynthetic traits in the present study was previously described by Singh et al. (2015). In brief, the Y/F genetic map is comprised of 496 DarT and 28 microsatellite (SSR) markers spanning 1,127cM across all seven chromosomes, 1H to 7H (Wenzl et al., 2006).

A subset of 127 lines were selected for QTL mapping analysis. Markers were selected every 10 cM so that the whole genome was evenly covered. Composite interval mapping (CIM) methods were used in QTL Cartographer version 2.5 (North Carolina State University, Raleigh, NC, USA), carrying out 1,000 iterations permutation analysis with steps at 1 cM, and with a 0.05 confidence level for all traits.

Statistical analyses

All other modelling and statistical analyses were performed in R (R Core Team, 2019).

Results

Photosynthetic induction kinetics

While specific photosynthetic induction kinetics were found to vary across individual leaves and genotypes, general trends were quite clear (representative induction curves from one day of measurements is shown in Figure 2). Net photosynthesis ($A$) increased immediately after transition from to saturating light for all leaves. Stomatal responses were more variable than those of photosynthesis but there tended to be an initial reduction in $g_s$ after transition to saturating light and then a gradual rise towards steady state. By normalising photosynthesis to a constant $c_i$ of 300 ppm, we were able to obtain a measure of photosynthesis limited by Rubisco carboxylation unobstructed by variation in stomatal kinetics ($A^*$). $A^*$ showed a similar trend to $A$, increasing immediately after the switch from low to high light.
QTLs for Rubisco activation rate

Rubisco activation rates of the parental lines Yerong and Franklin were found to differ, with within-genotype medians of 0.38 min\(^{-1}\) and 0.74 min\(^{-1}\) respectively. Wide variation in 1/\(\tau\) was found across the population (Figure 3), with within-genotype medians ranging from 0.099 min\(^{-1}\) to 0.74 min\(^{-1}\). Interestingly, the parental line Franklin was found to have the fastest rate of Rubisco activation. A frequency distribution of 1/\(\tau\) was plotted for the population and was found to follow a normal distribution suggesting that Rubisco activation rate was under complex genetic control (Figure S2). CIM analysis revealed the presence of a distinct QTL for Rubisco activation rate (Figure 4; further details in Table 1). Q1/\(\tau\).sun-7H, was located at 41.67 cM on chromosome 7H (proximal to DarT marker bPb-9601) accounting for 10.48% of the phenotypic variance in this trait.

Steady state photosynthesis and equilibration time tests

Variation was also found in steady state photosynthetic rates across the population (Figure 5). Median rates of \(A\) and \(g_s\) were 17.45 μmol m\(^{-2}\) s\(^{-1}\) and 0.31 mmol m\(^{-2}\) s\(^{-1}\), respectively. From this phenotyping data, there was no correlation found between steady state \(A\) and 1/\(\tau\) (correlation coefficient = 0.0026; \(p > 0.05\); Figure 6).

As hypothesized, “steady-state” photosynthetic rates were substantially underestimated if measurements were recorded without sufficient equilibration time (Table 2). This was more pronounced the earlier the measurements were recorded after enclosing the leaf in the chamber of the IRGA. Mean values of \(A\) and \(g_s\) were both underestimated by 21% at five minutes compared to steady state. It should be noted that although some of the fastest genotypes reached steady state after five minutes, most of the lines did not. In fact, \(g_s\) was underestimated by 82% for one of the genotypes and \(A\) was underestimated by 54% for another if measurements were recorded after just five minutes.

To assess the importance of equilibration time for accurate identification of steady state QTLs, QTL mapping was first performed for steady state \(A\) and \(g_s\). Frequency distributions were plotted for both traits and they followed a normal distribution suggesting they are under complex genetic control (Figure S3). Several QTL were found for both \(A\) and \(g_s\) (Table 1). Trait co-location was
observed on chromosome 7H whereby the position of the Q1/τ.sun-7H QTL was almost identical to QTL for both A and gₗ. This suggests a region on the short arm of chromosome 7H either carries a single gene or more likely a cluster of genes responsible for the genetic control of photosynthesis, stomatal conductance and Rubisco activation. For steady state A and gₗ, additional overlapping and distinct QTL were identified. A common overlapping QTL for both A and gₗ was identified, peaking at 27.03 cM on chromosome 2H, whilst distinct QTL were identified on chromosomes 4H (41.67 cM) and 5H (53.39 cM) for A and gₗ, respectively.

QTL mapping was then performed with data collected at five, ten and fifteen minutes after the start of induction for comparison with detected steady state QTLs (coloured traces in Figure 7). Although most QTL were still identified with non-steady state data, the significance these QTL peaks were found to be weakened under non-steady state conditions. This was particularly evident for the gₗ QTL identified on chromosome 7H (Figure 7h), with the LOD score of this QTL dropping from 6.8 when using steady state data to 4.9, 3.9 and 3.3 when using data collected at 15 min, 10 min and 5 min after the start of induction, respectively.

Discussion

We have identified QTLs for \textit{in planta} Rubisco activation rate for the first time in any species. As in other crops, we found Rubisco activation rate to be a highly variable trait across genotypes of barley, aiding in the discovery of a significant QTL in our doubled haploid population. QTLs were also identified for steady state photosynthetic parameters, including co-localised QTLs for A, gₗ and 1/τ on chromosome 7H. The 7H QTL was distinct from the location of both Rubisco activase isoforms that map to the same region on chromosome 4H (Saghai Maroof et al. 1994), suggesting other genetic factors are also important for the regulation of Rubisco activation. The importance of adequate equilibration time in the measurement of steady state gas exchange was highlighted by comparing these results to those obtained using arbitrary non-steady state rates at 5, 10 and 15 min after the start of induction. The significance of QTLs was reduced if steady state conditions had not been reached.
Improving whole canopy photosynthesis

It is well established that improving photosynthesis has the potential to increase crop yield (for a review of recent progress see Simkin et al., 2019). However, until now research has invariably focussed on only the uppermost leaves of the canopy under optimal conditions (i.e. continuous saturating light, 25°C). This approach has its merits because these leaves have the most light available to them and their contribution to whole canopy photosynthesis reflects this (Osborne et al. 1998). Yet, for monocot cereal species such as wheat and barley there have been few studies that have shown flag leaf photosynthesis to correlate well with crop yield (Richards et al., 2000). Whole canopy photosynthesis, and more specifically the cumulative rate of photosynthesis over the growing season, has been shown through modelling studies to be a much more reliable determinant of crop yield (Wu et al., 2019). Accordingly, there has been a recent shift in research focus towards dynamic photosynthetic traits. This is important because whilst some studies have found weak relationships between steady state and dynamic photosynthetic traits (Salter et al., 2019) other studies have not found any relationship (Soleh et al, 2017; Acevedo-Siaca et al., 2020). Our results also showed no link between steady state A and 1/τ (Figure 6), although the co-localisation of QTLs on chromosome 7H suggests they both may be controlled by the action of a single gene or a cluster of closely linked genes at the same chromosomal location.

Significant improvements in photosynthesis and resultant increases in plant growth have now been achieved under field conditions through genetic modification of model plant species (15% increased biomass production by accelerating recovery from photoprotection, Kromdijk et al., 2016; and 40% increased biomass via engineering of a photorespiratory bypass, South et al., 2019) and recent modelling has highlighted the potential of improving several dynamic photosynthetic traits on whole canopy photosynthesis (Wang et al., 2020). It is important now that we explore and exploit natural variation in photosynthetic traits across plant populations (for review see Furbank et al., 2020). As in previous studies with other species, we identified significant variation in Rubisco activation rate across barley genotypes. We identified a QTL for Rubisco activation rate, as well as several QTLs for steady state A and gs. Q1/τ.sun-7H was flanked by the bpb-9601 DArT marker which has previously been associated with both grain yield and crop spike number in the Yerong/Franklin population (Xue et al., 2010). This marker is of particular interest as it also flanks QTLs that we identified for steady state A and gs (QA.sun-7H and Qg.sun-7H in Table 1), highlighting its utility for marker assisted selection (MAS). MAS exploiting natural variation between barley genotypes can now be achieved through the development of a high throughput codominant marker using the sequence information
from the closely associated bpb-9601 DArT marker identified in this study. MAS for dynamic and steady state photosynthetic traits in barley now provides potential to improve daily photosynthetic carbon gain in both sporadically sunlit lower canopy and fully sunlit upper canopy leaves, respectively, bolstering whole canopy photosynthesis and contributing to yield potential.

Whilst we observed segregation for three different photosynthetic traits in the barley mapping population studied, and the V/D population presented in Figures S4 and S5, these populations were not specifically developed to investigate photosynthesis. Future work in this area would hugely benefit from phenotyping a diverse panel of barley accessions to either develop additional trait-specific mapping populations using parents with contrasting photosynthetic properties or use a genome wide association scan (GWAS) approach to mine for novel favourable alleles based on natural variation in photosynthetic traits. This may also include the investigation of crop wild relatives (Castañeda-Álvarez, 2016). Such approaches have already yielded promising outcomes for other desirable traits in crop species, including salinity (in barley, Saade et al., 2016) and drought tolerance (Venuprasad et al., 2009).

Due to the recent availability of multiple reference genomes for cultivated and wild barley, the precision of GWAS studies and ability to rapidly clone genes of interest from cereal crops is continually improving. Further studies are required to determine whether each of the traits studied are under control by a single gene or more complex genetic control within the QTL region on chromosome 7H. Further mendelisation of the 7H QTL by intercrossing select DH lines from the Y/F population will enable the development of a large segregating F2 fine-mapping population for positional cloning of the 7H QTL to unravel the underlying genetic and biological mechanisms involved.

Our study focussed on a step change from moderate (600 µmol m$^{-2}$ s$^{-1}$) to saturating light (1300 µmol m$^{-2}$ s$^{-1}$), rather than low to high light as has been reported previously (i.e. 50 – 1500 µmol m$^{-2}$ s$^{-1}$ in Taylor and Long, 2017). We felt this approach would provide more valuable information for plant breeding, as it more accurately represents the light regime experienced by the second youngest leaves in the canopy, which for wheat have been reported to receive between 300 – 700 µmol m$^{-2}$ s$^{-1}$ PPFD when not in a sunfleck (Townsend et al., 2018). Whilst leaves lower in the canopy receive much less light than this (< 300 µmol m$^{-2}$ s$^{-1}$), these leaves are also less likely to be exposed to
sunflecks and also have a much-reduced photosynthetic capacity (Townsend et al. 2018), so contribute considerably less to whole canopy photosynthesis. Our results show that Rubisco activation rates after a switch from moderate to high light in barley (median $1/\tau = 0.28 \text{ min}^{-1}$) are similar to those that have been reported from low to high light in other species ($0.3 - 0.45 \text{ min}^{-1}$ in rice, Yamori et al., 2012; $0.24 - 0.42 \text{ min}^{-1}$ in soybean, Soleh et al., 2016; and $0.25 - 0.33 \text{ min}^{-1}$ in wheat, Taylor and Long, 2017), albeit with greater variation. It would therefore seem that the same biochemical processes, likely related to the amount of and form of Rubisco activase present in the leaves (Carmo-Silva and Salvucci, 2013), are involved in photosynthetic induction under the two induction scenarios.

**Limitations and future directions**

Our study has focussed on Rubisco activation however this is only one part of the dynamic photosynthesis puzzle, in which all the pieces must be investigated to fully understand potential improvements that could be made to whole canopy photosynthesis. Responses of stomata can also limit photosynthesis in fluctuating light. Faster stomatal opening has now been shown to improve net photosynthesis and biomass production in overexpressing mutants of *Arabidopsis thaliana* compared to wild type plants (Kimura et al., 2020). And so, if improvements are made to Rubisco activation rate without also considering rates of stomatal opening/closure, the dominant limitation will likely shift in the direction of the stomata. In effect, this could nullify any improvements made to Rubisco activation in terms of net photosynthesis. On a positive note, it has been shown that stomatal traits can be linked to Rubisco kinetics during leaf development in some plant species (Conesa et al., 2019), and it has long been realised that stomata respond to photosynthetic activity in the mesophyll (Messinger et al., 2006). Glowacka et al. (2018) recently confirmed the intimate link between photosynthetic biochemistry and stomata in field grown crop plants. They showed that overexpression of *Photosystem II Subunit S (PsbS)*, which directly affects the rate of energy absorption in photosystem II, results in reduced stomatal opening in response to light. Kromdijk et al. (2019) subsequently incorporated this link into a mechanistic model of photosynthesis and stomatal conductance. It is therefore conceivable that improving Rubisco activation rate through targeted plant breeding could also inherently result in improved stomatal responses. Regardless, there is a definite need for future work in this area to address dynamic responses of stomata, Rubisco and other biochemical processes (i.e. non-photochemical quenching) of photosynthesis together, rather than focussing on each in isolation.
In this study, we measured photosynthetic induction and identified associated QTLs in plants grown under optimal and controlled conditions. The next important step is for photosynthetic induction traits to be investigated in field-grown plants with established canopies. Traditional gas exchange techniques combined with new higher throughput techniques based on thermography (for dynamic stomatal traits; Viallet-Chabrand & Lawson, 2020), hyperspectral imaging and chlorophyll fluorescence (for dynamic photosynthetic parameters; McAusland et al., 2019; Meacham-Hensold et al., 2020) may offer the potential to screen these two populations in the field and validate the QTLs we identified in this study. It is also important that we understand if these QTLS are strong under sub-optimal conditions (i.e. under drought or heat stress), as for most growers such conditions can be common during a growing season.

A note on gas exchange methodology

It is common practice to allow a leaf to stabilize to the chamber conditions of an IRGA, yet the recent push for “high throughput” and “big data” approaches in plant physiology may have made researchers complacent. We hypothesized that this complacency could impact detected QTLs for photosynthesis and stomatal conductance, and indeed we found that using non-steady state rates (i.e. before leaves had equilibrated to chamber conditions) resulted in less accurate detection of QTLs. It is likely that false QTL identifications are worsened by the high variability in photosynthetic induction kinetics that exists across this population (and has also been found in other crop species) and the fact that there is no clear relationship between steady state and dynamic photosynthesis. This result reinforces the importance of good gas exchange technique. The push for high-throughput measurements has resulted in new fast methods, such as the Rapid A/c method (Stinziano et al., 2017), being developed yet it must be highlighted that most of these methods still rely on the assumption of steady state conditions and these will therefore still be limited by equilibration time.

We suggest that plant physiologists treat this as a methodological opportunity instead of a hindrance. Rather than just waiting for the leaf to reach steady state and then recording a point measurement or photosynthetic response curve, the photosynthetic induction phase could always be logged continuously as soon as the leaf enters the chamber. Not only would this provide extra data on photosynthetic induction, it would also give a researcher more confidence in their data. Specifically, they would be able to backcheck to ensure that steady state conditions had been
reached. In the past, technical limitations may have prevented such an approach, but new gas exchange instruments have the computational power, storage capacity and environmental control to establish this as common practice. New data analysis software, including the R packages {Plantecophys} (Duursma, 2015) and {Photosynthesis} (Stinziano et al., 2020), that allow large gas exchange datasets to be quickly processed will further enable this change.

Conclusions

In this study, we found wide variation in photosynthetic induction to fluctuating light across a barley mapping population. This variation allowed us to identify a QTL for Rubisco activation rate, the position of which overlapped QTLs for steady state photosynthesis and stomatal conductance. These QTLs lie close to molecular markers that could be used for selection in plant breeding programs. Future work should aim to validate these QTLs under field conditions so that they can be used to aid plant breeding efforts.
Data availability

The following files are available in the Supporting information:

FileS1.xlsx – Yerong/Franklin dynamic and steady state gas exchange phenotypic data.

FileS2.xlsx – Results of composite interval mapping of dynamic and steady state photosynthetic traits.

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Figure legends

Figure 1 – Example of a typical leaf photosynthetic induction response and the linear regression used to calculate Rubisco activation rate (1/τ). The orange crosses represent the measured photosynthetic rate $A$; the grey squares the $c_i = 300 \ \mu$mol mol$^{-1}$ normalised photosynthetic rate $A^*$; and the blue circles the logarithmic difference between the fully induced photosynthetic rate $A_{\text{max}}^*$ and $A^*$. Filled circles represent the data points used in the linear regression to estimate 1/τ, the Rubisco activation rate. The slope of the regression represents 1/τ, in this case 0.31 min$^{-1}$.

Figure 2 – Induction curves for net photosynthesis, $A$ (red crosses); stomatal conductance, $g_s$ (blue triangles); $c_i$ (green circles); and $c_i = 300$ ppm normalised photosynthesis, $A^*$ (grey squares), after a switch from moderate to saturating light. Data shown in panels (a) – (i) are representative induction curves from one day of measurements in individual plants. The value of 1/τ is shown in each panel for reference.

Figure 3 – Distribution of Rubisco activation rate (1/τ) across genotypes of the Yerong/Franklin DH population. Each bar represents a single genotype. Parental lines are highlighted. Colours are arbitrary.

Figure 4 – Logarithm of odds (LOD) traces from composite interval QTL mapping analysis for 1/τ. LOD values are plotted against the position on the chromosomes. The significance threshold LOD of 2.5 is indicated by the dotted line in each plot. Vertical dashed red lines represent identified QTLs.

Figure 5 – Distribution of steady state (a) $A$ and (b) $g_s$ across genotypes of the Yerong/Franklin population. Each bar represents a single genotype. Parental lines are highlighted. Note that colours are arbitrary but are consistent for genotypes in panels (a) and (b).

Figure 6 – Relationship between steady state $A$ and 1/τ. Each point represents a genotype. Values are genotype means.

Figure 7 – Logarithm of odds (LOD) traces from composite interval QTL mapping analysis for $A$ and $g_s$ in the Yerong/Franklin DH population. LOD values are plotted against the cM position on chromosomes 2H, 4H, 5H and 7H. The threshold LOD of 2.5 is indicated by the horizontal dotted line in each plot. Note that LOD plots for chromosomes 1H, 3H and 6H are not shown as there were no significant QTLs identified on these chromosomes.
Table 1 – QTLs for dynamic and steady state photosynthetic traits identified in the mapping population.

| Trait   | QTL     | Chromosome | Position (cM) | Nearest Marker | Explained variance (%) | Additivity | LOD  |
|---------|---------|------------|---------------|----------------|------------------------|------------|------|
| 1/τ     | Q1/t.sun-7H | 7H        | 41.67         | bpb-9601       | 10.48                  | -0.07      | 4.40 |
| A       | QA.sun-2H  | 2H        | 27.03         | bpb-0003       | 9.20                   | -3.85      | 4.31 |
| A       | QA.sun-4H  | 4H        | 66.68         | bpb-2305       | 5.84                   | -4.37      | 2.64 |
| A       | QA.sun-7H  | 7H        | 41.67         | bpb-9601       | 10.80                  | -4.30      | 5.18 |
| g_s     | Qg_s.sun-2H | 2H        | 35.91         | bpb-8750       | 11.80                  | -0.09      | 5.41 |
| g_s     | Qg_s.sun-5H | 5H        | 53.39         | bpb-5532       | 6.49                   | 0.07       | 2.98 |
| g_s     | Qg_s.sun-7H | 7H        | 41.28         | bpb-4989       | 13.75                  | -0.11      | 6.80 |
Table 2 – Distribution features for photosynthetic rate (A) and stomatal conductance (g_s) across the population at 5, 10 and 15 minutes into photosynthetic induction.

| Trait | Time point (minutes) | Minimum | 25% Percentile | Median | 75% Percentile | Maximum | Mean |
|-------|----------------------|---------|----------------|--------|----------------|---------|------|
| A (µmol m⁻² s⁻¹) | 5 | 7.1 | 16.5 | 20.0 | 24.9 | 36.5 | 20.4 |
|       | 10 | 9.6 | 20.0 | 23.3 | 26.9 | 40.2 | 23.4 |
|       | 15 | 12.2 | 21.5 | 24.9 | 28.4 | 41.8 | 25.0 |
| g_s (mmol m⁻² s⁻¹) | 5 | 0.044 | 0.259 | 0.365 | 0.522 | 0.947 | 0.397 |
|       | 10 | 0.069 | 0.294 | 0.392 | 0.508 | 0.947 | 0.412 |
|       | 15 | 0.107 | 0.330 | 0.441 | 0.544 | 0.940 | 0.451 |
Figure 1

The graph shows the relationship between time after induction (minutes) and photosynthesis rate (A, µmol m⁻² s⁻¹) along with the natural logarithm of the difference between the maximum photosynthesis rate (A_max) and the current photosynthesis rate (A).

The equation for the line of best fit is:

\[ y = -0.31x + 3.1 \]

with a coefficient of determination \( R^2 = 0.996 \).

- Red x: A
- Gray square: A*
- Blue circle: \( \ln(A_{\text{max}} - A) \)
- Blue circle (filled): \( \ln(A_{\text{max}} - A^*) \) (used for regression)
Figure 2
Figure 4

(a) Chromosome 1
(b) Chromosome 2
(c) Chromosome 3
(d) Chromosome 4
(e) Chromosome 5
(f) Chromosome 6
(g) Chromosome 7

LOD = 2.5

Position (cM)
Figure 5

(a) Net photosynthesis

(b) Stomatal conductance
Figure 6

\[ y = 0.0026x + 0.245 \]

\[ R^2 = 0.0153 \]
Figure 7

(a) A on Chromosome 2H  
(b) A on Chromosome 4H  
(c) A on Chromosome 5H  
(d) A on Chromosome 7H  
(e) \( g_a \) on Chromosome 2H  
(f) \( g_a \) on Chromosome 4H  
(g) \( g_a \) on Chromosome 5H  
(h) \( g_a \) on Chromosome 7H

- **Steady state**
- **5 min into induction**
- **10 min into induction**
- **15 min into induction**
- **LOD = 2.5**