CSIRO PUBLISHING

Functional Plant Biology
https://doi.org/10.1071/FP17322

High-throughput chlorophyll fluorescence screening of *Setaria viridis* for mutants with altered CO_2 compensation points

Robert A. Coe[A,B,D], *Jolly Chatterjee*[A,*], *Kelvin Acebron*[C,*], *Jacqueline Dionora*[A,*], *Reychelle Mogul*[A], *HsiangChun Lin*[A], *Xiaojia Yin*[A], *Anindya Bandyopadhyay*[A], *Xavier R. R. Sirault*[D], *Robert T. Furbank*[B] *and W. Paul Quick*[A,E,F]

[A]C_4 Rice Centre, International Rice Research Institute (IRRI), Los Baños, Philippines.
[B]ARC Centre of Excellence for Translational Photosynthesis, Research School of Biology, Australian National University, GPO Box 1500, Canberra, ACT 2601, Australia.
[C]IBG-2, Forschungszentrum Jülich (FZJ), Jülich, 52425 Jülich, Germany.
[D]CSIRO Agriculture Flagship, High Resolution Plant Phenomics GPO Box 1500, Canberra, ACT 2601, Australia.
[E]Department of Animal and Plant Sciences, University of Sheffield, Sheffield, S10 2TN, UK.
[F]Corresponding author. Email: w.p.quick@irri.org

Abstract. To assist with efforts to engineer a C_4 photosynthetic pathway into rice, forward-genetic approaches are being used to identify the genes modulating key C_4 traits. Currently, a major challenge is how to screen for a variety of different traits in a high-throughput manner. Here we describe a method for identifying C_4 mutant plants with increased CO_2 compensation points. This is used as a signature for decreased photosynthetic efficiency associated with a loss of C_4 function. By exposing plants to a CO_2 concentration close to the CO_2 compensation point of a wild-type plant, individuals can be identified from measurements of chlorophyll *a* fluorescence. We use this method to screen a mutant population of the C_4 monocot *Setaria viridis* (L.) P.Beauv. generated using N-nitroso-N-methylurea (NMU). Mutants were identified at a frequency of 1 per 157 lines screened. Forty-six candidate lines were identified and one line with a heritable homozygous phenotype selected for further characterisation. The CO_2 compensation point of this mutant was increased to a value similar to that of C_3 rice. Photosynthesis and growth was significantly reduced under ambient conditions. These data indicate that the screen was capable of identifying mutants with decreased photosynthetic efficiency. Characterisation and next-generation sequencing of all the mutants identified in this screen may lead to the discovery of novel genes underpinning C_4 photosynthesis. These can be used to engineer a C_4 photosynthetic pathway into rice.

Additional keywords: C_4 photosynthesis, C_4 rice, forward genetics, high-throughput phenomics.

Received 17 November 2017, accepted 27 March 2018, published online 8 May 2018

Introduction

The introduction of a C_4 photosynthetic pathway into rice could potentially lead to increases in radiation use efficiency and yield of up to 50% (Hibberd *et al*. 2008). The C_4 Rice Consortium (https://c4rice.com, accessed 14 March 2018) is currently investigating the feasibility of engineering the leaf anatomy and biochemistry required to support a two-celled photosynthetic pathway (Kajala *et al*. 2011). To be fully functional, a greater understanding of the efficiencies, mechanisms and genes underpinning the C_4 photosynthetic process is required. To this end, the consortium has been screening large populations of *Sorghum*, *Setaria* and rice for mutants exhibiting a loss or gain of C_4 traits (Feldman *et al*. 2014; Rizal *et al*. 2015, 2017). A major challenge is extending the number and type of traits that can be screened in a rapid and efficient manner.

The inherent differences in the efficiencies of the C_3 and C_4 photosynthetic pathways provide several opportunities for high-throughput screening (Furbank *et al*. 2009). The CO_2 compensation point (Γ) – commonly measured as the intercept of CO_2 response curves at a saturating light intensity (Laing *et al*. 1974; Peisker 1974) – is the CO_2 partial pressure at which net CO_2 assimilation is zero. C_4 plants have a much lower Γ (0–5 μbar CO_2) compared with C_3 species (~50 μbar CO_2). This is primarily because, in C_4 plants, CO_2 is concentrated around ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in the bundle sheath cells. This leads to lower rates of photorespiration in comparison to C_3 plants where CO_2 is competitively fixed with oxygen (O_2) in the mesophyll cells. Here, photorespiration is referred to as CO_2 loss associated with RuBP oxygenase activity plus mitochondrial respiration of

*These authors contributed equally to this research.

CO_2 through the tricarboxylic acid cycle. However, variation in Γ can also be associated with differences in the kinetic properties of RuBisCO (the specificity for CO_2 and O_2), rates of dark-type mitochondrial respiration and the rate of fixation of photorespired CO_2 (von Caemmerer 2000).

Screening for differences in Γ of C_3 and C_4 plants can be achieved in a high-throughput manner by placing plants in airtight chambers under illumination and drawing the CO_2 concentration down to the Γ of a C_4 plant. The higher Γ of C_3 plants would lead to a net loss of carbon, which if sustained for long enough would eventually lead to the death of the plant. Bulk screens for mutant C_4 plants that can survive at ambient CO_2 but have increased Γ could be performed in a similar way. These individuals would, after increasing periods of time at CO_2 concentrations below their Γ, appear stunted and show signs of chlorosis. Rescuing these plants before they died by transfer to ambient or elevated CO_2 conditions would allow the physiological and genetic basis of the mutation to be characterised. An increased Γ in a C_4 plants would be a signature of a loss of C_4 function, i.e. reversion to C_3 photosynthesis. The nature of the mutation would determine the speed and severity of the physical response of plants to CO_2. To prevent this from becoming a limiting factor in throughput when screening, measurements of chlorophyll *a* fluorescence can be used to identified mutants before physical symptoms develop (Meurer *et al*. 1996; Niyogi *et al*. 1997; Shikanai *et al*. 1999; Badger *et al*. 2009).

Light energy absorbed by chlorophyll molecules can be used to drive photochemistry, dissipated as fluorescence or as heat. These three processes are in competition with each other, thus an increase in one is reflected by a decrease in the other two. In a healthy non-stressed dark-adapted plant there is no dissipation of light energy as heat or by photochemistry. Under these conditions, measurements of chlorophyll fluorescence reflect the maximum quantum efficiency of PSII photochemistry (F_v/F_m). Values are highly consistent at ~0.83 (Björkman and Demmig 1987), but decrease with the onset of 'stress' resulting in excess light energy (above the requirements of photochemistry). This decline can occur as a result of an increase in the dissipation of energy as heat (Horton and Ruban 2005; Demmig-Adams and Adams 2006; Belgio *et al*. 2014). This non-photochemical quenching (NPQ) is a photoprotective mechanism that safely dissipates light energy resulting from an imbalance between the absorption of light and its utilisation, such as those resulting from rapid fluctuations in the light environment. It consists of several components recognised by the rate of their relaxation kinetics in the dark (Quick and Stitt 1989). It includes components that lead to sustained quenching (do not relax even after a period in the dark) and decrease F_v/F_m values. NPQ is often accompanied by photoinhibition of the reaction centres (Long *et al*. 1994). Excess light energy that is neither used for phytochemistry nor dissipated as heat leads to the formation of reactive oxygen species (ROS) that damage PSII (Aro *et al*. 1993). This is widely considered to represent light-induced damage to the D1 protein within the PSII complex, a new protein has to be synthesised and inserted into the PSII complex for functionality to be restored (Aro *et al*. 1993; Long *et al*. 1994). If the rate of repair does not keep pace with the rate of damage F_v/F_m will decline. However it may also include photodamage leading to the loss of reaction centres.

Measurements of F_v/F_m can be used to distinguish between plants with different photosynthetic efficiencies based upon their response to CO_2 (Furbank and Walker 1986; Laisk and Edwards 1998). In C_3 plants photosynthesis is limited by the availability of CO_2 at ~200 ppm (<20 Pa), below this there is a linear response of photosynthesis to changes in CO_2 concentration until the Γ is reached. C_4 plants also respond to changes in CO_2 concentration; however, by comparison, they are able to maintain higher rates of photosynthesis due to the presence of a CO_2 concentrating mechanism (von Caemmer and Furbank 2003). Their lower Γ also means that photosynthesis is sustained at much lower CO_2 concentrations than in C_3 plants. These responses will be reflected by differences in the F_v/F_m values, as photosynthesis becomes increasingly limited by CO_2 F_v/F_m will decline. A mutant with an increased Γ could be distinguished from a wild type (WT) based upon the rapid and sensitive response of F_v/F_m to changes in CO_2 concentration. The exact nature of the response would depend upon the CO_2 concentration, duration of exposure and, critically, the intensity of the light.

Here, we used a forward-genetic approach to identify mutants with increased CO_2 compensation points in the C_4 monocot *Setaria viridis* (L.) P.Beauv. We screened a mutant population generated using N-nitroso-N-methylurea (NMU) using a combination of low CO_2 treatment and measurements of the maximum quantum efficiency of PSII photochemistry (F_v/F_m). Forty-six candidate lines were identified, and one line with a heritable homozygous phenotype selected for further characterisation. Growth of this mutant was reduced at ambient CO_2 and could be partial recovered at elevated CO_2 conditions. The CO_2 compensation point of this mutant was similar to values of C_3 rice; CO_2 assimilation was significantly reduced. Our results indicate that the screen is capable of identifying mutants with a loss of C_4 photosynthetic function. Characterisation and next-generation sequencing all of the mutants identified in this screen may lead to the identification of genes modulating key C_4 traits. These can be used to engineer a C_4 photosynthetic pathway into rice.

Materials and methods

Plant material

Seeds of *Setaria viridis* (L.) P.Beauv. A10.1 were a gift from Dr T Brutnell (Danforth Plant Science Centre). Seeds of rice (*Oryza sativa* L. cv. IR64) were obtained from The International Rice Genebank (IRRI, Philippines).

Mutagenesis experiment

Wild-type *Setaria* seeds were treated with N-nitroso-N-methylurea (NMU) and grown to obtain M_1 plants. Seeds were harvested from each individual plant to generate M_2 lines; each line was assigned a unique four-digit mutant line number (NM000 to NM08880). One hundred seeds were grown for each M_2 line and the seed bulk-harvested to generate M_3 lines. Seedlings from the M_3 generation were screened for altered CO_2 compensation points as described below. Candidate mutants were selfed to maintain homozygous mutant stocks.

Plant growth conditions

Dormancy of *Setaria* seeds was broken by soaking in 5% liquid smoke (Wright's Liquid Smoke, B & G Foods Inc.) for 24 h under constant shaking and then rinsed with water. Rice seeds were placed at 50°C for 5 days. All seeds were sown directly in 100 mL root trainers (http://rootrainers.co.uk/, 19 October 2017) containing sterilised soil from the IRRI upland farm mixed with 0.4 g L^{-1} of Osmocote Plus 15-9-12 (Scotts Co. Ltd). To ensure that each root trainer contained 40 mutant *Setaria*, five wild-type (WT) *Setaria* and five rice (*O. sativa* cv. IR64) plants, two seed were sown per cell: these were then thinned after germination. Plants were cultivated in a screen house at the International Rice Research Institute (IRRI) Los Baños, Philippines, 14°10′19.9″N, 121°15′22.3″E. For experiments at low CO_2 plants were transferred to custom-made chambers as described below. After low CO_2 treatment and measurement of F_v/F_m all candidate and WT plants were transferred to growth chambers at 2000 ppm CO_2 (high CO_2) under ambient irradiance and a constant temperature of 25°C. A total of 52 separate experiments were conducted between 2012 and 2015.

Low CO_2 treatment

Plants were exposed to low carbon dioxide (CO_2) concentrations in custom-made growth chambers (Fig. S1 available as Supplementary Material to this paper) designed to create a closed system for rapid screening of CO_2 compensation points. Plants were exposed to ~15 ppm CO_2 for 48 h at an air temperature of 25°C under ambient irradiance. Light intensity inside the screen house ranged between 500 and 2000 µmol m^{-2} s^{-1} with a daylength of between ~11 and 13 h. The chambers permitted transmission of ~63% of the ambient solar irradiance at the canopy level. Humidity inside the chambers was not controlled but was typically maintained at 60–70% (Fig. S2). Plants were watered twice daily at 0700 and 1600 hours through a hole in the side of the chamber. Low CO_2 treatment was applied to plants 10–16 DPG (2–3 leaf stage); chambers were loaded and unloaded at midday.

Chlorophyll fluorescence imaging

Chlorophyll fluorescence images were acquired within a PlantScreen Compact System (Photon System Instruments). Minimum fluorescence (F_o) was measured under a pulse amplitude-modulated measuring beam (1.06 µmol m^{-2} s^{-1}) and maximum fluorescence was induced with a 0.8 s pulse of white and red light. The system is capable of delivering a pulse with a maximum intensity of 3227 µmol m^{-2} s^{-1}, which may not be fully saturating for all C_4 plants. F_v/F_m was measured following 30 min dark adaptation inside PlantScreen. Measurements of rapid chlorophyll fluorescence kinetics were made following acclimation for 20 s at six different actinic light intensities between 25 and 1000 µmol m^{-2} s^{-1}.

Images were analysed using Fluorcam 7 ver. 1.024.2 (Photon Systems Instruments). The following parameters were calculated in the software according to Genty *et al*. (1989). The maximum quantum efficiency of PSII photochemistry (F_v/F_m) was calculated as: $(F_m - F_o)/F_m$, this is the maximum efficiency at which light absorbed by PSII is used for reduction of Q_A. The maximum efficiency of PSII in the light if all centres were open (F_v'/F_m'): $(F_m' - F_o')/F_m'$. PSII operating efficiency (φPSII): $(F_m' - F'/F_m')$, estimates the efficiency at which light absorbed by PSII is used for Q_A reduction. The fraction of the maximum PSII efficiency that is realised in the light (qP): $(F_m' - F')/(F_m' - F_o')$, relates PSII maximum efficiency to operating efficiency. Non-photochemical quenching (NPQ): $(F_m/F_m') - 1$ estimated the rate constant for heat loss from PSII. Where, F, F': fluorescence emission from dark- or light-adapted leaf, respectively; F_o, F_o': minimal fluorescence from a dark-adapted and light-adapted leaf respectively; F_m, F_m', maximal fluorescence from a dark- and light-adapted leaf respectively; F_q', difference in fluorescence between F_m' and F'; F_v, F_v', variable fluorescence from a dark- and light-adapted leaf, respectively. Average values for individual plants within images were then exported to an Excel (Microsoft Corp.) spread sheet.

Candidate selection criteria

In the M_3 generation each chlorophyll fluorescence image was artificially coloured using a rainbow scale to show a range in F_v/F_m between ~0.50 and 0.83. These images were visually interrogated to verify that the F_v/F_m values for (i) WT rice and *Setaria* plants were lower than ~~0.83, (ii) rice were lower than WT *Setaria* and (iii) the mutants were intermediate of the two. Any mutant plant with a reduced F_v/F_m (based on colour) compared with it siblings and the WT *Setaria* were classified as a reduce F_v/F_m candidate. Average values were exported for all images containing candidate plants. By considering only plants within a single image, variation in the reduction of F_v/F_m between individual trays or experiments (associated with the position of plants in the chambers or variation in ambient irradiance) could be compensated for. This approach enabled mutant plants with a reduced F_v/F_m to be rapidly identified. Individuals that were very severely stunted, pale, had visible damaged to the leaves or that died after the low CO_2 treatment were removed from the candidate list. All remaining candidate mutant plants that produced viable seed were advanced to the M_4 generation.

In the M_4, M_5 and M_6 generations average values were exported for every plant within an image.

The decrease in the F_v/F_m during the low CO_2 treatment was calculated by subtracting the value at ambient from the value from the same plant at low CO_2. For WT *Setaria* these values were averaged and used as a candidate threshold. A mutant plant was classified as a candidate if the decline in the F_v/F_m at low CO_2 was greater than the candidate threshold. This approach enabled mutant plants with an increased sensitivity to low CO_2 to be identified. In all generations, progeny where ~50% or more of the sibling were classified as candidates were prioritised and advanced to the next generation. For each mutant line the single best performing progeny (most responsive to low CO_2 and able to produce viable seed) was selected.

Gas-exchange measurements

Leaf gas-exchange measurements were made using a Li-6400XT infrared gas analyser (LI-COR Biosciences) fitted with a standard 2 × 3 cm leaf chamber and a 6400-02B light source. Measurements were made at a constant airflow rate of 400 µmol s^{-1}, leaf temperature 25°C, leaf-to-air vapour pressure

deficit 1.0 and 1.5 kPa and RH 60–65%. Data were acquired between 0800 and 1300 hours. Measurements were made during the vegetative stage on the mid-portion of a fully expanded leaf from three plants. Leaves were acclimated in the cuvette for ~30 min before measurements were made. The response curves of the net rate of assimilation (A, μmol m^{-2} s^{-1}) to changing intercellular CO_2 concentration (C_i, μmol CO_2 mol^{-1}) were acquired by increasing C_a (CO_2 concentration in the cuvette) from 20 to 2000 μmol CO_2 mol air $^{-1}$ at a photosynthetic photon flux density (PPFD) of 1800 μmol photons m^{-2} s^{-1}. The CO_2 compensation point (Γ) and maximum carboxylation efficiency (*CE*) were calculated from the intercept (Vogan *et al*. 2007) and initial slope (Wang *et al*. 2006) of the CO_2 response curves. Light response curves were acquired by increasing the PPFD from 0 to 2000 μmol photons m^{-2} s^{-1} at a C_a 400 μmol CO_2 mol^{-1}. The quantum yield (Φ) was calculated from the initial slope of the light-response curves (PPFD <100 μmol photons m^{-2} s^{-1}). Dark respiration rates (R_d) measurements were made between 0800 and 1300 hours on the mid-portion of the leaf blades after 30 min in the dark at a C_a of 400 μmol CO_2 mol^{-1}.

Results

The response of F_v/F_m *to low* CO_2

Transferring WT rice and *Setaria* from ambient to ~15 ppm CO_2 under continuous actinic illumination led to a rapid decline in F_v/F_m within the first six hours (Fig. 1). After 48 h F_v/F_m values had declined by 0.39 ± 0.09 in rice and 0.17 ± 0.08 in WT *Setaria* compared with values at ambient CO_2. When the lighting regime was changed to include a period of dark, following a period in the light at ~15 ppm CO_2, minimal changes in F_v/F_m were observed during the dark period (Fig. 2). F_v/F_m values started to recover in the light only once CO_2 concentrations were returned to ambient levels. During screening significant variation in the decline of F_v/F_m in response to the low CO_2 treatment between experiments was observed with F_v/F_m values for WT *Setaria* ranging between 0.55–0.75 and 0.35–0.55 for rice (Tables S1–S4 available as Supplementary Material to this paper).

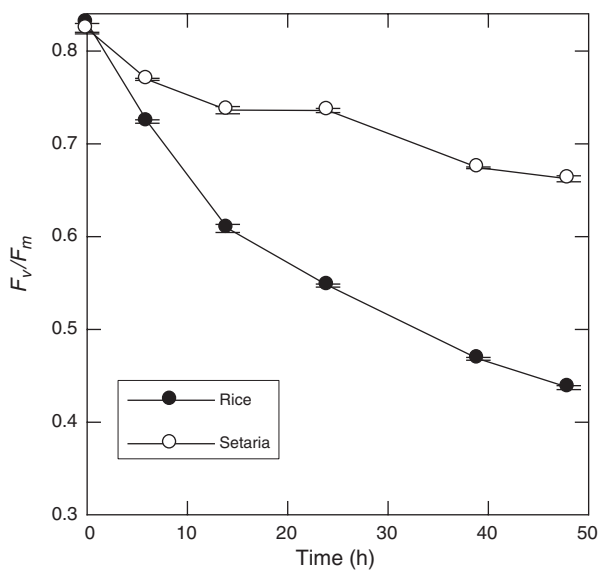

Fig. 1. Temporal response of F_v/F_m to low CO_2 conditions. Time course showing the decrease in F_v/F_m after exposure of WT *Setaria* and rice (2–3 leaf stage) to ~15 ppm CO_2 under continuous actinic illumination (1000 μmol m^{-2} s^{-1}). Values are the average ± s.e. of 50 plants.

Identification of low CO_2 *responsive* Setaria *mutants*

Mutants with a reduced F_v/F_m following exposure to low CO_2 could be easily identified within chlorophyll fluorescence images (Fig. 3). Of the 7325 M_3 generation mutant lines screened, a total of 1104 plants from 615 lines were classified as candidates with reduced F_v/F_m values after low CO_2 treatment. This was reduced to 46 candidate lines (240 plants; Table S1) by excluding individuals that were severely stunted, pale, had visible damaged to the leaves or that died after the low CO_2 treatment. The candidates exhibited reductions in F_v/F_m values of between 15 and 74% compared with WT *Setaria.* Significant variation in the F_v/F_m values of progeny of a single mutant line was observed, for example values of mutant NM01832 ranged between 0.24 and 0.55 (Table S1). In seven mutant lines (NM02144, 2175, 2456, 2845, 3021, 3891 and 4077) the F_v/F_m of all progeny were found to be lower than those of rice; in all other mutants values were typically intermediate of the two WT plants (Table S1).

A total of 136 M_3 candidate plants produced viable seed and were advanced to the M_4 generation; this included seed from at least one plant from each of the 46 mutant lines. All except one line (NM03966) was confirmed to be low CO_2 responsive (Table S2). With only two exceptions (NM02144-16 and NM04052-12), at least one progeny from 45 of the M_3 lines was classified as a candidate. Of the 15 lines advanced to the M_5 generation, 14 were confirmed as candidates (Table S3).

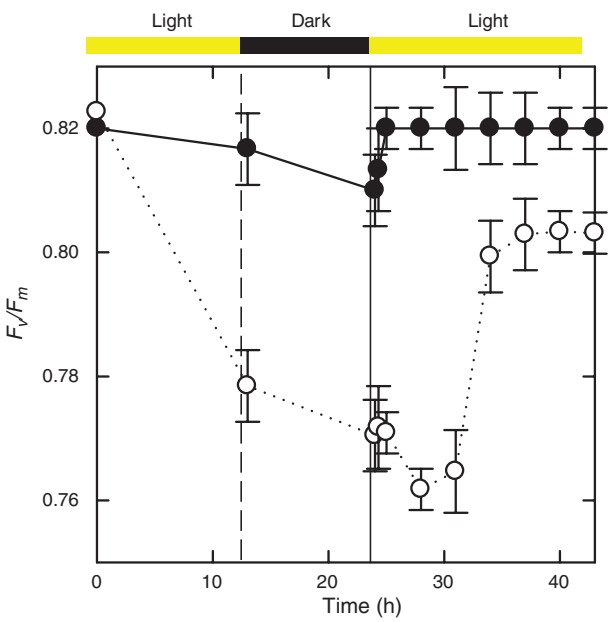

Fig. 2. Response of F_v/F_m in the dark. WT *Setaria* plants were placed in a chamber set at either 400 (black symbols) or 15 ppm CO_2 (white symbols) under 500 μmol photons m^2 s^{-1} of light. At 13 h the lights were turned off in both chambers. At 24 h the CO_2 concentration was increased from 15 to 400 ppm CO_2 and the lights were turned back on in both chambers. Values are the average ± s.e. of 150 plants per treatment.

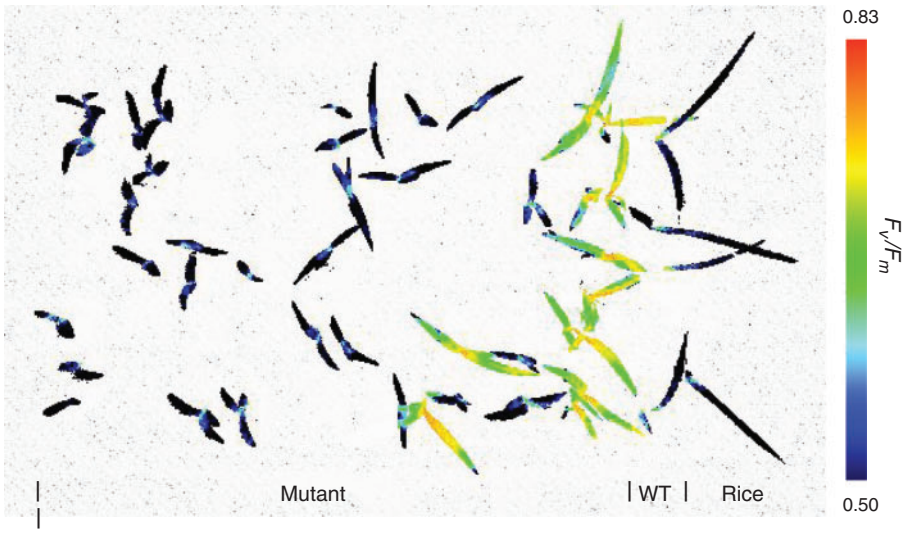

Fig. 3. Example chlorophyll fluorescence image. Measurements were made after 48 h exposure to 15 ppm CO_2. Abbreviations: mutant, *Setaria* M_3 mutant; WT, wild-type *Setaria* plants; rice, *Oryzsa sativa* IR64 wild type (WT).

Nine of these were advanced to the M_6 generation and all confirmed as low CO_2 responsive (Table S4).

Considerable variations in F_v/F_m values were observed within siblings of a single mutant line and between generations (Figs S3–S5). After low CO_2 treatment the F_v/F_m values of mutant plants ranged between those of WT *Setaria* to values less than those of rice (Tables S3, S4). Irrespective of the values and magnitude of the decline in F_v/F_m, low CO_2 responsive mutant could be clearly identified (Figs S3, S5). Segregation was observed (Fig. S5), and by the M_6 generation, mutants with a homozygous phenotype were obtained (Table S4). This was predominantly associated with a low F_v/F_m under ambient CO_2 conditions (Table S4).

Growth and photosynthesis is reduced in the mutants

The majority of mutants exhibited reduced growth at ambient CO_2 concentrations compared with WT *Setaria* (Fig. S6). The phenotypes observed ranged from a reduction in plant height and tiller number to sever stunting and chlorosis. Mutant NM04534 exhibited the most severe growth phenotype, with reduced height, tiller number, leaf size, chlorosis and senesce to the leaf tips (Fig. 4). Flowering was delayed by 3.0 ± 1.5 days. When grown under elevated CO_2 the only phenotypic difference between the mutant and WT *Setaria* was a reduction in tiller number (Fig. 4).

The Γ of the mutant was significantly higher than both WT *Setaria* and rice (Table 1). Photosynthesis was saturated at very low light intensities, above a PPFD ~100 µmol m^{-2} s^{-1} further increases in the light intensity did not lead to increases in CO_2 assimilation (Fig. 5*a*). There were no significance differences in the quantum yield for CO_2 assimilation compared with WT *Setaria* (Table 1). Rates of CO_2 assimilation were substantially lower at all CO_2 concentrations (Fig. 5*b*), CO_2 assimilation increased linearly up to a *Ci* of ~1600 µmol CO_2 m^{-2} s^{-1} before reaching a plateau (Fig. 5*b*). Stomatal conductance was lower in the mutant compared with WT *Setaria* at all CO_2 concentrations. Below 20 and above 600 µmol CO_2 mol air $^{-1}$ the difference in conductance between the mutant and WT *Setaria* was <15%. Between 50 and 400 µmol CO_2 mol air $^{-1}$ values for the mutant were 40–50% lower. The carboxylation efficiency (*CE*) was significantly decreased compared with both WT rice and *Setaria*, the rate of dark respiration was statistically significantly higher in the mutant (Table 1). Analysis of the rapid response of chlorophyll fluorescence kinetics revealed that non-photochemical quenching was increased in the mutant relative to WT *Setaria* between a PPFD of 200 and 500 µmol m^{-2} s^{-1} (Fig. 6*a*). Corresponding decreases were observed in the maximum efficiency of PSII in the light (Fig. 6*b*), PSII operating efficiency (Fig. 6*c*) and the fraction of the maximum PSII efficiency that is realised in the light (Fig. 6*d*). At 1000 µmol m^{-2} s^{-1} values of all parameters were similar reflecting an increase in NPQ in WT plants.

Discussion

A high-throughput screen for mutants with alterations CO_2 compensation points

The results presented here show that the screening protocol is able to distinguish plants with known differences in CO_2 compensation points. They also show the utility of the protocol for identifying a wide range of potentially interesting *Setaria* mutants in which photosynthetic function has been disrupted. Some of the mutants identified exhibited responses to low CO_2 that more closely resembled C_3 rice than WT *Setaria*. Mutants were identified with a high frequency in the population (one per 157 lines screened) and with a high frequency of heritability for the phenotype (98%). Although all the mutants identified have not yet been characterised in detail, to date, eight mutant lines with a homozygous phenotype have been generated. Taken together, these results indicate that the screen is sensitive, accurate and efficient at identifying photosynthetic mutants. Similar approaches to this have been used in the past to

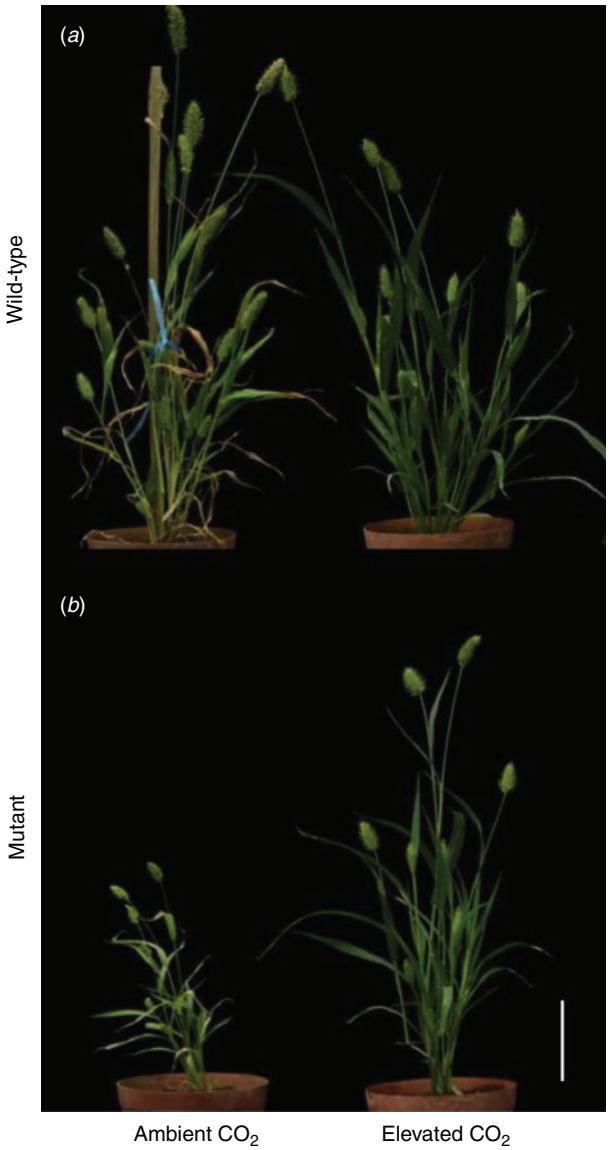

Fig. 4. Response of growth to changes in CO_2. Representative pictures of *Setaria* M_5 generation (*a*) wild-type (WT) and (*b*) mutant NM04534 plants grown at ambient (400 ppm CO_2) and elevated (2000 ppm CO_2) CO_2 concentrations; 37 days post germination (DPG). Scale bar = 5 cm.

identify C_3 photorespiratory mutants (Blackwell *et al.* 1988; Somerville 1986, 2001).

With our screening protocol it was difficult to reproducibly measure the response of F_v/F_m to low CO_2. Significant variation in the values of WT *Setaria* and rice were observed between different experiments. We attribute this to variation in the ambient irradiance as this strongly affects the energy allocation of PSII (Ishida *et al.* 2014). Had it been possible to conduct experiments under artificial illumination the F_v/F_m values of plants from separate experiments could have been directly compared. In part, this is also the reason why we do not examine the intrinsic causes for the decline in F_v/F_m or monitor its recovery following exposure to low CO_2. This means that we are unable to characterise the response of the mutants in detail. This is important because the majority of the mutants identified at low CO_2 appear to also exhibit a phenotype of reduced F_v/F_m at ambient CO_2 conditions. This may be indicative of the nature of mutation (Shikanai *et al.* 1999), a bias of protocol to identify mutants with specific response or artefact of the variable irradiance.

Candidate mutants exhibit a reduction in growth and photosynthesis

The majority of the mutants identified exhibited reduced growth compared with WT *Setaria* at ambient CO_2. This result is consistent with mutations leading to decreased photosynthetic efficiency at ambient CO_2, either through a reduction in CO_2 assimilation or increased loss of carbon through photorespiration. The increased CO_2 compensation point of NM04535 supports this hypothesis. Although the rate of dark respiration was increased in the mutant, this alone is unlikely to account for the difference in carbon allocated to growth compared with the WT. The stimulation of growth in the mutant at elevated CO_2 together with the almost the linear response of photosynthesis to increases in CO_2 concentration suggests that photosynthesis was RuBisCO limited. Differences in stomatal conductance alone do not account for this response because values were indistinguishable from WT plants above a CO_2 concentration of 500 µmol CO_2 mol air $^{-1}$. Although this has yet to be confirmed, this response is consistent with a reduction in the supply of CO_2 to RuBisCO in the bundle sheath (Furbank *et al.* 1996; von Caemmerer *et al.* 1997). This could arise from mutations disrupting the to the C_4 biochemical pump

Table 1. Comparison of photosynthetic parameters

F_v/F_m measurements were made at ambient CO_2 and after 48 h at ~15 ppm CO_2. Values are the average ± s.e. of 10 individual M_5 generation plants per line (2–3 leaf stage). Measurements of CO_2 compensation point (Γ), carboxylation efficiency (*CE*) and quantum yield for CO_2 assimilation (φ) were made at 400 µmol mol^{-1} CO_2 or 1800 µmol photons m^{-2} s^{-1} and a leaf temperature of 25°C. Measurements of dark respiration (R_d) were made 30 min after dark. Values are the average ± s.e. of one leaf from 4–8 plants per line 30 days post germination. Different letters denote statistically different based on a two-way ANOVA with a least significant difference (l.s.d.) Test for *post-hoc* pairwise comparison ($P < 0.05$). Dash means no measurements were made

	F_v/F_m (Ambient CO_2)	F_v/F_m (15 ppm CO_2)	Γ (µmol CO_2 m^{-2} s^{-1})	*CE* (µmol CO_2 m^{-2} s^{-1} µmol CO_2 mol^{-1})	φ (µmol CO_2 µmol^{-1} quanta^{-1})	R_d (µmol CO_2 m^{-2} s^{-1})
Rice	0.81 ± 0.020	0.41 ± 0.018	52.62 ± 0.98^a	0.13 ± 0.00^b	–	–
WT *Setaria*	0.80 ± 0.004	0.71 ± 0.009	2.27 ± 0.62^b	0.56 ± 0.04^a	0.06 ± 0.00^a	1.01 ± 0.18^b
NM04534	0.75 ± 0.002	0.37 ± 0.009	66.16 ± 11.53^a	0.02 ± 0.00^c	0.05 ± 0.00^a	1.46 ± 0.19^a

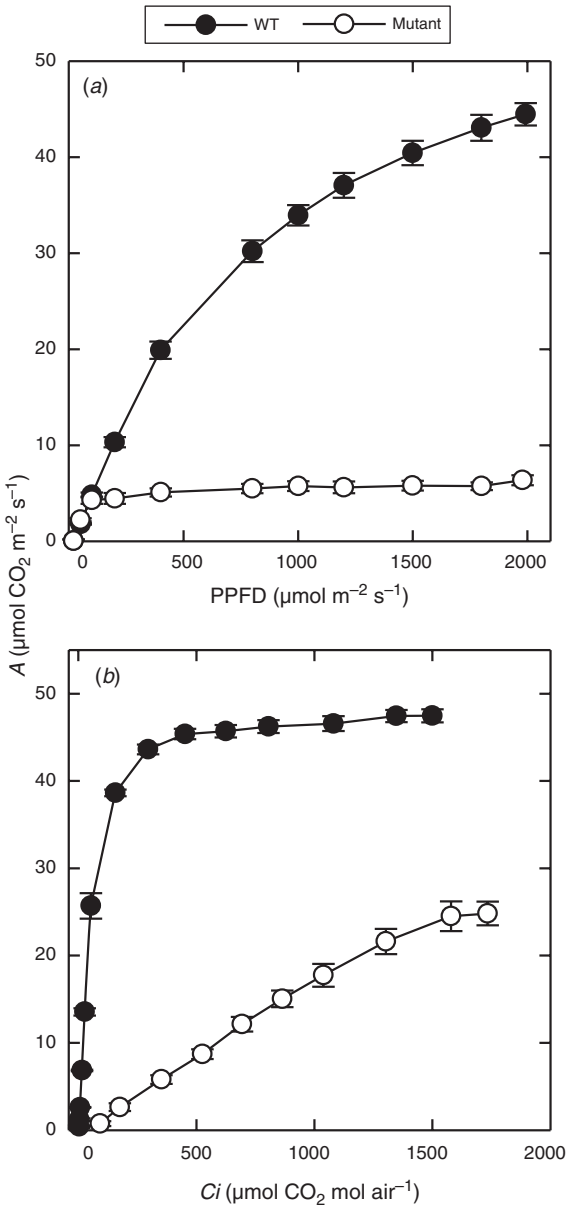

Fig. 5. CO_2 assimilation response curves of a *Setaria* wild type and mutant. (*a*) Net CO_2 assimilation rate (*A*) in response to photosynthetic photon flux density (PPFD) and (*b*) intercellular CO_2 concentration (C_i). Measurements were made at 400 µmol mol^{-1} CO_2 or 1800 µmol photons m^{-2} s^{-1} and a leaf temperature of 25°C. Values represent the mean ± s.e. of three leaves from three different M_5 generation plants.

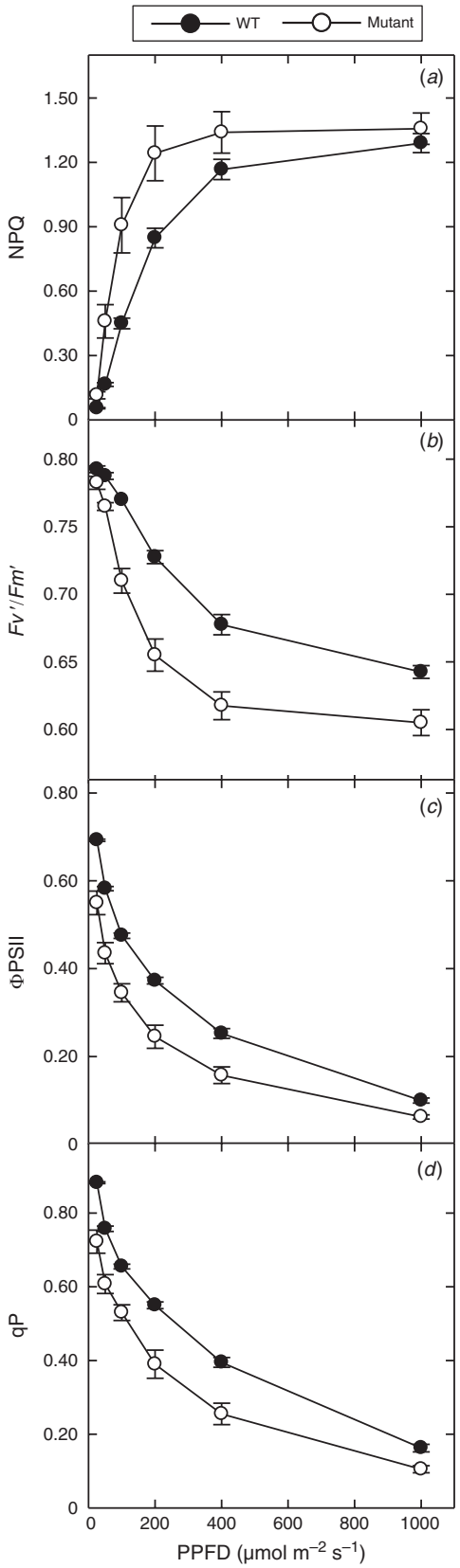

Fig. 6. (*continued*)

Fig. 6. Rapid chlorophyll fluorescence kinetics. The rapid response of PSII (*a*) operating efficiency (φPSII), (*b*) the fraction of the maximum PSII efficiency that is realised in the light (qP), (*c*) maximum PSII quantum efficiency (F_v'/F_m'), (*d*) the fraction of PSII reaction centres that are open (qL), and (*e*) non-photochemical quenching (NPQ) in wild type (WT) and mutant (NM04534) plants to increasing photosynthetically active photon flux density (PPFD). Values are average ± s.e. of nine individual M_5 generation plants per line grown at ambient CO_2; 20–22 days post germination (DPG).

(Ludwig *et al*. 1998), perhaps due to a change in the abundance or kinetic properties of photosynthetic enzymes (Dever *et al*. 1995; Hanson *et al*. 2003; Cousins *et al*. 2007).

The limitations affect the response of the mutant to light with CO_2 assimilation saturated at a very low light intensity. Further increases in light above this intensity led to an increase in NPQ and corresponding declines in photochemical quenching (qP) and the PSII operating efficiency (φPSII), which is the quantum efficiency of PSII electron transport. This indicates that less energy was being used as reducing power for CO_2 assimilation in the mutants compared with the WT. At 1000 µmol m^{-2} m^{-1} the difference in the values of these parameters between the WT and the mutant were negligible, reflecting an increase in NPQ in the WT rather than adaption of the mutant to high light. These changes were also reflected in the maximum efficiency of PSII in the light (F_v'/F_m') and dark (F_v/F_m). This is likely the result of sustained quenching of PSII and photoinhibition (Long *et al*. 1994; Demmig-Adams and Adams 2006). Under ambient conditions, except for at dawn and dusk, the light intensity is in excess of that required for photochemistry.

Future research

We have identified another 44 mutant lines that have yet to be characterised in detail. There are several other possible explanations for the phenotypes observed by the mutants identified in this screen. These include expression of RuBisCO in the mesophyll chloroplasts instead of, or in addition to, the bundle sheath chloroplasts; expression of glycine decarboxylase in the mitochondria of mesophyll cells instead of the bundle sheath cells; increase leakage of CO_2 from bundle sheath cell resulting from alterations in the positioning of chloroplasts site of C_4 acid decarboxylation or changes to BS cell wall composition.

All mutants that have been identified will be advanced and rescreened to confirm the F_v/F_m phenotype. Homozygous mutants will be characterised to establish the mechanistic basis leading to the phenotype. This may include quantification of the abundance, activity and location of C_4 biochemical enzymes, detailed gas-exchange, rapid chlorophyll fluorescence kinetics, dry carbon isotope discrimination analysis, growth responses and analysis of leaf anatomical structure. Promising mutants will be backcrossed with the WT parent and next-generation sequencing will be used in order to identify the genetic basis of the mutation. Promising candidate gene(s) will then be cloned and overexpressed in the current C_4 Rice Prototype (Kajala *et al*. 2011).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors thank Govinda Rizal and Nikki Arivel Larazo for curation of the *Setaria* mutant population, Julius Ranada and Richard Poiré for assistance developing the low CO_2 screen, Irma Canicosa for the gas-exchange measurements and Michael Orlina, Jeffren Punzalan, Rey Vergara and Joseph Langman for assistance with the screening. We also thank the reviewers for valuable suggestion on editing the manuscript. This work was supported by the International Rice Research Institute, Bill and Melinda Gates Foundation, Department for International Development (DFID) U.K and the ARC Centre of Excellence in Translational Photosynthesis in Australia.

References

Aro EM, Virgin I, Andersson B (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochimica et Biophysica Acta* **1143**, 113–134. doi:10.1016/0005-2728(93)90134-2

Badger MR, Fallahi H, Kaines S, Takahashi S (2009) Chlorophyll fluorescence screening of *Arabidopsis thaliana* for CO_2 sensitive photorespiration and photoinhibition mutants. *Functional Plant Biology* **36**, 867–873. doi:10.1071/FP09199

Belgio E, Kapitonova E, Chmeliov J, Duffy CDP, Ungerer P, Valkunas L, Ruban AV (2014) Economic photoprotection in photosystem II that retains a complete light-harvesting system with slow energy traps. *Nature Communications* **5**, 8–16. doi:10.1038/ncomms5433

Björkman O, Demmig B (1987) Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. *Planta* **170**, 489–504. doi:10.1007/BF00402983

Blackwell RD, Murray AJ, Lea PJ, Kendall AC, Hall NP, Turner JC, Wallsgrove RM (1988) The value of mutants unable to carry out photorespiration. *Photosynthesis Research* **16**, 155–176. doi:10.1007/BF00039491

Cousins AB, Baroli I, Badger MR, Ivakov A, Lea PJ, Leegood RC, von Caemmerer S (2007) The role of phosphoenolpyruvate carboxylase during C_4 photosynthetic isotope exchange and stomatal conductance. *Plant Physiology* doi:10.1104/pp.107.103390

Demmig-Adams B, Adams WW (2006) Photoprotection in an ecological context: the remarkable complexity of thermal energy dissipation. *New Phytologist* **172**, 11–21. doi:10.1111/j.1469-8137.2006.01835.x

Dever LV, Balckwell RD, Fullwood NJ, Lacuesta M, Leegood RC, Onek LA, Pearson M, Lea PJ (1995) The isolation and characterisation of mutant of the C_4 photosynthetic pathway. *Journal of Experimental Botany* **46**, 1363–1376. doi:10.1093/jxb/46.special_issue.1363

Feldman AB, Murchie EH, Leung H, Baraoidan M, Coe RA, Yu S-M, Lo S-F, Quick WP (2014) Increasing leaf vein density of mutagenesis: laying the foundations for C_4 rice. *PLoS One* **9**, e94947. doi:10.1371/journal.pone.0094947

Furbank RT, Walker DA (1986) Chlorophyll *a* fluorescence as a quantitative probe of photosynthesis: effects of CO_2 concentration during gas transients on chlorophyll fluorescence in spinach leaves. *New Phytologist* **104**, 207–213. doi:10.1111/j.1469-8137.1986.tb00645.x

Furbank RT, Chitty JA, von Caemmer S, Jenkins CLD (1996) Antisense RNA inhibition of RbcS gene expression reduced rubsico level and photosynthesis in the C_4 plant *Flaveria bidentis*. **111**, 725–734.

Furbank RT, von Caemmerer S, Sheehy J, Edwards G (2009) C_4 rice: a challenge for plant phenomics. *Functional Plant Biology* **36**, 845–856. doi:10.1071/FP09185

Genty B, Briantais J-M, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta. G, General Subjects* **990**, 87–92. doi:10.1016/S0304-4165(89)80016-9

Hanson DT, Franklin LA, Samuelsson G, Badger MR (2003) The *Chlamydomonas reinhardtii* cia3 mutant lacking a thylakoid lumen-localized carbonic anhydrase is limited by CO_2 supply to Rubisco and not photosystem II function *in vivo*. *Plant Physiology* **132**, 2267–2275. doi:10.1104/pp.103.023481

Hibberd JM, Sheehy JE, Langdale JA (2008) Using C_4 photosynthesis to increase the yield of rice-rationale and feasibility. *Current Opinion in Plant Biology* **11**, 228–231. doi:10.1016/j.pbi.2007.11.002

Horton P, Ruban A (2005) Molecular design of the photosystem II light-harvesting antenna: photosynthesis and photoprotection. *Journal of Experimental Botany* **56**, 365–373. doi:10.1093/jxb/eri023

Ishida S, Uebayashi N, Tazoe Y, Ikeuci M, Homma K, Sato F, Endo T (2014) Diurnal and developmental changes in energy allocation of absorbed

light and PSII in field-grown rice. *Plant & Cell Physiology* **55**, 171–182. doi:10.1093/pcp/pct169

Kajala K, Covshoff S, Karki S, Woodfield H, Trolley BJ, Dionora MJA, Mogul RT, Mabilangan AE, Danila FR, Hibberd JM, Quick WP (2011) Strategies for engineering a two-cell C_4 photosynthetic pathway into rice. *Journal of Experimental Botany* **62**, 3001–3010. doi:10.1093/jxb/err022

Laing WA, Ogren WL, Hageman RH (1974) Regulation of soybean net photosynthetic CO_2 fixation by the interaction of CO_2, O_2 and ribulose-1,5 diphosphate carboxylase. *Plant Physiology* **54**, 678–685. doi:10.1104/pp.54.5.678

Laisk A, Edwards GE (1998) Oxygen and electron flow in C_4 photosynthesis: Mehler reaction, photorespiration and CO_2 concentration in the bundle sheath. *Planta* **205**, 632–645. doi:10.1007/s004250050366

Long SP, Humphries S, Falkowski PG (1994) Photoinhibition of photosynthesis in nature. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**, 633–662. doi:10.1146/annurev.pp.45.060194.003221

Ludwig M, von Caemmerer S, Price GD, Badger MR, Furbank RT (1998) Expression of tobacco carbonic anhydrase in the C_4 dicot *Flaveria bidentis* leads to increased leakiness of the bundle sheath ad a defective CO_2-concentrating mechanism. *Plant Physiology* **117**, 1071–1081. doi:10.1104/pp.117.3.1071

Meurer J, Meierhoff K, Westhoff P (1996) Isolation of high-chlorophyll-fluorescence mutants of *Arabidopsis thaliana* and their characterization by spectroscopy, immunoblotting and northern hybridisation. *Planta* **198**, 385–396. doi:10.1007/BF00620055

Niyogi KK, Bjorkman O, Grossman AR (1997) *Chlamydomonas* xanthophyll cycle mutants identified by video imaging of chlorophyll fluorescence quenching. *The Plant Cell* **9**, 1369–1380. doi:10.1105/tpc.9.8.1369

Peisker M (1974) A model describing the influence of oxygen on photosynthetic carboxylation. *Photosynthetica* **8**, 47–50.

Quick WP, Stitt M (1989) An examination of factors contributing to non-photochemical quenching of chlorophyll fluorescence in barley leaves. *Biochimica et Biophysica Acta* **977**, 287–296. doi:10.1016/S0005-2728(89)80082-9

Rizal G, Thakur V, Dionora J, Karki S, Wanchana S, Acebron K, Larazo N, Garcia R, Mabilangan A, Montecillo F, Danila F, Mogul R, Pablico P, Leung H, Langdale JA, Sheehy J, Kelly S, Quick WP (2015) Two forward genetic screen for vein density mutants in sorghum converge on a cytochrome P450 gene in the brassinosteroid pathway. *The Plant Journal* **84**, 257–266. doi:10.1111/tpj.13007

Rizal G, Karki S, Thakur V, Wanachana S, Alonso-Cantabrana H, Dionora J, Sheehy JE, Furbank R, von Caemmerer S, Quick WP (2017) A sorghum (*Sorghum bicolor*) mutant with altered carbon isotope ratio. *PLoS One* **12**, e0179567. doi:10.1371/journal.pone.0179567

Shikanai T, Munekage Y, Shimizu K, Endo T, Hashimoto T (1999) Identification and characterization of *Arabidopsis* mutants with reduced quenching of chlorophyll fluorescence. *Plant & Cell Physiology* **40**, 1134–1142. doi:10.1093/oxfordjournals.pcp.a029498

Somerville CR (1986) Analysis of photosynthesis with mutants of higher plants and algae. *Annual Review of Plant Physiology and Plant Molecular Biology* **37**, 467–507. doi:10.1146/annurev.pp.37.060186.002343

Somerville CR (2001) An early *Arabidopsis* demonstration. Resolving a few issues concerning photorespiration. *Plant Physiology* **125**, 20–24. doi:10.1104/pp.125.1.20

Vogan PJ, Frohlich MW, Sage RF (2007) The functional significance of C_3-C_4 intermediate traits in *Heliotropium L.* (*Boraginaceae*): gas exchange perspectives. *Plant, Cell & Environment* **30**, 1337–1345. doi:10.1111/j.1365-3040.2007.01706.x

von Caemmerer S (2000) 'Biochemical models of leaf photosynthesis. Vol. 2.' (CSIRO Publishing: Melbourne)

von Caemmerer S, Furbank RT (2003) The C_4 pathway: an efficient CO_2 pump. *Photosynthesis Research* **77**, 191–207. doi:10.1023/A:1025830019591

von Caemmerer S, Millgate A, Farquhar GD, Furbank RT (1997) Reduction of ribulose-1,5-bisphosphate carboxylase/oxygenase by antisense RNA in the C_4 plant *Flaveria bidentis* leads to reduced assimilation rates and increased carbon isotope discrimination. *Plant Physiology* **113**, 469–477. doi:10.1104/pp.113.2.469

Wang Q, Zhang Q, Fan D, Lu C (2006) Photosynthetic light and CO_2 utilization and C_4 traits of two novel super-rice hybrids. *Journal of Plant Physiology* **163**, 529–537. doi:10.1016/j.jplph.2005.04.035