Photosynthetic linear electron flow drives CO2 assimilation in maize leaves

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Article

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Running head: Regulation of photosynthesis in C$_4$ plants

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Abstract

Photosynthetic organisms commonly develop the strategy to keep the reaction centre chlorophyll of photosystem I, P700, oxidised for preventing the generation of reactive oxygen species in excess light conditions. In photosynthesis of C₄ plants, CO₂ concentration is kept at higher levels around ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) by the cooperation of the mesophyll and bundle sheath cells, which enables them to assimilate CO₂ at higher rates and to survive under drought stress. However, the regulatory mechanism of photosynthetic electron transport for P700 oxidation is still poorly understood in C₄ plants. Here we assessed gas exchange, chlorophyll fluorescence, electrochromic shift, and near infrared absorbance in the intact leaves of NADP-malic enzyme subtype of C₄ plants maize in a comparison with the C₃ plant field mustard. Instead of the alternative electron sink due to photorespiration, photosynthetic linear electron flow was strongly limited between photosystems I and II dependent on the proton gradient across the thylakoid membrane (ΔpH) in response to the suppression of CO₂ assimilation in maize. The increase of ΔpH for P700 oxidation was caused by the regulation of proton conductance of chloroplast ATP synthase but not by promoting cyclic electron flow, which was supported by linear relationships among CO₂ assimilation rate, linear electron flow, P700 oxidation, ΔpH, and the oxidation rate of ferredoxin. At the scale of intact leaves, the ratio of PSI to PSII was estimated almost 1:1 in both C₃ and C₄ plants. Overall, the photosynthetic electron transport was regulated for P700 oxidation in maize through the same strategies as in C₃ plants only except for the capacity of photorespiration despite the structural and metabolic differences in photosynthesis between C₃ and C₄ plants.

Keyword: Photosynthesis; Linear electron flow; C₄ plants; P700 oxidation; Ferredoxin
**Introduction**

In chloroplasts of plant leaves, photosynthetic CO\(_2\) assimilation is driven in the Calvin-Benson cycle utilizing NADPH and ATP produced by light energy \(^1\). In the photosynthetic electron transport system, light energy is absorbed by chlorophyll in photosystems (PS) I and II, producing the photo-oxidised reaction centre chlorophylls, P700\(^+\) and P680\(^+\), to initiate photosynthetic linear electron flow (LEF) from PSII to PSI via the plastoquinone (PQ) pool, the cytochrome (Cyt) \(b_6/f\) complex, and plastocyanin (PC). On the electron acceptor side of PSI, NADP\(^+\) is reduced to NADPH using electrons from PSI via ferredoxin (Fd) and Fd-NADP\(^+\) reductase. In PSII, H\(^+\) is released by H\(_2\)O oxidation in the luminal side of the thylakoid membrane; in the Cyt \(b_6/f\) complex, the Q-cycle pumps stromal H\(^+\) to the luminal side. Both generate a proton gradient (ΔpH) across the thylakoid membrane to produce ATP via the chloroplast ATP synthase. In the Calvin-Benson cycle, ribulose 1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) catalyses the carboxylation of RuBP to produce two molecules of 3-phosphoglycerate (3-PGA), which are then metabolised in the Calvin-Benson cycle with NADPH and ATP. In this process, RuBP is regenerated. On the other hand, Rubisco competitively catalyses the oxygenation of RuBP, when available CO\(_2\) is limited in the presence of sufficient O\(_2\), to produce 3-PGA and 2-phosphoglycolate (2-PG). 2-PG is finally metabolised to 3-PGA in cooperation with the chloroplasts, peroxisomes, and mitochondria using Fd\(^-\) and ATP. This is the so-called photorespiration, in which CO\(_2\) is released from glycine in the mitochondria. The production and consumption of both NADPH and ATP are normally balanced to poise the redox state of the photosynthetic electron transport system in C\(_3\) plants \(^1, 3\). In C\(_4\) plants, the capacity of photorespiration had been degenerated during the evolutionary history \(^4, 5\), instead of the CO\(_2\) concentrating mechanism, where CO\(_2\) is incorporated into phosphoenolpyruvate (PEP) in mesophyll cells and then transported in the form of malate into bundle sheath cells that specifically express Rubisco \(^6\). Malate is converted to CO\(_2\) and pyruvate around Rubisco by NADP-malic enzyme (ME) with NADP\(^+\) as the electron acceptor, and PEP is regenerated in mesophyll cells theoretically with two additional ATP \(^7, 8\). Because of the structural and metabolic complexities, the regulation of photosynthetic electron transport has been poorly understood in C\(_4\) plants.

Oxidation of P700 is a universal physiological response in photosynthetic organisms to prevent photo-oxidative damage derived from reactive oxygen species (ROS) to PSI by dissipating excess light energy as heat \(^9\) because the inhibition of PSI can be a lethal event for photosynthetic organisms \(^10\). Actually, P700 is kept oxidised by a variety of molecular mechanisms in response to excess light conditions such as high light and CO\(_2\) limitation \(^11\). In C\(_3\) plants, P700 oxidation is supported on the donor side by the suppression of electron transport in the Cyt \(b_6/f\) complex, dependent on ΔpH.
On the acceptor side, photorespiration replaces CO\textsubscript{2} assimilation, to function as an electron sink for P700 oxidation under limited CO\textsubscript{2} conditions\textsuperscript{14}. In C\textsubscript{4} plants, there is little electron sink by photorespiration even at the CO\textsubscript{2} compensation point, different from C\textsubscript{3} plants\textsuperscript{5,15}. It is still not clear how P700 remains oxidised in C\textsubscript{4} plants when CO\textsubscript{2} assimilation is suppressed. One important hypothesis that should be tested is that cyclic electron flow around PSI (CEF) functions to make ΔpH to keep P700 oxidised in C\textsubscript{4} plants. Since CEF is mediated by the electron transport from Fd\textsuperscript{−} to PQ, theoretically it is capable of pumping H\textsuperscript{+} from the stroma to the lumen of the thylakoid membrane in the Q-cycle; this also results in an additional ATP production that is not linked to NADP\textsuperscript{+} reduction\textsuperscript{16}. Especially in C\textsubscript{4} plants, the ratio of PSI to PSII is much higher in isolated bundle sheath cells than in the mesophyll cells, which gives the hypothesis that CEF in bundle sheath cells contributes not only to keeping P700 oxidised but also to meeting the additional ATP demand for C\textsubscript{4} photosynthesis. Unfortunately, there is not currently a method available to directly measure the electron transport rate via CEF. The CEF activity has been indirectly estimated mainly from the quantum yield of PSI, which is inevitably under/overestimated dependent on the redox state of PC\textsuperscript{2}. Therefore, the extent of CEF activity remains controversial.

In this study, we evaluated gas exchange, chlorophyll fluorescence, electrochromic shift (ECS), and near infrared (NIR) absorbance in the NADP-ME subtype of C\textsubscript{4} plants maize to investigate how photosynthetic electron transport is regulated for P700 oxidation. Although maize did not show the significant capacity of photorespiration, P700 oxidation was tightly coupled with LEF like the C\textsubscript{3} plant field mustard (komatsuna, hereafter mustard), which was supported by the linear relationships among CO\textsubscript{2} assimilation rate, LEF, P700 oxidation, ΔpH, and the Fd\textsuperscript{−} oxidation rate. The ratio of PSI to PSII was estimated \textit{in vivo} approximately 1:1 in the both C\textsubscript{3} and C\textsubscript{4} plants. These results suggest that maize regulates photosynthetic electron transport for P700 oxidation tightly associated with LEF like C\textsubscript{3} plants but strongly relies it on the ΔpH-dependent suppression of electron transport on the donor side of PSI instead of photorespiratory electron sink on the acceptor side.
Results

Photosynthetic CO₂ assimilation and dark respiration were analysed in both the C₃ plant mustard and the C₄ plant maize simultaneously with chlorophyll fluorescence, ECS, and NIR absorbance. In this study, we analysed in vivo photosynthetic parameters at a constant light intensity and different CO₂ partial pressures to simply investigate the effects of limitation of electron sink on the photosynthetic electron transport. Additionally, the C₃ and C₄ intact leaves were measured at atmospheric (21 kPa) and low (1 kPa) O₂, where photorespiration is inhibited. We note that in maize CO₂ is first incorporated into PEP in mesophyll cells, different from C₃ plants, but the decrease of the CO₂ partial pressure in the intercellular space (Ci) finally results in the limitation of the carboxylation reaction of Rubisco in bundle sheath cells.

Fig. 1. Photosynthetic CO₂ assimilation and linear electron flow in the C₃ plant mustard (A–C) and the C₄ plant maize (D–F). (A, D) Net CO₂ assimilation rate at various intercellular CO₂ partial pressures (Ci). (B, E) Effective quantum yield of PSII, Y(II), at various Ci. (C, F) Relationship of Y(II) with CO₂ assimilation rate. Dark respiration rate is presented as Rd. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O₂ (open symbols). Solid lines represent estimated linear regression of the data at 1 kPa (C) and 21 kPa O₂ (F) (R², coefficient of determination). The y-intercepts (b) were tested based on the null hypothesis: **p<0.005.

In mustard, the net CO₂ assimilation rate was higher at 1 kPa than at 21 kPa O₂ when CO₂ assimilation was limited by Ci (Fig. 1A), whereas the effective quantum yield of PSII, Y(II), was kept high uncoupled from CO₂ assimilation (Fig. 1B and C). However, maize did not show any difference in net CO₂ assimilation and Y(II) between different O₂ partial pressures (Fig. 1D and E), which is in accordance with a small contribution, if any, of photorespiration to the capacity of electron sink. As a result, Y(II) has a linear relationship with the sum of net CO₂ assimilation rate and dark respiration rate (Rd) in maize (Fig. 1F). These typical photosynthetic characteristics of C₃ and C₄ plants have already
been established in previous studies, and extra Y(II) in mustard is known to be due almost exclusively
to photorespiration \(^5, 15, 17, 18, 19, 20\). In the other word, Y(II) reflects LEF mainly derived from CO\(_2\) assimilation and photorespiration in angiosperms \(^13, 21, 22\). The inferred reduction level of the PQ pool (1 – qL) and non-photochemical quenching (NPQ) were also calculated from chlorophyll fluorescence in mustard and maize, both of which increased at low Ci (Supplemental Fig. S1). In mustard, limiting photorespiration at 1 kPa O\(_2\) caused the further increase of 1 – qL at low Ci, whereas NPQ slightly decreased in this condition (Supplemental Fig. S1A and B).

Next, we evaluated the oxidation of P700 in the relationship with LEF reflected in Y(II) (Fig. 2). In mustard at 21 kPa O\(_2\), the oxidation level of P700 increased monotonously with the decrease in Y(II) (Fig. 2A) although the PQ pool was suggested to be reduced at low Ci (Supplemental Fig. S1B). That is, photosynthetic electron transport is limited between PSII and PSI, presumably at the Cyt \(b_6/f\) complex. However, at 1 kPa O\(_2\), P700 started to be kept reduced when Y(II) < 0.3 (Fig. 2A), which suggests that the electron sink by photorespiration is required for P700 oxidation \(^14\). Unlike mustard, P700 continued to remain oxidised in maize even at 1 kPa O\(_2\) except under an extreme CO\(_2\) limitation (< 1.5 Pa)(Fig. 2B). The inverse proportional relationship in the redox states of the PQ pool and P700 suggested that LEF was limited by the suppression of electron transport at the Cyt \(b_6/f\) complex in maize as in C\(_3\) plants (Fig. 2 and Supplemental Fig. S1).

Fig. 2. Relationship of P700 oxidation with effective quantum yield of PSII, Y(II), at various intercellular CO\(_2\) partial pressures in the C\(_3\) plant mustard (A) and the C\(_4\) plant maize (B). Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O\(_2\) (open symbols). Solid lines represent estimated linear regression of the data at 21 kPa O\(_2\) (\(R^2\), coefficient of determination).

To investigate molecular mechanisms for P700 oxidation in response to the limitation of electron sink, we evaluated the thylakoid membrane potential by ECS analysis in the transition from
light to dark at the steady-state photosynthesis (Fig. 3). We note that the ECS parameters are dependent on the properties of the leaves, not only the density of chloroplasts, but also the content of light-harvesting complexes that house the shifted pigments. Therefore, it is difficult to make any quantitative conclusions for the differences in the amplitudes of ECS parameters between mustard and maize. In the so-called dark-interval relaxation kinetics, proton motive force (pmf) in the light is defined as the total rapid (< 1 s) change in the ECS signal upon rapidly switching off the light, which increased with the limitation of CO$_2$ in both mustard and maize at 21 kPa O$_2$ (Fig. 4A and D). However, in mustard pmf was not enhanced in response to the suppression of CO$_2$ assimilation at 1 kPa O$_2$, where photorespiration is inhibited (Fig. 4A). That is, photorespiration functions as the electron sink to sustain LEF, supporting the increase of pmf in C$_3$ plants. Nevertheless, we found the increase of pmf independent of photorespiration in maize (Fig. 4D). Proton conductance of the ATP synthase ($g_{H^+}$) is calculated as the rate constant of the mono-exponential ECS decay, which increased with Ci and were then saturated as in the trend of CO$_2$ assimilation rate regardless of O$_2$ partial pressures in both mustard and maize (Fig. 4B and E). Further, the initial decay rate of the ECS changes is termed as relative light-driven proton flux through the chloroplast ATP synthase, the so-called $v_n$ (Fig. 4C). In mustard at 21 kPa O$_2$, a part of $v_n$ was uncoupled from the CO$_2$ assimilation rate (Fig. 4C), like the case of Y(II) (Fig. 1C), which suggested that $v_n$ has almost the linear relationship with LEF derived from CO$_2$ assimilation and photorespiration in C$_3$ intact leaves. In the case of maize leaves, the relationship of $v_n$ with photosynthetic CO$_2$ assimilation does not seem to be linear, different from that of Y(II), at
both 21 and 2 kPa O\textsubscript{2} (Fig. 4F). We note that $v_{\text{H}^+}$ decreased with photosynthetic CO\textsubscript{2} assimilation larger in lower Ci conditions (Fig. 4F), implying that ATP is utilized in a different manner at different Ci levels.

![Diagram](image)

**Fig. 4.** Electrochromic shift (ECS) parameters in the C\textsubscript{3} plant mustard (A–C) and the C\textsubscript{4} plant maize (D–F). (A, D) Proton motive force (pmf) at various intercellular CO\textsubscript{2} partial pressures (Ci). (B, E) Proton conductance of the chloroplast ATP synthase ($g_{\text{H}^+}$) at various Ci. (C, F) Relationship of proton efflux rate vs the ATP synthase ($\eta_{\text{H}^+}$) with CO\textsubscript{2} assimilation rate. Effective quantum yield of PSII, Y(II), are also shown in orange symbols. Dark respiration rate is presented as Rd. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O\textsubscript{2} (open symbols).

Theoretically, pmf includes two components: $\Delta$pH and transmembrane difference in the electric field ($\Delta\Psi$), which can be distinguished by the post-illumination transient change in ECS, and the former component triggers the suppression of electron transport in the Cyt $b_6/f$ complex\textsuperscript{25}. Like P700 oxidation, $\Delta$pH showed the linear relationship with LEF at 21 kPa O\textsubscript{2} in both mustard and maize. The formation of $\Delta$pH was disturbed at 1 kPa O\textsubscript{2} in mustard and also in maize at very low Ci (Fig. 5A and C). The increase of $\Delta$pH was associated with NPQ (Supplemental Fig. S1B), which was in agreement with that NPQ at PSII is stimulated by $\Delta$pH\textsuperscript{26}. There were two possibilities assumed for the lumen acidification: (1) H$^+$-pumping from stroma into the thylakoid lumen was promoted; or (2) H$^+$-leakage from the lumen to stroma was blocked. Both mustard and maize showed the linear relationship of $g_{\text{H}^+}$ with LEF reflected in Y(II) (Fig. 5B and D), which suggested that limiting $g_{\text{H}^+}$ leads to the increase in $\Delta$pH, resulting in P700 oxidation.

In this study, we investigated the redox state of Fd at the steady state of photosynthesis in both mustard and maize using a Klas-NIR spectrophotometer. The maximum amplitude of photo-reducible Fd was determined by the standard method in advance as shown in Supplemental Fig. S2. The decay of Fd$^-$ in the transition from light to dark was mono-exponentially fit, giving the amplitude and the oxidation rate of Fd$^+$ (Fig. 6). Exceptionally, a biphasic decay manner was recognised in the kinetics at 1 Pa CO\textsubscript{2} and 1 kPa O\textsubscript{2} in maize (Supplemental Fig. S3). The slow component remains to be
identified, but it was clearly negligible compared to LEF because the estimated half time was more than minutes. In mustard, Fd was strongly reduced under CO₂ limitation at 1 kPa O₂, whereas it was totally kept oxidised at 21 kPa O₂ (Fig. 6A and 7A), indicating that photorespiration functions as the electron sink to relieve the acceptor-side limitation of PSI. In maize, Fd⁻ was gradually accumulated with the decrease in Ci regardless of O₂ (Fig. 6B and 7B). The intact leaves of mustard showed a linear relationship of Fd⁻ oxidation rate with Y(II) and its y-intercept was close to zero (Fig. 7C), which is in agreement with the recent study on C₃ plants. Further, the same trend was observed also in maize leaves (Fig. 7D). That is, LEF estimated from Y(II) clearly corresponds to the electron transport via Fd in the situations where ΔpH increased and P700 was oxidised in response to the limitation of electron sink in both mustard and maize.

We also plotted effective quantum yield of PSI, Y(I), against Y(II), which is a conventional method to evaluate CEF activity used in numerous previous and recent reports. In mustard at 21 kPa O₂, we observed a linear relationship between Y(I) and Y(II) with extra Y(I) to Y(II) at lower Ci (Fig. 8A). Additionally, Y(I) showed no linear relationship with Y(II) in mustard at 1 kPa O₂ and maize at both 21 and 1 kPa O₂, resulting in the extra Y(I) (Fig. 8A and C), different from the relationship of Fd⁻ oxidation rate with Y(II). Here, we also evaluated the oxidation of PC and plotted it against Y(II). In mustard, PC was kept more oxidized at lower Ci, but it was reduced where photorespiration was inhibited (Fig. 8B), like P700 (Fig. 2A). In maize PC was reduced with the decrease in Y(II) less than 0.2, although it was
more oxidized at lower Ci if Y(II) was >0.2, finally giving a curved plot (Fig. 8D). Interestingly, the extra Y(I) to Y(II) was coincided with the oxidation of PC (Fig. 8).

Fig. 6. Dark-interval relaxation kinetics of ferredoxin (Fd⁺) in the C₃ plant mustard (A) and the C₄ plant maize (B) under ambient air (40 Pa CO₂, 21 kPa O₂, black), low CO₂ (1 Pa CO₂, 21 kPa O₂, purple), and low CO₂/O₂ (1 Pa CO₂, 1 kPa O₂, pink). Red actinic light (550 µmol photons m⁻² s⁻¹) was turned off at 0 ms for 600 ms during the steady-state photosynthesis. The kinetics were fit to mono exponential decay (R², coefficient of determination: 0.6628, 0.8816, and 0.9788 in A, 0.6612 and 0.8397 in B). Only Fd⁺ kinetics in maize under low CO₂/O₂ was fit to biphasic exponential decay (R², 0.9405).

Fig. 7. In vivo measurement for the redox state of ferredoxin (Fd) in the C₃ plant mustard (A, B) and the C₄ plant maize (C, D). (A, C) The Fd reduction during the steady-state photosynthesis at various intercellular CO₂ partial pressures (Ci). (B, D) Relationship of Fd⁺ oxidation rate with effective quantum yield of PSII, Y(II), at various Ci. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O₂ (open symbols). Solid lines represent the estimated linear regressions of the data at 1 kPa (B) and 21 kPa O₂ (D), respectively (R², coefficient of determination). The y-intercepts (b) were tested based on the null hypothesis: *p<0.05.
The linear relationships among CO₂ assimilation rate, Y(II), P700 oxidation, ΔpH, and Fd⁻ oxidation rate (Fig. 1, 2, 5, and 7) suggested that the regulation of photosynthetic electron transport is tightly associated with LEF in C₄ plants, similarly to C₃ plants, which is somehow unexpected if we assume that maize has a large activity of CEF. Originally, CEF in C₄ plants is hypothesized based on the biochemical fact that there is less grana structure containing PSII in the isolated bundle sheath cells. It should be noted that in this study we analysed a variety of photosynthetic parameters at the scale of intact leaves that is the mixture of mesophyll and bundle sheath cells. Here, we spectroscopically estimated the ratio of PSI to PSII in vivo in the intact leaves of mustard and maize. A short-saturation flash induces ECS dependent on the photochemical reaction at PSII and PSI. During far-red light illumination, where PSI is selectively excited, the short-saturation flash induces ECS presumably originated only from PSII. Actually, the ECS amplitude induced by the short-saturation flash decreased with the intensity of far-red light, finally reaching approximately 50% of the initial amplitude in both mustard and maize (Fig. 9), indicating that the ratio of PSII to PSI is about 1:1 at the scale of leaves. The same results were obtained from the field-grown sunflower (C₃ plant) and maize (Supplemental Fig. S4).

To further test if there is the significant amount of PSI uncoupled from PSII in bundle sheath cells of maize, the connectivity of PSI with PSII was roughly estimated in vivo using a Klas-NIR spectrophotometer. A short-saturation flash was applied to excite PSII and PSI after accumulating P700⁺ by the far-red light illumination, resulting in the decay kinetics of P700⁺ (Fig. 10). The P700⁺
reduction kinetics was likely to involve in more than two components, but the rapid decay within 100 ms should reflect the electron transport from PSII. Interestingly, a part of P700\(^+\) (10–20%) took more than second to be reduced (data not shown), which may be due to a redox equilibration between PC and P700 as implied from the reduction of a part of PC (ca. 20%) within 100 ms (Fig. 10A and D). The redox state of Fd did not change in response to the short-saturation flash (Fig. 10A and D). The ratio of P700\(^+\) rapidly reduced was rather larger in maize (ca. 80%) than in mustard (ca. 65%), both of which increased with the length of the short-saturation flash (5–50 μs) to approximately 90% (Fig. 10B, C, E, and F). These results implied that there is no significant amount of PSI having “less connectivity” with PSII uniquely to maize but not to mustard at the scale of the intact leaves. It should be noted that P700...
was kept more reduced in maize than in mustard during far-red light illumination (Fig. 10A and D, and Supplemental Fig. S2), and a part of P700* (ca. 20%) was immediately reduced within 50 μs just after the illumination with 5 μs short-saturation flash (Fig. 10D), which was likely to be too fast for CEF, considering the turnover of the Cyt b6/f complex 30.
We experimentally characterized the \textit{in vivo} regulatory mechanisms of photosynthetic electron transport for P700 oxidation in maize, which showed the different O$_2$ dependency but could be understood based on the same model for C$_3$ plants although photosynthesis in C$_4$ plants is driven in co-operation of mesophyll and bundle sheath cells, and metabolically different from that in C$_3$ plants. Oxidation of P700 is the universal strategy for photosynthetic organisms to suppress the generation of ROS at the acceptor side of PSI, which is mainly regulated in C$_3$ plants by the donor-side mechanism, i.e. the $\Delta$pH-dependent suppression of electron transport at the Cyt $b_{6}/f$ complex, and by the acceptor-side one, photorespiration. Since maize did not show the enough capacity of photorespiration as an alternative electron sink, LEF was linearly suppressed with the decrease in photosynthetic CO$_2$ assimilation (Fig. 1). Nevertheless, P700 is kept oxidised with the suppression of CO$_2$ assimilation, which is linearly associated with the increase in $\Delta$pH by limiting $g_{\text{H}^+}$ (Fig. 5). In these processes, the Fd$^{-}$ oxidation rate showed the linear relationship with $\text{Y(II)}$ (Fig. 7). The metabolic compartmentation in C$_4$ photosynthesis and the higher ratio of PSI to PSII in isolated bundle sheath cells have made it complicated to consider the regulation of photosynthetic electron transport in C$_4$ plants. However, the ratio of PSI to PSII was estimated almost 1:1 at the scale of the intact maize leaves, similarly to C$_3$ plants (Fig. 9). Further, the ratio of P700$^{+}$ rapidly reduced by a short-saturation flash was similar between mustard and maize (Fig. 10). All these results supported the robustness of P700 oxidation tightly associated with LEF in the C$_4$ plant maize, as previously proposed in C$_3$ plants, with the exception of little photorespiratory electron sink on the acceptor side of PSI.$^{13}$

Instead of the photorespiratory electron sink, maize strongly relies P700 oxidation on the $\Delta$pH-dependent suppression of electron transport at the Cyt $b_{6}/f$ complex (Fig. 2B and SC). The decrease in $g_{\text{H}^+}$ was linearly correlated with LEF reflected in $\text{Y(II)}$ (Fig. 5D) and Fd$^{-}$ oxidation rate (Fig. 7D). These results suggest that lumen acidification for P700 oxidation was attributed to the decrease of $g_{\text{H}^+}$ but not to an additional H$^+$-pumping from stroma to the thylakoid lumen, for example, by CEF. The different dependencies of P700 oxidation on the regulation of $g_{\text{H}^+}$ led to the different threshold of LEF for keeping P700 oxidised between mustard and maize. Contrary to mustard, which needed about 50% of the maximum $\text{Y(II)}$ for P700 oxidation, maize did not exhibit breakdown of P700 oxidation even when $\text{Y(II)}$ was close to zero (Fig. 2). The linear proportional relationship between $\text{Y(II)}$ and P700 oxidation indicated that the C$_4$ plant maize does not require an electron sink for P700 oxidation (Fig. 2B). The insensitivity to O$_2$ of P700 oxidation has been observed also at various irradiances at CO$_2$-saturated conditions.$^{31}$ Nevertheless, an extreme condition of 1 kPa O$_2$ and very low CO$_2$ (< 1.5 Pa) partially disturbed P700 oxidation in maize (Fig. 2B), which may be due to the slight but certain
electron flux via photorespiration or the Mehler reaction in C₄ plants ¹⁸, ³². In the other word, C₄ plants ultimately utilize O₂ for P700 oxidation maybe in some extremely stressed conditions. In C₃ plants, photorespiration relieves the electron transport limitation on the acceptor side of PSI and also sustains to produce ΔpH (Fig. 2A, 5A and C, and Supplemental Fig. S1A and B). It should be trade-off to rely P700 oxidation mainly on the regulations on the donor or acceptor sides of PSI. The contribution of the regulation of gH⁺ to P700 oxidation would be different among different types of C₄ plants and C₃-C₄ intermediates associated with the capacity of photorespiration ⁶. Whereas P700 oxidation with the suppression of photosynthesis is the phenomenon commonly observed in a variety of photosynthetic organisms, the dominant molecular mechanisms are rich in diversity, which has been already diversified among different species of cyanobacteria, the progenitor of oxygenic photosynthesis ¹⁰, and has changed during the evolutionary history of photosynthetic green and red plastid lineages. Interestingly, the strategy for P700 oxidation in maize can be categorized on the view of O₂-usage into the same type of that in some secondary algae derived from red algae, which do not need O₂ for P700 oxidation ³³.

What is the determinant for the strong contribution of the ΔpH-dependent donor side mechanism to P700 oxidation in maize? Lumen acidification should be controlled by both H⁺-pumping and H⁺-consumption rates in plant leaves. Based on the fact that the CEF activity via Fd was negligible (Fig. 7B and D), the H⁺-pumping rate into the thylakoid lumen is estimated from LEF with the rate constant (kH⁺), which is correlated with Y(II). Meanwhile, the H⁺-consumption rate is equal to the ECS parameter vH⁺, reflecting ATP consumption by CO₂ assimilation and photorespiration in C₃ plants. Finally, the change in pmf is presented as the following equation in C₃ plants ¹³:

\[
\frac{d(pmf)}{dt} = (kH^+ \times LEF) - (pmf \times gH^+)
\]

At the steady state,  

\[
pmf = \frac{(kH^+ \times LEF)}{gH^+}
\]

That is, lumen acidification occurs where LEF is sustained more than gH⁺. In mustard, the decrease in Y(II) (ca. 50%) was much smaller than that in gH⁺ (ca. 85%) at 21 but not at 1 kPa O₂ (Fig. 5B), indicating that photorespiration contributes to sustaining LEF, resulting in the H⁺ accumulation into the thylakoid lumen (Fig. 5A and 11)¹⁴, ³⁴. In maize LEF decreased almost concomitantly with gH⁺, different from mustard (Fig. 5D). Nevertheless, H⁺ was accumulated in the thylakoid lumen to cause P700 oxidation at both 21 and 1 kPa O₂ (Fig. 2B and 5C). Overall, different from C₃ plants, pmf in maize cannot be formulated as the above equation (Fig. 11). In the other words, there may be an additional mechanism to pump H⁺ into the thylakoid lumen independent of the electron transport via Fd, which remains to be further investigated in future works. One possibility that cannot be excluded is the effect of the
Mehler reaction on ΔpH at the steady state of photosynthesis even though the activity is very low. It should be also noted that the relationship of $v_{\text{H}^+}$ with photosynthetic CO$_2$ assimilation was different from that of Y(II) (Fig. 4F), which is presumably due to the different ATP consumption for regenerating PEP at various Ci. The strong contribution of the ΔpH-dependent donor side mechanism to P700 oxidation in maize should be also related to the different ATP utilization between C$_3$ and C$_4$ plants.

![Fig. 11. A brief illustration of mechanism for P700 oxidation in C$_3$ and C$_4$ plants. In C$_3$ plants, proton conductance of chloroplast ATP synthase ($\delta_{\text{H}^+}$) decreases with the suppression of photosynthetic CO$_2$ assimilation greater than photosynthetic linear electron flow reflected in effective quantum yield of PSI, Y(II), and ferredoxin (Fd) oxidation rate ($v_{\text{Fd}}$), resulting in the increase in proton motive force (pmf) to induce P700 oxidation. In C$_4$ plants, pmf increases with the suppression of photosynthetic CO$_2$ assimilation, although $\delta_{\text{H}^+}$ decreases concomitantly with Y(II) and $v_{\text{Fd}}$.](image)

The present results are unlikely to follow the hypothesis that CEF is driven in the bundle sheath cells of NADP-ME subtype of C$_4$ plants at a comparable flux to LEF in the mesophyll cells. Although there is the report that the PSII activities in the bundle sheath cells of NADP-ME subtype of C$_4$ plants, including maize, sorghum, and Flaveria, are almost equal to those in the mesophyll cells, numerous studies have followed and documented that PSII activity is nearly negligible in isolated bundle sheath cells in NADP-ME subtype of C$_4$ plants, especially in maize, having constructed the dogma that CEF is responsible for the additional ATP production in the bundle sheath cells of C$_4$ plants. Actually, recent modelling studies select the CEF model for simulating C$_4$ photosynthesis. However, it should be concerned that these studies follow the ratio of PSI to PSII in the experimentally differentiated mesophyll and bundle sheath cells, except for the semi-quantitative evaluation by immunocytology. Overall, the PSI:PSII ratio has yet not been quantitatively understood at the scale of intact leaves, and there is still no evidence for the energetic contribution of CEF to photosynthesis in C$_4$ plants. Another problem is that in vivo CEF activity has been ordinarily evaluated using the effective quantum yield of PSI that is easily under/overestimated (Fig. 8). Considering the experimental results by the previous and present studies, we propose that the amount of PSI in bundle sheath cells is presumably much smaller than that in mesophyll cells at the scale of C$_4$ intact leaves. It is still unclear how the additional ATP demand is met in C$_4$ photosynthesis. Chapman et al. (1980), one of important landmarks in C$_4$ photosynthesis research, has shown that in the presence of exogenously added malate photosynthetic CO$_2$ assimilation proceeds in the isolated bundle sheath cells of maize with PSII inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The PSII-
independent ATP production can reach about 80% of the ATP production of the illuminated cells in the in vitro system, which clearly suggests that there is a malate-dependent ATP source in the cells. It should be also considered that the NAD(P)H dehydrogenase complex plays an important role for C₄ photosynthesis. It has been recently accepted that the intermediates of the Calvin-Benson cycle shuttle between the mesophyll and bundle sheath cells with certainly large fluxes, which can make it more complicated how much ATP and NADPH are needed respectively in these two types of cells in the light. In both mustard and maize, pmf was formed to some extent even in the conditions where photosynthesis and photorespiration are almost completely suppressed under 1 kPa O₂ and very low Ci (Fig. 4A and D), which suggested that ATP is, at least, not limited. We also note that the stoichiometry of ATP and NADPH may not need to be necessarily satisfied because excess NADPH should not be accumulated as long as P700 oxidation system works.

For the last decades, CEF has been frequently evaluated by the comparison between Y(II) and Y(I). The extra Y(I) to Y(II) has been believed to be a conventional indicator to CEF activity. Nevertheless, recently it has been clearly shown that Y(I) can be easily over-estimated by the oxidation of PC. In this study, the extra Y(I) was totally coincided with the PC oxidation in both mustard and maize at various CO₂ and O₂ partial pressures (Fig. 8). These facts indicate that Y(I) must not be utilized to evaluate CEF activity. A variety of alternative methods, including the comparison of Fd⁻ oxidation rate with Y(II), should be considered in future works.
Materials and Methods

Plant materials

Field mustard (komatsuna, *Brassica rapa*) and maize (*Zea mays*) were grown under long-day conditions (14 h-light, 24 °C, 300 µmol photons m$^{-2}$ s$^{-1}$, white fluorescent lamp/10 h-dark, 22 °C). Seeds were planted in pots that contained a 5:3:2 mix of Metro-Mix 350 (Sun Gro Horticulture, Agawam, MA, USA), Akadama, and vermiculit with 1000-fold diluted Hyponex solution (Hyponex, Osaka, Japan) used as a watering solution. For the experiments in Supplemental Fig. S4, the field-grown sunflower (*Helianthus annuus*) and maize were used.

Gas exchange, chlorophyll fluorescence, and spectroscopic analyses

Exchanges of CO$_2$ and H$_2$O were measured using a GFS-3000 equipped with a 3010-DUAL gas exchange chamber (Walz, Effeltrich, Germany) in which ambient air was saturated with water vapor at 18.0 ± 0.1 °C, and the leaf temperature was maintained at 25 ± 2 °C. Ci was calculated based on the previous report.

Chlorophyll fluorescence and near infrared absorbance were simultaneously measured coupled with gas exchange analysis using a Klas-NIR spectrophotometer (Walz). Chlorophyll fluorescence parameters were calculated as follows: $F_o$, minimum fluorescence from a dark-adapted leaf; $F_m'$, maximum fluorescence from a light-adapted leaf; $F'$, fluorescence emission from a light-adapted leaf; $Y(II) = (F_m' - F')/F_m'$, effective quantum yield of PSII; $q_L = (F_m' - F')/(F_m' - F_o) 	imes (F_o/F')$, fraction of “open” PSII centres (with Q$_A$ oxidised) on the basis of a lake model for the PSII photosynthetic apparatus; NPQ = $(F_m - F_m')/F_m'$, non-photochemical quenching. Pulse-amplitude modulated green measuring light (540 nm, <0.1 µmol photons m$^{-2}$ s$^{-1}$) was used. To obtain $F_m'$, a saturation flash (630 nm, 8,000 µmol photons m$^{-2}$ s$^{-1}$, 300 ms) was applied. Red actinic light (630 nm, 550 µmol photons m$^{-2}$ s$^{-1}$) was supplied using a chip-on-board LED array. The signals for P700$^+$, PC$^+$, and Fd$^-$ were calculated based on the deconvolution of four pulse-modulated dual-wavelength difference signals in the near infrared region (780–820, 820–870, 840–965, and 870–965 nm). The redox state of P700 was evaluated as the ratio of P700$^+$ to the total P700, termed Y(ND). Both P700 and PC are kept completely reduced and Fd is fully oxidised in dark conditions. For the determination of total photo-oxidizable P700 and PC, the saturation flash was applied after 10 s illumination with a far-red light (740 nm; Supplemental Fig. S2). Total photo-reducible Fd was determined by the illumination with a red actinic light (450 µmol photons m$^{-2}$ s$^{-1}$) after plant leaves were adapted to the dark for 5 min (Supplemental Fig. S2). For the analysis of a dark-interval relaxation kinetics, the red actinic light (550 µmol photons m$^{-2}$ s$^{-1}$) was temporarily turned off for 600 ms at the steady-state.
The oxidation rate of Fd was estimated as relative values by a Klas-NIR spectrophotometer by a linear fitting for the initial decay of Fd. Recently, it has been shown that the reduced iron-sulphur clusters in PSI, F$_A$/F$_B$, can contribute to the Klas-NIR signal attributed to Fd$^-$ in almost the same manner \textit{in vitro}. In this study, we concluded that the F$_A$/F$_B$ contribution to the Fd signal was not critical problem in the measurement for the Fd$^-$ oxidation rate because Fd was kept oxidised more than 50% at the steady state of photosynthesis except for the condition where the electron acceptor side of PSI was extremely limited at low CO$_2$ and O$_2$ in mustard.

ECS was measured simultaneously with gas exchange using a Klas-100 spectrophotometer (Walz). The ECS signal was calculated from two pulse-modulated dual-wavelength difference signals using the following equation: \( \frac{\Delta I}{I_{521.4-507.6}} + \frac{\Delta I}{I_{520.2-534.8}} \)/2. Red actinic light was temporarily turned off for 600 ms in a dark-interval relaxation kinetics analysis during the steady-state photosynthesis to determine ECS parameters. The total rapid (<1 s) change in ECS signal upon rapidly switching off actinic light was defined as \( \text{pmf} \). The parameter \( v_{H^+} \) was estimated by a linear fitting for the initial phase of the mono-exponential decay of \( \text{pmf} \) in the transition from light to dark, giving the rate constant of the decay \( g_{H^+} \) by the calculation. The \( \Delta pH \) component of \( \text{pmf} \) was estimated also by turning red actinic light off for 30 s at the steady state of photosynthesis, which are used in Fig. 5A and C.

All of \textit{in vivo} spectroscopic measurements were based on the assumption that the absorption coefficient and the amplification factor of each targeted molecule are not different between mesophyll and bundle sheath cells. All statistical analyses were performed using Origin 2017 (Lightstone, Tokyo, Japan).
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**Figure legends**

*Fig. 1.* Photosynthetic CO₂ assimilation and linear electron flow in the C₃ plant mustard (A–C) and the C₄ plant maize (D–F). (A, D) Net CO₂ assimilation rate at various intercellular CO₂ partial pressures (Ci). (B, E) Effective quantum yield of PSII, Y(II), at various Ci. (C, F) Relationship of Y(II) with CO₂ assimilation rate. Dark respiration rate is presented as Rd. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O₂ (open symbols). Solid lines represent estimated linear regression of the data at 1 kPa (C) and 21 kPa O₂ (F) ($R^2$, coefficient of determination). The y-intercepts ($b$) were tested based on the null hypothesis: **p<0.005.

*Fig. 2.* Relationship of P700 oxidation with effective quantum yield of PSII, Y(II), at various intercellular CO₂ partial pressures in the C₃ plant mustard (A) and the C₄ plant maize (B). Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O₂ (open symbols). Solid lines represent estimated linear regression of the data at 21 kPa O₂ ($R^2$, coefficient of determination).

*Fig. 3.* Dark-interval relaxation kinetics of electrochromic shift (ECS) in the C₃ plant mustard (A) and the C₄ plant maize (B) under ambient air (40 Pa CO₂, 21 kPa O₂; black), low CO₂ (1 Pa CO₂, 21 kPa O₂; purple), and low CO₂/O₂ (1 Pa CO₂, 1 kPa O₂; pink). Red actinic light (550 μmol photons m⁻² s⁻¹) was turned off at 0 ms for 600 ms during the steady-state photosynthesis. The kinetics were fit to mono exponential decay ($R^2$, coefficient of determination: 0.7017, 0.9454, and 0.6868 in A; 0.8814, 9868, and 0.9801 in B respectively).

*Fig. 4.* Electrochromic shift (ECS) parameters in the C₃ plant mustard (A–C) and the C₄ plant maize (D–F). (A, D) Proton motive force (pmf) at various intercellular CO₂ partial pressures (Ci). (B, E) Proton conductance of the chloroplast ATP synthase ($g_{H+}$) at various Ci. (C, F) Relationship of proton efflux rate via the ATP synthase ($v_{H+}$) with CO₂ assimilation rate. Effective quantum yield of PSII, Y(II), are also shown in orange symbols. Dark respiration rate is presented as Rd. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O₂ (open symbols).

*Fig. 5.* Relationships of the proton gradient across the thylakoid membrane ($\Delta$pH; A, C) and the proton conductance of the chloroplast ATP synthase ($g_{H+}$; B, D) with effective quantum yield of PSII, Y(II), at
various intercellular CO$_2$ partial pressures in the C$_3$ plant mustard (A, B) and the C$_4$ plant maize (C, D). We note that $\Delta p$H and $g_{\text{H}^+}$ were separately measured from Y(II) at the same ambient CO$_2$ partial pressures. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O$_2$ (open symbols). Solid lines represent estimated linear regression of the data at 21 kPa O$_2$ ($R^2$, coefficient of determination).

**Fig. 6.** Dark-interval relaxation kinetics of ferredoxin (Fd$^-$) in the C$_3$ plant mustard (A) and the C$_4$ plant maize (B) under ambient air (40 Pa CO$_2$, 21 kPa O$_2$; black), low CO$_2$ (1 Pa CO$_2$, 21 kPa O$_2$; purple), and low CO$_2$/O$_2$ (1 Pa CO$_2$, 1 kPa O$_2$; pink). Red actinic light (550 $\mu$mol photons m$^{-2}$ s$^{-1}$) was turned off at 0 ms for 600 ms during the steady-state photosynthesis. The kinetics were fit to mono exponential decay ($R^2$, coefficient of determination: 0.6628, 0.8816, and 0.9788 in A; 0.6612 and 0.8307 in B). Only Fd$^-$ kinetics in maize under low CO$_2$/O$_2$ was fit to biphasic exponential decay ($R^2$: 0.9405).

**Fig. 7.** *In vivo* measurement for the redox state of ferredoxin (Fd) in the C$_3$ plant mustard (A, B) and the C$_4$ plant maize (C, D). (A, C) The Fd reduction during the steady-state photosynthesis at various intercellular CO$_2$ partial pressures (Ci). (B, D) Relationship of Fd$^-$ oxidation rate with effective quantum yield of PSII, Y(II), at various Ci. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O$_2$ (open symbols). Solid lines represent the estimated linear regressions of the data at 1 kPa (B) and 21 kPa O$_2$ (D), respectively ($R^2$, coefficient of determination). The $y$-intercepts ($b$) were tested based on the null hypothesis: $p>0.05$.

**Fig. 8.** Relationships of effective quantum yield of PSI, Y(I) (A, C), and plastocyanin (PC) oxidation (B, D) with effective quantum yield of PSII, Y(II), at various intercellular CO$_2$ partial pressures in the C$_3$ plant mustard (A, B) and the C$_4$ plant maize (C, D). Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O$_2$ (open symbols).

**Fig. 9.** Electrochromic shift (ECS) induced by a 5 $\mu$s-short saturation flash during far-red light illumination in the C$_3$ plant mustard (A) and the C$_4$ plant maize (B). Far-red light was provided at various intensities (0, black; 1, grey; 5, pink; 10, red; and the maximum 20, wine red; the values defined by the Walz software). (C) The flash-induced ECS changes normalized by the values without far-red light illumination as 100%. The data of mustard (light grey) and maize (dark grey) are shown as the mean with the standard deviation ($n=3$, biological replicates).
Fig. 10. Effects of a short-saturation flash on the redox state around PSI in the C₃ plant mustard (A–C) and the C₄ plant maize (D–F). (A, D) Reduction kinetics of P700⁺ (green) in response to a 5 μs-short saturation flash after far-red light illumination for 10 s. Far-red light was provided at the maximum intensity (20, the value defined by the Walz software). Deconvoluted signals to plastocyanin (PC⁺, blue) and ferredoxin (Fd⁻, red) are also shown. All the Klas-NIR signals were normalized within the range from the minimum 0 to the maximum 1. The maximum reduction/oxidation levels of each component were determined as shown in Supplemental Fig. S2. The signal to Fd⁻ has negative values. (B, E) Reduction kinetics of P700⁺ by a short saturation flash at different lengths (5, green; 10, light green; 20, light grey; and 50 μs, grey respectively) after far-red light illumination for 10 s. The kinetics are also shown with the logarithmic scale at y-axis (C, F). The representative traces of independent experiments (n = 3, biological replicates) are shown.

Fig. 11. A brief illustration of mechanism for P700 oxidation in C₃ and C₄ plants. In C₃ plants, proton conductance of chloroplast ATP synthase (g_H⁺) decreases with the suppression of photosynthetic CO₂ assimilation greater than photosynthetic linear electron flow reflected in effective quantum yield of PSII, Y(II), and ferredoxin (Fd⁻) oxidation rate (v_Fd⁻), resulting in the increase in proton motive force (pmf) to induce P700 oxidation. In C₄ plants, pmf increases with the suppression of photosynthetic CO₂ assimilation, although g_H⁺ decreases concomitantly with Y(II) and v_Fd⁻.
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Figures

Figure 1

Photosynthetic CO2 assimilation and linear electron flow in the C3 plant mustard (A–C) and the C4 plant maize (D–F). (A, D) Net CO2 assimilation rate at various intercellular CO2 partial pressures (Ci). (B, E) Effective quantum yield of PSII, Y(II), at various Ci. (C, F) Relationship of Y(II) with CO2 assimilation rate. Dark respiration rate is presented as Rd. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O2 (open symbols). Solid lines represent estimated linear regression of the data at 1 kPa (C) and 21 kPa O2 (F) (R², coefficient of determination). The y-intercepts (b) were tested based on the null hypothesis: **p<0.005.
Figure 2

Relationship of P700 oxidation with effective quantum yield of PSII, Y(II), at various intercellular CO2 partial pressures in the C3 plant mustard (A) and the C4 plant maize (B). Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O2 (open symbols). Solid lines represent estimated linear regression of the data at 21 kPa O2 (R2, coefficient of determination).
Figure 3

Dark-interval relaxation kinetics of electrochromic shift (ECS) in the C3 plant mustard (A) and the C4 plant maize (B) under ambient air (40 Pa CO2, 21 kPa O2; black), low CO2 (1 Pa CO2, 21 kPa O2; purple), and low CO2/O2 (1 Pa CO2, 1 kPa O2; pink). Red actinic light (550 μmol photons m−2 s−1) was turned off at 0 ms for 600 ms during the steady-state photosynthesis. The kinetics were fit to mono exponential decay (R2, coefficient of determination: 0.7017, 0.9454, and 0.6868 in A; 0.8814, 9868, and 0.9801 in B respectively).
Figure 4

Electrochromic shift (ECS) parameters in the C3 plant mustard (A–C) and the C4 plant maize (D–F). (A, D) Proton motive force (pmf) at various intercellular CO2 partial pressures (Ci). (B, E) Proton conductance of the chloroplast ATP synthase (gH+) at various Ci. (C, F) Relationship of proton efflux rate via the ATP synthase (vH+) with CO2 assimilation rate. Effective quantum yield of PSII, Y(II), are also shown in orange symbols. Dark respiration rate is presented as Rd. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O2 (open symbols).
Figure 5

Relationships of the proton gradient across the thylakoid membrane (ΔpH; A, C) and the proton conductance of the chloroplast ATP synthase (gH+; B, D) with effective quantum yield of PSII, Y(II), at various intercellular CO2 partial pressures in the C3 plant mustard (A, B) and the C4 plant maize (C, D). We note that ΔpH and gH+ were separately measured from Y(II) at the same ambient CO2 partial pressures. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O2 (open symbols). Solid lines represent estimated linear regression of the data at 21 kPa O2 (R2, coefficient of determination).
Figure 6

Dark-interval relaxation kinetics of ferredoxin (Fd−) in the C3 plant mustard (A) and the C4 plant maize (B) under ambient air (40 Pa CO2, 21 kPa O2; black), low CO2 (1 Pa CO2, 21 kPa O2; purple), and low CO2/O2 (1 Pa CO2, 1 kPa O2; pink). Red actinic light (550 µmol photons m−2 s−1) was turned off at 0 ms for 600 ms during the steady-state photosynthesis. The kinetics were fit to mono exponential decay (R2,
In vivo measurement for the redox state of ferredoxin (Fd) in the C3 plant mustard (A, B) and the C4 plant maize (C, D). (A, C) The Fd reduction during the steady-state photosynthesis at various intercellular CO2 partial pressures (Ci). (B, D) Relationship of Fd− oxidation rate with effective quantum yield of PSII, Y(II), at various Ci. Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O2 (open symbols). Solid lines represent the estimated linear regressions of the data at 1 kPa (B) and 21 kPa O2 (D), respectively (R2, coefficient of determination). The y-intercepts (b) were tested based on the null hypothesis: #p>0.05.
Figure 8

Relationships of effective quantum yield of PSI, Y(I) (A, C), and plastocyanin (PC) oxidation (B, D) with effective quantum yield of PSII, Y(II), at various intercellular CO2 partial pressures in the C3 plant mustard (A, B) and the C4 plant maize (C, D). Experiments were conducted independently three times as shown in different symbols (biological replicates) at 21 kPa (closed symbols) and 1 kPa O2 (open symbols).
Figure 9

Electrochromic shift (ECS) induced by a 5 μs-short saturation flash during far-red light illumination in the C3 plant mustard (A) and the C4 plant maize (B). Far-red light was provided at various intensities (0, black; 1, grey; 5, pink; 10, red; and the maximum 20, wine red; the values defined by the Walz software). (C) The flash-induced ECS changes normalized by the values without far-red light illumination as 100%. The data of mustard (light grey) and maize (dark grey) are shown as the mean with the standard deviation (n = 3, biological replicates).

Figure 10

Effects of a short-saturation flash on the redox state around PSI in the C3 plant mustard (A−C) and the C4 plant maize (D−F). (A, D) Reduction kinetics of P700+ (green) in response to a 5 μs-short saturation flash after far-red light illumination for 10 s. Far-red light was provided at the maximum intensity (20, the value defined by the Walz software). Deconvoluted signals to plastocyanin (PC+, blue) and ferredoxin (Fd−, red) are also shown. All the Klas-NIR signals were normalized within the range from the minimum 0 to the maximum 1. The maximum reduction/oxidation levels of each component were determined as shown in Supplemental Fig. S2. The signal to Fd− has negative values. (B, E) Reduction kinetics of P700+ by a short saturation flash at different lengths (5, green; 10, light green; 20, light grey; and 50 μs, grey respectively) after far-red light illumination for 10 s. The kinetics are also shown with the logarithmic scale at y-axis (C, F). The representative traces of independent experiments (n = 3, biological replicates) are shown.
Figure 11

A brief illustration of mechanism for P700 oxidation in C3 and C4 plants. In C3 plants, proton conductance of chloroplast ATP synthase (gH+) decreases with the suppression of photosynthetic CO2 assimilation greater than photosynthetic linear electron flow reflected in effective quantum yield of PSII, Y(II), and ferredoxin (Fd−) oxidation rate (vFd), resulting in the increase in proton motive force (pmf) to induce P700 oxidation. In C4 plants, pmf increases with the suppression of photosynthetic CO2 assimilation, although gH+ decreases concomitantly with Y(II) and vFd.

Supplementary Files

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