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Physiological basis of genetic variation in leaf photosynthesis among rice (*Oryza sativa* L.) introgression lines under drought and well-watered conditions

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Abstract

To understand the physiological basis of genetic variation and resulting quantitative trait loci (QTLs) for photosynthesis in a rice (*Oryza sativa* L.) introgression line population, 13 lines were studied under drought and well-watered conditions, at flowering and grain filling. Simultaneous gas exchange and chlorophyll fluorescence measurements were conducted at various levels of incident irradiance and ambient CO₂ to estimate parameters of a model that dissects photosynthesis into stomatal conductance (g_s), mesophyll conductance (g_m), electron transport capacity (J_{max}), and Rubisco carboxylation capacity (V_{cmax}). Significant genetic variation in these parameters was found, although drought and leaf age accounted for larger proportions of the total variation. Genetic variation in light-saturated photosynthesis and transpiration efficiency (TE) were mainly associated with variation in g_s and g_m . One previously mapped major QTL of photosynthesis was associated with variation in g_s and g_m , but also in J_{max} and V_{cmax} at flowering. Thus, g_s and g_m , which were demonstrated in the literature to be responsible for environmental variation in photosynthesis, were found also to be associated with genetic variation in photosynthesis. Furthermore, relationships between these parameters and leaf nitrogen or dry matter per unit area, which were previously found across environmental treatments, were shown to be valid for variation across genotypes. Finally, the extent to which photosynthesis rate and TE can be improved was evaluated. Virtual ideotypes were estimated to have 17.0% higher photosynthesis and 25.1% higher TE compared with the best genotype investigated. This analysis using introgression lines highlights possibilities of improving both photosynthesis and TE within the same genetic background.

Key words: drought, genetic variation, mesophyll conductance, modelling, *Oryza sativa* L., photosynthesis, rice, stomatal conductance.

Abbreviations: A, Net photosynthesis rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$); A_{max} , light saturated net photosynthesis at ambient CO₂ and O₂ level ($\mu\text{mol m}^{-2} \text{s}^{-1}$); A_c , Rubisco activity limited net photosynthesis rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$); A_j , electron transport limited net photosynthesis rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$); C_a , ambient air CO₂ concentration ($\mu\text{mol mol}^{-1}$) (refers to leaf-surface CO₂ level for model analyses, with boundary conductance already considered; $\sim 6.78 \text{ mol m}^{-2} \text{s}^{-1}$, from Li-Cor manual version 6.1); C_i , intercellular CO₂ concentration ($\mu\text{mol mol}^{-1}$); g_s , stomatal conductance for CO₂ ($\text{mol m}^{-2} \text{s}^{-1}$); g_m , mesophyll conductance ($\text{mol m}^{-2} \text{s}^{-1}$); g_{mo} , residual mesophyll conductance in the g_m model ($\text{mol m}^{-2} \text{s}^{-1}$); $g_{m(NRH-A)}$, g_m estimated by the NRH-A method ($\text{mol m}^{-2} \text{s}^{-1}$); g_t , diffusion conductance from ambient air to the site of carboxylation ($\text{mol m}^{-2} \text{s}^{-1}$); g_{to} , residual diffusion conductance in the g_t model ($\text{mol m}^{-2} \text{s}^{-1}$); I_{inc} , photon flux density incident on leaves ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$); J , e^- transport rate through photosystem II (PSII) used for NADP⁺ reduction ($\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$); J_{max} , maximum value of J under saturated light ($\mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$); K_m , Michaelis–Menten constant of Rubisco for CO₂ (μbar); K_{mo} , Michaelis–Menten constant of Rubisco for O₂ (mbar); O , oxygen partial pressure (mbar); R_d , day respiration (respiratory CO₂ release in the light other than by photorespiration) ($\mu\text{mol m}^{-2} \text{s}^{-1}$); R_{dk} , respiratory CO₂ release in the dark ($\mu\text{mol m}^{-2} \text{s}^{-1}$); s , lumped parameter; $S_{c/o}$, relative CO₂/O₂ specificity factor for Rubisco ($\text{mbar } \mu\text{bar}^{-1}$); V_{cmax} , maximum rate of Rubisco activity-limited carboxylation ($\mu\text{mol m}^{-2} \text{s}^{-1}$); δ_m , parameter in the g_m model, defining $C_c:C_i$ ratio at saturating light; δ_i , parameter in the g_t model, defining $C_c:C_a$ ratio at saturating light; δ_s , parameter in the g_s model, defining $C_i:C_a$ ratio at saturating light; κ_{2LL} , value of conversion efficiency of incident light into J at the strictly limiting light [$\text{mol e}^- (\text{mol photon})^{-1}$]; θ , convexity factor for response of J to I_{inc} ; Φ_2 , apparent quantum efficiency of PSII e^- flow on PSII-absorbed light basis [$\text{mol e}^- (\text{mol photon})^{-1}$]; N_a , leaf nitrogen per unit area ($\text{g N m}^{-2} \text{leaf}$); Γ , C_3 -based CO₂ compensation point in the absence of R_d (μbar); FS, flowering stage and drought-stressed treatment; FW, flowering stage and well-watered treatment; GS, grain-filling stage and drought-stressed treatment; GW, grain-filling stage and well-watered treatment; LMA, leaf mass per area ($\text{g m}^{-2} \text{leaf}$); TE, transpiration efficiency (mmol mol^{-1}).

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Introduction

The response of leaf photosynthesis to drought involves interactions between physical and metabolic mechanisms (Kramer & Boyer, 1995; Pinheiro & Chaves, 2011). The understanding of these physiological mechanisms is necessary to improve physiological dissection of the complexity of leaf photosynthesis in response to drought (Serraj *et al.*, 2008).

In general, the relationships among leaf photosynthesis (A), stomatal conductance (g_s), and transpiration are well understood, as g_s has been studied most when investigating photosynthetic responses to drought (reviewed by Israelsson *et al.*, 2006; Casson & Hetherington, 2010; Lawson *et al.*, 2011). However, g_s is not the only component of CO_2 diffusion in leaves. Mesophyll conductance (g_m), the conductance from substomatal cavities to the site of carboxylation, limits photosynthesis significantly as well, meaning that the CO_2 concentration in the chloroplast (C_c) is lower than in the intercellular space (C_i) (Lloyd *et al.*, 1992; Warren *et al.*, 2003; Warren, 2004, 2008; Flexas *et al.*, 2008). Ignoring g_m would erroneously attribute the decreased photosynthesis under drought to metabolic impairment (Delfine *et al.*, 1998; Flexas *et al.*, 2004; Centritto *et al.*, 2009).

The value of g_m is influenced by leaf traits such as leaf dry matter per unit area (LMA; Flexas *et al.*, 2008; Galmés *et al.*, 2011), but also by environmental variables, including water status (Delfine *et al.*, 1998; Galmés *et al.*, 2007; Niinemets *et al.*, 2009), temperature (Bernacchi *et al.*, 2002; Scafaro *et al.*, 2011), and nutrient supply (Warren, 2004). There is increasing evidence that g_m and g_s are tightly correlated (e.g. Evans, 1999; Flexas *et al.*, 2007a; Warren, 2008; Yin *et al.*, 2009; Barbour *et al.*, 2010; Douthe *et al.*, 2011) and follow the same pattern of variation: declining in response to short-term increases in CO_2 partial pressure and increasing with increases in irradiance. Thus, the relationship between g_m and g_s is worth exploring further when assessing genetic variation in leaf photosynthesis. Genetic variation in the $g_m:g_s$ ratio will allow breeding for high transpiration efficiency (TE) (Galmés *et al.*, 2011).

Photosynthesis is affected not only by diffusion components (g_s and g_m) but also by various biochemical capacities of protein complexes. The potential activity of Rubisco (V_{cmax}) limits photosynthesis at low C_c . As C_c increases, the chloroplastic electron transport capacity (J_{max}) can limit photosynthesis (Farquhar *et al.*, 1980). Both V_{cmax} and J_{max} are closely related to the amount of leaf nitrogen per unit area (N_a) (Makino *et al.*, 1984, 1985; Evans, 1989; Harley *et al.*, 1992a).

Whilst most studies have focused on photosynthetic responses to environmental factors, significant genotypic variation of A has long been reported among species of *Oryza* and among progeny plants derived from crosses between varieties. For example, variation was observed among varieties of *japonica* rice (Sasaki & Ishii, 1992; Ishii, 1995), and among varieties including *indica* and *japonica* rice and wild rice species (Cook & Evans, 1983; Dingkuhn *et al.*, 1989; Yeo *et al.*, 1994; Peng *et al.*, 1998; Masumoto *et al.*, 2004; Teng *et al.*, 2004). Moreover, quantitative trait loci (QTLs) responsible for the different photosynthetic parameters have been mapped successfully (Zhao *et al.*, 2008; Takai *et al.*, 2009; Xu *et al.*, 2009; Adachi *et al.*, 2011). Recently, Gu *et al.* (2012), using a population of introgression

lines (ILs) from a cross between upland rice and lowland rice, identified QTLs for light-saturated gas exchange and chlorophyll fluorescence parameters under both well-watered and drought conditions in the field. QTLs affecting these parameters tended to cluster in the same genomic regions, suggesting a common genetic basis and inherent physiological connections of photosynthesis parameters.

Few studies have investigated the physiological basis for these reported genetic variations and QTLs of leaf photosynthesis. Taylaran *et al.* (2011) showed that the higher N_a and higher g_s in *indica* cultivars could be the reason for higher A than observed in a *japonica* variety. Similarly, Adachi *et al.* (2011) reported that two mapped QTLs of net photosynthesis actually arose from an increased N_a and g_s . Scafaro *et al.* (2011) compared a cultivar of *Oryza sativa* with two wild *Oryza* relatives and found that the difference in mesophyll cell-wall thickness was responsible for differences in g_m , which resulted in substantial variation in A between the cultivated and the wild rice. Masle *et al.* (2005) isolated a TE-regulating gene, *ERECTA*, from a population of *Arabidopsis* and found that V_{cmax} , J_{max} , stomatal density, and mesophyll development caused the genetic variation in TE and A .

As a follow-up of our QTL mapping study (Gu *et al.*, 2012), the present study aimed to investigate the physiological basis of genetic variation and resulting QTLs identified for our IL population. Therefore, a model was used to analyse experimental data for complete curves of photosynthetic responses to CO_2 and to light measured on leaves in a representative subset of the ILs. Such a model analysis allowed us: (i) to identify the genetic variation in each biophysical and biochemical component; (ii) to analyse the physiological basis for the genetic variation in photosynthesis; and (iii) to evaluate the potential of utilizing the genetic variation in these components for improving A and TE under contrasting drought stress. The information obtained could have an important implication for developing drought-tolerant varieties.

Materials and methods

Plant growth conditions, treatments, and experimental design

A greenhouse experiment was conducted at the research facility UNIFARM, Wageningen, The Netherlands. Physiological dissection of photosynthesis requires complete curves of responses to various CO_2 and light levels, and it was practically infeasible to obtain these curves experimentally for all individual genotypes of the IL population of Gu *et al.* (2012). Eleven lines (IL7, IL37, IL42, IL69, IL84, IL100, IL130, IL157, IL159, IL161, and IL164) and two parents [Shennong265, *japonica*; Haogelao, *indica-japonica* intermediate] were therefore selected. The selection was based on two criteria: (i) the ILs should carry many QTLs to reflect as much as possible the genetic variation of the population; and (ii) the ILs should contain as few chromosome segments from the donor parent as possible, to remove the background noise (see also Eshed & Zamir, 1995). These 11 ILs had on average 6.5% of the genome introgressed. Their graphical genotypes are shown in Fig. 1.

The temperature in the greenhouse was set at 26 °C for the 12 h light period and at 23 °C for the 12 h dark period. The CO_2 level was about 380 $\mu\text{mol mol}^{-1}$, the relative humidity was set at 65%, and extra SON-T light was switched on when global solar radiation intensity outside the greenhouse was $<400 \text{ W m}^{-2}$ and then switched off once it exceeded 500 W m^{-2} . Pre-germinated seeds of the 13 genotypes were sown on sand beds twice (on 8 and 15 June 2010, respectively), to extend flowering and grain-filling

Fig. 1. Graphical genotypes of the eleven introgression lines in this study. The length of each linkage group is shown in centiMorgans (cM). The light blue regions indicate the introgression regions from the donor parent Haogelao; the red regions indicate the homozygous regions from the recurrent parent Shennong265. The figure was drawn using software GGT 2.0 (van Berloo, 2008). The seven regions responsible for variation of photosynthesis parameters (R_d , K_{2LL} , J_{max} , θ , δ_s , V_{cmax} , and δ_s ; see Abbreviation section for definitions), identified from regression analysis using Equation (14) with additive effects (i.e. a_1 , a_2 , a_3 , a_4 , a_5 , a_6 , and a_7), are indicated (vertical lines). Parameters on which genome alleles from Haogelao have positive effects and negative effects are shown in red and blue, respectively. (This figure is available in colour at JXB online.)

periods for a long enough time window of measurement. Seedlings were then transferred to containers (40 cm long $\times$ 30 cm wide $\times$ 20 cm high) in hydroponic culture using half-strength Hoagland's solution, according to a completely randomized block design. Sixteen plants of each genotype were grown with 7.5 $\times$ 7.5 cm² spaces between the plants. One week before flowering, eight plants per genotype were exposed to a moderate water stress [comparable to the stress level in the field experiment of Gu *et al.* (2012)], induced by adding 12.5% polyethylene glycol (PEG-8000) to the growth solution (Money, 1989). The stress was imposed continuously on plants until all measurements were completed. The remaining eight plants per genotype were maintained under non-stressed condition. The flowering period of the 13 genotypes of the two sowings lasted from the 15 August until 3 September. Measurements were conducted at flowering and at grain filling (~14 days after flowering). Therefore, there were four stage $\times$ treatment combinations, namely flowering/drought-stressed treatment (FS), flowering/well-watered treatment (FW), grain filling/drought-stressed treatment (GS), and grain filling/well-watered treatment (GW), for the measurements, as described below.

Gas exchange and chlorophyll fluorescence measurements

The flag leaves on the main stems of four representative plants (out of eight) per treatment of each genotype were used for measurements (except for IL42 at FS, because of a labour peak, as flowering of the late IL42 coincided with grain filling of some of the earlier genotypes). We used an open gas exchange system (Li-Cor 6400; Li-Cor Inc., Lincoln, NE, USA) and an integrated fluorescence chamber head (Li-Cor 6400-40; Li-Cor Inc., Lincoln, NE, USA) to simultaneously measure gas exchange and chlorophyll fluorescence parameters at 21% O₂. All measurements were made at a leaf temperature of 25 °C and a leaf-to-air vapour pressure difference (VPD) of 1.0–1.6 kPa. For C_i response curves, C_a was increased stepwise: 50, 60, 70, 80, 100, 150, 250, 380, 650, 1000, and 1500 $\mu\text{mol mol}^{-1}$, while keeping light intensity (I_{inc}) at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For the I_{inc} response curves, photon flux densities were in an increasing series: 10, 30, 50, 70, 100, 170, 500, 1000, 1500, and 2000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, while keeping C_a at 380 $\mu\text{mol mol}^{-1}$.

To properly estimate photosynthetic parameters, we also conducted measurements using a 2% O₂ gas mixture: a gas cylinder containing a mixture of 2% O₂ and 98% N₂ was used to blend with pure CO₂ to produce 2% O₂ in the leaf chamber. Under this condition, only the first half of the light or CO₂ response curves were measured: for C_i response curves, I_{inc} was kept at 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and C_a was increased stepwise: 50, 60, 70, 80, 100, and 150 $\mu\text{mol mol}^{-1}$; for I_{inc} response curves, I_{inc} was increased in the order of 10, 30, 50, 70, 100 and 170 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and this half curve of the light response was obtained using 2% O₂ combined with 1000 $\mu\text{mol mol}^{-1} C_a$ to ensure a non-photorespiratory

condition. Light and CO₂ responses for the two O₂ levels were measured on the same leaves.

Leaf respiration in darkness (R_{dk}) was measured ~15 min after leaves had been placed in darkness. For measurements at each irradiance or CO₂ step, A was allowed to reach the steady state, after which F_s (the steady-state fluorescence) was recorded. Next, a saturating light pulse (>8500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 0.8 s) was applied to determine F'_m (the maximum fluorescence during the saturating light pulse). The apparent PSII e^- transport efficiency was obtained as the following (Genty *et al.*, 1989) for each irradiance or CO₂ step:

$$\Phi_2 = \frac{F'_m - F_s}{F'_m}$$

Leakage of CO₂ into and out of the leaf cuvette was corrected for all gas exchange data, using heat-killed leaves according to Flexas *et al.* (2007b).

Leaf nitrogen content measurements

Photosynthesis measurements at the two stages were made on the same leaf positions. After measurements at grain filling, the portion of the flag leaves used for the above-described measurements was cut. The leaf material was weighed after drying at 70 °C to a constant weight, and LMA (g dry matter m⁻² of leaf) was determined. The fraction of total N in leaves was analysed using an element analyser based on the micro-Dumas combustion method. From these data, N_a (g N m⁻² of leaf) was calculated.

Model analysis

We use the model of Farquhar, von Caemmerer and Berry (1980) (FvCB model). The net CO₂ assimilation (A) is expressed as the minimum of the Rubisco limited rate (A_c) and the electron transport limited rate (A_j):

$$A = \min(A_c, A_j) \quad (1)$$

A_c is described, following Michaelis–Menten kinetics, as:

$$A_c = \frac{(C_c - \Gamma_s)V_{cmax}}{C_c + K_{mc}\left(1 + \frac{O}{K_{mo}}\right)} - R_d \quad (2)$$

where C_c and O are the CO₂ and O₂ levels at the carboxylation sites of Rubisco, V_{cmax} is the maximum rate of carboxylation, K_{mc} and K_{mo} are

Michaelis–Menten constants of Rubisco for CO₂ and O₂, respectively, and Γ^* is the CO₂ compensation point in the absence of day respiration (R_d). In the model, $\Gamma^* = 0.5O/S_{c/o}$. As the constants K_{mc} and K_{mo} are generally conservative for C₃ plants (von Caemmerer, 2000), their values were taken from Bernacchi *et al.* (2002).

A_j is described by:

$$A_j = \frac{(C_c - \Gamma^*)J}{4C_c + 8\Gamma^*} - R_d \quad (3)$$

where J is the potential PSII e⁻ transport rate that is used for CO₂ fixation and photorespiration, and can be described by the following (Ögren and Evans, 1993; von Caemmerer, 2000; Yin *et al.*, 2009):

$$J = \frac{\kappa_{2LL} I_{inc} + J_{max} - \sqrt{(\kappa_{2LL} I_{inc} + J_{max})^2 - 4\theta J_{max} \kappa_{2LL} I_{inc}}}{2\theta} \quad (4)$$

where κ_{2LL} is the conversion efficiency of incident light into J at strictly limiting light, J_{max} is the maximum value of J under saturated light, and θ is the convexity factor.

Model parameters were estimated according to the procedure described by Yin *et al.* (2009). Specifically, using data of the e⁻ transport-limited range under non-photorespiratory conditions (i.e. the irradiance response curve at 2% O₂ combined with 1000 μmol mol⁻¹ C_a), a simple linear regression can be performed for the observed A against $(I_{inc}\Phi_2/4)$. The slope of the regression yields the estimate of a lumped parameter s , and the intercept gives an estimate of R_d (Yin *et al.*, 2009, 2011). This allowed the actual rate of linear electron transport to be calculated as:

$$J = sI_{inc}\Phi_2 \quad (5)$$

The parameters J_{max} , κ_{2LL} , and θ were estimated by fitting Equation (4) to the calculated J .

$S_{c/o}$ was calculated by following the procedure described by Yin *et al.* (2009); see their Equation 10). Once all the above parameters were estimated, their values were used as input to the model described below, upon which V_{cmax} and coefficients related to diffusional conductances were estimated.

Modelling of g_m and g_s

To examine any variation in mesophyll conductance (g_m) in response to C_i and irradiance (at 21% O₂), the variable J method (Harley *et al.*, 1992b) was first applied:

$$g_m = \frac{A}{C_i - \frac{\Gamma^* [J + 8(A + R_d)]}{J - 4(A + R_d)}} \quad (6)$$

where A and C_i were taken from gas exchange measurements and J was calculated by Equation (5). This first analysis showed that g_m was variable (see Results). We therefore used a phenomenological equation of Yin *et al.* (2009) to model g_m :

$$g_m = g_{mo} + \frac{\delta_m (A + R_d)}{C_c - \Gamma^*} \quad (7)$$

where g_{mo} is the minimum mesophyll conductance if the irradiance approaches zero, δ_m is the coefficient that defines the C_c/C_i relationship under saturating light as: $(C_c - \Gamma^*)/(C_i - \Gamma^*) = 1/(1 + 1/\delta_m)$ (Yin *et al.*, 2009).

Combining Equation (7) with Equations (2) and (3) and replacing C_c with $(C_i - A/g_m)$ yields (Yin *et al.*, 2009):

$$A_c \text{ or } A_j = \frac{-b - \sqrt{b^2 - 4ac}}{2a} \quad (8)$$

where

$$\begin{aligned} a &= x_2 + \Gamma^* + \delta_m (C_i + x_2) \\ b &= -(x_2 + \Gamma^*)(x_1 - R_d) + (C_i + x_2)[g_{mo}(x_2 + \Gamma^*) + \delta_m (x_1 - R_d)] \\ &\quad + \delta_m [x_1(C_i - \Gamma^*) - R_d(C_i + x_2)] \\ c &= [g_{mo}(x_2 + \Gamma^*) + \delta_m (x_1 - R_d)][x_1(C_i - \Gamma^*) - R_d(C_i + x_2)] \end{aligned}$$

$$\text{with } x_1 = \begin{cases} V_{cmax} & \text{for } A_c \\ J/4 & \text{for } A_j \end{cases}$$

$$\text{and } x_2 = \begin{cases} K_{mc} \left(1 + \frac{O}{K_{mo}}\right) & \text{for } A_c \\ 2\Gamma^* & \text{for } A_j \end{cases}$$

The above model analysis showed that the pattern of variation of g_m is similar to that of g_s in response to CO₂ and irradiance levels (see Results). Therefore, Equation (8) is also valid to model the dynamics of an overall conductance (g_i) if places for C_i are replaced with C_a and those for δ_m are replaced with δ_i :

$$g_i = g_{to} + \frac{\delta_i (A + R_d)}{C_c - \Gamma^*} \quad (9)$$

where g_{to} is the minimum overall conductance and δ_i is the coefficient defining the C_c/C_a relationship under saturating light as: $(C_c - \Gamma^*)/(C_a - \Gamma^*) = 1/(1 + 1/\delta_i)$. Note that $g_i = 1/(1/g_s + 1/g_m)$, and that the value of C_a for our model analysis was adjusted to the CO₂ level at the leaf surface according to the boundary-layer conductance.

Assuming both g_{mo} and $g_{to} = 0$ (which is generally the case; see Results), and dividing Equation (7) by Equation (9), the following expression is obtained:

$$\frac{g_m}{g_s} = \frac{\delta_m}{\delta_i} - 1 \quad (10)$$

Equation (10) quantitatively indicates an overall relative limitation of g_m versus g_s to photosynthesis. From Equation (10), an equation for g_s is derived here:

$$g_s = \frac{\delta_s (A + R_d)}{C_c - \Gamma^*} \quad (11)$$

where $\delta_s = \delta_m \delta_i / (\delta_m - \delta_i)$.

Once A is calculated from Equation (8), g_m can be calculated using the equation obtained by replacing C_c in Equation (7) with $(C_i - A/g_m)$, and then by solving the equations for g_m (Yin *et al.*, 2009):

$$g_m = \frac{A + \delta_m (A + R_d)}{C_i - \Gamma^*} \quad (12)$$

Similarly, g_i can be calculated using the equation obtained by replacing C_c in Equation (9) with $(C_a - A/g_i)$ and then solving the equations for g_i :

$$g_i = \frac{A + \delta_i (A + R_d)}{C_a - \Gamma^*} \quad (13)$$

To allow comparisons across genotype × treatment × stage combinations, we also estimated the value of g_m as constant, using the so-called NRH-A method [$g_{m(NRH-A)}$], based on data obtained from high C_i of CO₂ response curves and low I_{inc} levels of light response curves at 21% O₂.

The rationale for this method and the choice of data has been discussed fully by Yin and Struik (2009). This estimate using the NRH-A method should represent the average value of g_m within its lower range of the variation.

Ideotype design

The 13 lines used in this study were selected based on the QTLs detected by single-point analysis (Gu *et al.*, 2012). In order to quantify the additive effect of the QTLs on each parameter in our model, a statistical covariant model was used, in which the value of a parameter X of introgression line k , containing N QTLs (as represented by the nearest marker loci), for a specific stage (S_i) $\times$ treatment (T_j) combination was presented as:

$$X_{ijk} = \mu + S_i + T_j + \sum_{n=1}^N a_n \times M_{k,n} + e_{ijk} \quad (14)$$

where μ is the intercept; S_i is growth stage effect, which stands for either of the two stages (flowering or grain filling); T_j is the treatment effect, which stands for either well watered or drought stressed; a_n is the additive effect of the n th QTL; $M_{k,n}$ is the genetic QTL scores of the individual introgression line k that take the value either -1 (allele coming from Shennong265) or 1 (Haogelao allele present), and e_{ijk} is an error term.

For the ideotype design, only QTLs with significant enhancing additive effects ($P < 0.05$) were kept in Equation (14). For example, an ideotype for improved photosynthesis was the virtual genotype of which parameter values were estimated as the sum of the allele effects that enhanced A for all QTLs of each FvCB model component. To construct the A response of ideotype to irradiance, estimated parameters were used as inputs in Equation (8). To calculate the TE response to irradiance, the following equation (Farquhar and Richards, 1984) was used:

$$TE = \frac{C_a - C_i}{1.6(e_i - e_a)} \quad (15)$$

where $(e_i - e_a)$ is leaf-to-air VPD and C_i is calculated from our model using values of A and estimates of the parameters δ_m and δ_s .

Statistics and curve fitting

A three-way analysis of variance of genotype $\times$ treatment $\times$ growth stage for the photosynthesis parameters was calculated. Non-linear fitting was carried out using the GAUSS method in PROC NLIN, and multiple linear regression fitting for Equation (14) was performed using PROC GLM, of SAS (SAS Institute Inc., Cary, NC, USA).

Results

Estimates of photosynthesis parameters

The estimated values for $S_{c/o}$ did not differ among genotypes, nor among treatment $\times$ stage combinations; so a single value for $S_{c/o}$ was obtained from the pooled data ($=3.02 \pm 0.03$ mbar μbar^{-1}). As reported by Yin *et al.* (2009), the estimated values for R_d did not differ between 21 and 2% O_2 levels, and a common R_d across the O_2 levels was obtained. However, the estimated values for R_d and s were genotype, treatment, and stage specific (Supplementary material Table S1 at JXB online), and the values of R_d were generally lower than those of R_{dk} (Table S1). After parameter s was estimated, J was obtained from Equation (5) and $J_{\max}$, κ_{2LL} , and θ were then estimated by fitting Equation (4) (see Table S1).

Once values of J , $S_{c/o}$, and R_d were known, we used Equation (6) to evaluate the effects of variations of CO_2 concentration and light intensity on g_m for four stage $\times$ treatment combinations (FS, FW, GS, and GW) of each genotype. In general, g_m strongly declined with an increase in C_i and increased with an increase in light intensity, following the same response as g_s to CO_2 concentration and light intensity; thus, a proportional relationship between g_m and g_s was obtained (Fig. S1 at JXB online). The slope of the proportional relationship, indicating the average $g_m:g_s$ ratio, differed among the stage $\times$ treatment combinations and was higher for drought-stressed plants than for well-watered plants, and for flowering than for grain filling.

The variation of g_m across I_{inc} and across C_i levels was confirmed by the curve-fitting based on Equation (8), as the value of parameter g_{m0} in the equation was found to be close to zero, whereas δ_m was found to vary from 0.452 to 1.571 (a zero g_{m0} combined with a non-zero δ_m would mean that g_m varies with C_i and I_{inc} ; see Yin *et al.*, 2009). This method allowed solving of δ_m and V_{cmax} simultaneously (Table S1; Fig. 2), when using the earlier estimated $S_{c/o}$, R_d , $J_{\max}$, θ , and κ_{2LL} as inputs. In this method, a universal parameter, δ_m (rather than specific g_m values), across whole photosynthesis light- and CO_2 -response curves was estimated. A further analysis based on Equation (9) also showed that parameter g_{t0} did not differ significantly from zero ($P > 0.05$). Therefore, an overall $g_m:g_s$ ratio (Equation 10) was calculated for each introgression line at each stage $\times$ treatment combination (Fig. 2D). The overall average $g_m:g_s$ ratio obtained from this method for most of the stage $\times$ treatment combinations (Table S1) was slightly higher than those values shown in Fig. S1, probably because the variable J method assumes no alternative e^- transport, whereas the curve-fitting method does account for any alternative e^- transport (Yin *et al.*, 2009).

Components of variation in and correlations among photosynthetic parameters

The variation in each estimated photosynthetic parameter can be statistically partitioned into genetic, environmental (stress versus non-stress), and developmental (i.e. flowering versus grain filling) components, and their two-way interactions. However, as most interactions were not significant ($P > 0.05$; results not shown), we omitted all interaction terms (Table 1). Significant genetic differences were found for R_d , κ_{2LL} , $J_{\max}$, θ , and V_{cmax} as well as for δ_s and the $g_m:g_s$ ratio ($P < 0.05$; Table 1, Fig. 2), although environmental and developmental components contributed most to the variation in most parameters (Table 1).

The parameters of the photosynthesis model were partly correlated (Table S2). In particular, the correlations between $J_{\max}$ and V_{cmax} , δ_m and V_{cmax} , δ_m and δ_s were significant in each stage $\times$ treatment combination ($P < 0.05$). These correlations may suggest that these traits are, at least partly, under common genetic control.

Physiological basis of the genetic variation

Significant genetic differences of some model parameters ($P < 0.05$, Table 1, Fig. 2) hinted a physiological basis for genetic

Fig. 2. Values of photosynthesis parameters estimated for flag leaves of introgression lines, including two parents, Haogelao (H) and Shennong265 (S), at four stage $\times$ treatment combinations: FS (filled bars); FW, striped bars; GS, open bars; GW, hatched bars). (A) maximum rate of Rubisco activity-limited carboxylation (V_{cmax}); (B) maximum value of electron transport rate used for NADP^+ reduction (J_{max}); (C) mesophyll conductance [$g_{\text{m(NRH-A)}}$],

variation in A found earlier by Gu et al. (2012), who identified QTLs for light-saturated A (A_{max}) under field conditions.

Using our model approach, A_{max} can be dissected into four physiological components: g_s , g_m , electron transport, and Rubisco activity. To quantitatively analyse the effects of each component, A_{max} (at $380 \mu\text{mol mol}^{-1} \text{CO}_2$, $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance, 25°C , and 1.5 kPa VPD) was first plotted against each component here. Within each stage $\times$ treatment combination, the correlation between A_{max} and each component (g_s , g_m , J_{max} , and V_{cmax}) could be observed (Fig. S2 at JXB online), providing the evidence about where genetic differences in A_{max} possibly came about. In order to quantify the main sources of genetic variation in A_{max} , a multiple regression analysis was carried out (Table 2). For each stage $\times$ treatment combination, the genetic variation in g_s and g_m had the largest impact on the genetic variation in A_{max} . Under well-watered treatment, g_m caused more genetic variation in A_{max} than g_s did, while under drought-stressed treatment, g_s accounted for more genetic variation.

We also analysed TE under the same measurement conditions (Fig. S3 at JXB online). When we inspected the relationship within each stage $\times$ treatment combination, the correlation appeared very weak, except for g_s (Fig. S3A) and $g_m:g_s$ (Fig. 3). Multiple regression analysis (Table 2) also showed that genetic variation in g_s and g_m , relative to that in V_{cmax} and J_{max} , contributed more to TE in this genetic background, and, not surprisingly, g_m and g_s affected TE in the opposite direction.

Physiological basis of a major photosynthesis QTL

Of the ILs used, IL161 is unique in that it has the background of the recurrent parent Shennong265 except for a single introgression segment on chromosome 9 from the donor parent (Fig. 1). Compared with the recurrent parent, IL161 significantly increased A_{max} across stages and treatments; thus, a major QTL was consistently detected for A_{max} on chromosome 9 (Gu et al., 2012). CO_2 and light response curves measured in the present study indicated that the QTL contributed to a higher photosynthesis rate across all irradiance and CO_2 levels (Fig. 4). Through our analysis, seven parameters of both IL161 and Shennong265 were estimated for each stage $\times$ treatment combination (Table 3). There was no significant difference between them for R_d , $\kappa_{2\text{LL}}$, and θ ($P > 0.05$). At flowering, IL161 showed significantly higher g_m , g_s , V_{cmax} and J_{max} than Shennong265 across the two treatments. At grain filling, however, only higher diffusional conductance (larger g_m and g_s) could be the reason for higher A , as V_{cmax} was even lower in IL161 than in Shennong265 for the stress treatment (Table 3). Therefore, there was a greater difference between IL161 and Shennong265 at flowering than at grain filling (Fig. 4). Our whole-curve measurements are consistent with the results of Gu et al. (2012) that larger additive effects of the QTL on A_{max} were obtained at flowering than at grain filling.

calculated by the NRH-A method of Yin & Struik (2009); (D) mesophyll conductance:stomatal conductance ratio ($g_m:g_s$), calculated by Equation (10); (E) ratio of $J_{\text{max}}:V_{\text{cmax}}$.

Table 1. A three-way analysis of variance of genetic effect versus treatment versus growth stage for the estimated photosynthesis parameters

Parameters		F value (probability of significance)		
		Genetic effect	Treatment	Stage (ontogeny)
Primary parameters of the model	R_d	4.82 (<0.0001)	3.06 (0.0721)	15.45 (0.0004)
	κ_{2LL}	6.14 (<0.0001)	9.34 (0.0042)	38.93 (<0.0001)
	J_{max}	2.00 (0.0494)	0.00 (0.9850)	80.19 (<0.0001)
	θ	6.09 (<0.0001)	9.78 (0.0035)	100.85 (<0.0001)
	δ_m	1.69 (0.1110)	5.55 (0.0241)	1.99 (0.1671)
	V_{cmax}	2.44 (0.0191)	6.96 (0.0122)	29.21 (<0.0001)
	δ_t	1.70 (0.1090)	20.65 (<0.0001)	0.10 (0.7587)
Other parameters	δ_s	2.39 (0.0218)	53.46 (<0.0001)	9.46 (0.0040)
	$g_{m(NRH-A)}$	0.82 (0.6314)	9.39 (0.0041)	50.24 (<0.0001)
	$g_m:g_s$	2.79 (0.0085)	19.27 (<0.0001)	31.38 (<0.0001)
	$J_{max}:V_{cmax}$	1.49 (0.1721)	25.85 (<0.0001)	1.88 (0.1787)

F and P values significant at a level of $P < 0.05$ are shown in bold.

Table 2. Multiple linear regression analysis of light-saturated photosynthesis (A_{max}) or TE as a function of g_s , g_m , J_{max} , and V_{cmax} (i.e. A_{max} or $TE = b_0 + b_1g_s + b_2g_m + b_3J_{max} + b_4V_{cmax}$), based on data of 11 introgression lines and their parents, for each stage×treatment combination

Trait	Stage × treatment	Intercept (b_0)	Regression coefficient (probability of significance)			
			b_1	b_2	b_3	b_4
A_{max}	FS	1.21	46.99 (4.4×10^{-5})¹	27.24 (3.0×10^{-4})²	0.04 (0.0058)³	0.00 (0.6866) ⁴
	FW	1.26	31.19 (1.3×10^{-5})²	32.82 (4.9×10^{-6})¹	0.02 (0.0857) ⁴	0.02 (0.0260)³
	GS	0.63	30.85 (7.0×10^{-6})¹	46.59 (1.5×10^{-7})²	0.04 (0.0001)³	0.00 (0.9929) ⁴
	GW	1.39	22.45 (7.7×10^{-5})²	53.15 (2.8×10^{-5})¹	0.00 (0.8682) ⁴	0.04 (0.0147)³
TE	FS	6.02	-20.70 (6.7×10^{-7})¹	6.33 (0.0003)²	0.01 (0.0024)³	0.00 (0.1739) ⁴
	FW	4.29	-15.77 (1.1×10^{-6})¹	8.11 (8.0×10^{-5})²	0.00 (0.3442) ⁴	0.01 (0.0523) ³
	GS	4.84	-21.31 (1.0×10^{-7})¹	12.89 (0.0002)²	0.01 (0.0595) ³	0.00 (0.7278) ⁴
	GW	4.47	-13.21 (3.2×10^{-7})¹	10.00 (0.0005)²	0.00 (0.4847) ⁴	0.01 (0.0394)³

^{1, 2, 3, 4} The comparative importance of each parameter, determined from the level of significance. Coefficient values significant at a level of $P < 0.05$ are in bold.

Fig. 3. Relationship between transpiration efficiency (TE) ($380 \mu\text{mol mol}^{-1} \text{CO}_2$, $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 25°C , and 1.5 kPa VPD) and ratio of mesophyll conductance and stomatal conductance ($g_m:g_s$). Linear regressions were fitted for overall data (solid grey line) and each stage×treatment combination: FS (circles and dotted line), FW (squares and dashed line), GS, (triangles and dashed line), and GW (diamonds and dotted-dashed line). The significance of each correlation is indicated: ** $P < 0.01$.

Relationships between photosynthesis parameters and leaf morpho-physiological characteristics

The variation in $g_{m(NRH-A)}$, either across genotypes or across treatments, was negatively correlated with LMA (Fig. 5A). Similar relationships were found between LMA and g_m or g_s calculated for the condition of measuring A_{max} , despite lower r^2 values (results not shown). As expected, drought stress induced thicker leaves (increased LMA, Fig. 5A) and the increased LMA led to an increased N_a ($r^2=0.40$). However, there was a poor correlation between $g_{m(NRH-A)}$ and N_a ($r^2=0.08$; Fig. 5B). Instead, the variation in J_{max} and V_{cmax} , either across genotypes or across water-supply treatments, was found to be positively correlated with N_a (Fig. 6A, 6B), but less correlated with LMA (results not shown). Analysis with an F test demonstrated that generally there was no significant difference between well-watered and drought-stressed plants at grain filling on the relationships shown in Figs 5 and 6 ($P > 0.05$), although the slope of the relationship between J_{max} and N_a was significantly lower ($P=0.012$) for plants under drought conditions.

Fig. 4. Photosynthesis response curves of IL161 (squares) and Shennong265 (circles) under 21% O₂ at four stage × treatment combinations: FS (A, B), FW (C, D), GS (E, F), and GW (G, H). The curves are drawn from the model using fitted parameter values: dashed lines for IL161 and solid lines for Shennong265. Left panels (A, C, E, and G) show the response of net photosynthesis *A* to ambient CO₂ (*C*_a) under a light intensity of 1000 μmol m⁻² s⁻¹. Right panels (B, D, F, and H) show the response of photosynthesis *A* to light intensity under 380 μmol mol⁻¹ CO₂. Values are means ± SD (*n* = 4).

Ideotype design based on physiological understanding

Given the significant genetic difference in each of the model component traits (Table 1, Fig. 2) and their significant effects on *A*_{max} and TE (Table 2, Fig. S2 and S3), it was considered worthwhile exploring the potential to improve *A* and TE using the genetic variation observed. We therefore estimated the additive effects of individual genome loci, based on Equation (14). Of the loci differing among the ILs, seven loci were identified as significantly affecting the seven primary model parameters (Fig. 1, Table 4). These seven loci were also identified or in close proximity to

those mapped for *A*_{max} using the whole IL population (Gu *et al.*, 2012), suggesting that our selected 11 ILs did represent the population well. There was no one-to-one locus–parameter relationship. Instead, each model parameter was controlled by one to three loci, and most loci had an effect on multiple parameters (Table 4), providing a genetic basis of significant correlations between model parameters in Table S2.

The ideotype for high *A* requires high *g*_s and *g*_m and improved photosynthetic efficiency (*κ*_{2LL} and *θ*) and capacities (*V*_{cmax} and *J*_{max}), while the ideotype for high TE requires low *g*_s, high *g*_m, and improved photosynthetic efficiency and capacities. Thus, the

Table 3. Parameter values ($\pm$ SE of the estimate) of the photosynthesis model, estimated for IL161 and Shennong265 (S) that differ in a single introgression region on chromosome 9 (see Gu *et al.*, 2012), for the four stage-treatment combinations

Environment	Genotype	Model parameters					Other derived parameters		
		R_d	K_{2LL}	J_{max}	θ	V_{cmax}	δ_m	δ_t	g_s^a g_m^b
FS	IL161	0.669 $\pm$ 0.193	0.344 $\pm$ 0.044	214.8$\pm$14.9*	0.790 $\pm$ 0.151	213.3$\pm$12.0*	0.524$\pm$0.019*	0.267 $\pm$ 0.008	0.145
	S	0.539 $\pm$ 0.177	0.321 $\pm$ 0.029	174.6 $\pm$ 7.6	0.806 $\pm$ 0.098	173.3 $\pm$ 14.5	0.572 $\pm$ 0.031	0.279 $\pm$ 0.013	0.120
FW	IL161	0.474 $\pm$ 0.175	0.332 $\pm$ 0.033	222.7$\pm$13.5*	0.744 $\pm$ 0.136	204.0$\pm$15.4*	0.697$\pm$0.036*	0.401$\pm$0.016*	0.221
	S	0.407 $\pm$ 0.139	0.326 $\pm$ 0.038	151.1 $\pm$ 8.1	0.764 $\pm$ 0.143	120.7 $\pm$ 7.2	1.044 $\pm$ 0.103	0.574 $\pm$ 0.032	0.163
GS	IL161	0.317 $\pm$ 0.147	0.322 $\pm$ 0.053	153.2 $\pm$ 10.4	0.863 $\pm$ 0.137	133.4$\pm$4.6*	0.767$\pm$0.039*	0.450$\pm$0.016*	0.190
	S	0.365 $\pm$ 0.124	0.316 $\pm$ 0.020	151.4 $\pm$ 3.9	0.852 $\pm$ 0.054	141.1 $\pm$ 5.5	0.694 $\pm$ 0.035	0.376 $\pm$ 0.012	0.186
GW	IL161	0.059 $\pm$ 0.235	0.297 $\pm$ 0.031	143.9 $\pm$ 6.0	0.884 $\pm$ 0.078	101.5 $\pm$ 4.7	1.340 $\pm$ 0.158	0.723$\pm$0.026*	0.146
	S	-0.147 $\pm$ 0.172	0.312 $\pm$ 0.050	142.2 $\pm$ 9.7	0.823 $\pm$ 0.160	102.0 $\pm$ 4.9	1.211 $\pm$ 0.139	0.635 $\pm$ 0.021	0.200
									0.173

^a g_s and ^b g_m were derived from the fitted model at saturated light of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, CO_2 concentration of 380 $\mu\text{mol mol}^{-1}$.

* Statistically significantly different between IL161 and the recurrent parent Shennong265 (S) ($P < 0.05$).

Fig. 5. Relationship between (A) mesophyll conductance [$g_{m(NRH-A)}$] calculated by the non-rectangular hyperbolic method (Yin & Struik, 2009) and LMA; (B) $g_{m(NRH-A)}$ and leaf nitrogen per unit area (N_a). Values are means $\pm$ SD of four replicates. Linear regressions were fitted for overall data (solid grey lines) and each stage $\times$ treatment combination: GS (triangles and dashed lines) and GW (diamonds and dotted-dashed lines). The significance of each correlation is indicated: * $P < 0.05$; ** $P < 0.01$.

Fig. 6. Relationship between (A) electron transport capacity (J_{max}) and leaf nitrogen content (N_a), and (B) Rubisco carboxylation capacity (V_{cmax}) and N_a . Symbols and significance levels are as described in Fig. 5.

ideotype of high A carried the alleles having positive effects on κ_{2LL} , J_{max} , θ , δ_m , δ_s , and V_{cmax} , and negative effects on R_d , whereas the ideotype of high TE carried the alleles having positive effects on κ_{2LL} , J_{max} , θ , δ_m , and V_{cmax} , and negative effects on δ_s and R_d . The ideotype of high A showed an increase in A of 15.2% (FS), 15.5% (FW), 20.6% (GS), and 17.1% (GW) compared with the mean A of the 13 ILs (Fig. 7, solid lines), while the ideotype of high TE showed an increase of 32.2% (FS), 14.8% (FW), 26.1% (GS), and 17.3% (GW) compared with the mean TE of the 13 ILs (Fig. 8, solid lines).

The above estimated improvement in A or TE was moderate, because the same alleles at some loci had contradicted effects on different photosynthesis parameters (Table 4). Assuming that these contradicted effects were not due to pleiotropy, but rather to tight gene linkage that could be broken through further rounds of introgression and a higher density marker map to develop near-isogenic lines carrying fine-mapped QTLs, we evaluated virtual ideotypes for A and TE that contained only positive effects in all the photosynthesis parameters. For A , this virtual ideotype showed an average improvement of 29.9% (FS), 29.3% (FW), 36.4% (GS), and 34.5% (GW) compared with the mean A of the 13 ILs (Fig. 7, dotted lines). For TE, the virtual ideotype showed an average improvement of 46.9% (FS), 28.2% (FW), 42.0% (GS), and 31.6% (GW) when compared with the mean TE of the 13 ILs (Fig. 8, dotted lines). When compared with the best genotype we investigated in each stage $\times$ treatment combination, for A , the virtual ideotype

showed an improvement of 11.0% (FS), 9.6% (FW), 18.7% (GS), and 28.5% (GW); for TE, the virtual ideotype showed an improvement of 38.3% (FS), 12.3% (FW), 33.9% (GS), and 15.8% (GW).

The above analysis examined the ideotypes for A and TE separately. To explore the potential of selecting a genotype with both improved TE and photosynthesis, A_{max} was plotted against TE (Fig. 9). There were negative correlations for all the stage $\times$ treatment combinations, and the negative correlations were more significant under drought conditions than in a well-watered environment. These relationships suggested that simultaneous improvement of A and TE is difficult, especially under drought conditions. The opportunities for simultaneous selection for improved A and TE are discussed below.

Discussion

Physiological basis of genetic variation in photosynthesis

Our model approach allowed to quantitatively dissect photosynthesis into different physiological components: g_s , g_m , and biochemical efficiency (κ_{2LL} , θ) and biochemical capacity (J_{max} and V_{cmax}). In our analysis, most of the model parameters showed significant genetic differences (Table 1). For example, parameters κ_{2LL} and θ both affected the electron transport efficiency under limited light. Thus, the genetic variation in κ_{2LL} and θ (Table 1)

Fig. 7. Constructed response curve of net photosynthetic rate (A) to light intensity at ambient CO_2 concentration ($380 \mu mol mol^{-1}$) at four stage $\times$ treatment combinations: FS (A), FW (B), GS (C), and GW (D). The rate of photosynthesis of the 13 lines (circles, values are means $\pm$ SD of the 13 lines) were calculated from the model using fitted parameter values. The ideotype response (solid lines) and the potential virtual ideotype curves (dotted lines) of photosynthesis were drawn using parameter values, which were calculated by methods described in the Materials and methods and Results sections.

Fig. 8. Constructed transpiration efficiency TE (red) and photosynthesis (A ; green) response curve to light intensity at four stage×treatment combinations: FS (A), FW (B), GS (C), and GW (D). All data were estimated at $380 \mu\text{mol mol}^{-1} \text{CO}_2$ and 1.5 kPa VPD . The TE (red circles, values are means $\pm$ SD) and rate of photosynthesis (green circles, values are means $\pm$ SD) and of 13 lines were calculated from the model using fitted parameter values. The ideotype response (solid red lines), the potential virtual ideotype curves (dotted red lines) of TE values, and corresponding A of the virtual ideotype (dotted green lines) were drawn. These curves were drawn using parameter calculated by methods described in the Materials and methods and Results sections. (This figure is available in colour at JXB online.)

Fig. 9. Relationships between TE and light-saturated net photosynthesis rate (A_{max}) (at $380 \mu\text{mol mol}^{-1} \text{CO}_2$, $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 25°C , and 1.5 kPa VPD). Symbols and accessions as follows: IL7 (upright filled triangles), IL37 (upright open triangles), IL42 (filled squares), IL69 (open squares), IL84 (filled circles), IL100 (open circles), IL130 (filled diamonds), IL157 (open diamonds), IL159 (upside-down filled triangles), IL161 (upside-down open triangles), IL164 (circle/cross), Haogelao (diamond with line), and Shennong265 (egg-timer shape). To distinguish between stage×treatment combinations, different colours were used: FS (red); FW (green); GS (purple); and GW (blue). The linear regression lines (solid lines) were fitted for each stage×treatment combination. The diagonal dashed line was

could potentially be used to improve photosynthetic efficiency before light intensity reaches saturation.

As our previous analysis identified QTLs for A_{max} (Gu *et al.*, 2012), we specifically analysed the relative contribution of photosynthesis parameters (g_s , g_m , V_{cmax} , and J_{max} ; Table 2) relevant for the condition under which A_{max} was measured. The value of g_s was found to be most associated with genetic variation in A_{max} in our IL population (Table 2, Fig. S2A) under drought conditions. This was in line with reported results showing that mapped QTLs of net photosynthesis (Adachi *et al.*, 2011) were related to g_s . These results are not surprising, given that g_s controls diffusion of CO_2 from ambient air into intercellular airspace and that stomata have evolved into physiological control mechanisms to maximize carbon gain while minimizing water loss (Lawson *et al.*, 2011). However, g_m was also important for the expression of genetic variation in A_{max} (Table 2, Fig. S2B). In fact, under well-watered conditions, g_m contributed most to the genetic variation in A_{max} (Table 2).

We found that V_{cmax} and J_{max} contributed comparatively less to genetic variation in A_{max} in each stage×treatment combination (Table 2). This is surprising, given that V_{cmax} and J_{max} reflect

fitted for all the stage×treatment combinations, when forcing the regression line to go through the origin. This dashed line shows the trend line for both high TE and A_{max} . (This figure is available in colour at JXB online.)

Table 4. Effects of growth stages (S_f , flowering; S_g , grain filling), treatments (T_s , drought stressed; T_w , well watered), and additive effects of QTLs (i.e. a_1 , a_2 , a_3 , a_4 , a_5 , a_6 , and a_7) on seven modelled traits. The results were estimated from regression analysis using Eqn (14): $X_{ijk} = \mu + S_i + T_j + \sum_{n=1}^N a_n \times M_{k,n} + e_{ijk}$. QTL positions and their additive effect coefficients are shown in Fig. 1. Empty cells in this table indicate that the corresponding effect was not significant ($P > 0.05$) and was therefore not included in the regression model.

Trait	Intercept	Growth stage		Treatment		Additive effect (a_n) of QTLs ^a						
	μ	S_f	S_g	T_s	T_w	a_1	a_2	a_3	a_4	a_5	a_6	a_7
R_d	0.6071	0.2454	0						0.1240	0.2055		
K_{2LL}	0.3097	0.0219	0	0.0104	0		0.0088		-0.0213			0.0182
J_{max}	151.81	47.81	0				8.42				6.85	
θ	0.8637	-0.0890	0	0.0284	0	0.0277	-0.0304		0.0386			
δ_m	0.8189			-0.1390	0	-0.0890						
V_{cmax}	131.14	37.78	0	18.42	0		12.74				8.84	
δ_s	1.1547	-0.1488	0	-0.3630	0	-0.0899		0.0971		-0.0878		

^a The positive value for the allelic effect from donor parent Haogelao.

Rubisco carboxylation and e^- transport capacities, respectively. The weak correlation between biochemical capacities and A_{max} within each stage $\times$ treatment (Fig. S2D,E) could be due to the small range of variation in V_{cmax} and J_{max} . A comparison of IL161 versus Shengnong265 (whose difference was due to a single introgression on chromosome 9) showed (Table 3) that V_{cmax} and J_{max} together with g_m and g_s did explain the difference in photosynthesis light and CO_2 response curves (Fig. 4), at least for the flowering stage.

It is known that a long-term environmental adaptation results in a change in leaf morphology, and LMA as a morphological trait has a high plasticity in adjusting to environmental conditions (Westoby *et al.*, 2002; Poorter *et al.*, 2009). For example, Pons and Pearcy (1994) showed that plants that switched from a high-light environment to low light can substantially (30–50%) decrease LMA within days. The change in LMA was also shown in our data obtained after the grain-filling stage measurements, where the average LMA for drought-stressed leaves was higher than the average for non-stressed leaves (Fig. 5A). Our results agree with the literature (Flexas *et al.*, 2008; Niinemets *et al.*, 2009; Galmés *et al.*, 2011) that g_m decreases with increasing LMA (Fig. 5A). Interestingly, this relationship also holds for the genetic variation across 13 lines within either stress or non-stress treatment, and the stress treatment did not change the relationship (Fig. 5A). This suggests that LMA plays an important role in the plant's adaptation to environmental conditions as well as in the plant's genotypic strategies within the same environment.

Similar to LMA, N_a also varied between treatments and among genotypes (Fig. 5B). V_{cmax} and J_{max} , rather than g_m or g_s , were linearly correlated with N_a (Figs 5 and 6). Furthermore, V_{cmax} and J_{max} were less correlated with LMA (results not shown). Again, water supply treatments hardly affected these relationships across the 13 genotypes. As V_{cmax} and J_{max} affected genetic variation of A_{max} (Table 2), especially at flowering stage (Table 3), the elevated capacity of nitrogen accumulation in the leaf should be a preferred trait for improving leaf photosynthetic capacity, as suggested in the literature (Peng *et al.*, 1998; Shiratsuchi *et al.*, 2006; Taylaran *et al.*, 2011).

Physiological basis of genetic variation in transpiration efficiency

TE is another important breeding target for drought tolerance (Condon *et al.*, 2002, 2004). Our data showed that genetic variation in g_s was best correlated with genetic variation in TE in our genetic material (Table 2, Fig. S3A). This was in line with reported results that the gene for TE, *ERECTA*, is related to g_s (Masle *et al.*, 2005).

From a theoretical perspective, however, Condon *et al.* (2004) indicated that, under certain environment conditions, TE could be improved not only by lowering g_s but also by higher photosynthetic potential, or a combination of these two. In particular, a greater $g_m:g_s$ ratio results in a higher TE without a negative impact on carboxylation (Barbour *et al.*, 2010; Galmés *et al.*, 2011). We found significant genetic difference for the $g_m:g_s$ ratio in this population ($P < 0.01$; Table 1), and the genetic variation in TE was strongly correlated with the variation in this ratio (Fig. 3). A further improvement of TE may be achieved by improving biochemical activities, resulting in improved A with the same transpiration. An ideal plant in drylands would have low g_s , high g_m , and improved biochemical efficiency (Flexas *et al.*, 2010). However, our data showed little association between TE and V_{cmax} or J_{max} (Table 2, Fig. S3).

Potential of using genetic variation to improve photosynthesis and TE

Our model analysis revealed a strong physiological basis of the genetic variation in photosynthesis and in TE; therefore, the model was used to design ideotypes for an improved A or TE based on their physiological components. This kind of bottom-up approach has been successful in the past for yield component analysis. For example, more insights could be obtained from analysing QTLs or genes for yield components rather than for grain yield per se (Yin *et al.*, 2002), and the component-trait QTLs could be explored to improve yields. Based on this ideotype idea, recent genomic studies have successfully identified genes for

one or a few yield components (reviewed by Miura *et al.*, 2011; Xing and Zhang, 2011). However, very few studies have been performed using the same approach for photosynthesis.

Based on the genetic variation from our study, we could significantly improve A and TE by manipulating alleles of loci influencing different physiological components of photosynthesis (Figs 7 and 8), suggesting that an understanding of the physiological basis of photosynthesis will benefit marker-assisted selection. Some gene linkage limited further improvement. For example, a locus from Shenong265 has positive effects on both g_m and g_s , which will benefit breeding for high A , while it has a contradictory effect for high TE. High g_s will increase photosynthesis at the expense of high transpiration. Any further improvement of these rice ideotypes of our IL background for higher photosynthetic performance and TE requires further steps of marker-assisted selection. For example, further backcrossing using markers is needed to reduce the size of introgression segments and develop near-isogenic lines carrying fine-mapped QTLs to break any gene linkage. Through this approach, a potential improved ideotype could be achieved as shown by the dotted lines in Figs 7 and 8.

Can A and TE be improved simultaneously?

As expected from existing physiological understanding, our data showed general negative correlations between A_{max} and TE among ILs in each stage $\times$ treatment combination (Fig. 9). This agrees with the observation that selection for higher TE often hampers plant growth and results in smaller plants (Blum, 2005). The negative correlations were stronger under drought conditions than in a well-watered environment (Fig. 9), consistent with the result shown in Table 2 that A_{max} was most limited by genetic variation in g_s under drought. Thus, under drought, any genetic variation resulting in decreased g_s will improve TE but will decrease photosynthesis. Under well-watered conditions, however, A_{max} was most limited by genetic variation in g_m (Table 2), so it is comparatively easier to select for higher TE and higher A_{max} . The small R^2 in Fig. 9 on the one hand may reflect the small range of the data set, but on the other may imply the potential extent to select for both higher A_{max} and TE (i.e. selecting genotypes that follow the dashed line in the figure). In ideotype design analysis, we assessed the trade-off between A and TE, and found that improving A would be achieved at the expense of, on average, a 35.3% decrease in TE when comparing the best virtual ideotype of A with the best virtual ideotype of TE. Similarly, the average A would decrease by 12.4% when comparing the best virtual ideotype of TE with the best virtual ideotype of A (data not shown). However, if the linkage between g_m and g_s (as shown by the correlation between δ_m and δ_s in Table S2, and co-location of QTLs of δ_m and δ_s in Table 4 and Fig. 1) could be broken (reflected by the dotted lines in Fig. 8), the best virtual ideotype could have both improved TE (red lines) and A (green lines) compared with the average of ILs (dotted lines versus circles in Fig. 8). Similar results were given by Barbour *et al.*, (2010) for barley varieties, in which variety Dash with a higher g_m and comparatively lower g_s resulted in the highest A and TE across the six varieties examined. Our analysis using ILs highlights the possibility of improving both A and TE within the same genetic background.

Concluding remarks

In this study, combined gas exchange and chlorophyll fluorescence data of CO_2 and light response curves of photosynthesis were measured for two stages on leaves of 13 ILs under moderate drought and well-watered conditions. These curves showed that our previously reported QTLs, especially the major QTL on chromosome 9 (Fig. 4), identified for the condition of A_{max} measurements (Gu *et al.*, 2012), also affected A across all irradiance and CO_2 levels. Using these curves, we estimated seven parameters of a combined conductance–FvCB model as proposed by Yin *et al.* (2009). We then quantitatively dissected photosynthesis into different physiological components: stomatal conductance, mesophyll conductance, and biochemical efficiency and capacity. Our model method, Equation (10), presents a novel approach to quantitatively analyse an overall relative limitation of stomatal versus mesophyll diffusion on photosynthesis of a genotype under a given condition.

Our data and analysis confirmed the literature reports in several areas. Firstly, we confirmed that g_m strongly declined with an increase in C_i and increased with an increase in light intensity, a response to CO_2 concentration and light intensity similar to that of g_s (Centritto *et al.*, 2003; Flexas *et al.*, 2007a; Yin *et al.*, 2009; Douthe *et al.*, 2011). Therefore, there was strict $g_m:g_s$ proportionality (Fig. S1), although independence of g_m on I_{inc} and C_i levels was also found (Tazoe *et al.*, 2009, 2011). Secondly, our results confirmed that there was little significant influence of drought on V_{cmax} and J_{max} ($P > 0.01$), suggesting that no metabolic impairment but increased diffusional resistances happened under moderate drought (Centritto *et al.*, 2003; Grassi & Magnani, 2005; Galmés *et al.*, 2007). Our result of a decrease in J_{max}/V_{cmax} under drought is in line with that of Galle *et al.* (2011), suggesting that drought stress could cause downregulation of linear electron transport (Kohzuma *et al.*, 2009). Thirdly, we confirmed the decrease in photosynthetic parameters with leaf ageing (e.g. Harley *et al.*, 1992a; Ethier *et al.*, 2006; Flexas *et al.*, 2007c). The ageing decreased g_m , R_d , κ_{2LL} , J_{max} , V_{cmax} , and $g_m:g_s$, and increased θ . These changes of parameters may be associated with leaf nitrogen loss through protein degradation as a result of retranslocation of nitrogen to the grains.

However, the main aims of our study were to analyse the effect of genotypes arising from segregation of photosynthetic QTLs detected by Gu *et al.* (2012) and to identify the physiological basis of genetic variation and the QTLs. Although the effects of leaf stage and water supply on photosynthesis were predominant, the effect of genotype was significant enough to allow examination of the physiological basis of the genetic variation by use of the combined conductance–FvCB model. Genetic variation in A_{max} as well as in TE was mainly caused by genetic variation in g_s and g_m (Table 2), in line with significant stomatal and mesophyll limitations when plants face environmental stress (e.g. drought stress; Flexas *et al.*, 2004; Grassi & Magnani, 2005). Thus, more efforts should be focused on g_s and g_m in breeding programmes for improving photosynthesis and TE. Furthermore, the relationships between photosynthetic parameters (g_m , V_{cmax} , and J_{max}) and morpho-physiological measurements (LMA, and N_a), which

were usually found across environmental treatments (e.g. Harley *et al.*, 1992a; Flexas *et al.*, 2008; Galmés *et al.*, 2011), were shown here, for the first time, to be valid for the variation across genotypes of the same genetic background (Figs. 5A and 6). Therefore, variation in photosynthesis due to environmental conditions and the variation in photosynthesis due to genetic variation within the same environment may share common physiological mechanisms.

Based on the genetic variation of physiological components underlying *A* and TE, we explored the ideotype design by constituting alleles that contained loci influencing different components of the physiological process of photosynthesis. The suggested virtual ideotypes could be obtained by more rounds of introgression to break any gene linkage within the genome segments of our present ILs. Model calculation showed that these ideotypes could potentially improve *A* and TE by 17.0 and 25.1%, respectively, compared with the best genotype we investigated. In addition, our analysis using ILs highlights the possibility of improving both *A* and TE simultaneously within the same genetic background. Further experimental data with more ILs, especially under field conditions, can strengthen this conclusion. Of course, improvements in *A* and TE could also be achieved by broadening the genetic background. Recent advances in genome-wide association studies (e.g. Huang *et al.*, 2010) will enhance this approach.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. List of photosynthesis parameter values estimated for the ILs.

Supplementary Table S2. Simple correlation coefficients among seven parameters of the photosynthesis model at four stage $\times$ treatment combinations.

Supplementary Fig. S1. Relationships between stomatal conductance (g_s) and mesophyll conductance (g_m).

Supplementary Fig. S2. Relationships between light-saturated net photosynthesis rate ($A_{\max}$) and (A) stomatal conductance to CO_2 (g_s), (B) mesophyll conductance to CO_2 (g_m), (C) total diffusion conductance to CO_2 , including g_s and g_m (g_t), (D) electron transport capacity ($J_{\max}$), and (E) Rubisco carboxylation capacity ($V_{\max}$).

Supplementary Fig. S3. Relationships between transpiration efficiency (TE) and (A) stomatal conductance to CO_2 (g_s); (B) mesophyll conductance to CO_2 (g_m); (C) electron transport capacity ($J_{\max}$); and (D) Rubisco carboxylation capacity ($V_{\max}$).

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