The existence of $C_4$-bundle-sheath-like photosynthesis in the mid-vein of $C_3$ rice

Weijun Shen¹, Luhuan Ye¹, Jing Ma¹, Zhongyuan Yuan¹, Baogang Zheng¹, Chuangen LV², Ziqiang Zhu¹, Xiang Chen³, Zhiping Gao¹* and Guoxiang Chen¹*

Abstract

Background: Recent studies have shown that $C_4$-like photosynthetic pathways partly reside in photosynthetic cells surrounding the vascular system of $C_3$ dicots. However, it is still unclear whether this is the case in $C_3$ monocots, especially at the molecular level.

Results: In order to fill this gap, we investigated several characteristics required for $C_4$ photosynthesis, including $C_4$ pathway enzymes, cyclic/non-cyclic photophosphorylation rates, the levels and assembly state of photosynthetic machineries, in the mid-veins of $C_3$ monocots rice with leaf laminae used as controls. The signature of photosystem photochemistry was also recorded via non-invasive chlorophyll a fluorescence and reflectance changes at 820 nm in vivo. Our results showed that rice mid-veins were photosynthetically active with higher levels of three $C_4$ decarboxylases. Meanwhile, the linear electron transport chain was blocked in mid-veins due to the selective loss of dysfunctional photosystem II subunits. However, photosystem I was sufficient to support cyclic electron flow in mid-veins, reminiscent of the bundle sheath in $C_4$ plants.

Conclusions: The photosynthetic attributes required for $C_4$ photosynthesis were identified for the first time in the monocotyledon model crop rice, suggesting that this is likely a general innate characteristic of $C_3$ plants which might be preconditioned for the $C_4$ pathway evolution. Understanding these attributes would provide a base for improved strategies for engineering $C_4$ photosynthetic pathways into rice.

Keywords: $C_3$ and $C_4$ photosynthesis, Cyclic/linear electron flow, Dysfunctional PSII, Mid-vein, Rice

Background

$C_4$ plants partition photosynthetic reactions between two distinct cell types: vascular bundle sheath (BS) and mesophyll (M) cells, a structure called ‘Kranz’ anatomy (Majeran and van Wijk 2009). Atmospheric CO₂ is firstly fixed as $C_4$ acids by phosphoenolpyruvate carboxylase (PEPC) in M cells, then the $C_4$ acids are transferred into BS cells to be degraded by decarboxylating enzymes. The released CO₂ is fixed by ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBiSCO) and incorporated into the $C_3$ cycle (Furbank 2011). This structural specialization of leaf tissue allows optimum operation of the $C_4$ photosynthetic pathway to concentrate CO₂.

$C_4$ plants are divided into three biochemical subtypes based on different decarboxylating mechanisms: nicotinamide adenine dinucleotide phosphate-dependent malic enzyme (NADP-ME), nicotinamide adenine dinucleotide-dependent malic enzyme (NAD-ME), and phosphoenolpyruvate carboxykinase (PEPCK) types (Yoshimura et al. 2004). The majority of $C_4$ crop species belong to the NADP-ME group (Furbank 2011) in which BS cells utilize malate as the $C_4$ acid. Since malate decarboxylation results in a donation of reductive power, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), substantial production of additional ATP would be required in BS cells (Finazzi et al. 2002). Therefore, NADP-ME $C_4$ monocots such as sorghum (Sorghum bicolor) have completely agranular BS. They lack linear electron flow for NADPH generation due to photosystem II (PSII) deficiency and only function to generate ATP by photosystem I (PSI)-mediated cyclic electron flow (Voznesenskaya et al. 1999).

* Correspondence: 1053803594@qq.com; ketty.gao@gmail.com; guoxiangchen@njnu.edu.cn
1 College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210023, China
2 College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210023, China
Full list of author information is available at the end of the article

© 2016 Shen et al. Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.
suppression of PSII activity mainly results from selective losses of subunits PsbP and PsbQ of the oxygen evolving complex (OEC) as well as PsbR, rather than the entire PSII complex (Meierhoff and Westhoff 1993).

It has been reported that a C₄-like pathway existed in photosynthetic cells surrounding the vascular system (PCSVS) of C₄ dicots tobacco and celery (Hibberd and Quick 2002). PCSVs of C₃ plants is a more spatially separated version of the C₄ photosynthetic pathway with high activity levels of three C₃ photosynthesis decarboxylases, allowing to decarboxylate malate from the transpiration stream. This is also the case in mid-veins of *Arabidopsis*, a model C₃ dicot (Brown et al. 2010). These findings suggest that essential biochemical components and the regulatory elements controlling the cell-specific gene expression required for C₄ photosynthesis are already present in C₃ plants. Thus, C₃ photosynthesis can evolve from C₃ plants with some modifications (Hibberd and Quick 2002).

Furthermore, PCSVs internal microenvironment and metabolic demands are similar to BS cells in NADP-ME C₄ plants rather than C₃ leaves. Because of fewer stomata and intercellular air spaces, but more layers of surrounding cells, the vascular tissue has been previously noted to reduce solubility and diffusivity of oxygen (Raven 1991; Hibberd and Quick 2002). Those further suppress mitochondrial respiration and result in ATP losses (Geigenberger 2003) in conjunction with the NADP⁺ requirement for high C₄-acid decarboxylating activity (Yiotis et al. 2009). Therefore, the compensatory metabolic demands of higher ATP/NADPH ratios in mid-veins are comparable to that found in BS cells of NADP-ME type C₄ plants.

These properties may require qualitative and quantitative adjustments of the photosynthetic attributes in such organs for both light and biochemical reactions (Yiotis and Manetas 2010). As observed in NADP-ME type C₄ plants, chloroplasts in PCSVs of C₃ plants deficient in linear but sufficient in cyclic electron flow would act as electron valves restoring the ATP/NADPH ratio (Yiotis et al. 2009). For example, Kotakis et al. (2006) show that twigs of *Eleagnus angustifolius* display low dark-adapted PSII photochemical efficiency and linear electron transport rates. Kalachanis and Manetas (2010) further demonstrate that the innately low linear flow is limited in the donor side (OEC) of PSII and the acceptor side of both PSII and PSI.

The origin, function and selective advantages of PCSVs in C₃ lineages are critical for the understanding of the environmental, molecular and phylogenetic determinants for C₄ evolution (Griffiths et al. 2012). With no doubt, more studies of PCSVs in C₃ species are required to fill this fundamental gap (Leegood 2008) with molecular evidence. In particular, the neglected investigation on the monocot model plant rice (*Oryza sativa*), may have more significant impacts in Asia, because it would potentially simplify the approaches to generate a two-celled C₄ shuttle in rice by expressing the classical enzymes of the NADP-ME C₄ cycle (Kajala et al. 2011).

Following up the preliminary findings as mentioned above, we took advantage of the *O. sativa* cv. Liangyoupeijiu as the plant material, which had large mid-veins. We have elucidated a wealth of photosynthetic traits of leaf laminae in the super hybrid rice (Zhang et al. 2007). In order to examine whether rice mid-veins had innate properties of C₄-like photosynthesis, physiological traits, biochemical parameters, and spectral indicators were compared between leaf laminae and mid-veins in *O. sativa*.

### Results

**Rice mid-veins had photosynthesizable chloroplasts**

The presence of chlorophyll (Chl) in chloroplasts caused greenness and bright red fluorescence under optical and epifluorescence microscopy, respectively (Berveiller and Damesin 2007). To examine the distribution of chlorophyllous cell in rice leaves, transverse sections of leaves were subjected to the microscopy (Fig. 1b and c). Our results demonstrated that both mid-veins and leaf laminae showed green regions under optical microscopy (Fig. 1b). Under epifluorescence microscopy, the red fluorescence signals emitted by the Chl also surrounded the xylem vessels in mid-veins (Fig. 1c).

**Rice mid-veins accumulated high levels of C₄ acid decarboxylases**

To test whether rice mid-veins possessed some enzymatic features of C₄ BS cells or not, we evaluated various key enzymatic activities in either C₃ or C₄ cycles. The key enzyme of C₄ cycle, PEPC, exhibited a little higher activity levels for three C₄ acid decarboxylases (NADP-ME, NAD-ME and PEPCK) and pyruvate phosphate dikinase (PPDK) per Chl unit: 6.2 to 7.6-fold greater than that of the leaf laminae (Fig. 2a). Immunoblot results showed that the enzyme protein levels were consistent with the enzymatic data (Fig. 2b). Mid-veins enriched more than 3.5-fold three C₄ acid decarboxylases than leaf laminae, while the large subunit of RuBisCO was reduced by 42 % in mid-veins (Fig. 2c).

**Rice mid-veins showed unusual fluorescence signature of photosynthetic machineries**

Fast Chl a fluorescence transients (F₆₇₀) can provide information for the whole photosynthetic process from water splitting to PSI electron acceptor (ferredoxin and NADP⁺) (Strasser et al. 2010). To study whether mid-veins operated...
unique photosynthetic machineries, fluorescence analysis was employed.

As shown in Fig. 3, both leaf laminae and mid-veins displayed a typical and distinct polyphasic \( F_t \) rise, which meant that they were both photosynthetically active and not interfered by rectangular window fitted on the clip.

Various additional normalizations and difference kinetics were employed to reveal bands that were hidden in \( F_t \) (Fig. 4). \( F_t \) was firstly normalized between the step O and P and presented as relative variable fluorescence, \( V_t \) (Fig. 4a). The O, L, K, J, I and P steps were marked in the plot. Positive \( \Delta L- \), \( \Delta K- \), \( \Delta J- \) and \( \Delta I- \) bands were displayed in \( \Delta V_t \) (Fig. 4a), and the most distinct peak appeared at K step in mid-veins. Compared to leaf laminae, mid-veins had positive L-bands (Fig. 4b) and K-bands (Fig. 4c), as well as less half time and the maximum amplitude of IP rise (Fig. 4d).

To provide further quantitative and precise information, radar plot graphs were presented in Fig. 5a, b. The behavior of structural and functional parameters was analyzed according to the JIP-test (Strasser and Srivastava 1995; Strasser et al. 2004). The parameter definition and derivation were shown in Additional file 1: Table S1.

Although specific fluxes parameters (ABS/RC, TR\(_O/RC\), ET\(_O/RC\) and RE\(_O/RC\)), \( V_K/V_p \), \( V_p \), \( M_0 \) and the parameters about heat dissipation (DL\(_O/ABS\) and DL\(_O/RC\)) increased in mid-veins, \( S_m/\gamma_{3M} \), \( \gamma_{RC} \), \( 1/V_p \), \( t_{1/2}^{(1-P)} \), ET\(_O/\)TR\(_O\), ET\(_O/ABS\), EC\(_O/ABS\), RE\(_O/ABS\), RE\(_O/\)TR\(_O\) and PI\(_{total}\) in mid-veins exhibited a smaller value than those in leaf laminae. Other parameters, such as \( S_m \), TR\(_O/ABS\) and RE\(_O/ET\(_O\) did not differ significantly between two tissues.

To test PSI activity and the connectivity of the two photosystems, the kinetics of the normalized modulated reflection at 820 nm (MR/MR\(_O\)) were recorded according to Strasser et al. (2010). As shown in Fig. 6a, the amplitude of MR/MR\(_O\) diminished in mid-veins as compared to leaf laminae. To further characterize MR/MR\(_O\), the derived parameters (\( \Delta MR_{fast}/MR_O \) and \( \Delta MR_{slow}/MR_O \)) from MR/MR\(_O\) were shown in Fig. 6b and c. The fast phase (\( \Delta MR_{fast}/MR_O \)) had no significant difference in both tissues, whereas the slow phase (\( \Delta MR_{slow}/MR_O \)) was distinctly higher in leaf laminae than mid-veins.

**Rice mid-veins possessed a lower linear electron flow**

To further confirm that mid-veins had an unbalanced requirement for the electron transport flows, the cyclic and non-cyclic (linear) photophosphorylation rate of the chloroplasts were measured. Non-cyclic phosphorylation rate of mid-veins was lower than that of leaf laminae, while cyclic photophosphorylation exhibited no obvious changes (Fig. 7a and b). Hence, mid-veins were superior in cyclic/non-cyclic photophosphorylation ratio (Fig. 7c).

**Rice mid-veins had a lower accumulation of PSII supercomplexes and selective subunits**

Thylakoid multi-subunit complexes can be separated in their native form with high resolution in Blue native polyacrylamide gel electrophoresis (BN-PAGE), whereas
specific subunit stoichiometry of these complexes can be detected with immunoblots (Takabayashi et al. 2009). To understand the molecular mechanism for the modified photosystems in mid-veins, the assembly status of thylakoid membrane complexes was determined by BN-PAGE (Fig. 8a). Seven major bands were resolved, apparently corresponding to PSII supercomplexes, PSI-light harvesting complex (LHC) I, PSI core, ATP synthase-Cytb₆f-PSII core, CP43 less PSII core, LHCII trimer, and dimer.

As compared to leaf laminae, we did not observe any obvious differences for PSI core + LHCI, PSI core, and ATP synthase-Cytb₆f complex-PSII core, whereas PSII supercomplexes and CP43 less PSII core in mid-veins were far below detection in the leaf laminae. LHCII trimer and LHCII dimer were also reduced in the mid-vein (Fig. 8b and c).

In order to fingerprint the composition of these complexes, representative subunits were examined by immunoblots (Fig. 9). In PSII supercomplexes, the level...
**Fig. 4** The different expressions of relative variable fluorescence (V or W, left vertical axis) of rice mid-veins (▲) and leaf laminae (□). The difference fluorescence kinetics of mid-veins to leaf laminae was calculated using the equation: \( \Delta V \) (or \( \Delta W \)) = V (or W) \(_{\text{mid-vein}}\) – V (or W) \(_{\text{leaf laminae}}\) (right vertical axis). Each curve represents the average kinetics derived from 15 independent measurements of \( F_t \). a Normalized between \( F_O \) and \( F_M \): \( V \) \(_{\text{t}}\) = (\( F_t \) – \( F_O \)) / (\( F_M \) – \( F_O \)), and \( \Delta V \) was marked by the O, L, K, J, I, P steps. The graph was plotted on a logarithmic time scale (0.02 ms to 1 s). b Normalized between \( F_O \) and \( F_K \): \( W_{\text{ok}} \) = (\( F_t \) – \( F_O \)) / (\( F_K \) – \( F_O \)), and \( \Delta W_{\text{ok}} \) reveals L-bands which indicate the degree of energetic dis-connectivity (grouping) of the PSII units. The graph was plotted on a linear time scale (0.02 ms to 0.3 ms). c Normalized between \( F_O \) and \( F_J \): \( W_{\text{oJ}} \) = (\( F_t \) – \( F_O \)) / (\( F_J \) – \( F_O \)), and \( \Delta W_{\text{oJ}} \) reveals K-bands which indicate the degree of inactivation of OEC. The graph was plotted on a linear time scale (0.02 ms to 2 ms). d In the main panel, normalized between \( F_I \) and \( F_P \): \( W_{\text{IP}} \) \( (\geq 1) = (F_t - F_I) / (F_P - F_I) \), and horizontal dashed line at 0.5 indicates half time needed to reduce pool of the end electron acceptor with electrons donated by intermediate carriers. In the inserted panel, normalized between \( F_O \) and \( F_I \): \( W_{\text{OI}} \) \( (\geq 1) = (F_t - F_O) / (F_I - F_O) \), and the maximum amplitude of IP phase illustrates the differences in the pool size of the end electron acceptors. The graph was plotted on a linear time scale (30 ms to 400 ms).

**Fig. 5** Radar plot representation of the behavior of structural and functional parameters. a Quantum yields, flux ratios, and specific energy fluxes per absorption flux (ABS) and reaction center (RC). Mid-veins (▲) and leaf laminae (□). b Performance indexes, density of RCs and other fluorescence parameters. Each parameter was derived from JIP-test of the corresponding \( F_t \), and then normalized to the leaf laminae (with value of 100% = 1). The significant difference between two tissues (\( P < 0.05 \)) is indicated by the asterisk. The parameters represent the average kinetics collected from 15 independent measurements.
of PsbP, PsbQ of OEC and PsbR were reduced most significantly than other subunits. PsbO of OEC, PsbA (PSII core subunit), Lhcb1 and Lhcb2 (LHCII subunits) were also decreased in mid-veins. Lhcb3 level was not altered significantly. In contrast, the relative level of the Cytb \(_6\)f subunits, Cyt b\(_6\) and Cyt f, and ATP synthase subunits, Atp\(_{\beta}\) were not vulnerable in rice mid-veins. In respect of PSI supercomplex, PsaA (PSI core subunits), Lhca1 and Lhca2 (LHCI subunits) were also not changed in mid-veins (Fig. 9b).

**Discussion**

**Rice mid-veins are photosynthetically active with high enrichment of C\(_4\) acid decarboxylases**

With transverse section of rice leaves under epifluorescence or optical microscopy, both mid-veins and leaf laminae were proved to be photosynthesizable with chloroplasts (Fig. 1b, c). The chlorophyllous cells also border the vascular system in dicots such as celery, tobacco (Hibberd and Quick 2002), and woody species (Berveiller and Damesin 2008). Therefore, the cells around the veins in C\(_3\) plants (PCSVS) can also be termed ‘BS’ cells as C\(_4\) plants (Kinsman and Pyke 1998; Hibberd and Quick 2002).

The stems and petioles of tobacco (Hibberd and Quick 2002) and mid-veins of Arabidopsis thaliana (Brown et al. 2010) possess high activities of decarboxylating enzymes (NADP-ME, NAD-ME and PEPCK) and PPDK. Thus the ability of PCSVs to decarboxylate organic acids is phylogenetically widespread among C\(_3\) dicotyledons (Aubry et al. 2011). Our results firstly provided direct molecular evidence to the accumulation of decarboxylating enzymes in monocot rice mid-veins by immunoblot analyses (Fig. 2). The preferential accumulation of decarboxylating enzymes in rice mid-veins is analogous with that in BS cells of C\(_4\) plants beyond the typical range observed for C\(_3\) leaf laminae (Marshall et al. 2007; Kocurek and Pilarski 2011). Therefore, the location of chlorophyllous cells near PCSVs could be advantageous in terms of the carbon assimilation, which allow the decarboxylation of malate from the xylem and phloem, thus releasing CO\(_2\) for C\(_3\) cycle.

Indeed, these enzymes recruited into C\(_4\) photosynthesis fulfill conserved roles in distantly related C\(_3\) plants. During
C₃ plant defense response, PEPCK provides PEP to the shikimate pathway for the biosynthesis of aromatic compounds (Leegood et al. 1999; Lai et al. 2002). PPDK increases in rice roots during anoxia (Moons et al. 1998). NADP-ME2 also appears to be involved in the generation of reactive oxygen species (Voll et al. 2012). Especially, those members abundant in the PCSVS play an indispensable role in C₃ plants. For example, in Arabidopsis, cucumber, and grape, PEPCK is suggested to be localized in phloem companion cells, where it may function in nitrogenous metabolism and pH regulation (Walker et al. 1999; Delgado-Alvarado et al. 2007; Malone et al. 2007); NAD-ME, NADP-ME and PPDK would also be linked with vascular bundles of C₃ plants. NADP-ME and NAD-ME operate in the decarboxylation direction to supply CO₂ to photosynthesis in mid-veins, and regulate the flux of carbon into soluble sugars, amino acids and glucosamine (Hibberd and Quick 2002). Hence, mid-veins similarly encounter the particular metabolic demands of a higher ATP/NADPH ratio as BS cells of C₄ plants (Kotakis et al. 2006; Kalachanis and Manetas 2010). Since the demands would further shape the structure and function of photosystems and the associated electron flow (Kalachanis and Manetas 2010), we decided to further evaluate the differences of photosystems between mid-veins and leaf laminae by the non-invasive spectral technology.

Vₜ from Fₜ was higher in mid-veins than leaf laminae (Fig. 4a), indicating that the fraction of closed PSII RCs is higher at any time (Kalachanis and Manetas 2010; Yiotis and Manetas 2010) in rice mid-veins. The significantly increased initial slope of the fluorescence transient (M₀/tFM) and decreased average redox state of QA/Q₀ in the time span from 0 to tFM (Sₘ/tFM) (Fig. 5b) in mid-veins also demonstrate that mid-veins have a higher proportion of closed RCs of PSII (Chen and Cheng 2009). The positive value of L-bands indicates a low energetic connectivity/grouping of PSII units (Strasser et al. 2004). Hence, the higher L-bands in mid-veins (Fig. 4b) further suggest that the closed RCs of PSII lose stability and become inefficient to transfer energy.

ΔVₜ (Fig. 4a) uncovered the main bottleneck of PSII occurring at K step. The appearance of positive K-bands in mid-veins (Fig. 4b) further suggest that the closed RCs of PSII lose stability and become inefficient to transfer energy. 

Rice mid-veins are equipped with C₄-like photosystems, with lower linear electron transport

Leaf laminae chloroplasts of C₃ plants function primarily in linear electron transport, which produces 3 ATP and 2 NADPH per O₂ evolved to meet the requirements for C₃ cycle (Finazzi et al. 2002). In contrast, in the NADP-ME type C₄ plants, the decarboxylation of malate in BS cells results in a donation of NADPH. Therefore, Chl a fluorescence results from BS cells of C₄ maize show the closure of PSII reaction center (RC) and a low PSII activity (Ivanov et al. 2005). BS cells lack PSII-initiated linear electron transport and only function to generate ATP by PSI-mediated cyclic electron flow. The ATP production per NADPH is about 2-fold higher in BS chloroplasts than that in M chloroplasts (Voznesenskaya et al. 1999).

Higher activity of three C₄ acid decarboxylases also allows PCSVS in C₃ mid-veins to decarboxylate malate for generating NADPH (Hibberd and Quick 2002). Hence, mid-veins similarly encounter the particular metabolic demands of a higher ATP/NADPH ratio as BS cells of C₄ plants (Kotakis et al. 2006; Kalachanis and Manetas 2010). Since the demands would further shape the structure and function of photosystems and the associated electron flow (Kalachanis and Manetas 2010), we decided to further evaluate the differences of photosystems between mid-veins and leaf laminae by the non-invasive spectral technology.

Vₜ from Fₜ was higher in mid-veins than leaf laminae (Fig. 4a), indicating that the fraction of closed PSII RCs is higher at any time (Kalachanis and Manetas 2010; Yiotis and Manetas 2010) in rice mid-veins. The significantly increased initial slope of the fluorescence transient (M₀/tFM) and decreased average redox state of QA/Q₀ in the time span from 0 to tFM (Sₘ/tFM) (Fig. 5b) in mid-veins also demonstrate that mid-veins have a higher proportion of closed RCs of PSII (Chen and Cheng 2009). The positive value of L-bands indicates a low energetic connectivity/grouping of PSII units (Strasser et al. 2004). Hence, the higher L-bands in mid-veins (Fig. 4b) further suggest that the closed RCs of PSII lose stability and become inefficient to transfer energy. 

ΔVₜ (Fig. 4a) uncovered the main bottleneck of PSII occurring at K step. The appearance of positive K-bands in mid-veins (Fig. 4b) reflects an inactivation of the OEC at the donor side of PSII (Yusuf et al. 2010). The destruction of OEC, also supported by higher Vₜ/Vₗ quantitation (Fig. 5b) and a decrease in ΔMRslow/MRO (Oukarroum et al. 2012), would allow light to over-energize PSII and...
deactivate PSII RCs. Meanwhile, the lower $\gamma_{RC}$ (the fraction of PSII Chl a molecules that function as RCs), and higher ABS/RC (a relative measure of antenna size feeding active RCs) (Fig. 5) in mid-veins suggest that the harvesting antenna complexes (LHC) of PSII were relatively larger than active RCs (Kirst et al. 2012). Therefore, higher proportions of absorbed energy in mid-veins needs to be dissipated as heat, which was supported by the higher DIo/RC (dissipated energy flux per RC) and DIo/ABS (quantum yield for energy dissipation, Fig. 5a). An inactivated fraction of RCs also forced each RC to bear more specific fluxes of absorbed, trapped energy, electron transfer and reduction of PSI end electron acceptors (ABS/RC, TRO/RC, ET/RC and RE/RC, Fig. 5a) in mid-veins.

IP phase depends on electron transfer through PSI and is caused by the transient block at the acceptor side of PSI (NADP$^+$) (Schansker et al. 2005). The maximal amplitude of IP phase, parameterized by $1/V_{I}$, represents the relative pool size of the final electron acceptors of PSI (Yusuf et al. 2010). As NADP$^+$ is continuously consumed by C$_4$ acid decarboxylases, mid-veins are supposed to have a smaller pool of PSI end electron acceptors with the lower maximal amplitude of IP (Fig. 4d) and $1/V_{I}$ (Fig. 5b).

On the other hand, the operation of cyclic electron flow in C$_4$ BS chloroplasts is also studied by the redox kinetics of P700$^+$, which predominantly excites PSI. Since the electron flow solely from PSII is insufficient to reduce the active PSI RCs, the decarboxylation of malate generates NADPH as an electron donor to PSI for a larger cyclic electron flow (Ivanov et al. 2005). Our results similarly showed that the activation status of PSI RC (P700$^+$), as indicated by $\Delta M_{R_{fast}}/M_{RO}$ (Fig. 6b), in rice mid-veins was kept as high as in leaf laminae. Consequently, filling of PSI acceptors with electrons proceeded faster in mid-veins (Fig. 4d), resulting in the shorter time needed for half saturation of these pools with electrons donated by intermediate carriers ($t_{1/2}^{(I-P)}$, Fig. 5b).
phenomenon is also observed in pericarps of *Nerium oleander* where the completion of electron flow toward the acceptor side of PSI is likely facilitated due to the reduced final electron acceptor pools of PSI and their high affinities for electrons (Kalachanis and Manetas 2010). Therefore, the quantum yield for reduction of end electron acceptors at the PSI acceptor side ($REO/O_{ABS}$), and efficiency/probability that a trapped excitation can move an electron into the electron transport chain from $Q_A^-$ to the PSI end electron acceptors ($REO/O_2$) were lower in mid-veins (Fig. 5a). Better-preserved pools of intermediate carriers ($S_m$, Fig. 5b) and activation status of PSI RC ($P700^+$; $\Delta MR_{fast}/MR_O$, Fig. 6c) further ensured mid-veins to achieve higher cyclic electron flow (Fig. 7a) by diverting electrons back to intermediate carriers around PSI (Kalachanis and Manetas 2010), while the maximal performance for linear electron transport from water to reduce the end electron acceptor, PI total ($ETO/O_2$ and $ETO/O_{ABS}$ accordingly, Fig. 5a).

**Rice mid-veins lacking linear electron flow is attributed to a selective loss of PSI-bound subunits**

In C4 plants, M thylakoids have a complete linear electron transport chain, containing PSII, Cytb6f, and PSI complexes, similar to C3 leaf laminae. In contrast, BS thylakoids contain fewer functional PSII, but normal number of PSI, Cytb6f and ATP synthase complexes which primarily participate in cyclic electron transport. Subunits of PSI, LHCl, Cyt b6f, and ATP synthase complexes show BS/M accumulation ratios of 1.6, 1.72, 1.0, and 1.33, respectively, whereas ratios for the PSII and LHCl were 0.45 and 0.68, respectively (Wojciech et al. 2008).

As direct molecular evidence for the physiological data, BN-PAGE and immunoblot analysis of the thylakoid photosynthetic apparatus prove an uneven distribution of photosystems between mid-veins and leaf laminae in rice. Either the integrated forms or CP43-less core of PSII supercomplexes was far below detections in leaf laminae (Fig. 8). The loss of PSII complexes explains the dysfunction of PSII and the hindered linear electron transport rates in mid-veins as mention above. Considering the sub-sets of PSII supercomplexes, the accumulation of LHCl trimers and their subunits (Lhcb1 and Lhcb2) was reduced less than PsbA (PSII core subunit) in rice mid-veins (Fig. 9b). It corresponds well to the fluorescence results that LHCl is larger than active PSII RCs.

Early studies in NADP-ME type C4 species maize (*Zea may*) and sorghum (*Sorghum bicolor*) show that limited PSII activities in chloroplasts of BS cells are mainly caused by the depletion in PsbP and PsbQ of OEC and PsbR, which play an important role in water oxidation (Meierhoff and Westhoff 1993). Likewise, the loss of PsbP, PsbQ and PsbR in mid-veins was more than other...
PSII subunits (Fig. 9b), although not as significant as that in BS cells of C₄ plants (Meierhoff and Westhoff 1993). Hence, K-bands (inactivation of OEC, Fig. 4c) in rice mid-veins also perhaps result from the selective loss of distinct OEC polypeptides (PsbP and PsbQ). An RNAi-induced Arabidopsis mutant lacking detectable PsbP proteins exhibits a significant defect in electron transfer from Qₓ to Qᵇ with loss of the J to I transition of Fᵥ and shows seriously retarded charge recombination between Q₃ and OEC (Andréasson et al. 1995). Therefore, removals of PsbP and PsbQ of OEC are speculated to be the most important reasons for introducing linear electron transport defects in the PSII.

Furthermore, rice mid-veins assembled intact PSI core complex (Fig. 8), PsaA (PSI core subunit), and Lhca1 and Lhca2 (LHCl subunits, Fig. 9b) into the oligomeric forms of PSI-LHCI (Fig. 8). Cyt b₅f and ATP synthase complexes (Fig. 8), as well as their subunits (Cytf, Cytb₅, and ATPf, Fig. 9b) were also well-preserved in rice mid-veins. If most of the excitation energy is utilized by PSI, cyclic electron transport around PSI and Cytb₅f may prevail over linear electron flow mediated by both PSII and PSI (Finazzi et al. 2002). Therefore, the particular photosystems in mid-veins ensured the cyclic electron flow to work efficiently at the expense of the linear one (Fig. 7).

C₄ photosynthesis is considered as one of the most convergent of the complex evolutionary phenomena on Earth, and the majority of C₄ crop species have quite a degree of mechanistic flexibility and circumstantial superiority (Furbank 2011). Introducing their multigenic trait into rice has been recognized as an ambitious and multinational project for increasing rice yields. Without doubt, it still faces enormous challenges, because it is not clear how C₄ photosynthesis has evolved independently from the ancestral C₃ pathway (Sage et al. 2011), and how the metabolism of the rice leaf will be changed after introduction (Aubry et al. 2011). However, our finding that the existence of C₃-like photosynthesis (high enrichment of C₄ acid decarboxylases, lower linear electron transport and a selective loss of PSII-bound subunits) in rice mid-veins makes it seem more feasible to introduce components of the C₄ pathway into rice and gives some indication that the evolution of C₄ photosynthesis may not be as difficult as first appears (Kajala et al. 2011).

Conclusions
In summary, this was the first study showing higher levels of C₄ cycle key enzymes and special adjustments in the photosynthetic machinery in rice mid-veins. The linear electron transport chain was mostly blocked by a “traffic jam” occurring on PSII complexes, whereas PSI in mid-veins is sufficient to support a larger cyclic one. The loss-of-function of PSII was primarily attributed to selective subunits, such as PsbP and PsbQ of OEC and PsbR. These attributes of photosynthetic machinery in rice mid-veins partly resemble that in the BS cells of C₄ plants, as mentioned in Introduction. Hence, our findings indicate that the photosynthetic cells surrounding mid-veins in the rice, a typical C₃ monocot, innately possess C₄-like features as do other C₃ dicots. Of course, further research is needed to extend these findings to more C₃ plants. Such studies would provide a simple explanation for the polyphyletic evolution of C₄ photosynthesis (Hibberd and Quick 2002).

Methods
Plant materials and growth conditions
Oryza sativa. cv. Liangyoupeijiu, a typical monocot C₃ plant was pot-cultivated, watered and fertilized routinely in a net house under natural conditions at the Institute of Agricultural Sciences of Jiangsu Nanjing, China (32° 03’ N, 118° 47’ E). Air temperature, rainfall and global radiation were available in previous studies (Yu et al. 2012). The flag leaves of the main culm were sampled during mature stage (24 days after leaf emergence). To avoid regional discrepancy due to developmental stages, only midsections of flag leaves were utilized. For in vitro experiments, mid-veins were pulled out from leaf laminae according to a method developed by Brown et al. (2010), and both mid-veins and residual leaf laminae were immediately frozen in liquid nitrogen, and stored at −80 °C. Several plant samples were pooled to obtain sufficient material for further analyses. For fast Chl a fluorescence transients and modulated 820 nm reflection experiments in vivo, leaf laminae and mid-veins were still attached to the rice, and the measurements were operated at ambient temperature and 8–10 a.m.

Determination of chlorophyllous cell distribution
Transverse sections of leaves containing mid-veins and leaf laminae were cut manually using razor blades, and mixed with a glycero-PBS solution (50 % glycerol, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH = 7.2) for optical and epifluorescence microscopy (Nikon CFI60, Tokyo, Japan). Chlorophyllous cells were visualized as bright red fluorescence (LP 520) when excited with blue light (BP 450–490) in epifluorescence microscopy.

Enzyme-linked assays
Enzymes were isolated according to Ivanov et al. (2006). Briefly, samples were ground in liquid nitrogen and extracted with 1 ml of a buffer containing 100 mM Tris/HCl (pH 7.6), 5 % (w/v) PVP-40, 0.85 % (w/v) BSA, and 10 mM DTT.

The enzyme-linked assays for detection of PEPC (EC 4.1.1.32), NAD-ME (EC 1.1.1.39), NADP-ME (EC 1.1.1.40)
and PPDK (EC 2.7.9.1) were conducted as described previously (Ashton et al. 1990; Marshall et al. 2007). Enzymatic activities of PEPC (EC 4.1.1.31) and Rubisco (EC 4.1.1.39) were determined according to Tietz and Wild (1991) and Berveiller and Damesin (2007), respectively. Enzyme activity was expressed as μmol substrate consumed or product generated per second to total Chl content. Experiments were carried out at 25 °C.

**Measurement of fast Chl a fluorescence transients (JIP curve, F<sub>1</sub>) and modulated 820 nm reflection (MR)**

Light intensity actually falling on a cylindrical mid-vein cannot be accurately determined and small sized mid-veins cannot fill the leaf clip space. Therefore, photon exchange between the instrument and mid-veins or leaf laminae was through a 3 mm × 15 mm rectangular window. The window was a non-fluorescing black tape aligned according to the method of Manetas (2004). Leaf laminae and mid-veins, from the midsection of the same leaf and still attached to plants, were mounted in a leaf clip fitted by the window and dark adapted for 60 min before measurements.

F<sub>1</sub> was captured by a Handy Plant Efficiency Analyzer (Hansatech Instruments Ltd, Norfolk, UK) according to Strasser and Srivastava (1995) after excitation by a band of three red light emitting diodes (650 nm, 3000 μmol photons m<sup>−2</sup> s<sup>−1</sup>). Data were recorded from 20 μs to 1 s, and analyzed according to the JIP test (Strasser et al. 2004; Jiang et al. 2008) for calculation of structural and functional parameters. The corresponding definitions and calculations of the parameters were given in Additional file 1: Table S1. The average values were expressed as ratios to that of leaf laminae in radar plots. Extended analysis of F<sub>1</sub> was done by normalization as various relative variable fluorescence (V or W) between different time points, according to previous studies (Strasser et al. 2007; Tsimilli-Michael and Strasser 2008). The difference fluorescence kinetics (ΔV or ΔW) between mid-veins and leaf laminae were calculated through the equation:

\[ ΔV(W) = V(W)_{mid-vein} - V(W)_{leaf lamina}. \]

See details below:

(A) normalized between F_O and F_M:

\[ V_I = (F_I - F_O)/(F_M - F_O), \]

and ΔV<sub>I</sub> was marked by the O, L, K, J, I, P steps. The graph was plotted on a logarithmic time scale (0.02 ms to 1 s).

(B) normalized between F_O and F_K:

\[ W_{ok} = (F_I - F_O)/(F_K - F_O), \]

and ΔW<sub>ok</sub> revealed L-bands which indicated the degree of energetic dis-connectivity (grouping) of the PSII units. The graph was plotted on a linear time scale (0.02 ms to 0.3 ms). (C) normalized between F_O and F_F:

\[ W_{of} = (F_I - F_O)/(F_F - F_O), \]

and ΔW<sub>of</sub> revealed K-bands which indicated the degree of inactivation of OEC. The graph was plotted on a linear time scale (0.02 ms to 2 ms).

(D) normalized between F_I and F_F:

\[ W_{jp} = (F_I - F_F)/(F_F - F_I), \]

and horizontal dashed line at 0.5 indicated half time needed to reduce pool of the end electron acceptor with electrons donated by intermediate carriers. (E) normalized between F_O and F_F:

\[ W_{O1} ≥ (F_I - F_O)/(F_F - F_O), \]

and the maximum amplitude of IP phase illustrated the differences in the pool size of the end electron acceptors of PSI. The graph was plotted on a linear time scale (30 ms to 400 ms).

Modulated reflection at 820 nm (MR) was recorded on a Multifunctional Plant Efficiency Analyzer M-PEA (Hansatech Instrument Ltd., Norfolk, UK), according to an operating protocol elucidated by Strasser et al. (2010). MR was normalized and expressed by MR/MR<sub>O</sub> on logarithmic time scale from 0 ms to 10 s, where MR<sub>O</sub> was the value at 0.7 ms (taken at the onset of the red light illumination, the first reliable MR measurement). The definition of characteristic parameters of MR/MR<sub>O</sub> kinetics (MR<sub>min</sub> and MR<sub>max</sub>) was shown on an inserted graph (Fig. 6a), where MR<sub>min</sub> represented the minimal MR reached during the fast phase (i.e., between 0.7 ms and 10–200 ms) and MR<sub>max</sub> was the maximal MR reached by the end of the slow phase (taken at 1 s). Each curve represented the average kinetics collected from 15 independent measurements of 15 individual plants and was used to calculate relevant parameters.

**Isolation of thylakoid membrane and total soluble protein**

Thylakoid membrane was isolated from mid-veins and leaf laminae as described earlier (Kang et al. 2012). The Chl content was determined spectrophotometrically in 80 % acetone (Arnon 1949). Total soluble proteins were isolated as described by Ku et al. (1999). PPDK proteins needed to be concentrated before immunoblot (Chastain et al. 2002). Protein concentration was determined using the method of Lowry et al. (1951), with bovine serum albumin as a standard.

**Immunodetection of photosynthetic proteins**

The isolated thylakoid membrane (for thylakoid membrane subunits) and total soluble protein (for key enzymes in C<sub>3</sub> or C<sub>4</sub> cycles) were pretreated with the loading buffer (0.5 M Tris–HCl, pH 6.8, 1 % SDS, 24 % glycerol, 4 % β-mercaptoethanol, and 0.001 % w/v bromophenol blue) and denatured for 10 min at 90 °C. Thylakoid membrane polypeptides (2 μg Chl per spot) or total soluble protein (15 μg protein per spot) from mid-veins and leaf laminae were separated by 12 % SDS-PAGE. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 or transferred electrophoretically to PVDF membranes (0.2 μm pore size, BioRad, USA). PVDF membranes were subsequently incubated with primary antibodies raised in rabbits against key enzymes in C<sub>3</sub> or C<sub>4</sub> cycles [the large subunit of Rubisco (Agrisera, Sweden, http://www.agrisera.com/), NAD-ME and NADP-ME (Beijing
Blue native polyacrylamide gel electrophoresis (BN-PAGE)

For the separation of thylakoid membrane complexes, BN-PAGE was carried out in the procedure described by Chen et al. (2007) with some modifications. Briefly, the thylakoid membranes were washed with 330 mM sorbitol and 50 mM BisTris-HCl (pH 7.0), and then resuspended in buffer containing 20 % glycerol, 25 mM BisTris-HCl (pH 7.0) at 1.0 mg Chl ml$^{-1}$. The suspension was solubilized with an equal volume of resuspension buffer containing 2 % (w/v) dodecyl-$\beta$-D-maltoside. After incubation at $4^\circ$C for 30 min, insoluble material was removed by centrifugation at $40,000 \times g$ for 10 min. The supernatant equal to 10 mg Chl was mixed with one-tenth volume of 1 % Coomassie brilliant blue G-250 in 100 mM BisTris-HCl (pH 7.0), 0.5 M 6-amino-n-caproic acid, and 30 % glycerol. The mixture was then applied to 1-mm-thick $5\sim12$ % polyacrylamide gradient gels for electrophoresis. Electrophoresis was performed at 4 °C, 120 V for 5 h. High molecular weight native protein marker kit (GE Healthcare, Amersham-Pharmacia, 17-0445-01, UK) was used to determine the molecular mass of these complexes.

Quantitation of band signal in BN-PAGE and immunoblot analyses was performed using Quantity One, version: 4.52 (Bio-Rad, Hercules, USA) based on densitometric analysis. The experiments were repeated three times and the representative images were taken. The amount of protein was expressed as the percentage of that in leaf laminae.

Chloroplast isolation and cyclic/non-cyclic (linear) photophosphorylation rate assays

Chloroplast isolation was performed according to Ketcham et al. (1984). The cyclic/non-cyclic (linear) photophosphorylation activity of chloroplasts was assessed by using the luciferin-luciferase method to measure the amount of ATP synthesized within 2 min at a saturating irradiance of about 1,500 $\mu$mol quanta $m^{-2} \cdot s^{-1}$ at $25^\circ$C (Allnutt et al. 1991).

Statistical analyses

Statistical analyses were carried out using SPSS 15.00 statistical package (Chicago, USA). Parametric one-way ANOVA was used to determine statistical significance between leaf laminae and mid-veins. Differences in the measured parameters were considered significant at $p < 0.05$. 

Additional file

**Additional file 1: Table S1.** Formulas and definitions of the selected JIP-test fluorescence parameters used in this study. (DOCX 38 kb)

**Abbreviations**

ABS: absorption flux; BN-PAGE: blue native polyacrylamide gel electrophoresis; BS: bundle sheath; Chl: chlorophyll; DI$^\circ$C: dissipated energy flux; EC$^\circ$C: total electron carriers; ET$^\circ$C: electron transport flux; $F_2$: fast chlorophyll fluorescence kinetics; M: mesophyll; MR: modulated reflection at $820 \text{nm}$; NAD-HE: nicotinamide adenine dinucleotide-dependent malic enzyme; NADPH: the reduced form of nicotinamide adenine dinucleotide phosphate; NADP-ME: nicotinamide adenine dinucleotide phosphate-dependent malic enzyme; OEC: oxygen evolving complex; PCSV: photosynthetic cells surrounding the vascular system; PEPC: phosphoenolpyruvate carboxylase; PEPPK: phosphoenolpyruvate carboxykinase; PPDK: pyruvate phosphate dikinase; PSI: photosystem I; PSII: photosystem II; RC: reaction center; RE$^\circ$C: reduction of end acceptors of PSII; RuBisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase; TR$^\circ$C: trapped energy flux.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

WS and ZG participated in the design of the study, the analysis and interpretation of data, and performed the determination of chlorophyllous cell distribution and pigments. GC carried out the immunoassays. LY carried out chloroplast isolation and cyclic/non-cyclic (linear) photophosphorylation rate assays. ZY performed fast chlorophyll a fluorescence transient assays. CL carried out enzyme-linked assays. ZZ and XC participated in results interpretation and the manuscript revision. All authors read and approved the final manuscript.

**Acknowledgements**

This work was funded by the National Natural Science Foundation of China (grant no.31271621/C1302), the Natural Science Foundation of Jiangsu Province (grant no.11KJA180001), the Youth Natural Science Foundation of Jiangsu Province (grant no. BK20140916), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (grant no.14KJB180011), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We are also very grateful to Dr. Tang, R.H (Eshelman School of Pharmacy, University of North Carolina) who reviewed the manuscript and Dr. Chen S.G (Weed Research Laboratory, Nanjing Agricultural University) who provided the M-PEA instrument.

**Author details**

1College of Life Sciences, Nanjing Normal University, 1 Wenyuan Road, Nanjing 210023, China. 2Institute of Food and Crops, Jiangsu Academy of Agricultural Sciences, 50 Zhongjing Street, Nanjing 210014, China. 3University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA.

Received: 9 November 2015 Accepted: 30 April 2016

**References**

Allnutt FCT, Ewy R, Renganathan M, Pan RS, Dilley RA (1991) Nigericin and hexylamine effects on localized proton gradients in thylakoids. Biochim Biophys Acta Biocatal 1059(1):28–36. doi:10.1016/S0005-2728(09)80184-7

Andréasson L-E, Vass I, Styling S (1995) Ca$^{2+}$ depletion modifies the electron transfer on both donor and acceptor sides in photosystem II from spinach. Biochim Biophys Acta Bioenerg 1230(3):155–164. doi:10.1016/0005-2728(95)00047-M

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in Beta vulgaris. Plant Physiol 24(1):1–15

Ashton A3, Burrell JN, Furbanck RT, Jenkins CLD, Hatch MD (1990) Enzymes of C$\text{4}$ photosynthesis. In: Methods in Plant Biochemistry. Academic, London

Aubry S, Brown NJ, Hibberd JM (2011) The role of proteins in C$\text{4}$ plants prior to their recruitment into the C$\text{4}$ pathway. J Exp Bot 62(9):3049–3059. doi:10.1093/jxb/erl012
Kinsman EA, Pyke KA (1998) Bundle sheath cells and cell-specific plastid development in Arabidopsis thaliana leaves. Development 125(10):1815–1822
Kirst H, Garcia-Cerdan XG, Zurbriggen A, Ruehle T, Melis A (2012) Truncated photosystem chlorophyll antenna size in the green microalga Chlamydomonas reinhardtii upon deletion of the TLAT-3-CPSR43 gene. Plant Physiol 160(4):2251–2260. doi:10.1104/pp.112.206572
Kocurek M, Pilanski J (2011) Activity of C4 enzymes in C3-type herbaceous plants. Photosynthetica 49(3):473–477. doi:10.1007/s11091-011-0053-8
Kotakis C, Petropoulou Y, Stamatakis Y, Yiotis C, Manetas Y (2006) Evidence for active cyclic electron flow in twig chlorenchyma in the presence of an extremely deficient linear electron transport activity. Planta 225(1):245–253. doi:10.1007/s00425-006-0322-7
Ku MSB, Agarie S, Nomura M, Fukayama H, Tsuchida H, Ono K, Hirose S, Toki S, Miyao M, Matsuoka M (1999) High-level expression of maize photosynthetic pathway in transgenic rice plants. Nat Biotechnol 17(1):76–80
Lai LB, Tausta SL, Nelson TM (2002) Differential regulation of transcripts encoding cytosolic NADP-malic enzyme in C3 and C4 Flaveria species. Plant Physiol 128(1):140–149
Leegood RC, Acheson R, Tétil L, Walker R (1999) The many-faceted function of photosynthetic pyruvate carboxylase in plants. In: Kruger N, Hill S, Ratcliffe RG (eds) Regulation of Primary Metabolic Pathways in Plants, vol 42. Proceedings of the Phytochemical Society of Europe. Springer, Netherlands, pp 37–51. doi:10.1007/978-94-011-4818-4_3
Leegood RC (2008) Roles of the bundle sheath cells in leaves of C4 plants. J Exp Bot 59(7):1663–1673. doi:10.1093/jxb/erm335
Liehl M, Tabaik S, Czeke L, Piekarsky E, Anderson B, Adam Z (1996) Identification, characterization, and molecular cloning of a homologue of the bacterial Fsh protease in chloroplasts of higher plants. J Biol Chem 271(46):29229–29334
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193(1):265–275
Majeran W, van Wijk KJ (2009) Cell-type-specific differentiation of chloroplasts in C4 plants. Trends Plant Sci 14(10):109–115. doi:10.1016/j.plants.2008.11.006
Malone S, Chen ZH, Bahrari AM, Walker RP, Gray JE, Leegood RC (2007) Phosphoenolpyruvate carboxylase in Arabidopsis: changes in gene expression, protein and activity during vegetative and reproductive development. Plant Cell Physiol 48(3):441–450. doi:10.1093/pcp/pcm014
Manetas Y (2004) Probing cotyledon photosynthesis through in vivo chlorophyll fluorescence measurements: evidence that high internal \( \text{CO}_2 \) levels suppress electron flow and increase the risk of photoinhibition. Physiol Plantarum 119(3):509–517. doi:10.1111/j.1399-3054.2004.00326.x
Marshall DM, Muhaidat R, Brown NJ, Liu Z, Stanley S, Griffiths H, Sage RF, Hibberd JM (2007) C4e, a gene closely related to Arabidopsis, contains species spanning a developmental progression from \( \text{C}_3 \) to \( \text{C}_4 \) photosynthesis. Plant J 51(5):886–896. doi:10.1111/j.1365-311X.2007.03188.x
Meierhoff K, Westhoff P (1993) Differential biosynthesis of photosystem II in mesophyll and bundle-sheath cells of monocotyledonous NADP-malic enzyme-type \( \text{C}_4 \) plants: the non-stoichiometric abundance of the subunits of photosystem II in the bundle-sheath chloroplasts and the active participation of the plasto-enzyme encoded genes. Planta 191(1):23–33. doi:10.1007/bf00240892
Moomi A, Valicke R, Van Montagu M (1998) Low-oxygen stress and water deficit induce cytosolic pyruvate orthophosphate dikinase (PPOD) expression in roots of rice, a \( \text{C}_3 \) plant. Planta 1(158):89–98
Okurakor A, Strasser RJ, Schansker G (2012) Heat stress and the photosynthetic electron transport chain of the lichen Pannellina rilacea (Hoffm.) Ach. in the dry and the wet state: differences and similarities with the heat stress response of higher plants. Photosynth Res 113(3):303–314. doi:10.1007/s11120-012-9728-7
Raven JA (1991) Long-term functioning of enucleate sieve elements: possible mechanisms of damage avoidance and damage repair. Plant Cell Environ 14(2):139–138
Sage RF, Christin PA, Edwards EJ (2011) The \( \text{C}_4 \) plant lineages of planet Earth. J Exp Bot 62(9):3195–3199. doi:10.1093/jxb/err048
Schansker G, Toth SZ, Strasser RJ (2005) Methylviologen and dibromomethoxyquinone treatments of pea leaves reveal the role of photosystem I in the \( \text{C}_3 \) chloroplast fluorescence rise. Plant Physiol Biochem 43(6):250–261. doi:10.1016/j.plaphy.2004.11.006
Strasser R, Tisslimmil-Michael M, Dangre D, Rai M (2007) Biophysical phenomenons reveals functional building blocks of plants systems biology: a case study for the evaluation of the impact of mycorrhization with Piriformospora indica. In:
et al. Rice (2016) 9:20

Zantedeschia aethiopica

Brassica juncea

plants: quantification of organelles and glycine

pathway key enzymes in flag leaves of a super-high-yield hybrid rice

Haberlea: evidence for innately high

differentiation for chloroplast membrane proteomes in

Zhang CJ, Chen L, Shi DW, Chen GX, Lu CG, Wang P, Wang J, Chu HJ, Zhou QC, Zuo M, Sun L (2007) Characteristics of ribulose-1,5-bisphosphate carboxylase and C4 pathway key enzymes in flag leaves of a super-high-yield hybrid rice and its parents during the reproductive stage. S Afr J Bot 73(1):22–28. doi:10.1016/j.sajb.2006.05.002

Takabayashi A, Ishikawa N, Obayashi T, Ishida S, Obokata J, Endo T, Sato F (2009) Three novel subunits of Arabidopsis chloroplastic NAD(P)H dehydrogenase identified by bioinformatic and reverse genetic approaches. Plant J 57(2):207–219. doi:10.1111/j.1365-313X.2008.03980.x

Tietz S, Wild A (1991) Investigations on the phosphoenolpyruvate carboxylase activity of spruce needles relative to the occurrence of novel forest decline. J Plant Physiol 137(3):327–331. doi:10.1016/S0176-1617(91)80140-9

Tsimilli-Michael M, Strasser RJ (1995) Polyphasic chlorophyll a fluorescence transient in plants and cyanobacteria*. Photochem Photobiol 61(1):32–42. doi:10.1111/j.1751-1097.1995.tb09240.x

Tsimilli-Michael M, Qiang S, Goltsiev V (2010) Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant Habellaea rhodopensis. Biochim Biophys Acta 1797(8):1431–1438. doi:10.1016/j.bbabio.2010.02.002

Submit your next manuscript at ►.springeropen.com

Submit your manuscript to a SpringerOpen journal and benefit from:

► Convenient online submission
► Rigorous peer review
► Immediate publication on acceptance
► Open access: articles freely available online
► High visibility within the field
► Retaining the copyright to your article

Submit your next manuscript at ►springeropen.com