Rapid temperature responses of photosystem II efficiency forecast genotypic variation in rice vegetative heat tolerance

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SUMMARY
A key target for the improvement of Oryza sativa (rice) is the development of heat-tolerant varieties. This necessitates the development of high-throughput methodologies for the screening of heat tolerance. Progress has been made to this end via visual scoring and chlorophyll fluorescence; however, these approaches demand large infrastructural investments to expose large populations of adult plants to heat stress. To address this bottleneck, we investigated the response of the maximum quantum efficiency of photosystem II (PSII) to rapidly increasing temperatures in excised leaf segments of juvenile rice plants. Segmented models explained the majority of the observed variation in response. Coefficients from these models, i.e. critical temperature (Tcrit) and the initial response (m1), were evaluated for their usability for forecasting adult heat tolerance, measured as the vegetative heat tolerance of adult rice plants through visual (stay-green) and chlorophyll fluorescence (高效的PSII) approaches. We detected substantial variation in heat tolerance of a randomly selected set of indica rice varieties. Both Tcrit and m1 were associated with measured heat tolerance in adult plants, highlighting their usability as high-throughput proxies. Variation in heat tolerance was associated with daytime respiration but not with photosynthetic capacity, highlighting a role for the non-photorespiratory release of CO2 in heat tolerance. To date, this represents the first published instance of genetic variation in these key gas-exchange traits being quantified in response to heat stress in a diverse set of rice accessions. These results outline an efficient strategy for screening heat tolerance and accentuate the need to focus on reduced rates of respiration to improve heat tolerance in rice.

Keywords: chlorophyll fluorescence, stay-green, photosynthesis, heat stress, Oryza sativa, high-throughput phenotyping, technical advance.

INTRODUCTION
Global climatic change is a key contributor to the multifaceted challenge of achieving food security. The increase in average Earth surface temperatures is a fundamental aspect of climate change and is well understood to be particularly detrimental to agricultural productivity. In concurrence, independent model estimates have indicated that with each incremental increase in surface temperature (per °C) there are concurrent decreases in rice yields of up to 3.2% (Zhao et al., 2017). Moreover, empirical evidence from field-based temperature manipulation studies have demonstrated that an increase in air temperature of approximately 3°C can significantly reduce carbon fixation and grain yield (Chaturvedi et al., 2017). When this evidence is taken in the context of the forecasted increases in average surface temperatures of 0.2-°C per decade (IPPC, 2007), it is evident that the development of elite rice varieties that produce stable yields during heat stress events is a key priority for future crop improvement.

Heat stress upregulates the expression of intrachloroplastic proteases that perturb chloroplast structure and function through protein degradation (Sinvany-Villalobo et al., 2004). The degradation of Rubisco and other proteins involved in carbon fixation with intensifying temperatures reduces photosynthesis (Jagadish et al., 2015; Chen et al., 2019). As photosynthesis is the ultimate basis of yield (Zhu et al.,
et al., 2012), reductions herein are of critical importance for crop production. Prolonged instances of temperatures above optimal for typical plant functionality disrupt photosynthetic carbon fixation. This disruption of photosynthesis commonly co-occurs with early-onset and accelerated leaf senescence, which results from chlorophyll degradation caused by reactive oxygen species (ROS; Khanna-Chopra, 2012; Jajic et al., 2015), as well as through vascular collapse and the disruption of cellular homeostasis (Lim et al., 2007; Cossani and Reynolds, 2012). These processes are developmentally pre-programmed to initiate and control senescence during reproductive growth (Sekhon et al., 2019). Delayed and/or reduced senescence is typically referred to as ‘stay green’ (SG). SG is recognized as a key physiological trait and physical marker for stress adaptation, as it permits the maintenance of photosynthesis (Lim et al., 2007). Furthermore, as SG can be evaluated rapidly across a population, it has been phenotypically and genetically linked to yield in key crops, such as Triticum aestivum (wheat; Kumar et al., 2010; Vijayalakshmi et al., 2010), Sorghum bicolor (sorghum; Rama Reddy et al., 2014) and Oryza sativa (rice; Yoo et al., 2007; Fu et al., 2011). Despite this link, it is important to note that the developmental timing of reductions in foliar carbon fixation and concurrent senescence can define yield consequences. For example, senescence triggered or accelerated by heat stress during grain filling is typically important for stabilizing yield, as it facilitates the remobilization of carbon from vegetative sources or stores to reproductive sinks (Uauy et al., 2006). Selection on SG pre-anthesis, and sometimes during and post-anthesis, can afford yield benefits under heat stress, however, through the assimilation of extra photosynthates that are directly translocated to reproductive processes or are stored as water-soluble carbohydrates in the stem and subsequently remobilized (Blum, 2009; Jagadish et al., 2015).

Heat stress is also particularly damaging to the oxygen-evolving complex of photosystem II (PSII), the initial site of light-dependent photosynthetic reactions (Murata et al., 2007). The repair mechanism of PSII is inhibited by heat-induced ROS production, leading to increased photoinhibition, which impairs photosynthesis (Allakhverdiev et al., 2008). PSII efficiency can be quantified in vivo through chlorophyll fluorescence techniques (Baker, 2008), where the associated methodologies are relatively accessible and can provide a general measure of the photosynthetic response to stress (Murchie et al., 2018). The usefulness of chlorophyll fluorescence for understanding crop heat tolerance has been successfully employed in a series of studies focusing on natural variation in wheat (Sharma et al., 2012, 2015; Sharma et al., 2017). In these studies, the response of the maximum quantum efficiency of PSII \( F_{v}/F_{m} \) to heat stress was observed to correlate with traits linked to heat tolerance, such as chlorophyll content and biomass accumulation after heat stress (Sharma et al., 2015). Subsequently, genetic loci underlying variation in the response of \( F_{v}/F_{m} \) to heat stress were detected through quantitative trait loci (QTLs) mapping, and were observed to co-localize with known heat-tolerance genes (Sharma et al., 2017).

Introducing and developing heat tolerance into modern germplasm requires the ability to screen large populations of natural genotypes, or mutants, for breeding or forward-genetic screens (Driedons et al., 2016). Visual assessments of SG or measurements of chlorophyll fluorescence are somewhat amenable to this end, as they are relatively quick, do not require expensive equipment and provide a good indication of the maintenance of carbon fixation and heat tolerance that can be associated with yield. As physical markers of heat tolerance they can be limiting, however, in that they require substantial infrastructure investment, such as facilities to induce heat stress or access to multiple field sites to leverage naturally occurring heat stress. Additionally, adequate space to grow plants to adult and reproductive developmental stages can be substantially limiting. For example, Sharma et al. (2012) required considerable glasshouse and climate chamber space in order to quantify the heritable variation of the heat response of \( F_{v}/F_{m} \) in a panel of over 1200 wheat varieties at adult developmental stages. Consequently, employing SG or chlorophyll fluorescence as physical markers for selection can represent a phenotyping bottleneck for adapting crops to future environments.

With this study we sought to develop a novel and rapid methodology for understanding the temperature response of PSII photochemistry. Through broken-line analyses we hypothesized that it would be possible to accurately characterize this response and that this characterization could be used to forecast the heat tolerance of foliar tissue, through the relationship between the maintenance of photosynthesis under heat stress and SG. Consequently, we tested the efficiency of the initial response of \( F_{v}/F_{m} \) to temperature and the critical temperature of \( F_{v}/F_{m} \) in excised juvenile tissue segments for predicting SG in adult plants. Finally, we explored how this related to traditional metrics of photosynthetic responses to heat stress, demonstrating the first published example of the assessment of the genetic variation of key photosynthetic traits in response to heat stress in rice to date.

RESULTS

Genotypic variation in the response of excised juvenile leaf segments to rapidly increasing temperatures

A small portion of leaf tissue was excised from the youngest leaf of 10-day-old rice plants in order to measure \( F_{v}/F_{m} \) at incrementally increasing temperatures (approx. 21–50°C) within a closed chlorophyll fluorescence system
We modelled the response of \( F_o \) to temperature as an exponential increase in \( F_m \), where small increases were induced by the initial lower temperatures and rapid increases were induced by the higher temperatures (Figure 2a). The response of \( F_o \) to temperature was reflected by \( F_v/F_m \), where all genotypes demonstrated a response to temperature similar to a logistic decay (Figure 2b). We tested whether the process of removing the plant material encased in glass plates into and out of a water bath before measuring chlorophyll fluorescence impacted the values of \( F_o \) and \( F_v/F_m \), irrespective of temperature changes, but noted no change in either parameter when performing the regular experimental procedure without incremental temperature changes (Figure S1).

At the initial temperatures there was little variation in chlorophyll fluorescence between the genotypes (Figure 2a,b). As temperatures increased, significant genetic variation was detected: for example, \( F_v/F_m \) at 35°C varied from 0.62 for Black Gora (SE = 0.03) to 0.80 for BJI (SE = 0.00). A repeated-measures one-way analysis of variance (ANOVA) demonstrated that there were significant genotype and temperature effects on both \( F_o \) and \( F_v/F_m \) (Figure 2a,b). Additionally, a significant interaction between genotype and temperature was detected (Figure 2a,b), suggesting that genotype differences in fluorescence parameters were dependent on temperature. This is evident when comparing the \( F_o \) response to temperature of IR 64: for example, IR 64 had the highest value of all genotypes at 30°C (149.14; SE = 11.42) but the lowest value at 48°C (262.05; SE = 10.51). A similar effect is seen with the BJI genotype and the \( F_v/F_m \) parameter, where BJI demonstrated the highest value of all genotypes at 30°C (0.81; SE = 0.00) but the second lowest value at 48°C (0.04; SE = 0.02).

We modelled the response of \( F_v/F_m \) to temperature of each individually excised leaf segment through a linear model and a quadratic model. The coefficient of determination (\( R^2 \)) of the linear models varied moderately within genotypes, where within-genotype standard errors ranged from 0.02 to 0.05, suggesting that linear models do not describe the relationship between \( F_v/F_m \) and temperature in a wholly consistent manner (Figure 3). Moreover, the variation in the linear model \( R^2 \) between genotypes was significantly different (\( P < 0.01 \)), where the mean ranged from 0.57 to 0.81 (Figure 3), thereby indicating that linear models are better at describing this relationship for certain genotypes than others. Conversely, intragenotypic variation in the \( R^2 \) values of quadratic models varied little, where within-genotype standard errors were consistently \( \leq 0.01 \) (Figure 3). Furthermore, the variation between genotypes for \( R^2 \) varied much less for quadratic than linear models, where the lowest genotype mean value was 0.91 and the highest was 0.96. This still represented a significant difference according to a one-way ANOVA, however (\( P < 0.01 \); Figure 3).

The fitted linear models were used to produce segmented models through broken-line analyses (see 'Experimental procedures'). Segmented models out-performed linear and quadratic models with respect to minimizing intra- and intergenotypic variation (Figure 3). The within-genotype standard errors of \( R^2 \) were \( < 0.00 \) for all genotypes, and the genotype mean values varied from 0.97 to 0.99 (\( P = 0.06 \)). Therefore, this suggests that segmented models do not bias certain genotypes in their modelling of the response of \( F_v/F_m \) to temperature to the same extent as linear or quadratic models.

Fitting segmented models to the response of \( F_v/F_m \) to temperature allowed us to characterize this relationship in three ways. For all individual models only one breakpoint in the relationship was ever detected. We term this so-called breakpoint as the critical temperature of \( F_v/F_m \) (\( T_{\text{crit}} \)), as it defines the temperature point at which \( F_v/F_m \) transitions from a slow to a rapid decline (Figure 2c). Genotype mean values for \( T_{\text{crit}} \) varied from 36.48°C (SE = 1.18°C) for...
Black Gora to 39.89°C (SE = 0.32°C) for BJI (Figure 4a). The slopes of the linear regressions fitted before and after \(T_{\text{crit}}\) were extracted and designated as \(m_1\) and \(m_2\), respectively. \(m_1\) and \(m_2\) define the strength of the relationship of \(F_v/F_m\) and temperature before and after \(T_{\text{crit}}\). The genotype mean values of \(m_1\) varied from 0.0008 (SE = 0.0001) for Aus Kushi to 0.0125 (SE = 0.0016) for Black Gora (Figure 4b). A linear model regressing \(T_{\text{crit}}\) on \(m_1\) demonstrated a negative relationship between the two parameters (Figures 4d and 5; Table S1), where the lines that respond more strongly to initial temperature changes had the lowest \(T_{\text{crit}}\). Genotype means for \(m_2\) varied from 0.051 (SE = 0.008) for Black Gora to 0.088 (SE = 0.006) for BJI (Figure 4c). \(T_{\text{crit}}\) and \(m_2\) did not show a discernible association (Figure 5; Table S1); however, \(m_1\) and \(m_2\) demonstrated a significant negative association, suggesting that the lines that respond most strongly to temperature before \(T_{\text{crit}}\) respond least strongly after \(T_{\text{crit}}\), and vice versa (Figure 5; Table S1).

Genotypic variation in vegetative heat tolerance in adult plants

We subjected adult plants to an 8-day-long period of heat stress (42°C day temperature) at 60 days post sowing. At this point, the majority of plants were transitioning from the tillering to the stem elongation growth stage (Lancashire et al., 1991). This stage was chosen because of the importance of the heat tolerance of foliar tissue and the maintenance of photosynthesis just prior to reproductive growth (Jagadish et al., 2015). During the heat-stress period, the operational efficiency of PSII in light (\(\Phi_{\text{PSII}}\)) was assessed every day to determine the impact of heat stress on the photosynthetic biochemistry of all genotypes (Figures 6 and S3). \(\Phi_{\text{PSII}}\) decreased across the duration of the heat-stress period for all genotypes except BRRI 28. The most substantial reductions were observed from 6 days after heat-stress initiation (Figure S3). We calculated the percentage reduction in \(\Phi_{\text{PSII}}\) from day 1 to day 8 of the heat-stress experiment (Figure S3). BRRI 28 did not...
respond to heat stress in terms of $\Phi_{\text{PSII}}$ (Figures 6 and S3), indeed $\Phi_{\text{PSII}}$ actually increased marginally during this period for BRRI 28, yielding a negative percentage decrease (Figure S3d). The remainder of the genotypes showed significant reductions in $\Phi_{\text{PSII}}$, leading to a range in percentage declines from 8.06 for IR 64 to 34.59 for Black Gora (Figure S3).

After 7 days from heat-stress initiation, SG was visually scored as the stay-green rating (SGR) on all plants. SGR describes the extent of fully expanded foliar tissue that is senesced and varies from 1 (no senescence) to 5 (total leaf and stem death). Genotype means of SGR varied from 1.50 (SE = 0.29) for BRRI 28 to 3.75 (SE = 0.25) for Black Gora. Four statistically significant different genotype groups were established for SGR (Figure 7a).

Figure 3. Effectiveness of the broken-line analyses for describing the relationship between the maximum efficiency of photosystem II ($F_v/F_m$) and temperature. Box plots describe the variation in the coefficient of determination ($R^2$) of models – linear (red), quadratic (green) and segmented (blue) – that predict $F_v/F_m$ from temperature for each genotype. Each box plot denotes the interquartile range (IQR; 25th–75th percentile), with the 50th percentile marked. The whiskers extend to maximum values within 1.5 times the IQR, with values outside of this range being indicated by black dots. The $P$ values from a one-way analysis of variance describing $R^2$ as a function of genotype for each model are indicated within parentheses in the inset legend.

Figure 4. Characterization of the response of maximum quantum efficiency of photosystem II ($F_v/F_m$) to temperature in the seven genotypes. (a–c) Dot plots demonstrating the variation in the critical temperature ($T_{\text{crit}}$) of $F_v/F_m$ (a), the initial rate of response of $F_v/F_m$ to increasing temperature ($m_1$, b) and the secondary rate of response of $F_v/F_m$ to increasing temperature ($m_2$, c). Individual dots represent means of each genotype and error bars extend to the maximum and minimum value. Statistically significant differences between genotypes are denoted by different colours and by different letter groups above each genotype. (d) The relationship between $m_1$ and $T_{\text{crit}}$. The blue crosses indicate individual data points and the linear regression for $T_{\text{crit}}$ as a function of $m_1$ is denoted by the solid red line. The associated $P$ value and $R^2$ value of the linear model are inset.
Temperature driven plasticity of photo-physiological traits

We measured the response of net photosynthesis ($A_n$) to incrementally increasing intracellular CO$_2$ concentrations ($c_i$) for all genotypes 3 days before and 5 days after heat-stress initiation. This allowed us to model the maximum rate of carboxylation by Rubisco ($V_{c_{\text{max}}}$) and the maximum rate of electron transport for RuBP regeneration ($J_{\text{max}}$). These parameters reflect the CO$_2$-limited and electron transport-limited rates of photosynthesis, respectively. We also calculated the ratio $J_{\text{max}}:V_{c_{\text{max}}}$ to provide information on electron usage per Rubisco carboxylation event. Additionally, $A_n-c_i$ response measurements were used to model
daytime respiration ($R_d$) as non-photorespiratory release of CO$_2$ (Figure 8a,b).

Significant variation was detected in $V_{cmax}$ between pre- and post-heat-stress initiation (Figures S4a and S5a). Heat stress was also observed to have a significant impact on $V_{cmax}$, reducing rates substantially in all genotypes except Pachodi 427 (Figure 8c). Heat stress did not alter the rank order of genotypes for $V_{cmax}$ significantly, as no
DJ 123 showed the opposing response to heat stress and only minor increases were noted. Conversely, the majority of genotypes, except for IR 64 and BRRI 28 where S4b and S5b. The heat-stress period had a significant genetic variation with no G\textsuperscript{T} interaction demonstrated marginally insignificantly (Figures S4c and S5c). Combining this variation before and after heat-stress initiation (Figures S4d and S5d), but was upregulated significantly under heat stress in all genotypes without demonstrating any G \times T interaction (Figure S6a).

When testing the variation for R\textsubscript{d} before and after heat-stress initiation separately, significant genotypic variation was detected (Figures S4c and S5c). Combining this variation into a multiple test demonstrated marginally insignificant genetic variation with no G \times T interaction, however, but heat stress was observed to increase the rate of R\textsubscript{d} significantly in all genotypes (Figure 8e).

With respect to the non-modelled photo-physiological parameters, only intrinsic water-use efficiency (iWUE) demonstrated a significant response to heat stress (Figure S6), and this was primarily associated with the >50% reduction experienced by IR 64 (Figure S6e). In concurrence, iWUE was the only parameter wherein a significant G \times T interaction was observed (Figure S6).

We calculated the plasticity of photo-physiological traits using the phenotypic plasticity index (PPI) in order to gauge the response of these traits to 5 days of heat stress, where values close to 0 denote a lack of response and values close to 1 denote a strong response. The non-modelled metrics of photosynthetic assimilation of CO\textsubscript{2} demonstrated the lowest phenotypic plasticity for all genotypes, where plasticity for A\textsubscript{400} ranged from 0.03 to 0.19 (Figure S7e) and plasticity for A\textsubscript{max} ranged from 0.02 to 0.23 (Figure S7f), suggesting a relatively reduced response to temperature of instantaneous rates of photosynthesis. Conversely, when focusing on particular photosynthetic processes, the modelled rates of V\textsubscript{cmax} and J\textsubscript{max} demonstrated much higher degrees of phenotypic plasticity, ranging between 0.01 and 0.41 (Figure S7a) and between 0.04 and 0.37 (Figure S7b), respectively. The final trait modelled from A\textsubscript{400} to C\textsubscript{1} response measurements, R\textsubscript{d}, demonstrated by far the greatest plasticity to heat stress, where the phenotypic plasticity index ranged from 0.64 to 1.00 (Figure S7c), suggesting a substantial response in leaf-level respiration to elevated temperature.

**Vegetative heat tolerance can be predicted from novel coefficients extracted from segmented models of the F\textsubscript{v}/F\textsubscript{m} temperature response**

To determine the efficiency of the coefficients extracted from the segmented models, i.e. T\textsubscript{crit} and m\textsubscript{f} for forecasting heat tolerance, we compared the variation in these parameters obtained from juvenile leaf tissue to variation in heat tolerance parameters resulting from the heat-stress experiment performed on adult plants. m\textsubscript{f} demonstrated a tight positive correlation with SGR (P = 0.02; R\textsuperscript{2} = 0.72; Figures 5 and 7c; Table S1), thereby suggesting that the genotypes in which the leaf tissue responds less strongly, in terms of F\textsubscript{v}/F\textsubscript{m}, to the initial incremental temperatures in the chlorophyll fluorescence assay were the same as those that demonstrated an enhanced maintenance of chlorophyll content and heat tolerance. Similarly, T\textsubscript{crit} demonstrated a negative correlation with SGR (P = 0.08; R\textsuperscript{2} = 0.50; Figures 5 and 7b; Table S1), highlighting that the genotypes that demonstrated the highest temperature breakpoint in the response of F\textsubscript{v}/F\textsubscript{m} to temperature were the most heat tolerant.

We also gauged heat tolerance in adult plants as the percentage reduction in \textit{\textphi}PSII from the first to the last day of the heat-stress period. The percentage reduction in \textit{\textphi}PSII was positively associated with SGR (P = 0.08; R\textsuperscript{2} = 0.50; Figure 5; Table S1), suggesting a good level of agreement between the two metrics of heat tolerance. Moreover, the percentage reduction in \textit{\textphi}PSII was negatively correlated with T\textsubscript{crit} (P = 0.02; R\textsuperscript{2} = 0.72; Figure 5; Table S1), further highlighting the efficiency of T\textsubscript{crit} for forecasting heat tolerance.
Daytime respiration ($R_d$) was the photo-physiological trait that demonstrated the greatest plasticity to heat stress (Figure 5c), indicating a role for respiration in heat tolerance. In concurrence, $R_d$ after heat-stress initiation demonstrated a positive association with SGR ($P = 0.10$; $R^2 = 0.45$; Figure 5; Table S1). Furthermore, $R_d$ after heat-stress initiation was positively associated with $T_{crit}$ ($P = 0.01$; $R^2 = 0.76$; Figure 5; Table S1) and negatively associated with $m_1$ ($P = 0.07$; $R^2 = 0.40$; Figure 5; Table S1), suggesting that the lines forecast to have elevated heat tolerance had the most reduced rates of respiration during heat stress. Variation in $R_d$ before the initiation of heat stress did not correlate with any of these aforementioned parameters, notwithstanding the lack of a $G \times T$ interaction, suggesting that $R_d$ under optimal conditions is not a suitable indicator of future heat tolerance.

**DISCUSSION**

$m_1$ and $m_2$ as proxies of heat tolerance and heat resistance, respectively

Assessing heat tolerance across many genotypes is constrained by issues related to space for growing plants and facilities and equipment for experimentally increasing the temperature, both in controlled (Wang et al., 2012) and field environments (Thomey et al., 2019). Here we detail a rapid methodology for assaying heat tolerance that does not require substantial space to grow plants and that is correlated with independent estimates of heat tolerance in genetically diverse adult rice plants. The novel coefficients $m_1$ and $T_{crit}$ represent high-throughput proxies for heat tolerance that can be used as specific targets for developing climate-resilient rice.

Photo-physiological screening of excised leaf material through chlorophyll fluorescence has recently been demonstrated to be equivalent to screening intact material (McAusland et al., 2019), even over extended time periods (>3 h) similar to those employed in the present study. In addition, the lack of response in $F_o$ and $F_v/F_m$ to the experimental procedure performed without temperature changes (Figure S1a,b) suggests that the demonstrated responses observed for these parameters primarily result from the rapid incremental temperature changes (Figure 2a,b). In general, very few plant species exhibit deleterious effects on photosynthesis at temperatures below $30^\circ$C (Zhang and Sharkey, 2009), indeed the optimum temperature for rice cultivation can be as high as $35^\circ$C (Ghadirnezhad and Falah, 2014). Indeed, the majority of the accessions studied here did not demonstrate substantial changes in $F_v/F_m$ below $30^\circ$C (Figure 2b). Interestingly, IR 64 did show a reasonable response in this range, explainable in part by it being a mega indica variety developed in the Philippines, where quarterly average temperatures have never exceeded $27^\circ$C (Stuecker et al., 2018). Conversely, the remaining accessions in this study are aus indica varieties from the Indian subcontinent that are typically cultivated during much warmer summer periods (Travis et al., 2015). Furthermore, IR 64 has been demonstrated to be fairly susceptible to moderate temperature increases (Kilasi et al., 2018). The heat tolerance of the aus varieties employed in this study have never previously been empirically tested.

The slow reduction in $F_v/F_m$ up to $T_{crit}$ can be used to assess the reduction in photosynthesis that can be considered manageable for maintaining growth and reversible upon the return of optimal temperatures (Crafts-Brandner and Salvucci, 2000; Sage and Kubien, 2007; Zhang and Sharkey, 2009). At these temperatures, reductions in photosynthesis result from reduced carbon metabolism and reduced electron transport. The reduction in carbon metabolism is primarily caused by decreased Rubisco activation (Perdomo et al., 2017). The reduction in photosynthesis quantified by our rapid assay is caused by perturbed electron transport, however, in line with long-standing work that recognizes PSII as a heat-labile component of photosynthesis (Berry and Bjorkman, 1980; Williams et al., 1985). More specifically, the initial reduction in $F_v/F_m$ as a result of moderate temperatures (Figure 2b) can reflect an increase in the leakiness of thylakoid membranes (Havaux, 1996), which in turn accelerates photophosphorylation (Bukhov et al., 1998), denoted here by the concurrent rise in $F_o$ (Figure 2a) indicating a reduction in the plastoquinone pool. As PSII complexes are embedded in the lipid bilayers of thylakoid membranes, their functional efficiency is affected by the condition of the membranes. Under these moderate initial temperatures, however, thylakoid membranes can easily unfold and allow PSII repair machinery to access damaged protein complexes (Theis and Schroda, 2016; Yoshioka-Nishimura, 2016). For this reason, it is appropriate to consider $m_1$ as a metric of the adaptive photosynthetic response to temperature, as it represents the slope of the initial $F_v/F_m$ response (Figure 2c). Genotypes with reduced $m_1$ can therefore be considered heat tolerant, as they are able to maintain close-to-optimal photosynthesis under heat stress.

As temperatures increase beyond the adaptive range, tolerating heat and maintaining close-to-optimal photosynthesis is no longer viable, and the response of $F_v/F_m$ to temperature reflects the innate susceptibility of photosynthetic biochemistry to elevated temperatures. The $T_{crit}$ parameter reflects this transition in response (Figure 2c). The response of $F_v/F_m$ to temperatures beyond $T_{crit}$ represents a sequence of well-characterized steps in heat-induced disassembly and denaturation of chlorophyll-containing protein complexes (Lipová et al., 2010). For PSII complexes, this initially involves the release of the manganese-stabilizing protein perturbing the oxygen evolution reaction (Thompson et al., 1989; Yu et al., 2006), and
culminates in the formation of a complex that is non-fluorescent and cannot return to an active state (Satoh et al., 1998). As \( m_2 \) represents the rapid response of \( F_v/F_m \) to temperature that occurs after moderate heat stress (Zhang and Sharkey, 2009), it can be considered a measure of the rate of deconstruction of PSII (Thompson et al., 1989). To this end, lines that demonstrate reduced rates of \( m_2 \) can be considered heat resistant, which in this case is distinct from heat tolerance as benchmarked by \( m_1 \), in that it gauges the capacity to restrain permanent damage as opposed to maintaining typical plant function.

We observed an expected negative correlation between \( m_1 \) and \( T_{\text{crit}} \) (Figures 4d and 5; Table S1), which highlights how lines that are not able to tolerate moderate heat stress efficiently transition to the \( m_2 \) phase more rapidly. Conversely, \( m_2 \) was not correlated with \( T_{\text{crit}} \) (Figure 5; Table S1), which demonstrates that the temperature point at which leaf segments enter the rapid-response phase does not influence this secondary rate. Interestingly, we also observed a highly significant negative correlation between the two response phases (Figure 5; Table S1). This suggests that the lines with improved heat tolerance have reduced heat resistance. That is to say, the lines that are better equipped to maintain photosynthetic biochemistry under moderate heat stress are the same lines in which PSII protein complexes are disassembled more quickly following the transition to the rapid-response phase, i.e. \( m_2 \) and vice versa. As \( m_2 \) is not a function of \( T_{\text{crit}} \) (Figure 5; Table S1), the uncoupling of \( m_1 \) and \( m_2 \) is potentially related to the three-dimensional structure of thylakoid membranes within chloroplasts. Photoinhibition stimulated through increasing temperatures (Murata et al., 2007) can be addressed by the PSII repair machinery. As noted previously, this repair system requires alterations to the thylakoid membrane, and consequently the PSII repair cycle and thylakoid membrane dynamics share a close relationship (Yoshioka-Nishimura, 2016). To this end, it is conceivable that membranal characteristics, e.g. curvature, thickness, stromal gaps, etc., that determine the ease of access to reversibly damaged PSII complexes under moderate heat stress, thus determining \( m_1 \), may also accelerate non-reversible protein complex damage after \( T_{\text{crit}} \) thereby also defining \( m_2 \). This represents an interesting avenue for future research and crop improvement, as many of the genes that control these membranal characteristics have been elucidated (Fristedt et al., 2009; Samole et al., 2012; Armbuster et al., 2013). Future work to this end could, for example, perform incremental temperature-response assays of thylakoid structure between wild-type lines and lines transformed to differentially express key genes involved in thylakoid membranal characteristics. Similarly, a key signal of moderate heat stress is the de-phosphorylation of important PSII component proteins (Sharkey 2005), and consequently it would also be highly interesting to determine how incremental temperature increases impact this de-phosphorylation, and whether this stimulates cyclic electron flow around PSI more in transgenic lines with altered thylakoid membrane structures than in wild-type lines.

It is important to note that previous work has adopted similar strategies to assess the temperature response of \( F_v \) and/or \( F_v/F_m \) (Schreiber and Berry, 1977; Lazár and Iilik, 1997; Xu et al., 2014; Ribeiro et al., 2015; Marias et al., 2017); however, these studies were constrained by equipment issues, meaning at best only three species (Schreiber and Berry, 1977), genotypes (Xu et al., 2014) or developmental stages (Marias et al., 2017) were able to be compared in terms of their photosynthetic response to incrementally increasing temperatures. A fundamental aim of this present study was to determine the efficacy of the coefficients extracted from the segmented models for forecasting heat tolerance. As a result of the commonly encountered space constraints for performing heat experiments on adult plants, we limited the number of genotypes in this study to seven. Despite this, the experimental procedure to assay the response of chlorophyll fluorescence to incrementally increasing temperatures could easily be employed to screen >200 leaf segments at a time (McAuland et al., 2019), thereby opening up the possibility of surveying large populations or many species. A further advance in this present study compared with the aforementioned studies is with respect to the determination of \( T_{\text{crit}} \) or its equivalent. In all cited studies, \( T_{\text{crit}} \) is calculated manually by the authors selecting the points that they best believe represent the slow and fast portions of the temperature-dependent response, fitting linear models to those points and determining where those models transect (Schreiber and Berry, 1977; Lazár and Iilk, 1997; Xu et al., 2014; Ribeiro et al., 2015; Marias et al., 2017). Through this approach, these studies introduce substantial human bias in the calculation of \( T_{\text{crit}} \). This bias is alleviated in our study through the broken-line analysis that computationally determines \( T_{\text{crit}} \). This approach could be applied to screen for improved heat tolerance in other species, including crops.

As shown by the segmented models, the rapid response \( m_2 \) is not a function of \( T_{\text{crit}} \) (Figure 2c). Furthermore, these cited studies do not attempt to characterize the linear portion of the models for any purpose, nor define their relevance.

\( m_1 \) and \( T_{\text{crit}} \) effectively forecast vegetative heat tolerance in adult rice plants

To determine the usefulness of the coefficients extracted from the segmented models for forecasting heat tolerance in adult plants, we subjected 60-day-old rice plants to a heat-stress period of 7 days (Figures 6, S2 and S3). At this point, all accessions were transitioning from the tillering to the stem elongation growth phase. This developmental timing was selected because of the importance of the SG phenotype at this stage for assessing abiotic stress tolerance, as it relates to the maintenance of photosynthesis and thus contributes to floral development and eventual
seed set and seed filling (Cossani and Reynolds, 2012; Jagadish et al., 2015; Pinto et al., 2016). SG is best defined as ‘heritable delayed foliar senescence’ (Thomas and Stoddart, 1975). It has been demonstrated to be a highly effective physical marker for chlorophyll content and has been used as a selection tool for crop breeding (Xu et al., 2000; Cossani and Reynolds, 2012; Thomas and Ougham, 2014; Pinto et al., 2016). We detected substantial variation in SGR 7 days after heat-stress initiation (Figure 7a). Our visual assessment of SGR follows on from those well-established and cited studies in providing low-variance estimates of senescence; however, we recommend that future work to characterize plant- or leaf-level senescence could be improved in throughput and objectivity through the development and adoption of software facilitating machine-based image assessments of SG. Multiple types of SG have been characterized in cereals and these can essentially be divided into two forms: cosmetic and functional. Cosmetic SG refers to the capacity to delay senescence without maintaining photosynthesis, in contrast to the functional form (Thomas and Howarth, 2000; Thomas and Ougham, 2014). The strong correlation between the percentage decline in ϕPSII and SGR demonstrates that the SG variation assessed in this study was of the functional form (Figure 5; Table S1). That is to say, the visual assessment of SG screened for the capacity to stabilize photosynthesis. This corroborates previous work in wheat that demonstrated strong links between SG, biomass accumulation and PSII efficiency (Sharma et al., 2012, 2015; Sharma et al., 2017).

Variation in SGR was associated with both $T_{\text{crit}}$ (Figures 5, 7b, Table S1) and $m_1$ (Figures 5, 7c, Table S1), thereby demonstrating the effectiveness of these parameters determined from juvenile leaf segments for forecasting vegetative heat tolerance in adult plants. The negative association between $T_{\text{crit}}$ and SGR (Figures 5 and 7b; Table S1) demonstrates that the lines that enter the rapid temperature response phase at higher temperatures also have improved vegetative heat tolerance as adult plants. As previously discussed, we interpret $m_1$ to be the most applicable predictor of heat tolerance, as it directly relates to the ability to maintain close-to-optimal photosynthetic biochemistry before heat resistance to restrain PSII disassembly becomes necessary as the heat stress intensifies. This is reflected by the variation in SGR being more explainable by variation in $m_1$ ($R^2 = 0.72$) than by variation in $T_{\text{crit}}$ ($R^2 = 0.50$). Furthermore, we did not observe any association between $m_2$ and SGR or the percentage reduction in ϕPSII; this is probably because these gold-standard assessments performed on adult plants during heat stress quantify heat tolerance and not heat resistance. It is important to note that this study was limited to just seven accessions for determining the efficiency of $m_1$ as a proxy for heat tolerance. Despite this, we still demonstrated a very strong correlation between $m_1$ and SGR, and we propose that this minor limitation could be addressed in future studies that employ this methodology for screening diverse germplasm, such as mapping or mutagenized populations, through the selection of a set of accessions that fall along a wide range of $m_1$ values, and testing them for measured vegetative heat tolerance (SGR) for confirmation purposes. The assessment of such populations in this manner offers a clear strategy for future crop development via forward genetics and would also unequivocally confirm the efficiency of this methodology as a high-throughput phenotyping platform.

Respiration is more responsive to heat stress than photosynthetic capacity, and is linked to forecasted and measured heat tolerance

We measured the response of $A_\text{c}$ to incrementally increasing $c_1$ before and after heat-stress initiation in order to test the response of photosynthetic capacity to temperature (Figure 8a,b). From the $A_{\text{c}}$-$c_1$ response measurements, we modelled $V_{\text{cmax}}$ and $J_{\text{max}}$ to gauge the efficiency of carboxylation by Rubisco and the rate of electron transport, respectively. These parameters are frequently assessed to determine the photo-physiological response to heat stress (Perdomo et al., 2016; Haworth et al., 2018; Thomey et al., 2019; Chen et al., 2019); however, we believe that this is the first instance of genetic variation in these parameters being tested under heat stress in rice in a substantial and genetically diverse set of lines. All accessions demonstrated a downregulation in $V_{\text{cmax}}$ (Figure 8a), which is likely to be linked to Rubisco activase thermosensitivity (Feller et al., 1998; Makino and Sage, 2007), as well the degradation of other Calvin-cycle enzymes (Sharkey, 2005) and general metabolic reprogramming in the chloroplasts (Wang et al., 2018). Despite this, the observed reduction in the rate of consumption of RuBP by Rubisco did not appear to limit $A_\text{c}$ (Figure 6b,c). This is in agreement with previous work that has demonstrated that when operating away from the thermal optimum, the capacity of Rubisco is not a rate-limiting factor for light-saturated CO$_2$ assimilation (Sharkey, 1985; Kubien and Sage, 2008). Curiously, in all but one of the seven accessions we observed a significant upregulation in $J_{\text{max}}$ after heat-stress initiation, suggesting a substantial alteration to the energy sink balance. Under optimal conditions, the vast majority of energy is supplied to photosynthetic carbon reduction (Dani et al., 2014); however, heat and other abiotic stressors can substantially alter this balance as the electron demand from non-photosynthetic processes, primarily elevated photorespiration (Noctor et al., 2002) and isoprenoid emissions (Dani et al., 2014), increases. To this end we calculated the ratio of $J_{\text{max}}$ to $V_{\text{cmax}}$ to determine the balance of electron transport to carboxylation events, and noted an increase in this ratio (Figure 6a) in response to heat stress, thereby
confirming an increase in non-photosynthetic electron demand and highlighting an adjustment in leaf nitrogen investments in response to temperature (Benomar et al., 2019).

Reflecting the increased non-photosynthetic energy demand, we also observed a hugely significant upregulation in \( R_{n} \), modelled as non-photorespiratory release of \( \text{CO}_2 \) from \( A_{n-c} \), response curves, in response to heat stress. Indeed, of all the photo-physiological traits measured pre-and post-heat-stress initiation, \( R_{n} \) demonstrated by far the greatest degree of phenotypic plasticity in all accessions (Figure S7). It is worth noting, however, that the \( A_{n-c} \) response measurements of heat-stressed plants were performed 5 days after heat-stress initiation, which is before substantial reductions in \( \text{F}_{\text{PSII}} \) were observed (Figures 6 and S3). Consequently, this demonstrates that at the leaf level, respiration appears to respond earlier to heat stress than photosynthetic processes. We propose that future work should seek to better understand how and why respiration responds earlier to heat stress in rice than photosynthesis does. This will allow for the determination of direct photo-physiological targets for mitigating heat stress perturbations. For example, diurnal measurements of dark respiration would elucidate the extent to which both nighttime and daytime respiration contribute to senescence and yield reductions. Additionally, more specific estimates of respiration, via gas-exchange measurements under low \( \text{O}_2 \) or via direct measurements of leaf level \( \text{O}_2 \) exchange, would allow for a higher resolution overview of natural variation in heat stress-induced respiration within diverse germplasm. For this reason, respiration may represent a more efficacious physical marker than photosynthesis for the early detection of heat-stress sensitivity. The upregulation of \( R_{n} \) here reflects our understanding of the temperature sensitivity of respiration (Kruse et al., 2011; Gauthier et al., 2014), which is a major limiting factor for ecosystem and agricultural productivity because it increases the percentage of fixed \( \text{CO}_2 \) that is re-released into the atmosphere, thereby reducing the portion of total photosynthates available for growth and productivity (Huntingford et al., 2013). Indeed, leaf-level and whole-plant respiration have been attributed to substantial yield losses in rice (Mohammed and Tarpley, 2009) and other species (Nadeem et al., 2018; Posch et al., 2019). Reflecting the detrimental impact of heat-induced respiration on plant growth and productivity, we observed a positive association between \( R_{n} \) measured during heat stress and SGR, implying that the lines that demonstrated greater visual symptoms of senescence were respiring more. Furthermore, this association reflects the fact that senescence is a hugely energy-intensive process that requires substantial biochemical and metabolic reprogramming (O’Leary et al., 2017). Interestingly, the \( m_1 \) and \( T_{\text{crit}} \) parameters were also significantly associated in the expected direction with \( R_{n} \) during heat stress, but not prior to heat stress, further highlighting the efficiency of these parameters for forecasting overall heat tolerance. Conversely, the variation in photosynthetic capacity assessed as \( \text{V}_{\text{max}} \) and \( \text{J}_{\text{max}} \) during heat stress was not associated with the percentage decline in \( \text{F}_{\text{PSII}} \) or SGR.

**Conclusion**

In summary, this article details the development of a high-throughput phenotyping methodology for assessing the response of \( \text{F}_{\text{V}}/\text{F}_{\text{m}} \) to temperature and demonstrates the use of a novel modelling approach to characterize this response in terms of heat tolerance and heat resistance. Moreover, we have demonstrated that the coefficients extracted from these segmented models that pertain to heat tolerance represent accurate proxies of measured heat tolerance in adult plants. Additionally, our forecasting and assessment of adult heat tolerance was closely linked to daytime respiration, but not photosynthetic capacity, thereby highlighting the importance of non-photorespiratory \( \text{CO}_2 \) release as a target for developing heat tolerance in rice. A natural progression for this work is to employ this platform for forward genetics by screening populations of natural or induced genetic variants with the ultimate aim of elucidating genetic loci linked to heat tolerance. Similarly, this platform represents a strategy for varietal selection in a plant breeding context.

**EXPERIMENTAL PROCEDURES**

**Plant material and growing conditions**

A random selection of six genotypes from the Bengal and Assam Aus Panel (BAAP; Norton et al., 2018) and the reference rice genotype IR 64 were selected for this study (Table 1). We had no prior information regarding the response of any of these genotypes to heat stress. Six biological repeats of each genotype were sown directly into a specialized rice compost (50:50; John Innes 3:Levington M3, The Scotts Company, Ipswich, UK). Prior to sowing, all seeds were heat treated to prevent fungal infection by submerging them in water within a 2.0-ml screwcap tube and heating them to 50°C for 40 min. Plants were initially sown just beneath the soil surface in 5-cm × 5-cm cell trays before being transplanted into 2-L pots filled with the same soil type 2 weeks after germination.

All plants were grown in the same GEN1000 reach-in growth chamber (Conviron, https://www.conviron.com) at the University of Nottingham in 2019. The growth chamber was set to a 12-h photoperiod (06:00–18:00 h) with the following conditions: 32°C day temperature, 27°C night temperature, 65% day relative humidity (RH) and 60% night RH. The light level was set to maximum, where the average light level above the plants increased from 600 to 1000 \( \mu\text{mol m}^{-2} \) photosynthetic photon flux density (PPFD) as the plants grew. On day 60 post sowing, when all plants were in the stem elongation growth stage, the day temperature of the growth chamber was increased to 42°C and the night temperature was increased to 37°C.

At 7 days after heat-stress initiation, the SG of five biological repeats of each genotype was scored via a visual rating system. Scoring used a scale from 1 to 5 based on the proportion of leaf
Table 1 List of genotypes used in this study. For each genotype, where applicable, ID codes corresponding to the Bengal and Assam Aus Panel (BAAP; Norton et al., 2018), the International Rice Germplasm (IRGC) and Genetic Stocks Oryza (GSOR) collections are provided, along with the country of origin

| Genotype | BAAP ID | IRGC ID | GSOR ID | Country of origin |
|----------|---------|---------|---------|-------------------|
| BRRI 28  | 256     | –       | –       | Bangladesh        |
| Aus Kushi | 111     | 66688   | –       | Bangladesh        |
| BJ 123   | 200     | –       | 301006  | India             |
| Pachodi  | 274     | –       | 311588  | India             |
| Black Gora | 201   | –       | 301017  | India             |
| IR 64    | 285     | 117268  | –       | Philippines       |

area of normal sized leaves that had prematurely senesced or died. An SGR of 1 indicates senescence, an SGR of 3 indicates approximately 50% leaf death and an SGR of 5 indicates complete leaf and stem death. This visual assessment of SG has been successfully employed for rice and has been shown to be highly correlated with chlorophyll content (Xu et al., 2000; Jiang et al., 2004; Hoang and Kobata, 2009).

Chlorophyll fluorescence

At 10 days post germination, a leaf segment approximately 8 mm x 20 mm was excised from the youngest leaf of five biological repeats of each genotype. Each leaf segment was placed on top of a damp filter paper in a random order defined using a random list generator. The filter paper and leaf segments were then encased between glass plates (approx. 1 cm thick) on either side. Glass plates containing leaf segments (adaxial side up) were then arranged inside a closed 800C FluorCam chlorophyll fluorescence imager (Photon System Instruments, https://psi.cz). Leaf segments were dark adapted for 1 h before measurements. After dark adaptation, the standard F0/Fm protocol of the associated FluorCam 7 software (Photon System Instruments) was run (McAusland et al., 2019). Here, a measuring light pulse (PPFD 0.09 μmol m−2 s−1) provides a measurement of minimal chlorophyll fluorescence (F0) and a follow-up saturating light pulse (PPFD 5500 μmol m−2 s−1) provides a measurement of maximum chlorophyll fluorescence (Fm). Variable fluorescence (Fv) is calculated as Fm – F0 and the maximum quantum efficiency of PSII is calculated as Fv/Fm. All fluorescence parameters measured in this way were averaged across the entire leaf segment.

The initial measurements of Fv/Fm were performed at room temperature (approx. 21°C). Following this, the glass plates were removed from the closed chlorophyll fluorescence imager and the room was kept dark to maintain the dark-adapted states of the leaf segments. The glass plates were then placed inside sealed plastic bags. The sealed plastic bags were then placed into a water bath set to 25°C for 15 min. The plates were then removed from the water bath and plastic bags and re-positioned within the closed chlorophyll fluorescence imager. Fv/Fm was then measured again using the protocol described above. This process was then repeated at the following temperatures: 27.5, 30, 32, 34, 35, 37, 38, 39, 40, 42, 44, 46, 48 and 50°C. The only adjustment being the water bath incubation time, which was temperature dependent and selected based on the time taken for the leaf segments to reach the target temperature. The period of time needed to reach target temperature was defined by placing a thin-wire thermometer next to leaf segments during protocol development. Sampling of leaf material occurred between 07:30 and 08:00 h. Dark adaptation of leaf material occurred between 08:15 and 09:15 h and the chlorophyll fluorescence measurements at the incremental temperatures occurred between 09:20 and 13:20 h.

To test whether the above protocol without any temperature changes initiated chlorophyll fluorescence responses, we performed the protocol exactly as above but without altering the water bath temperature until the final three steps, where it was changed to 30, 40 and 45°C. This testing was performed using five biological repeats of the IR 64 rice genotype.

To characterize the response of Fv/Fm to the incrementally increasing temperatures we employed segmented (or broken-line) relationships using the ‘segmented’ function from the Σ package (Muggeo, 2017). Initially, and for each individual leaf segment, a linear model is built where Fv/Fm is expressed as a function of temperature using the base ‘lm’ function in Σ. The ‘segmented’ function then estimates a new model based on the initial linear model. The new model is characterized by a segmented relationship through the introduction of breakpoint(s) based on changes in the relationship of Fv/Fm and temperature. Individual linear models are then fitted before and after any breakpoint(s). It is possible to define the approximate location and number of breakpoints; however, we opted against this to avoid introducing any human bias. Despite this, only one breakpoint was ever detected in all of the segmented models constructed. Three coefficients were extracted from each segmented model (Figure 2c): (i) the breakpoint or critical temperature of Fv/Fm (Tcrit); (ii) the slope of the initial response of Fv/Fm to temperature before Tcrit (m1); and (iii) the slope of the secondary response of Fv/Fm to temperature after Tcrit (m2). The Σ code to generate segmented models across multiple independent Fv/Fm temperature responses is available at: github.com/johnferguson1989/tpj_paper_Ferguson2020.

Chlorophyll fluorescence measurements made during the adult heat-stress experiment were performed using a FluorPen portable fluorometer (Photon System Instruments). The ‘QY’ protocol was used to initially achieve a measure of steady-state fluorescence in light (F0) via a measuring light pulse (PPFD 0.09 μmol m−2 s−1). Subsequently, a measure of maximal fluorescence in light (Fm) was achieved through a saturating light pulse (3000 μmol m−2 s−1). The operating efficiency of PSII photochemistry (φPSII) was then calculated as: Fm – F0/Fm. This protocol was performed on five biological repeats of each genotype every day during the heat-stress period. For each biological repeat (n = 5 per genotype), four technical repeat measurements of φPSII were performed on the youngest fully expanded leaf of the main tiller and then averaged. These measurements were performed between 12:00 and 13:00 h each day. We determined the percentage decrease in φPSII of all plants measured based on the φPSII values on day 0 and day 7 of the heat stress. These percentage decrease values were then averaged for each genotype.

Leaf gas exchange

Infra-red leaf level gas exchange was performed using three LI-8800 gas exchange systems (LI-COR, https://www.licor.com). To minimize time and instrument effects, the order of measurements and the system used for each plant was determined by a random list generator.

Initial gas-exchange chamber conditions were set as follows: 32°C heat-exchange temperature; 65% RH; 400 μmol mol−1 reference CO2 concentration; and 1000 μmol m−2 sec−1 PPDF. For the gas-exchange measurements performed 5 days after heat-stress initiation, the heat-exchange temperature was set to 42°C. The middle portion of the youngest fully expanded leaf of the main
tiller of each measured plant was selected for gas exchange. Once clamped on, the leaves were allowed to equilibrate to the chamber conditions, which typically took between 30 and 40 min. Upon stability, rates of photosynthesis ($\mathcal{A}_{\text{can}}$) and stomatal conductance to water ($g_s$) were logged, from which the intrinsic water-use efficiency (IWUE) was calculated as $\frac{\mathcal{A}_{\text{can}}}{g_s}$. Subsequently, an $A_{\text{can}}-\varphi$ response curve was initiated, where the reference CO$_2$ concentration was altered incrementally to the following steps: 300, 200, 100, 50, 400, 600, 800, 1000, 1200, 1400 and 1600 $\mu$mol mol$^{-1}$. At each incremental step, rates of gas exchange were allowed to stabilize for a minimum of 90 sec and a maximum of 120 sec according to standard error stability criteria based on $g_s$ and $A_{\text{can}}$ at the final $A_{\text{can}}-\varphi$ step was logged as $A_{\text{can, max}}$. These measurements were performed between 08:00 and 15:00 h 3 days before heat-stress initiation and 5 days after heat-stress initiation. Between four and six biological repeats of each genotype were measured.

The photosynthesis model of Farquhar et al. (1980) was fitted to all $A_{\text{can}}-\varphi$ response curves using the bilinear method of the ‘fitacis’ (l) function from the R package PLANTECOPHYS (Duursma, 2015). This fitting method provides estimates of Rubisco carboxylation capacity ($V_{\text{cmax}}$), potential electron transport rate ($J_{\text{max}}$) and daytime respiration ($R_d$), also referred to as the non-photorespiratory CO$_2$ release rate. We also calculated the ratio $J_{\text{max}}/V_{\text{cmax}}$. The ‘photosyn’() function from PLANTECOPHYS was used to obtain photosynthesis model estimates of $A_{\text{can}}$ from 50 to 1000 $\varphi$ for visualization purposes.

The phenotypic plasticity of photosynthesis-related traits to heat stress was calculated according to the PPI (Valladares et al., 2006). Here, the mean values for each photosynthetic trait of each genotype 3 days before and 5 days after heat-stress initiation was used to calculate plasticity as: (maximum mean – minimum mean)/maximum mean.

A glossary of all chlorophyll fluorescence and leaf gas-exchange parameters measured and modelled in this study can be found in Table 2.

Statistical analyses

All data processing, analyses and figure generation were performed within the R software environment (R Core Team, 2014). Additional post-processing of figures was performed in AFFINITY DESIGNER ( Serif, https://www.serif.com).

A repeated-measures two-way analysis of variance (ANOVA) was performed to determine the effect of genotype and temperature, and their interaction, on $F_o$ and $F_o/F_{\text{m}}$. This was achieved using the ‘anova_test’() function from the RSTATX package.

To determine the effectiveness of the segmented model approach for characterizing the response of $F_o/F_{\text{m}}$ to temperature, we additionally characterized this response through linear and quadratic models. For each individual biological repeat, the coefficient of determination ($R^2$) of the segmented, linear and quadratic models was extracted. All models were fitted using the base ‘lm()’ function in R. One-way ANOVA comparison of means testing was used to determine whether there were significant genotype effects on the $R^2$ value for all three model types using the base ‘aov()’ function in R.

For all measured traits, except $\Phi_{\text{PSII}}$ (on all days measured), a single one-way ANOVA was performed to determine whether there were significant genotype effects for traits of interest. Subsequently, post-hoc Tukey tests were performed to facilitate multiple comparisons between genotypes. Post-hoc Tukey tests were performed using the ‘HSD.Test()’ function from the R package AGRICOLAE (Mendiburu et al., 2015), with the alpha significance threshold being set to 0.10. For the traits measured through infra-red gas analysis, a three-way ANOVA was initially performed to determine whether the LI-6800 instrument used and the hour the measurement was performed, as well as genotype, had a significant effect on the trait of interest. Neither instrument nor hour of measurement were observed to have a noticeable effect on any measured trait ($P > 0.05$). In addition, and to test for genotype x treatment ($G \times T$) interactions for the photosynthetic traits measured before and after heat-stress initiation, a two-way ANOVA with an interaction term was performed for all traits.

To test for associations between putative dependent and explanatory variables, e.g. SGR and $T_{\text{crit}}$ (Figure 7a), or $\Phi_{\text{PSII}}$ and days following heat-stress initiation (Figures 6a and S3), linear models were fitted. To more generally determine the existence of trait associations, tests for associations between paired samples using the Pearson’s product-moment correlation coefficient were performed for all pairwise trait interactions using the ‘corrplot()’ function from the R package HMSC, which was subsequently visualized using the ‘corrplot()’ function from the R package CORRPLT (McKenna et al., 2016).

| Trait     | Units          | Description                                      |
|-----------|----------------|--------------------------------------------------|
| $F_o/F_{\text{m}}$ | Dimensionless ratio | Maximum quantum efficiency of photosystem II (PSII) |
| $F_o$    | Arbritary      | Minimum chlorophyll fluorescence                  |
| $T_{\text{crit}}$ | °C             | Critical temperature point for $F_o/F_{\text{m}}$, as determined by the segmented analyses |
| $m_1$ | Slope of regression (m) | Slope of linear response of $F_o/F_{\text{m}}$ to temperature before $T_{\text{crit}}$ |
| $m_2$ | Slope of regression (m) | Slope of linear response of $F_o/F_{\text{m}}$ to temperature after $T_{\text{crit}}$ |
| SGR      | Arbitrary      | Visual stay-green rating                          |
| $\Phi_{\text{PSII}}$ | Dimensionless ratio | Apparent efficiency of PSII                        |
| $V_{\text{cmax}}$ | $\mu$mol CO$_2$ m$^{-2}$ sec$^{-1}$ | Maximum carboxylation efficiency of Rubisco       |
| $J_{\text{max}}$ | $\mu$mol electrons m$^{-2}$ sec$^{-1}$ | Maximum electron transport rate                    |
| $R_d$    | $\mu$mol CO$_2$ m$^{-2}$ sec$^{-1}$ | Day respiration                                   |
| $J_{\text{max}}/V_{\text{cmax}}$ | $\mu$mol electrons $\mu$mol CO$_2$$^{-1}$ | Ratio of $J_{\text{max}}$ to $V_{\text{cmax}}$ |
| $A_{\text{can}}$ | $\mu$mol CO$_2$ m$^{-2}$ sec$^{-1}$ | Net photosynthetic rate                           |
| $A_{400}$ | $\mu$mol CO$_2$ m$^{-2}$ sec$^{-1}$ | Light-saturated net photosynthetic rate at ambient CO$_2$ |
| $A_{\text{max}}$ | $\mu$mol CO$_2$ m$^{-2}$ sec$^{-1}$ | Light-saturated net photosynthetic rate at CO$_2$ saturation |
| $g_s$    | $\mu$mol H$_2$O m$^{-2}$ sec$^{-1}$ | Light-saturated rate of stomatal conductance to water at ambient CO$_2$ |
| IWUE     | $\mu$mol CO$_2$ mol H$_2$O$^{-1}$ | Intrinsic water-use efficiency, calculated as the ratio of $A_{400}$ to $g_s$. |
PSII temperature response forecasts rice heat tolerance

DATA AVAILABILITY STATEMENT
Data included in this paper are available at github.com/johnferguson1989/tjp_paper_Ferguson2020.

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AUTHOR CONTRIBUTIONS
JNF, LM, AHP, ZAW and EHM conceived and designed the study. AHP provided the germplasm. JNF, LM and KES performed the experiments. JNF analysed the data. JNF wrote the article, with input from all of the authors.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Response of $F_o$ and $F_Fo/F_o$ to consistent temperatures.

Figure S2. Photographs of representative plants after heat stress.

Figure S3. ϕPSII response to heat stress.

Figure S4. Variation in photosynthetic parameters before heat-stress initiation.

Figure S5. Variation in photosynthetic parameters after heat-stress initiation.

Figure S6. Interaction plots of genotype-specific heat-stress responses.

Figure S7. Photo-physiological plasticity to heat stress.

Table S1. Pairwise trait correlation matrix.

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PSII temperature response forecasts rice heat tolerance

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