Title: Anatomical features, efficient photosystem, and Rubisco activity contribute to high leaf photosynthesis in wild rice

Running Title: Efficient Leaf Photosynthesis in Wild Rice

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Highlight

Distinct leaf developmental, photochemical and biochemical features contribute to efficient photosynthesis in wild rice that could potentially be exploited for increasing rice leaf photosynthesis.

Abstract

The importance of increasing photosynthetic efficiency for sustainable crop yield increases to feed the growing world population is well recognized. The natural genetic variation for leaf photosynthesis in crop plants is an overlooked and untapped resource. The genus *Oryza*, including cultivated rice and wild relatives, offers tremendous genetic variability to explore photosynthetic differences, and underlying developmental, photochemical, and biochemical basis. We quantified leaf photosynthesis and related physiological parameters for ten cultivated and wild rice genotypes to identify photosynthetically efficient wild rice species. Wild rice species with high leaf photosynthesis per unit area had striking anatomical features, such as larger mesophyll cells with more chloroplasts, larger and closer veins, and a smaller number of mesophyll cells between two consecutive veins. In addition, photosynthetically efficient wild rice species showed an efficient Photosystem II as well as higher carboxylation activity of Rubisco compared to less efficient cultivated varieties, such as IR 64. Our results show the existence of desirable variations in mesophyll and vein features, Photosystem II efficiency, and Rubisco activity in the rice system itself that could possibly be targeted for higher leaf photosynthesis. Detailed genetic and molecular understanding of these traits shall be instrumental in increasing photosynthetic efficiency of cultivated rice.

Keywords: Chlorophyll fluorescence, Leaf anatomy, Photosynthesis, Photosystem II (PSII), Rubisco, Wild rice.

Introduction

A remarkable increase in food grain production is needed to meet the demand of the growing world population that is expected to reach 10 billion by 2050 (Alexandratos and Bruinsma, 2012). Crop yield increases due to extensive breeding and an agronomic practice is approaching a plateau over the last decade (Lawson *et al.,* 2012). Yield increases due to the breeding for semi-dwarf varieties, resulting in optimization of photosynthates' allocation, during the green revolution have also largely been saturated (Jansson *et al.,* 2018). The further increases in crop yield and productivity, therefore, requires improving photosynthetic efficiency of crop plants (Lawson *et al.,* 2012; Ort *et al.,* 2015; Simkin *et al.,* 2019).

Photosynthesis, the key biochemical process fixing atmospheric CO_2 in the form of carbohydrates, is a multifaceted process that has contributions from leaf physiological, developmental, and biochemical features (Long *et al.,* 2006; Mathan *et al.,* 2016). Stomatal conductance (g_s), carboxylation efficiency (CE), transpiration (E), and water-use efficiency (WUE) are critical physiological parameters determining leaf gaseous exchange and water relationship that eventually affect the leaf photosynthesis (Farquhar and Sharkey, 1982). Extrinsic factors, such as light and CO_2 concentration, influence photosynthetic performance by regulating the activity of light-harvesting complexes (LHCs) and Rubisco as well as stomatal opening (Eichelmann and Laisk, 2002; Murchie and Niyogi, 2011; Hiyama *et al.,* 2017). In addition, the diurnal timing of the day influences the photosynthesis by mid-day stomatal closure and photorespiration (Zhao *et al.,* 2010).

Developmental characteristics of leaf are strong determinants of photosynthesis (Mathan *et al.,* 2016). Flag leaf width, flag leaf thickness, and specific leaf area showed a positive correlation with photosynthetic capacity, thus effecting overall crop performance (Liu *et al.,* 2015). Anatomical features such as size, lobbing, and surface area of mesophyll cells, are critical for proper light distribution and CO_2 diffusion within a leaf (Sage and Sage, 2009; Tholen *et al.,* 2012). Similarly, leaf vasculature serves as a channel for distribution of water and minerals for photosynthesis and mobilizes photoassimilated CO_2 from leaves to sink organs (Sack and Scoffoni, 2013). Vein density and interveinal distance have been shown to influence CO_2 fixation efficiency (Amiard *et al.,* 2005; Brodribb *et al.,* 2007). Thus, investigating relationships between leaf anatomy and photosynthetic features, and identification of genetic regulators of desirable anatomical features could help in optimizing crop photosynthetic efficiency (Evans, 2013).

Photochemical features, such as organization of light-harvesting complexes, quantum efficiency of photosystem, electron transport rate, and photochemical and non-photochemical

quenching are key factors for photosynthetic efficiency (Murchie and Lawson, 2013; Wu *et al.,* 2019). Maintaining a higher level of chlorophyll content for longer duration in a leaf is one of the suggested strategies for increasing net photosynthesis and crop production (Huang *et al.,* 2013). Quantification of chlorophyll fluorescence is used to differentiate photochemical quenching and non-photochemical quenching, and to evaluate the efficiency of Photosystem II (PSII) reaction centres (Kalaji *et al.,* 2016). Approaches to increase light harvesting by PSII reaction centres and photochemical quenching shall be effective in increasing crop photosynthetic efficiency (Cardona *et al.,* 2018).

Different steps of CO_2 photoassimilation are under complex genetic and environmental regulation. Transcriptional control of photosynthesis and associated leaf features is suggested to have potential for improving photosynthetic assimilation (Wang *et al.,* 2017). Transcription factors, such as GLK1, HY5, ATAF1, ORE1, and PIF, have been shown to activate the photosynthetic gene expression in crop plants (Toledo-Ortiz *et al.,* 2014; Wang *et al.,* 2017). Golden2-like (GLK) transcription factor positively regulates the chloroplast development and also binds directly to the promoter of LHC genes (Waters *et al.,* 2009). Similarly, HY5 and PIF4/5 are important regulators controlling the regulatory network governing photosynthetic gene expression and senescence, respectively (Kindgren *et al.,* 2012; Toledo-*Ortiz et al.,* 2014). However, genetic rewiring of transcription factors towards improving crop photosynthetic performance remains elusive.

Over the past years, the biochemical understanding of photosynthesis has been extensively elaborated. There have been examples for increasing photosynthetic efficiency, resulting in increased yield and/or biomass. One such example is the evolution of C_4 plants, where specific developmental and biochemical features promote efficient photoassimilation of CO_2 (Kajala *et al.,* 2011; Koteyeva *et al.,* 2011). Free air CO_2 enrichment increases leaf photosynthesis in C_3 plants (Rogers *et al.,* 2004). Optimization of additional parameters, such as decreasing Rubisco oxygenase activity, increasing RuBP regeneration, suppressing photorespiration, and increasing photoprotection of the photosynthesis have also been suggested to improve photosynthetic efficiency (Long *et al.,* 2006; Murchie and Niyogi, 2011).

Engineering C_4 photosynthesis in C_3 plants has been envisioned as a potential strategy towards enhancing crop photosynthetic performance. However, this is challenging due to evolutionary, developmental, and biochemical constraints (von Caemmerer *et al.,* 2012; Schuler *et al.,* 2016; Reeves *et al.,* 2017). The natural genetic variation in leaf photosynthesis, associated developmental, photochemical, and biochemical features, and underlying genetic

regulation in crop plants is an overlooked and untapped resource (Flood *et al.,* 2011; van Bezouw *et al.,* 2019). Rice, with two cultivated species: *Oryza sativa* (Asian rice with two subspecies: *indica and japonica*) and *Oryza glaberrima* (African rice), and many related wild rice species, provides an excellent system to investigate photosynthetic differences, and underlying genetic basis. Many of the wild rice species have been used in crop breeding programs towards improving biotic and abiotic stress tolerance of the cultivated rice (Sanchez *et al.,* 2013; Atwell *et al.,* 2014; Wing *et al.,* 2018). Interestingly, some of the wild rice species (*O. rufipogon*, *O. latifolia*, and *O. australiensis*) are reported to accumulate higher biomass than cultivated varieties, and introgression from a wild *Oryza* species to cultivated rice has also been shown to increase biomass (Yeo *et al.,* 1994; Zhao *et al.,* 2008). However, a systematic effort to identify the photosynthetic differences, linking the differences to developmental, photochemical, and biochemical features, and investigating the underlying genetic and molecular basis is warranted.

We examined a set of cultivated (including both subspecies of *Oryza sativa* and *Oryza glaberrima*) and wild rice species, differing in biomass accumulation, to find out strong differences in leaf photosynthesis. We, further, identified the developmental, photochemical, and biochemical differences among the selected photosynthetically different accessions. Expression pattern of genes encoding photosynthetic components, chlorophyll-biosynthetic enzymes and transcription factors, and Rubisco enzyme activity corroborated the leaf photosynthesis differences across cultivated and wild rice.

Materials and methods

Plant material

Ten rice genotypes, consisting of four varieties of *Oryza sativa* ssp. *indica* (IR 64, Swarna, Nagina 22, and Vandana), a variety from *Oryza sativa* ssp. *japonica* (Nipponbare), African cultivated rice *Oryza glaberrima*, and four wild rice species, *Oryza rufipogon*, *Oryza punctata*, *Oryza latifolia*, and *Oryza australiensis* were grown in Kharif 2016 and 2017 at NIPGR research farm (Supplementary Table S1). These genotypes were also grown in pots under controlled growth chambers with the temperature (28 ºC), humidity (70-90 %), and day length (14h: 10h, light: dark). Physiological, developmental, photochemical, and biochemical traits were evaluated at heading stage in the widest part of the flag leaves.

Photosynthesis rate and related physiological trait measurements

Flag leaf photosynthesis was measured at the full heading stage with a portable photosynthesis system (LI-6400XT equipped with a 6400-40 Leaf Chamber Fluorometer (LI-COR Biosciences). Measurement was conducted on a clear day between 9.00 h to 11.00 h under ambient CO_2 level (400 µmol mol^{-1}), air temperature (30 ºC), leaf temperature (27 ºC), and PAR (1,000 µmol m^{-2} s^{-1}) conditions. Net photosynthesis rate (P_N), and related physiological parameters such as stomatal conductance (g_s), intercellular CO_2 concentration (Ci), transpiration rate (E), water use efficiency (WUE; ratios P_N : E), and carboxylation efficiency (CE; ratios P_N : Ci) were recorded once the expanded leaf was enclosed in the LI-COR leaf chamber. In order to examine the effect of light intensity and CO_2 concentration, leaf photosynthesis was measured at different photosynthetically active radiations (PAR; 0, 300, 500, 700, 1000, 1200, and 1500 µmol m^{-2} s^{-1}), and at intercellular CO_2 concentration (Ci; 0, 400, 600, 800, 1000, 1200, and 1400 µmol mol^{-1}). Further, leaf photosynthesis was measured at every hour from 9.00 h to 15.00 h to examine the diurnal effect on P_N for the selected cultivated and wild rice as described in Feldman *et al.* (2017).

Visualization and quantification of leaf developmental traits

Morphological trait such as flag leaf width (FLW), leaf thickness (FLT) was measured from the widest part of the leaves from ten individual rice plants for each accession. For anatomical traits visualization, transverse sections were cut from the mid-portion of four leaves of different plants, and stained with toluidine blue with some modification of the method as described in Lux *et al.* (2005). Briefly, thin sections were mounted with 50 % glycerol on a tissue culture plate, chlorophyll pigments were cleared using 85 % (w/v) lactic acid saturated with chloral hydrate, heated on a water bath at 70 ºC for 1 hour and washed several times with distilled water. To visualize mesophyll cells, sections were incubated in absolute alcohol for overnight, stained with 1% toluidine blue in 1% (w/v) disodium tetraborate for 15 sec. Finally, thin sections were visualized using a bright field microscope (LMI, UK). The quantification of leaf anatomical features related to minor vein, mesophyll cell and, bundle sheath cells were performed at 10X and 100X magnification (ten images per leaf) using Fiji-Image J software (Wayne Rasband, National Institute of Health, USA) as described in Chatterjee *et al.* (2016) and Ivanova *et al.* (2017).

Chloroplast ultrastructure

Freshly harvested flag leaf segments (1 × 1 mm; four leaves from four different plants) were fixed under fixative buffer (3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2) for 2 hours and then treated with 1% osmium tetroxide overnight at 4 ºC. The fixed segments were dehydrated in a graded acetone series and embedded in epoxy resin. Ultrathin sections were cut with diamond knives, stained with uranyl acetate and lead citrate double staining procedure as described by Reynolds (1963). Chloroplasts of the uppermost part of the leaf sections were viewed using a digital camera (BH-2, Olympus) equipped in transmission electron microscopy at 1000X magnification (JEM 1230; JEOL).

Leaf chlorophyll measurement

Total chlorophyll content (TCC) were measured in 80% acetone following the procedure described by Kiran *et al.* (2013). Briefly, flag leaf (50 mg) was incubated in 5 ml ice-cold 80 % acetone for 48 h, centrifuged at 5,000 × g for 5 min and absorbance was recorded at 663 nm and 645 nm. The pigment concentration was calculated using the method described by van Campen *et al.* (2016) and expressed in mg g^{-1} FW.

Leaf Chlorophyll Fluorescence Measurements

Leaf chlorophyll (chl) fluorescence was measured using the LI-6400XT fluorometer (LI-COR Biosciences) according to the method described in van Campen *et al.* (2016). Plants were grown under controlled growth conditions, dark-adapted for at least 45 min before the measurement of F_v/F_m (ratio of the variable to maximum fluorescence - the quantum efficiency of open PSII centers). After the initial or minimum fluorescence (F_o) was measure, plants were illuminated with a 0.8 sec saturated pulse of light provided by an array of light-emitting diodes (LEDs) [mixture of red (90 %) and blue (10 %) lights at 650 nm peak wavelength, 1000 µmol m^{-2} s^{-1}] focused on a circular 2.0 cm^2 leaf surface area. Maximum fluorescence (F_m) was measured, and F_v/F_m calculated as (F_m-F_o/F_m). Plants were then placed under natural irradiance, and the steady-state of fluorescence (F_s) was recorded. A second saturating pulse at 1000 µmol m^{-2} s^{-1} was imposed to determine the maximal fluorescence level in light-adapted leaves (F_m´). Then, the actinic light was removed, and the minimal fluorescence level in the light-adapted state (F_o´) was determined through of illuminating the leaf with 1 sec of far-red light. Using light fluorescence parameters, we calculated: (a) ratio of variable to maximum fluorescence - the quantum efficiency of open PSII centers (F_v/F_m) in dark-adapted state, (b) the actual PSII efficiency (φPSII), (c) photochemical quenching

coefficient (qP), and (d) the photosynthetic electron transport rate (ETR) in light-adapted state as described in Murchie and Lawson (2013).

RNA isolation and expression analysis

Total RNA was isolated from flag leaf and leaf 4 at primordial 4 stages (P4) (van Campen *et al.,* 2016). Tissues were harvested, immediately frozen in liquid nitrogen. Total RNA extraction (TRIzol reagent), DNase treatment (DNase enzyme), and cDNA synthesis (RevertAid First-strand cDNA synthesis kit) were performed according to the manufacturer protocol (Thermo Fisher Scientific, USA). The PowerUp™ SYBR® Green fluorescence dye and specific primer sets (Supplementary Table S6) were used for quantitative gene expression analysis on CFX connect Real-time PCR detection system (Bio-Rad, CA, USA). The expression analysis was confirmed by analyzing two biological replicates, with three technical repeats each. Rice actin was used as an internal control gene for normalization of gene expression. The relative change or fold change was calculated following the method of (Livak and Schmittgen, 2001).

Rubisco activity assay

Rubisco extraction and activity (Vcmax) was performed following the procedure described in Kubien *et al.* (2011) and Scales *et al.* (2014). Briefly, leaf tissue (3.6 × 1.0 cm^2) from illuminated leaves were excised from five plants of each species and homogenized in extraction buffer [EB, 5 mM DTT, 10 mg ml^{-1} PVPP, 2 mg ml^{-1} BSA, 2 mg ml^{-1} PEG, 2% (v/v) Tween-80, 12 mM 6-aminocaproic acid, 2.4 mM benzamidine in HEPES stock) at 4 ºC. The homogenate centrifuged for 1 min at ~16,000 × g and pellet was discarded. To assess the activity of Rubisco from leaf extract, 50 µl of leaf extract was incubated with 1,550 ml of the EME buffer, 40 µl 10 mM of NADH (final concentration 200 µM), 80 µl of 250 mM $NaH^{12}CO_3$ (10 mM), 200 µl of ATP/phosphocreatine (1 and 5 mM, respectively), 40 µl of coupling enzymes (Carbonic anhydrase 500U, Glyceraldehyde-3-P dehydrogenase 50U, 3-PGA kinase 50U, Triose-P Isomerase/Glycerol-3-P dehydrogenase 400/40U), 40 µl of 25 mM RuBP (500 µM), mixed gently, and decrease of absorbance was monitored at 340 nm continuously for 0 to 180 sec using a UV–Vis spectrophotometer. Linear region of spectrophotometer absorbance were used to determine rate of RuBP-use using equations given below:

$$RuBP - use\ \left(\frac{\mu mol}{s}\right) = \frac{A(t0 - t1) * vol}{6.18 * 4 * (t0 - t1)}$$

Where A is absorbance (340 nm) at time 0 or time 1, vol (assay volume in ml), t time in sec, and 6.18 is an extinction coefficient in µmol $mL^{-1}cm^{-1}$ for NADH oxidation at 340 nm. Further, the maximum rate of RuBP-use was also used to calculate Rubisco activity in terms of Vcmax.

Statistical analysis

One-way analysis of variance (ANOVA) followed by statistical significance subscripts were grouped according to post-hoc Tukey HSD calculation (*P < 0.05, **P < 0.01). Least significant difference was calculated by Fisher's test (α = 0.05). Pearson correlation matrices were calculated based on mean values of each leaf developmental and physiological trait for each accession to evaluate the trait-to-trait associations. The level of significance (α) of correlation coefficients was determined at *P < 0.05 (± 0.666; degree of freedom (df; n-2; 9-2 = 7).

Results

Quantification of leaf photosynthesis and physiological parameters

Cultivated and wild rice species, showing remarkable differences for biomass accumulation for the same crop growth period, were examined for leaf photosynthesis (P_N) and related physiological features (Supplementary Fig. S1). The mean P_N value for the wild rice species, *O. latifolia*, *O. australiensis*, and *O. rufipogon*, and African cultivated rice *Oryza glaberrima* were found to be significantly higher than the cultivated rice varieties, such as IR64, Nagina 22 and Vandana (Fig. 1A). Among the selected accessions, *O. australiensis* and *O. latifolia* showed highest P_N, 23.50 and 24.18 µmol CO_2 m^{-2} s^{-1}, respectively. Also, we observed remarkable variations for P_N among the cultivated varieties. For instance, one of the high yielding *indica* variety Swarna and *japonica* variety Nipponbare displayed higher P_N compared to other cultivated varieties.

We further examined other important physiological traits of the selected accessions. As expected, wild rice species, *O. latifolia* and *O. australiensis* with very high P_N, showed higher stomatal conductance (g_s), and intercellular CO_2 concentration (Ci) (Fig. 1B, C). In addition, these species along with *O. rufipogon* displayed higher carboxylation efficiency (CE) than those of cultivated rice (Fig. 1F). Interestingly, *O. australiensis*, one of the two wild species with highest P_N, had relatively lower transpiration rate (E), resulting in highest water use efficiency (WUE) for the species (Fig. 1D, E). Wild species *O. punctata* and *O.*

sativa ssp *indica* cv. Vandana also exhibited lower E compared to other genotypes (Fig. 1D), and corresponding higher WUE of Vandana (Fig. 1E). WUE of *O. punctata* was still relatively lower due to lower P_N of the species (Fig. 1A, E).

Pearson correlation analysis showed a strong positive correlation between P_N with g_s ($r = 0.665$), Ci ($r = 0.632$), and CE ($r = 0.656$) (Supplementary Fig. S2). Higher WUE results from higher P_N coupled with lower E, which is evident from the strong positive correlation between WUE and P_N ($r = 0.618$) and a negative correlation between WUE and E (r = -0.589). A poor correlation was observed between P_N and E ($r = 0.248$) (Supplementary Fig. S2). We, further, quantified leaf photosynthesis and related physiological traits at early vegetative stage under controlled conditions in two independent experiments. Similar to our observations at full heading stage under field conditions, wild rice species (excluding *O. punctata*) and *O. glaberrima* showed higher P_N and g_s compared to cultivated rice since the beginning of vegetative stage (Supplementary Table S2). Taken together, these results showed significant differences in photosynthetic traits across cultivated and wild rice, with *O. australiensis* showing higher WUE along with remarkably higher leaf photosynthesis than cultivated rice varieties. We then selected representative cultivated rice accessions (IR 64, Nipponbare, and *O. glaberrima*) and wild rice species (*O. rufipogon, O. latifolia,* and *O. australiensis)* for detailed analyses.

Effects of light intensity, CO_2 concentration, and diurnal time on photosynthesis and related physiological traits

Light intensity and CO_2 concentration are the two most important factors determining leaf photosynthesis. Therefore, we examined the effect of different light intensity and CO_2 concentration on P_N, g_s, E, WUE, P_N (max), Ci and CE of selected wild and cultivated rice accessions (Fig. 2 and Supplementary Fig. S3). All the species showed a steeper initial slope, instantaneous enhancement followed by a plateau for leaf photosynthesis per unit area with an increasing photosynthetically active radiation (PAR) and CO_2 concentration (Fig. 2A and 2B-I). Wild rice species, *O. latifolia* and *O. australiensis*, together with *O. glaberrima* and *O. sativa* cv. Nipponbare exhibited higher P_N at the beginning, with clearly higher P_N at PAR > 1000 μmol m^{-2} s^{-1} (Fig. 2A-I). Similarly, P_N in response to increasing CO_2 concentration segregated the selected genotypes under two distinct groups: *O. australiensis, O. latifolia* and *O. glaberrima* with higher P_N, and cultivated varieties with lower P_N. The genotypes showed the pattern of g_s similar to that of P_N in response to changing light intensity (Fig. 2A-II). The g_s, however, showed a decreasing trend over increasing CO_2 concentration, likely due to the

closure of stomata (Fig. 2B-II). Nonetheless, P_N showed an increasing trend at higher concentration of CO_2. Interestingly, wild rice species were able to maintain higher g_s at different light intensity and CO_2 concentration. Cultivated varieties showed increased E, thus reduced WUE, in response to increasing light intensity (Fig. 2A, III –IV). In contrast, the wild species, *O. australiensis* and *O. latifolia*, showed lower E, and hence higher WUE, compared to cultivated varieties at different light intensities. Reducing E, and hence increased WUE, was detected for all the genotypes beyond 400 µmol mol^{-1} CO_2 concentration (Fig. 2B, III –IV).

In order to investigate the effect of diurnal timing, P_N and other physiological traits were quantified for the selected genotypes at different times of the day (Fig. 2C). P_N increased for all the genotypes until mid-day (9.00 h to 12.00 h) and decline thereafter (12.00 h to 15.00 h) (Fig. 2C-I). However, the extent of the decline was lesser in wild rice species, indicating that the wild species were able to maintain higher photosynthetic activity throughout the day. The g_s also followed the similar pattern to that of P_N for the selected species at different times of a day (Fig. 2C-II). Interestingly, E was found to be higher for the wild species, with an associated decrease in WUE in the immediate afternoon (13.00-14.00 h), likely due to leaf and stomatal features (Fig. 2C, D-III).

Taken together, all these results showed that the selected wild rice species were photosynthetically more efficient than cultivated rice varieties at different light intensities, CO_2 concentrations, and time points of a day.

Leaf developmental traits underpinning photosynthetic variations

Leaves are the prime photosynthetic organs, and expectedly the selected rice accessions exhibited striking differences in overall leaf architecture (Supplementary Fig. S1). At the morphological level, cultivated and wild rice species showed considerable variation for flag leaf width (FLW) and flag leaf thickness (FLT) (Supplementary Table S3). Wild rice species, except *O. punctata* that showed lower P_N, had thicker and wider leaves. A strong positive association of P_N were found with FLW ($r = 0.829$) and FLT ($r = 0.806$) (Fig. 3A).

At the anatomical level, remarkable differences in the vascular architecture and mesophyll cell features were observed. Wild rice species, with higher net photosynthesis, had larger and closely spaced veins with fewer number of mesophyll cells between two consecutive veins (Fig. 3B, Supplementary Table S3, S4). Those wild species also had larger mesophyll cell size, and thus lesser number of mesophyll cells per unit leaf area (Fig. 3C, Supplementary Fig. S4A-B and Supplementary Table S4). We, then, quantified total

mesophyll cell surface area per unit leaf area of the selected accessions. Wild rice species with higher P_N, *O. australiensis* and *O. latifolia*, showed remarkably higher mesophyll cell surface area per unit leaf area as compared to cultivated varieties (Fig. 3D). We also investigated the organization of chloroplasts in mesophyll cells of the representative wild and cultivated leaf. Photosynthetically efficient wild rice exhibited larger and numerous chloroplasts, systematically distributed along the mesophyll cell walls (Supplementary Fig. S5). Furthermore, wild species with higher P_N exhibited a larger vein height and width (Fig. 3E and Supplementary Table S3). Nipponbare and Swarna that showed relatively higher P_N compared to other cultivated varieties also showed larger vein height and width (Supplementary Table S3). Swarna, further, had wider leaf and higher vein number. Pearson correlation coefficient analysis showed a strong positive correlation of P_N with mesophyll area ($r = 0.905$), mesophyll length ($r = 0.874$), mesophyll width ($r = 0.930$), and mesophyll lobbing ($r = 0.771$) (Fig. 3A). The P_N was also strongly correlated with total number of veins ($r = 0.857$), minor veins height ($r = 0.906$), and minor vein width ($r = 0.758$) (Fig. 3A). However, P_N was poorly correlated with bundle sheath features, number, and area (Fig. 3A).

Photochemical differences among the selected rice accessions

Most of the accessions with higher photosynthesis also had higher total chlorophyll content (TCC; $r = 0.54$) (Fig. 4A). The *O. australiensis*, *O. latifolia* and *O. rufipogon* among wild rice species, and *O. glaberrima*, *O. sativa* cv. *Swarna*, and *O. sativa* cv. *Nipponbare* among cultivated rice had higher leaf chlorophyll content. The *O. punctata*, despite having highest chlorophyll content, showed lower P_N, suggesting developmental and/or biochemical constraints for efficient photosynthesis in the species. We, then, quantified chlorophyll fluorescence (F_v/F_m) of dark-adapted leaves to determine the differences in quantum photochemical efficiency of Photosystem II (PSII). Significant variations for F_v/F_m, which ranged from 0.73 to 0.83, were found across the selected cultivated and wild rice species (Fig. 4B). Wild rice species, *O. australiensis*, and *O. latifolia*, and *O. glaberrima* had higher F_v/F_m, indicating the better quantum yield of PSII chemistry in those species. *O. latifolia* and *O. australiensis* showed a significantly higher value of efficiency of PSII (□PSII), photochemical quenching (qP), and electron transport rate (ETR) compared to other accessions (Fig. 4C-E). *O. glaberrima*, the African cultivated rice with reasonably higher P_N, also showed relatively higher □PSII, qP, and ETR than *O. sativa* cultivated varieties. Cultivated accessions Nipponbare and Swarna didn't show significantly different □PSII, qP, and ETR compared to IR64, despite showing relatively higher P_N, suggesting contribution of

anatomical features and/or efficiency of photoassimilation reaction towards higher P_N in the two accessions (Fig. 1A, 4C-E). A strong correlation was observed between P_N with F_v/F_m ($r = 0.789$), □PSII ($r = 0.981$), qP ($r = 0.980$), and ETR ($r = 0.981$) (Supplementary Fig. S6). These results showed that photochemical features of wild rice species, as well as *O. glaberrima*, contribute to the higher leaf photosynthesis in those species.

Expression pattern of genes encoding core photosynthetic and chlorophyll biosynthesis enzymes

To envisage any differences in photosynthetic gene expression across the genotype, we checked the expression pattern of representative genes encoding key photosynthetic components, LHC1 (Light harvesting chlorophyll protein complex), Rubisco LS (Large Subunit), Rubisco SS (Small Subunit), and TPT (Triose phosphate translocator) (Supplementary Table S5). The expression of the selected photosynthetic genes was significantly higher in wild rice species (Fig. 5A), suggesting higher photosynthesis in those species could, at least in part, be due to the higher abundance of these gene products. These genes could possibly increase net photosynthetic efficiency by increasing the efficiency of light capture, CO_2 assimilation, and transport of triose phosphate from chloroplast to cytoplasm. Since wild rice species with higher photosynthesis also showed higher chlorophyll content, we checked the expression level of chlorophyll biosynthesis genes. The expression of chlorophyll biosynthesis genes, such as *GluTR*, *PPOX*, *Mg-CHLD*, *POR*, and *CHLSYN*, were found to be significantly higher in *O. latifolia* and *O. australiensis* (Fig. 5B). *O. rufipogon* also had significantly higher expression of selected photosynthetic and chlorophyll-biosynthetic genes compared to Nipponbare and IR 64.

We then investigated the involvement of transcription factors in the regulation of expression of the selected photosynthetic and chlorophyll-biosynthetic genes. A comprehensive list of transcription factors regulating photosynthetic and chlorophyll biosynthetic-genes has been reported in Wang *et al.* (2017). The differentially expressed photosynthetic and chlorophyll biosynthetic-genes were hierarchically clustered with the expression of potential transcription factors in rice using Genevestigator (https://genevestigator.com/gv/). We found a number of transcription factors co-expressed with the selected differentially expressed genes in flag leaves (Supplementary Fig. S7). We, then, selected ten such transcription factors and profiled their gene expression at primordia 4 (P4) stage of leaf 4 (Supplementary Table S5). At least six of those ten genes, including *GLK1*, *GLK2*, and *SIGMA* factors, were expressed at higher levels in wild rice with higher

leaf photosynthesis (Fig. 5C). *O. glaberrima* and *O. sativa cv. Nipponbare*, with moderately high P_N, also showed higher expression of some of these transcription factors. Differential expression of these transcription factors suggested that differences in the transcriptional module regulating photosynthetic gene expression could be critical for determining leaf photosynthetic efficiency.

Rubisco activity differences across the selected rice genotypes

The catalytic properties of Rubisco vary considerably from diverse plant sources, suggesting that differences in the Rubisco activity might contribute to differences in photosynthetic activity (Parry *et al.,* 2013). To test the possible Rubisco activity differences, we quantified the RuBP-use and Rubisco activity (Vcmax) across selected cultivated and wild rice species. The CO_2-saturated RuBP-use was found to be significantly higher for wild rice species, *O. latifolia*, *O. australiensis*, and *O. rufipogon*, compared to *Oryza sativa cv.* IR 64 (Fig. 5D). Interestingly, *Oryza sativa cv. Nipponbare*, a *japonica* variety, also had higher RuBP-use compared to *indica* variety IR 64. The photosynthesis rate per unit area, as discussed earlier, was found to be higher in wild rice species as well as Nipponbare compared to IR 64 (Fig. 1A). Regression analysis between P_N and Rubisco enzyme activity (Vcmax at 180 sec.) showed strong association between P_N and Vcmax ($R^2 = 0.835$, $P < 0.001$) (Fig. 5E). These results indicated the existence of variation in Rubisco carboxylation activity within the rice system, cultivated and wild rice, which could possibly be exploited for increasing CO_2 assimilation.

Discussion

The importance of investigating variations in leaf photosynthesis within and across species to be exploited for sustainable increases in crop yield and productivity has been emphasized (Ort *et al.,* 2015; Simkin *et al.,* 2019; van Bezouw *et al.,* 2019). A number of studies have shown the variation in photosynthesis and related traits within species, such as maize (Yabiku and Ueno, 2017), wheat (Driever *et al.,* 2014), rice (Giuliani *et al.,* 2013; Qu *et al.,* 2017), and even C_4 species *Gynandropsis gynandra* (Reeves *et al.,* 2018). Extensive comparative genetic and genomic studies have been undertaken to investigate the variation in photosynthetic efficiency across C_3 and C_4 species (Külahoglu *et al.,* 2014; Wang *et al.,* 2014). Based on these studies, global efforts to engineer C_4 photosynthesis in rice are being made, which is challenging due to extensive genetic, developmental, and biochemical

differences between C_4 plants and rice. An alternative and efficient strategy would be exploiting the photosynthetic variations within rice system itself, including cultivated and wild rice. There had been a few studies highlighting the photosynthetic differences within cultivated, and among cultivated and wild rice accessions (Kiran *et al., 2013;* Kondamudi *et al.,* 2016; Haritha *et al.,* 2017). In this study, we not only quantified leaf photosynthetic differences across cultivated and wild rice accessions, but also identified the underlying anatomical, biochemical, and photochemical traits mediating higher leaf photosynthesis in rice. In addition, we evaluated the relationship between photosynthesis and other physiological traits, such as gas exchange, carboxylation efficiency, transpiration, and water use efficiency for the wild and cultivated rice accessions.

Wild relatives of rice, *Oryza australiensis*, *Oryza latifolia*, and *Oryza rufipogon*, as well as African domesticated rice *Oryza glaberrima* showed higher leaf photosynthesis per unit area (P_N) compared to cultivated varieties of *Oryza sativa* (Fig. 1A). Stomatal conductance (g_s) is known to be an important factor determining the leaf photosynthesis rate (Gago *et al.,* 2016). Two wild rice species, *O. australiensis* and *O. latifolia*, with highest P_N also had highest values of g_s, which in turn resulted in higher internal CO_2 concentration (Ci) in those species (Fig. 1B, C), supporting a strong correlation between P_N and g_s (Kanemura *et al.,* 2007; Giuliani *et al.,* 2013). *O. rufipogon* with reasonably higher P_N, however, had lower g_s and Ci. The higher P_N in *O. rufipogon* was likely due to highest carboxylation efficiency (CE) in the species among the selected accessions (Fig. 1F). Further, *O. australiensis* and *O. latifolia* also had reasonably higher CE. The P_N has been shown to be positively associated with CE in rice (Yeo *et al.,* 1994). Indeed, quantification of photosynthesis and related physiological parameters for introgressions from *O. rufipogon* in a cultivated background showed the influence of both CE as well as g_s on higher leaf photosynthesis (Haritha *et al.,* 2017). Furthermore, higher g_s and CE could also explain higher P_N across different PAR in the wild rice (Fig. 2A-II and Supplementary Fig. S3A-III). Higher P_N in the wild species at different times of a day, even in the afternoon when stomata tend to close, compared to cultivated varieties could also be attributed to relatively higher g_s and CE in those species (Fig. 2C and Supplementary Fig. S3C). Notably higher photosynthetic efficiency in the wild rice species suggests that leaf photosynthesis in cultivated rice has not yet reached the saturation level, and therefore, can possibly be achieved by targeting higher g_s and/or higher CE. Moreover, increments in g_s might also help in optimizing photosynthetic performance under environmental stress conditions. *O. australiensis*, interestingly, appeared to be the most water-use efficient among the selected accessions due to high P_N coupled with low

transpiration rate (E), making it an ideal genotype to investigate for maintaining higher leaf photosynthesis under drought conditions (Fig. 1A, D, and E). Many of the wild rice species, including *O. australiensis*, have been marked for their potential usage towards tolerance to heat and drought (Sanchez *et al.*, 2013; Menguer *et al.*, 2017). The differences in g_s and E are likely related to differences in the stomatal features or regulation of stomatal opening across the genotypes, as stomatal features are critical for photosynthesis, gas exchange, and water use efficiency (Farquhar and Sharkey, 1982). Kondamudi *et al.* (2016) showed that leaves with larger-sized stomata had higher photosynthesis rate than the leaves with smaller-sized stomata due to higher g_s. Similarly, rice species with broader leaves have been shown to exhibit higher P_N due to increased g_s than species with narrow leaves (Giuliani *et al.*, 2013). Differences in internal CO_2 concentration and/or biochemical differences, such as Rubisco activity, may mediate the observed differences in the CE (Eichelmann and Laisk, 2002). Nonetheless, efforts to increase g_s and CE in the cultivated rice varieties comparable to the high photosynthetic wild species shall help in increasing photosynthetic efficiency.

The strong correlation of leaf developmental traits with P_N is not surprising considering the strong influence of leaf traits on light perception, gas exchange, CO_2 assimilation, and downstream photoassimilate partitioning. We observed a strong positive association of P_N with leaf width and thickness (Fig. 3A). Leaf thickness has been suggested to influence photosynthesis through efficient absorption and utilization of light by homogenizing the leaf internal light distribution as well as maintaining leaf temperature (Tholen *et al.*, 2012). The association of leaf width with photosynthetic performance and yield in rice has been observed earlier. *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes *NAL1*, *NAL2*, and *NAL3* have been shown to be associated with increased leaf width, which in turn results in increased photosynthetic efficiency and yield (Fujita *et al.*, 2013; Ishiwata *et al.*, 2013). Introgression of a natural variant of *NAL1* from a *japonica* to *indica* cultivar led to thicker and wider leaves with higher leaf photosynthesis rate and yield (Takai *et al.*, 2013).

Rice system also displays significant inter-species variation for anatomical traits, suggesting strong genetic control over leaf anatomical features (Chatterjee *et al.*, 2016). Photosynthetically efficient wild rice species have mesophyll cell features that promote both higher accesses to available CO_2 as well as efficient light harvesting. Larger sized and lobed mesophyll cells in wild rice species likely provides increased surface area to access more internal CO_2 (Fig. 3C, D), whereas the organized pattern and systematic distribution of chloroplasts in the mesophyll cells of those species may promote efficient light capture to enhance photosynthesis (Supplementary Fig. S5 and Fig. 6). Interestingly, *NAL1* (discussed

above) promotes leaf width and thickness by increasing mesophyll cell number and area. An increased vein density has been suggested to facilitate higher leaf photosynthesis by promoting efficient export of photosynthates (Amiard *et al.,* 2005; Feldman *et al.,* 2017). Lesser number of mesophyll cells between two consecutive veins in photosynthetically efficient wild species, a feature similar to C_4 plants, should be promoting efficient transport of photoassimilate. Larger vein size in the wild rice species shall, further, be adding to efficient transport, thus increasing the leaf photosynthesis. The larger mesophyll cells with bigger chloroplasts and larger veins in photosynthetically efficient wild rice species are reminiscent of "Proto-kranz anatomy," an evolutionary step towards C_4 photosynthesis and have been suggested to be an essential step towards increasing photosynthetic efficiency (Osborne and Sack, 2012). Photosynthetically efficient wild rice species with desirable anatomical features provide a potential genetic resource towards the identification of genes for desirable manipulation in mesophyll and vein features.

Wild species with higher P_N showed significantly higher F_v/F_m compared to cultivated varieties, indicating better quantum efficiency of Photosystem II in those species (Fig. 4B). The F_v/F_m is used as the most common parameter for monitoring 'stress' in leaves as stress results in inactivation damage of PSII by lowering of F_v/F_m (Murchie and Niyogi, 2011). The comparative higher values for quantum efficiency of PSII (□PSII), photochemical quenching (qP), and Electron Transport Rate (ETR) in the wild rice indicate PSII reaction centres are more receptive for electron transport from PSII to PSI, making an efficient link between light and light-independent reactions to increase P_N (Fig. 4C-E) (Kramer and Evans, 2011). A positive correlation has been reported between chlorophyll fluorescence in leaf and grain yield (von Kroff *et al.,* 2008). The wild species with better photochemical features and higher P_N accumulates more biomass, and not grain yield, likely due to defects in photoassimilate partitioning to the grain. Nonetheless, the desirable photochemical features, at least in part, contribute to higher photosynthetic efficiency of the wild species. Gene expression studies also showed higher expression of *LHC1* gene, which is involved in organization of reaction centre, in photosynthetically efficient wild species.

Rubisco is the rate-limiting enzyme for photoassimilation of CO_2, and Rubisco abundance and activity strongly determines the photosynthetic performance of a plant (Parry *et al.,* 2013). High photosynthetic wild species had higher expression of genes encoding Rubisco large subunit and small subunit, indicating the higher abundance of Rubisco in those species (Fig. 5A). Those wild species also had higher expression of chlorophyll biosynthetic genes (Fig. 5B). The photosynthetic process as such, and genes encoding key components of

photosynthesis are under strong regulation of transcription factors (Wang *et al.,* 2017). Differences in the expression of transcription factors, such as *GLKs* involved regulation of chlorophyll biosynthesis and *SIGMA* factors regulating the chloroplastic gens *Rubisco LS*, suggested the rewiring of transcriptional module regulating photosynthesis as a potential target to increase rice leaf photosynthesis. Moreover, we also observed a higher RuBP-use and Rubisco carboxylation activity (Vcmax) in photosynthetically efficient wild species (Fig. 5D, E). The prospects of improving Rubisco catalysis to increase photosynthetic efficiency have been explored. It has been suggested that engineering maize Rubisco in rice would improve CO_2 assimilation (Parry *et al.,* 2013; Sharwood *et al.,* 2016). This study suggests a possible existence of an efficient Rubisco in photosynthetically efficient wild rice. Further studies are needed to investigate whether Rubisco abundance or Rubisco kinetic properties contribute to differences in Rubisco carboxylation activity across cultivated and wild rice.

In summary, we characterized photosynthetically efficient wild rice species for leaf developmental, photochemical, and biochemical differences. Leaf developmental features (larger and lobbed mesophyll cells, larger veins, and lesser number of mesophyll cells between two consecutive veins), photochemical features (quantum efficiency of PSII, Photochemical quenching, and Electron Transport Rate), and Rubisco activity contribute to higher leaf photosynthesis in wild rice (Fig. 6). The study highlights the existence of significant exploitable variation in leaf photosynthesis and underlying traits across cultivated and wild rice. Future efforts to dissect the genetic and molecular basis of the underlying developmental, photochemical, and biochemical traits shall be instrumental in tailoring photosynthetically efficient rice.

Supplementary data

Fig. S1. Representative images of cultivated and wild rice species showing remarkable differences in plant architectural traits, leaf features, and biomass. Shown are the field grown plants (A), and controlled growth chambers grown plants (B) of the selected genotypes.

Fig. S2. Pair-wise Pearson correlation coefficient (*r*) analysis among net photosynthetic rate (P_N) and related physiological traits.

Fig. S3. Quantification of P_N (max), Ci and CE in response to increasing PAR (A, I-III), increasing CO_2 concentration (B, I-III), and at different diurnal time (C, I-III) for selected cultivated and wild rice species.

Fig. S4. Quantification of average mesophyll cell area (A) and mesophyll cell number per unit leaf area (cm^{-2}) (B) of the selected genotypes.

Fig. S5. TEM images of organization of chloroplasts in mesophyll cells.

Fig. S6. Pair-wise Pearson correlation coefficient (r) analysis among net photosynthetic rate (P_N), chlorophyll fluorescence traits, and photochemical features.

Fig. S7. An *in silico* generated hierarchical clustering dendrogram for the expression pattern of key photosynthetic and chlorophyll-biosynthetic genes, and transcription factor regulating photosynthetic machinery across nine anatomical parts of rice plants (seedling, coleoptile, seedling leaf, stem, vegetative leaf, blade, collar, sheath, flag leaf). The data were collected and dendrograms was generated using Genevestigator (https://genevestigator.com/gv/).

Table S1. Details of rice accessions used to investigate the leaf photosynthesis and related physiological features.

Table S2. Variation in leaf photosynthesis and related physiological traits among cultivated and wild rice species under controlled conditions.

Table S3. Quantitative measurement of flag leaf width (FLW, cm), flag leaf thickness (FLT, mm), Minor vein width (µm), Minor vein height (µm), and total veins (n) in the fully expanded flag leaf of cultivated and wild rice species.

Table S4. Quantitative measurement of mesophyll lobes (n), length (µm), width (µm), and area (μm^2) and bundle sheath number (n), and area (μm^2) in the fully expanded flag leaf of cultivated and wild rice species.

Table S5. Details of genes and transcription factors used for gene expression analysis.

Table S6. List of primers for qRT-PCR analyses.

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Figure Legends

Fig. 1. Variations in leaf photosynthesis and related physiological parameters across cultivated and wild rice species. Quantification of net photosynthesis per unit area of flag leaf surface area (P_N, A), stomatal conductance (g_s, B), intercellular CO_2 concentration (Ci, C), transpiration rate (E, D), water use efficiency (WUE, E), and carboxylation efficiency (CE, F) for six cultivated rice accessions and four wild rice species. Cultivated rice accessions and wild species are represented with blue and green shading box plots, respectively. Asterisks indicate significant differences between genotypes (one-way ANOVA, n = 15, $*P < 0.05$). Different letters indicate significant groupings according to post-hoc Tukey HSD calculation.

Fig. 2. Effects of PAR intensity, CO_2 concentration, and diurnal time on leaf photosynthesis (P_N), stomatal conductance (g_s), transpiration (E), and water use efficiency (WUE) of different rice genotypes.

(A) Quantification of P_N (I), g_s (II), E (III), and WUE (IV) in response to increasing PAR from 0 to 1500 µmol m^2 s^{-1}.

(B) Quantification of P_N (I), g_s (II), E (III), and WUE (IV) in response to increasing CO_2 concentration from 0 to 1400 µmol mol^{-1}.

(C) Quantification of P_N (I), g_s (II), E (III), and WUE (IV) at different time points of a day from 9.00 h to 15.00 h

Each value represents the mean of 5 data points ± SD from different plants.

Fig. 3. Leaf anatomical traits effecting photosynthesis rate and related physiological traits of rice.

(A) Pearson correlation analysis of net photosynthesis rate (P_N) and related physiological traits with leaf developmental features. The level of significance (α) of correlation coefficients at $*P < 0.05$ (± 0.666; degree of freedom (df; n-2): 9-2 = 7).

(B) Bright-field images of a flag leaf of the selected genotypes showing variations in mesophyll cells and vasculature. Arrows indicate bulliform cells (bc), bundle sheath (bs),

epidermis (ep), mesophyll cells (mc), and minor veins (miv). Scale bars represent 100 µm at 10X magnification.

(C) Bright-field microscopy images showing variations in mesophyll cell features, including lobbing. Scale bar represents 100 µm at 100X magnification.

(D-E) Quantification of total mesophyll cell surface area per unit leaf area (D) and minor vein height (E) of selected cultivated and wild rice species. Cultivated rice accessions and wild species are represented with blue and green shading box plots, respectively. Asterisks indicate significant differences between genotypes [one-way ANOVA, n = 4 leaves (10 images per leaf), $*P < 0.05$]. Different letters indicate significant groupings according to post-hoc Tukey HSD calculation.

Fig. 4. Quantification of photochemical features of selected cultivated and wild rice species. Total chlorophyll content (TCC) per gram of fresh weight (A), F_v/F_m in dark-adapted leaves (B), Efficiency of PSII (ΦPSII, C), Photochemical quenching (qP, D), and Electron transport rate (ETR, E) of the selected genotypes. Cultivated rice accessions and wild species are represented with blue and green shading box plots, respectively. All the measurements were performed in fully developed vegetative stage leaves of controlled growth chambers grown plants. Asterisks indicate significant differences between genotypes (one-way ANOVA, n = 7, $*P < 0.05$). Different letters indicate significant groupings according to post-hoc Tukey HSD calculation.

Fig. 5. Expression analysis of genes and transcription factors regulating photosynthesis, and differences in Rubisco activity for selected cultivated and wild rice species.

(A) Expression levels of genes encoding key component of light harvesting complex, Rubisco, and Triose phosphate/phosphate translocator.

(B) Expression levels of chlorophyll biosynthetic genes in flag leaves.

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(E) Regression analysis between P_N and Vcmax at 180 sec of the assays (µmol m^2 s^{-1}) generated from the maximum rate of RuBP-use of the selected genotypes.

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Gene abbreviations: GluTR, *Glutamyl-tRNA reductase*; PPOX, *Protoporphyrinogen oxidase*; Mg-CHL, *Magnesium chelatase*; CHLSYN, *Chlorophyll synthase*; CAO, *Chlorophyllide a oxygenase;* LHCI, *Light-harvesting chlorophyll protein complex I*; Rubisco SS, *Ribulose bisphosphate carboxylase small subunit*; Rubisco LS, *Ribulose bisphosphate carboxylase large subunit*; TPT, *Triose phosphate/phosphate translocator*; GLK, *Golden like* (1 and 2); SIG, *Sigma factors* (1, 2, 5, and 6); ZML2, *ZINC FINGER PROTEIN 2*; ORE1, *ORESARA1*; CGA1, *Cytokinin responsive GATA Factor*; GRF5, *Growth regulating factor 5*.

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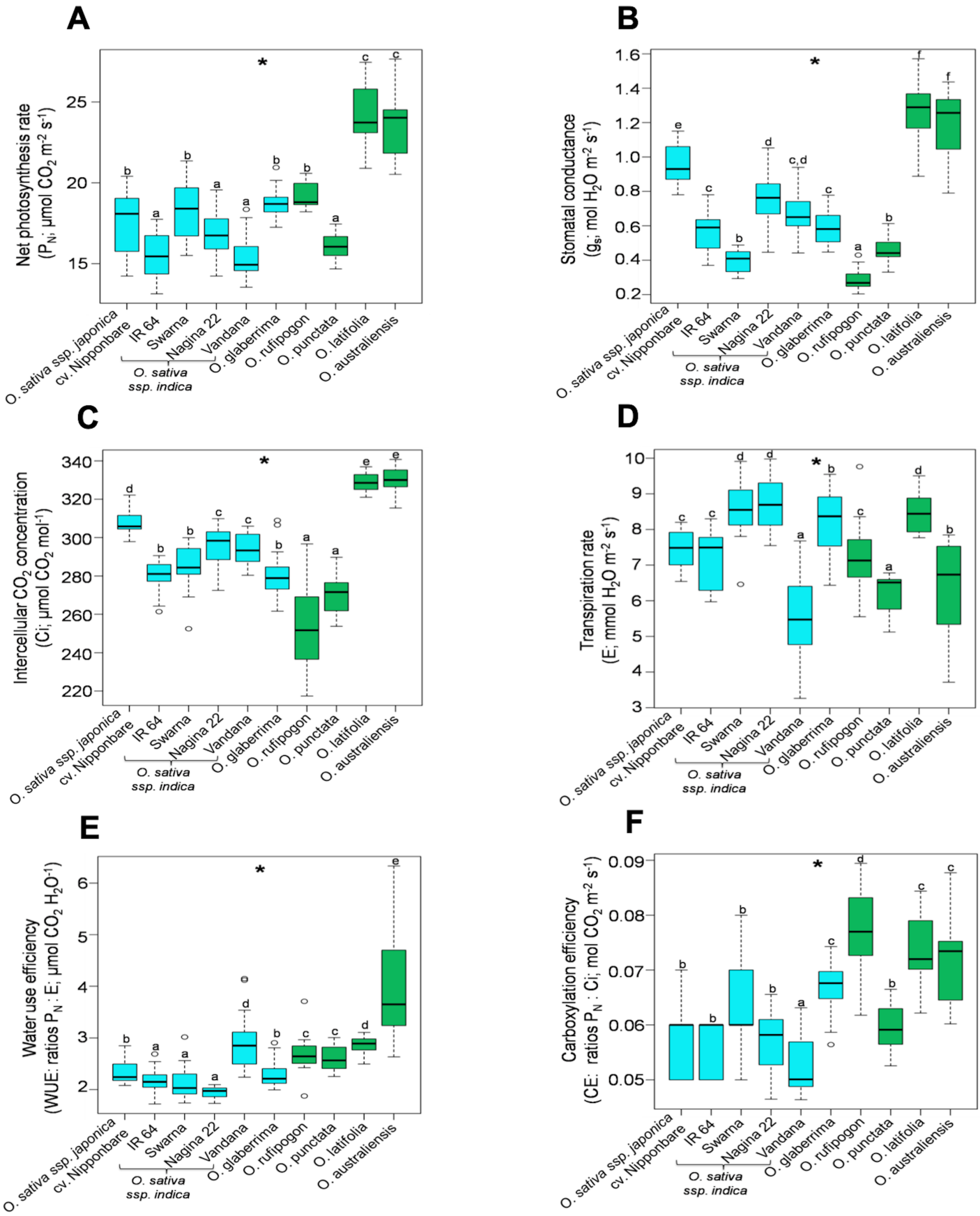

Fig. 1. Variations in leaf photosynthesis and related physiological parameters across cultivated and wild rice species. Quantification of net photosynthesis per unit area of flag leaf surface area (P_N, A), stomatal conductance (g_s, B), intercellular CO_2 concentration (Ci, C), transpiration rate (E, D), water use efficiency (WUE, E), and carboxylation efficiency (CE, F) for six cultivated rice accessions and four wild rice species. Cultivated rice accessions and wild species are represented with blue and green shading box plots, respectively. Asterisks indicate significant differences between genotypes (one-way ANOVA, n = 15, $*P < 0.05$). Different letters indicate significant groupings according to post-hoc Tukey HSD calculation.

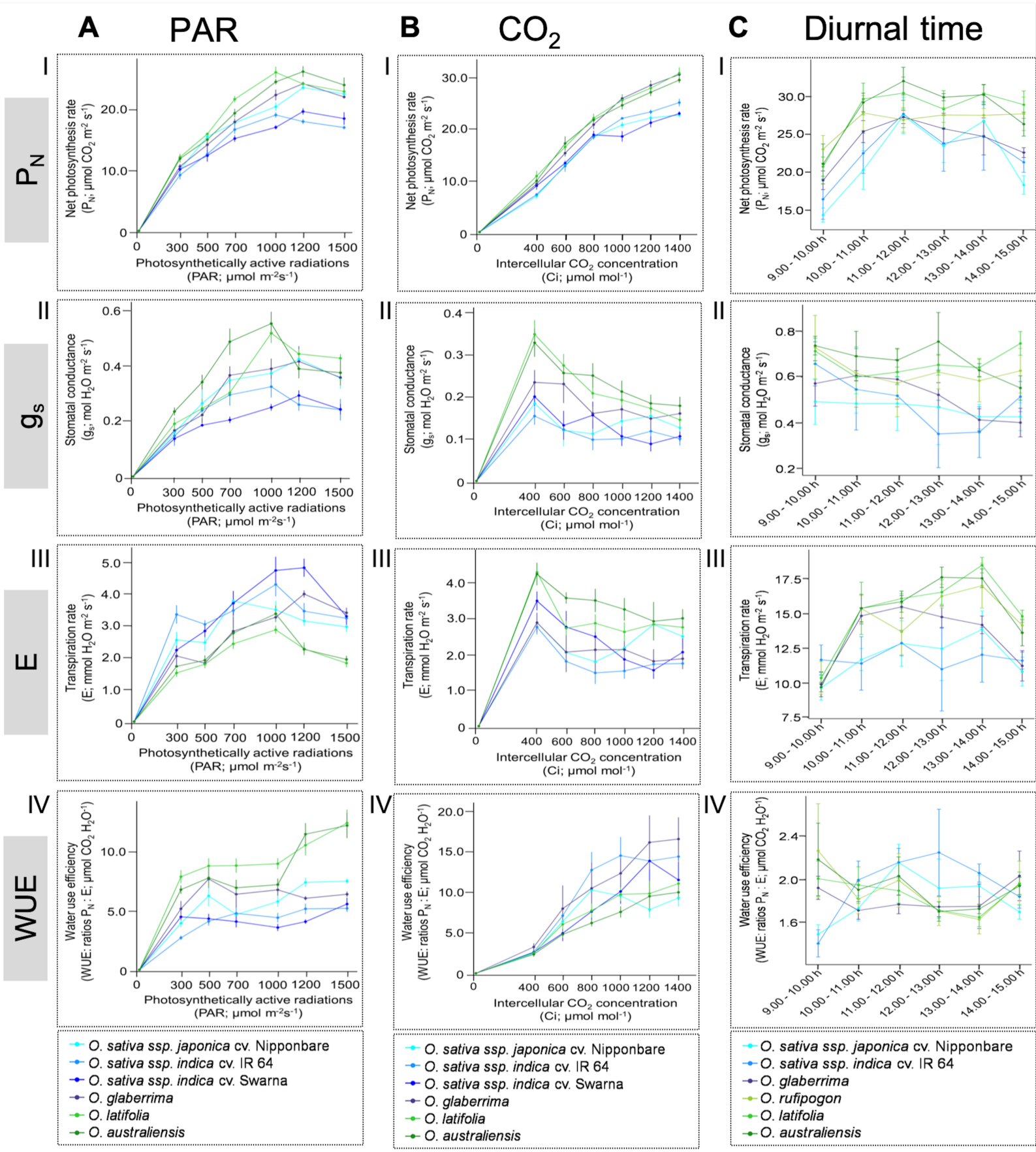

Fig. 2. Effects of PAR intensity, CO_2 concentration, and diurnal time on leaf photosynthesis (P_N), stomatal conductance (g_s), transpiration (E), and water use efficiency (WUE) of different rice genotypes.
(A) Quantification of P_N (I), g_s (II), E (III), and WUE (IV) in response to increasing PAR from 0 to 1500 µmol $m^2 s^{-1}$.
(B) Quantification of P_N (I), g_s (II), E (III), and WUE (IV) in response to increasing CO_2 concentration from 0 to 1400 µmol mol^{-1}.
(C) Quantification of P_N (I), g_s (II), E (III), and WUE (IV) at different time points of a day from 9.00 h to 15.00 h
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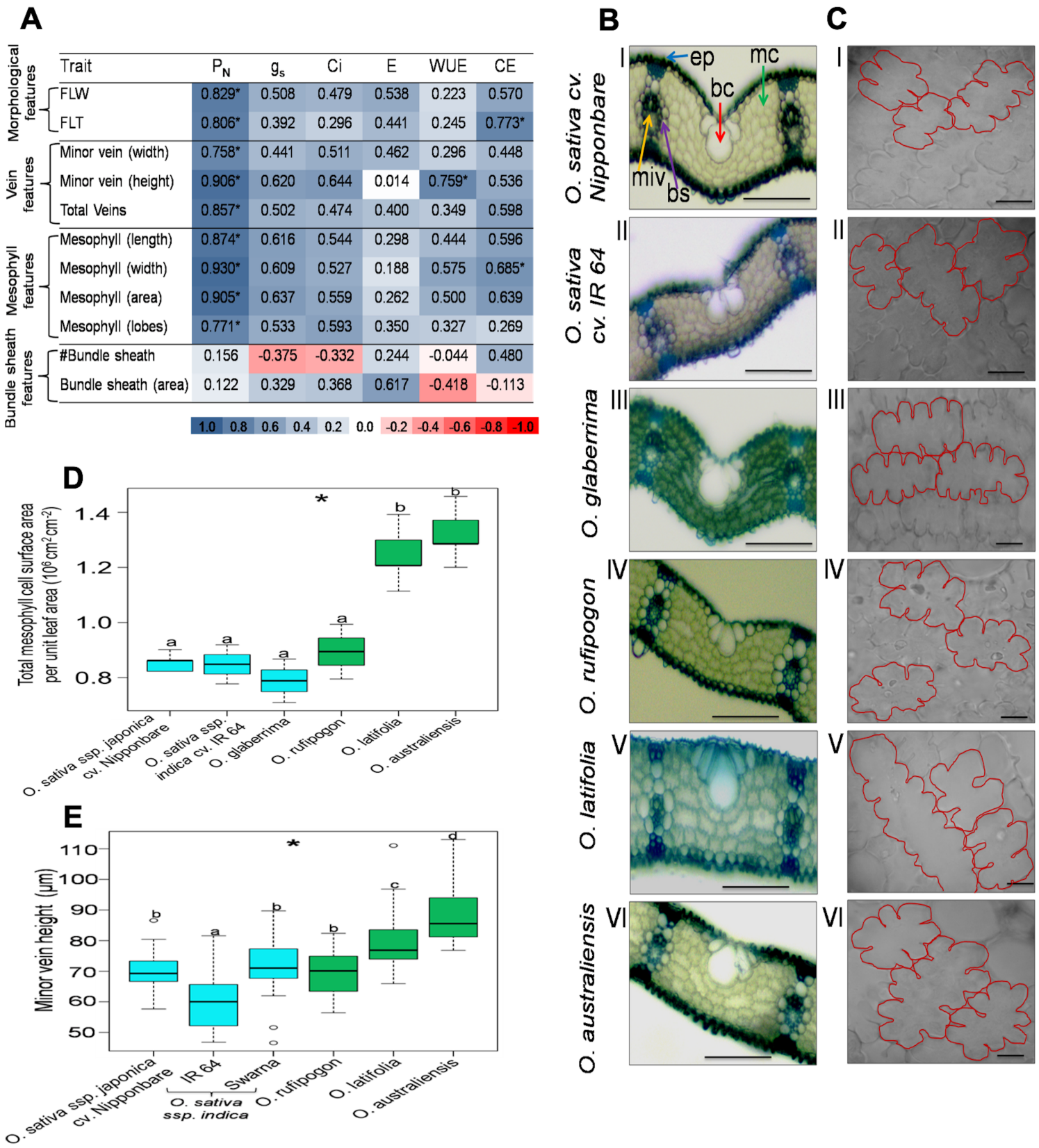

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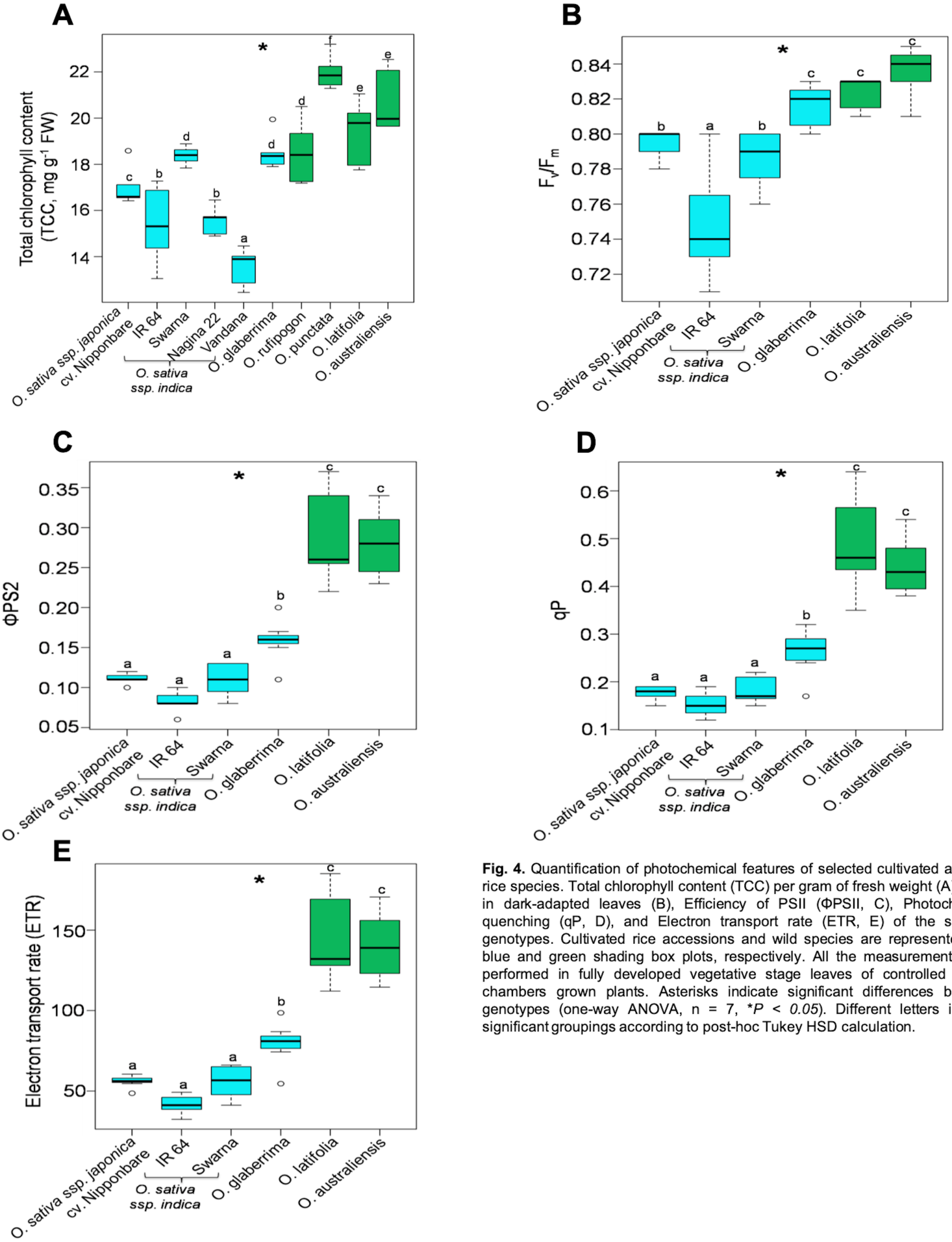

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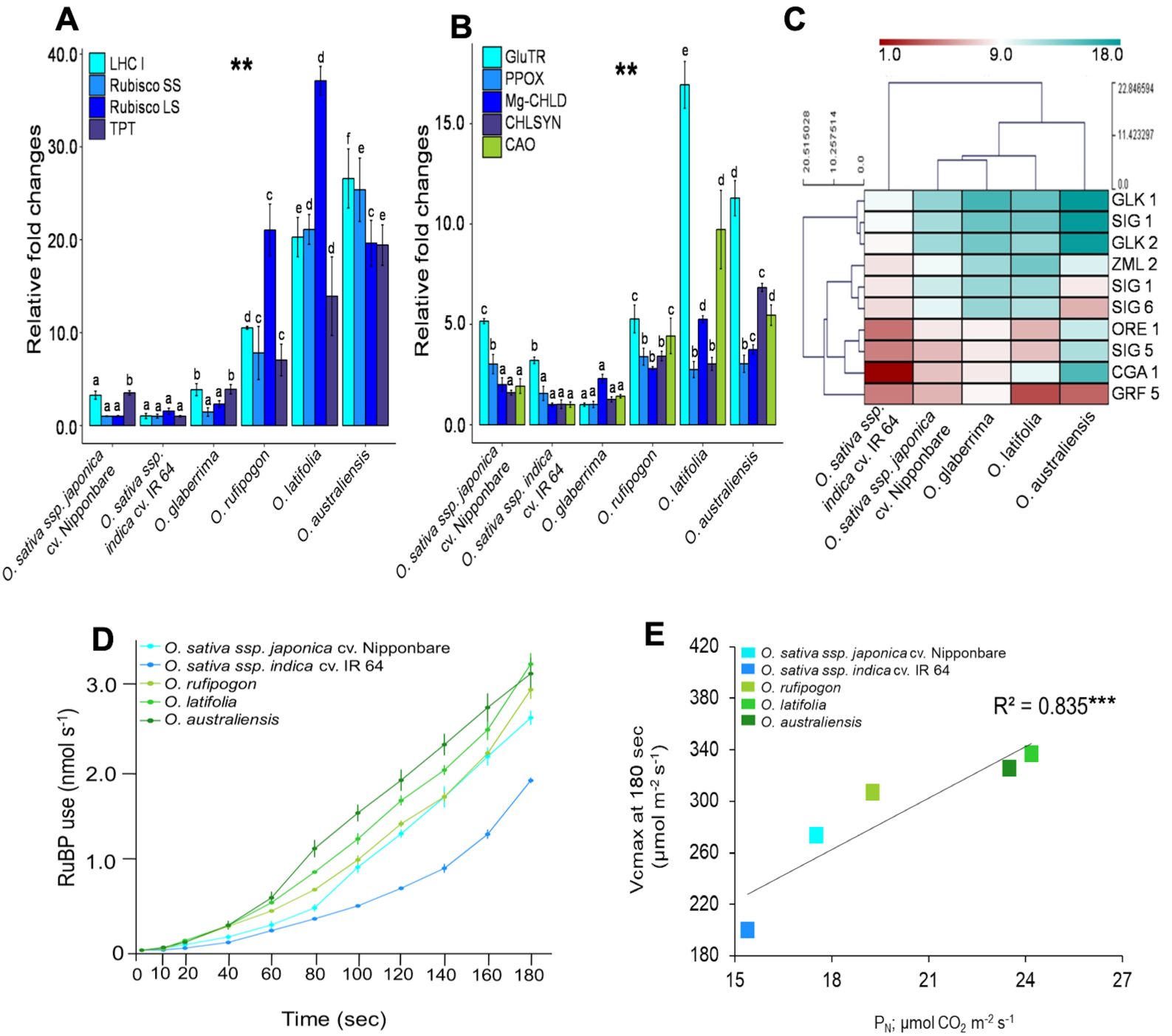

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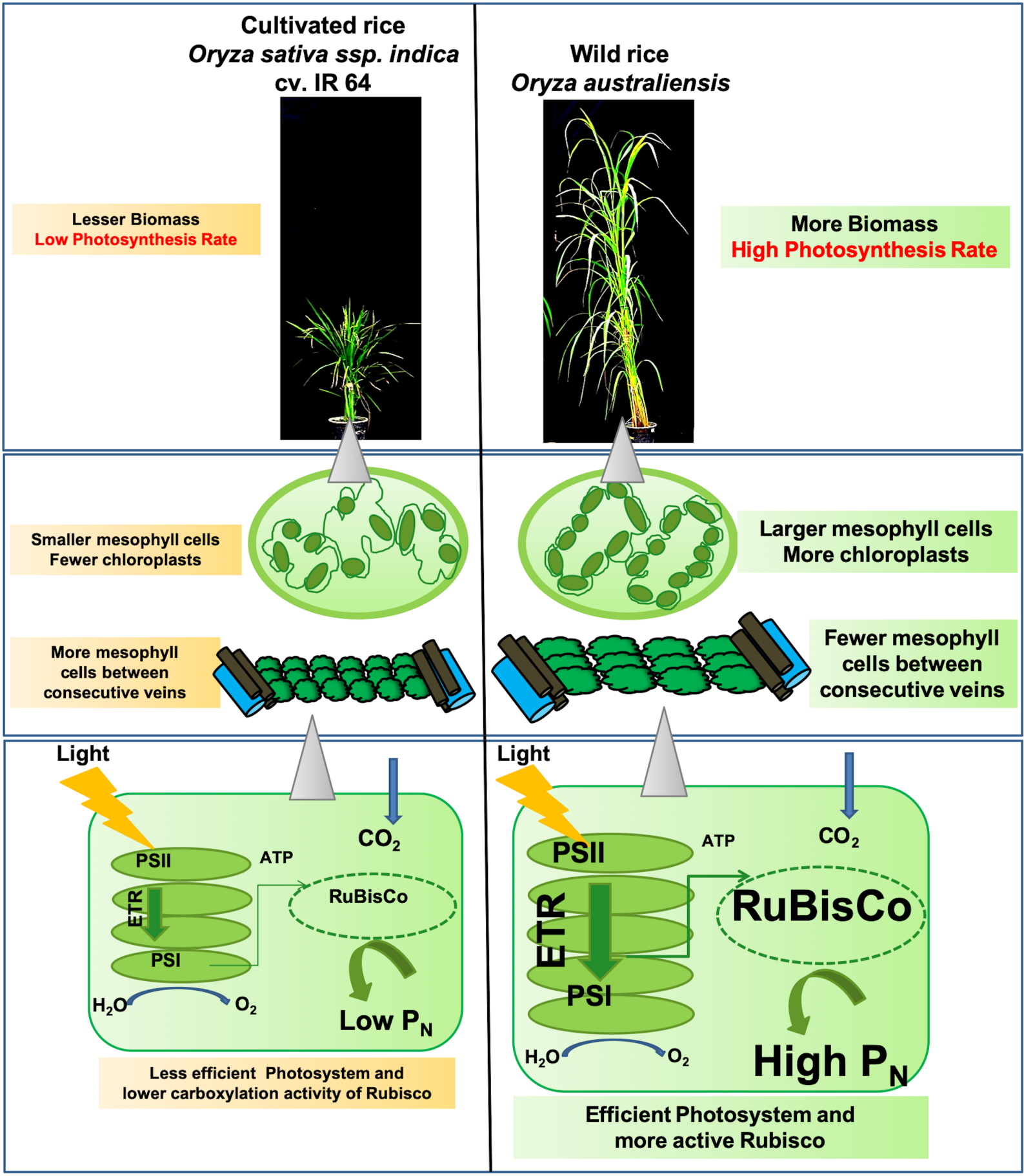

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