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Gymnosperms Have Increased Capacity for Electron Leakage to Oxygen (Mehler and PTOX reactions) in Photosynthesis Compared with Angiosperms

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Oxygen plays an important role in photosynthesis by participating in a number of O_2-consuming reactions. O_2 inhibits CO_2 fixation by stimulating photorespiration, thus reducing plant production. O_2 interacts with photosynthetic electron transport in the chloroplasts' thylakoids in two main ways: by accepting electrons from PSI (Mehler reaction); and by accepting electrons from reduced plastoquinone (PQ) mediated by the plastid terminal oxidase (PTOX). In this study, we show, using 101 plant species, that there is a difference in the potential for photosynthetic electron flow to O_2 between angiosperms and gymnosperms. We found, from measurements of Chl fluorescence and leaf absorbance at 830 nm, (i) that electron outflow from PSII, as determined by decay kinetics of Chl fluorescence after application of a saturating light pulse, is more rapid in gymnosperms than in angiosperms; (ii) that the reaction center Chl of PSI (P700) is rapidly and highly oxidized in gymnosperms during induction of photosynthesis; and (iii) that these differences are dependent on oxygen. Finally, rates of O_2 uptake measured by mass spectrometry in the absence of photorespiration were significantly promoted by illumination in dark-adapted leaves of gymnosperms, but not in those of angiosperms. The light-stimulated O_2 uptake was around 10% of the maximum O_2 evolution in gymnosperms and 1% in angiosperms. These results suggest that gymnosperms have increased capacity for electron leakage to oxygen in photosynthesis compared with angiosperms. The involvement of the Mehler reaction and PTOX in the electron flow to O_2 is discussed.

Keywords: Angiosperms • Electron flow • Gymnosperms • Mehler reaction • Photosynthesis.

Abbreviations: A_{max}, maximum A_{830}; A_{830}, leaf absorbance at 830 nm; ANOVA, analysis of variance; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; Fd, ferredoxin; FNR, ferredoxin-NADP reductase; *n*-PG, *n*-propyl gallate; PC, plastocyanin; PQ, plastoquinone; PTOX, plastid terminal oxidase; ROS, reactive oxygen species.

Introduction

In photosynthesis, electrons are transported in the thylakoid via the linear electron transport pathway from PSII to PSI and finally to $NADP^+$ in the stroma. Besides linear electron flow, other types of electron flow occur, such as those associated with the Mehler reaction (Asada 2000, Badger et al. 2000), chlororespiration (Peltier and Cournac 2002), nitrate assimilation and electron cycling around PSI. Electron flow dependent on photorespiration (Stitt et al. 2010) and the malate valve might also be included. These alternative electron fluxes have been considered to serve as a sink or valve for electrons to avoid over-reduction of the electron transport chain (Niyogi 2000, Ort and Baker 2002).

In the Mehler reaction, oxygen is reduced at the reducing side of PSI, by ferredoxin (Fd) or iron–sulfur acceptors Fx. Reduction of O_2 results in production of reactive oxygen species (ROS) such as superoxide (O_2^-), which can be dealt with in the scavenging system (in the thylakoid and stroma) that has been referred to collectively with the O_2-reducing Mehler reaction as the Mehler–ascorbate peroxidase (APX) pathway or the water–water cycle (Asada 2000). In this system, O_2^- is converted to hydrogen peroxide (H_2O_2) via a disproportionation reaction catalyzed by a Cu/Zn superoxide dismutase (SOD), and then H_2O_2 is reduced to water by an APX using ascorbate as a reductant. The oxidized form of ascorbate, monodehydroascorbate (MDA), is reduced back into ascorbate by reduced Fd or NADPH. If ROS scavenging does not keep up with ROS production, plants suffer photoinhibition (Murata et al. 2007, Takahashi and Badger 2011). However, as long as the ROS generated are properly detoxified, plants can reduce

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the possibility of photoinhibiton (Niyogi 2000, Ort and Baker 2002).

Following completion of whole-genome sequencing in the model higher plant *Arabidopsis thaliana*, mutants impaired in the alternative electron flow such as cyclic electron flow have been isolated and molecular mechanisms involved have been studied (e.g. Shikanai 2007). However, this has not been the case for the Mehler reaction where key mutants impaired in oxygen reduction in and around PSI have not been obtained. In contrast, mutants defective in reactions serving to degrade and detoxify ROS in the Mehler–ascorbate peroxidase pathway have been defined (Rizhsky et al. 2003). Unfortunately, these latter mutants have not necessarily shown a clear phenotype under physiological growth conditions (Giacomelli et al. 2007, Maruta at al. 2010). For these reasons, our knowledge of the Mehler reaction or the Mehler–ascorbate peroxidase pathway has not been significantly advanced for more than a decade (Heber 2002).

Extensive studies have shown that the Mehler reaction is of significant magnitude in algae and cyanobacteria. The rate can reach up to 50% (Badger et al. 2000) or 100% (Asada 2000) of that of whole-chain electron transport in these organisms. On the other hand, the rate in C_3 plants is likely to be lower than that in cyanobacteria, but varies greatly depending on materials, conditions and instruments used, making it difficult to generalize or build consensus on its magnitude or even on its occurrence. The potential rate has been estimated to range from being negligibly small (Ruuska et al. 2000, Laisk et al. 2006) to almost 30% of whole-chain electron transport (Asada 2000, Badger et al. 2000).

Chlororespiration is another oxygen-related process in chloroplasts' thylakoids (Peltier and Cournac 2002, McDonald et al. 2010). It has been defined as electron transport from the stromal reductant NAD(P)H to oxygen via the plaatoquinone (PQ) pool in the dark. The reduction of PQ is assumed to be mediated by the NAD(P)H dehydrogenase complex (the NDH complex), and the oxidation of PQH_2 is assumed to be mediated by plastid terminal oxidase (PTOX). PTOX had been identified from analysis of an Arabidopsis mutant, *immutans* (*im*), with a variegated phenotype (Carol et al. 1999, Wu et al. 1999): *PTOX* (= *IM*) is the gene responsible for the *im* mutant, and is assumed to be involved in carotenoid biosynthesis, in which PQ is reduced by electrons from phytoene, the precursor of colored carotenoids, and then PQH_2 is oxidized by PTOX. The function of PTOX is crucial during the early stages of chloroplast development, when PSI cannot yet function effectively. The variegation, i.e. white sectors in leaves, was proposed to be due to photoinhibition caused by the deficiency of photoprotective colored carotenoids (Wetzel et al. 1994).

PTOX has also been hypothesized to act as a safety valve for electrons under excess excitation (to prevent over-reduction of the intersystem electron carriers) (Niyogi 2000, Ort and Baker 2002, Peltier and Cournac 2002). The involvement of PTOX in electron flow was demonstrated in PSI-deficient *Chlamydomonas* (Cournac et al. 2000), in which a homolog of PTOX in the algae sustained the electron flow from PSII. In tobacco transformants overexpressing Arabidopsis *PTOX*, although the oxidation of the PQ pool during photosynthetic induction was enhanced in low light, no such effect was seen during steady state in low light or during either induction or the steady state in high light (Joët et al. 2002). Similarly, overexpression of *PTOX* in Arabidopsis did not lower the redox state of the PQ pool or P700 during the steady state at 25 and 5°C (Rosso et al 2006). Whereas these observations raise doubts about the above hypothesis for PTOX, it was also shown that the PTOX content is extremely high in alpine *Ranunculus glacialis* acclimatized to high light and low temperature (Streb et al. 2005). In addition, in *Thellungiella halophila*, a salt-tolerant model plant of the Brassicaceae along with Arabidopsis, salt treatment induced a large increase in PTOX abundance and concomitant stimulation of linear electron flow that was sensitive to oxygen and *n*-propyl gallate (*n*-PG), a PTOX inhibitor (Josse et al 2003, Stepien and Johnson 2009). In comparison, Arabidopsis could not induce such adaptive responses (Stepien and Johnson 2009).

The action of oxygen in photosynthesis would be similar in most oxygenic phototrophs, at least in C_3 plants (Asada 2000). However, the impact of O_2 on electron flow in the thylakoid has been debated, as above. Normally, measurement of the O_2-dependent alternative electron flow has been conducted using a specific or a limited number of species in individual studies while testing various conditions such as low CO_2, drought and high light which are expected to promote the reaction. In this study, we focused on fluorescence decay kinetics after application of a 1 s saturating light pulse in a dark-adapted leaf, from which a potential for the O_2-dependent alternative electron flow was estimated. We show in a survey of 101 plant species that the potential is different within C_3 plants and is larger in gymnosperms than in angiosperms. This is reinforced by studies of O_2 photoreduction using mass spectrometry. In addition, analyses using the PTOX inhibitor *n*-PG imply that the contribution of PTOX to the higher potential for the O_2-dependent electron flow in the gymnosperms is limited.

Results

Rapid electron drain from PSII in gymnosperms as measured by Chl fluorescence

To investigate alternative electron flow, we analyzed the decay kinetics of Chl fluorescence after excitation by a saturating light pulse in dark-adapted leaves (**Fig. 1A**). After cessation of illumination, the fluorescence yield (F) declines toward the minimum (F_o) in the dark. This decline reflects oxidation of the secondary electron acceptor in PSII (Q_A), caused by electron drain from PSII presumably via alternative electron flow (see the Discussion) (Cao and Govindjee 1990, Bukhov et al. 1992, Bukhov et al. 2001), because linear electron flow is inhibited after dark adaptation owing to inactivation of

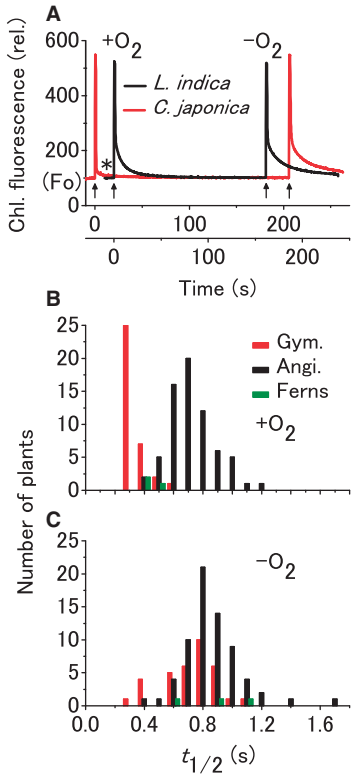

Fig. 1 Electron drain from PSII. (A) Decay kinetics of Chl fluorescence after excitation by a saturating light pulse (~1 s, 5,500 µmol photons m^{-2} s^{-1}) in a dark-adapted leaf of *Lagerstroemia indica* (black) and *Cryptomeria japonica* (red). The light pulses were applied as indicated by arrows. Oxygen was removed from air approximately 2 min before the application of the second pulse. (B, C) Histograms of the $t_{1/2}$ (s) of fluorescence decay in gymnosperms (red), angiosperms (black) and ferns (green) in the presence (B) and absence (C) of oxygen. Data are from **Supplementary Tables S1** and **S2**.

Fd-NADP reductase (FNR) (Arakaki et al. 1997) and several Calvin cycle enzymes such as RubisCO (Portis et al. 2008).

In gymnosperms, fluorescence declined quickly to a point close to F_o, as shown in *Cryptomeria japonica* (**Fig. 1A**, *). In angiosperms, the corresponding decline was smaller, and a subsequent slow decline was notable, as shown in *Lagerstroemia indica* (**Fig. 1A**). The quick decay phase could be fitted satisfactorily by a single exponential function with a first-order rate constant, and allowed calculation of the half-time ($t_{1/2}$) for decay. The value of $t_{1/2}$ was determined in leaves of various angiosperms, gymnosperms and ferns (**Table 1**; **Supplementary Table S1**; see the Materials and Methods). Although there has been a taxonomic debate on the Gnetales (so-called anthophyte theory), we treated *Welwitschia mirabilis* and *Gnetum gnemon* as gymnosperms according to results supported by many molecular studies that the taxon is grouped into the conifers as a sister group of the Pinaceae (Winter et al. 1999, Bowe et al. 2000, Chaw et al. 2000, Palmer et al. 2004).

In the gymnosperms, $t_{1/2}$ was around 0.3 s on average (**Table 1**; **Supplementary Table S1**). In the angiosperms, on the other hand, $t_{1/2}$ was around 0.68 s on average (**Table 1**; **Supplementary Table S1**). This difference is shown clearly in a frequency distribution histogram (**Fig. 1B**): histograms peaked at 0.3 s in the gymnosperms and at 0.7 s in the angiosperms, although there is some overlap in the distribution of properties. These results suggest that in general electrons are more promptly drained from PSII to downstream acceptors in the gymnosperms than in the angiosperms, probably via alternative electron transport.

Table 1 Differences in fluorescence decay kinetics between angiosperms and gymnosperms

	$t_{1/2}$ (s)	
Taxon	Plus O_2	Minus O_2
Gymnosperms	0.299 ± 0.065ac	0.667 ± 0.185dg
Angiosperms	0.675 ± 0.158be	0.817 ± 0.198fh

Data are total means ± SD of the half-time of Chl fluorescence decay, $t_{1/2}$ (**Supplementary Tables S1** and **S2**).
Letters indicate significance of differences between data: a–b, c–d, e–f, g–h (ANOVA, $P < 0.05$).

Rapid oxidation of PSI in gymnosperms as measured by 830 nm leaf absorbance

Fig. 2A shows changes in the redox state of P700 and plastocyanin (PC) during the induction of photosynthesis in strong light as measured by leaf absorbance at 830 nm (A_{830}) and as expressed by the ratio A_{830}/A_{max}, where A_{max} is the maximum A_{830} (Tsuyama and Kobayashi 2009). In *L. indica* (**Fig. 2A**), A_{830} first increased (i.e., P700 and PC were oxidized), followed by a sudden drop (i.e. re-reduction of $P700^+$ and PC^+). This drop (re-reduction) is attributable to both electron flow from PSII and a back-reaction in PSI, involving $P700^+$ and the iron–sulfur acceptors F_A^-/F_B^- (Tsuyama and Kobayashi 2009). Lowering of A_{830} below its dark level is attributed to the reduction of Fd (Klughammer and Schreiber 1994, Oja et al. 2003). Thereafter, A_{830} increased slowly throughout the illuminated period (20 s; **Fig. 2A**). These changes were basically common among the angiosperms tested. To express the extent of the increase in A_{830}, we evaluated A_{830} at 20 s ($A_{830,\ 20}$) as the ratio $A_{830,\ 20}/A_{max}$ (vertical double-headed arrow in **Fig. 2A**). The mean $A_{830,\ 20}/A_{max}$ of all the angiosperms tested was approximately 30.2 ± 26.6% (**Supplementary Table S2**). Reflecting the large standard deviation, species could be found at all intervals in the frequency distribution of $A_{830,\ 20}/A_{max}$ (**Fig. 2C**). A histogram peak arose at 10–20%, and in a few species the ratio was negative, which means that $A_{830,\ 20}$ was below the initial dark level of A_{830}. These results suggest that oxidation of P700 and PC was suppressed in the angiosperms during the induction period.

In contrast, in *C. japonica*, A_{830} increased rapidly to its maximum, following a sudden drop as noted above, after the onset of illumination (**Fig. 2A**), indicating full oxidation of P700 and PC. This was true of all the gymnosperms tested, and, thus, by definition, $A_{830,\ 20}/A_{max} = 100\%$ for the gymnosperms in the

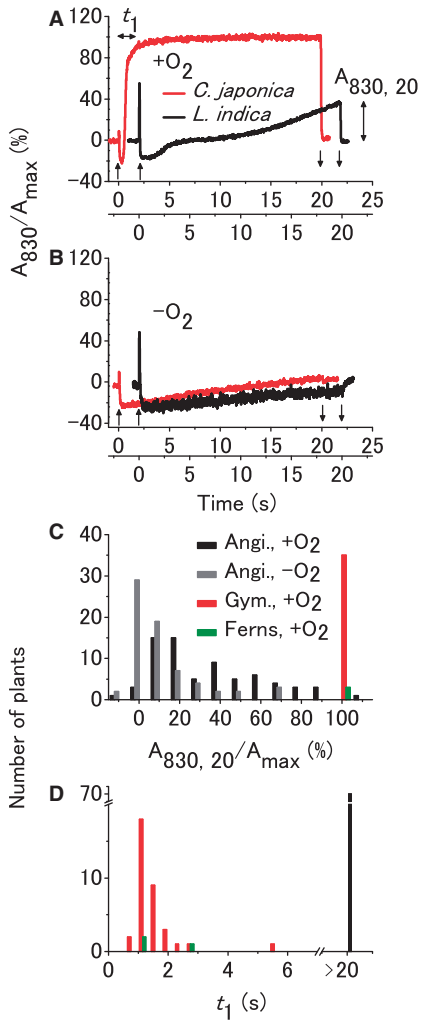

Fig. 2 Oxidation of P700 and PC. (A, B) Changes in A_{830} in *L. indica* (black) and *C. japonica* (red) during photosynthetic induction (1,600 µmol photons m^{-2} s^{-1}) in (A) the presence and (B) the absence of oxygen. (C) Histogram of A_{830} 20 s after onset of illumination ($A_{830, 20}/A_{max}$) in angiosperms (black), gymnosperms (red) and ferns (green) in the presence of oxygen. Gray bars: results of angiosperms in the absence of oxygen. (D) Histograms of the t_1 (s) in the presence of oxygen. Bars as in (C). Others as in **Fig. 1**.

histogram (**Fig. 2C**). To express the rapid P700/PC oxidation in the gymnosperms, we evaluated the time (t_1) required for A_{830} to reach maximum (horizontal double-headed arrow in **Fig. 2A**). The value of t_1 was approximately 1.4 s on average for the gymnosperms (**Supplementary Table S1**), and a histogram peak appeared at 1.2 s (**Fig. 2D**; note that t_1 for angiosperms is >20 s). These results suggest that P700 and PC were more quickly oxidized during photosynthetic induction in the gymnosperms than in the angiosperms, probably due to rapid transport to an alternative electron acceptor other than $NADP^+$.

Among the ferns (Pteridophyta; Nos. 36–38), both the fluorescence decay and the A_{830} rise were rapid, as observed in the gymnosperms (**Figs. 1B**, **2D**).

Effects of oxygen on electron drain from the electron transport chain

To analyze the potential involvement of O_2 as an alternative electron acceptor, we tested the effects of O_2 removal on the kinetics observed in **Figs. 1** and **2**. In O_2-free air (400 p.p.m. CO_2), the fluorescence decay was slowed in both *L. indica* and *C. japonica*, but more so in *C. japonica* (**Fig. 1A**). O_2 removal generally increased the $t_{1/2}$ of the fluorescence decline, making the $t_{1/2}$ values closer between the taxa (i.e. means of 0.667 s for the gymnosperms and 0.817 s for the angiosperms; **Table 1**; **Supplementary Tables S1**, **S2**). The histogram peaks of $t_{1/2}$ shifted to 0.8 s in both taxa (**Fig. 1C**).

O_2 removal also delayed the A_{830} rise, which was suppressed strongly in both species (**Fig. 2B**). The histogram of $A_{830, 20}/A_{max}$ of the angiosperms shifted to lower values in the absence of O_2 (**Fig. 2C**): for instance, the ratio was <0% in 31 plants (29 species) out of 68 plants (65 species) tested in the absence of O_2, compared with only four plants (four species) out of 70 plants (67 species) tested in the presence of O_2 (**Fig. 2C**; **Supplementary Table S2**). A_{830} was also suppressed strongly in the other gymnosperms, as in *C. japonica* (**Fig. 2B**): A_{830} did not reach A_{max} within 20 s (i.e. t_1 >20 s) in at least one out of three experiments in 16 plants (16 species) out of 33 plants (31 species) tested in the absence of O_2, as compared with 0 out of 35 plants (33 species) tested in the presence of O_2 (**Supplementary Table S1**). The effects of O_2 removal on $t_{1/2}$ and t_1 in the ferns were similar to those in the gymnosperms (**Fig. 1C**; **Supplementary Table S1**). These results suggest that O_2-related alternative electron flow was responsible for the rapid electron drain from PSII and the rapid P700/PC oxidation in the gymnosperms and the ferns.

Quantitative analysis of electron drain to oxygen in vivo

To define the involvement of O_2 as an electron acceptor in the light, membrane inlet mass spectrometry and the use of O_2 stable isotopes were employed to measure gross O_2 exchange directly in the light (Ruuska et al. 2000). **Fig. 3** shows a typical result of measurements of leaf gas exchange using this approach. The measurements were conducted in well dark-adapted leaves (>1–2 h), and started in air with 5–6% CO_2 and O_2 (see the Materials and Methods and the Discussion). In *Eucalyptus pauciflora* (an angiosperm), the rate of CO_2 uptake increased in the light in parallel with an increase of the rate of gross O_2 evolution, and subsequently reached a maximum (**Fig. 3A**). The CO_2 uptake rate remained at this level while CO_2 was sufficient in the chamber to saturate RubisCO, and declined toward zero as the leaf consumed most of the CO_2 (**Fig. 3C**). This decline was accompanied by an increase of the rate of gross O_2 uptake due to stimulation of RubisCO oxygenase and photorespiration, which was the only clear stimulation of gross O_2 uptake in the light under the experimental conditions. These changes (the decrease of CO_2 uptake and the increase of O_2 uptake) were accompanied by a

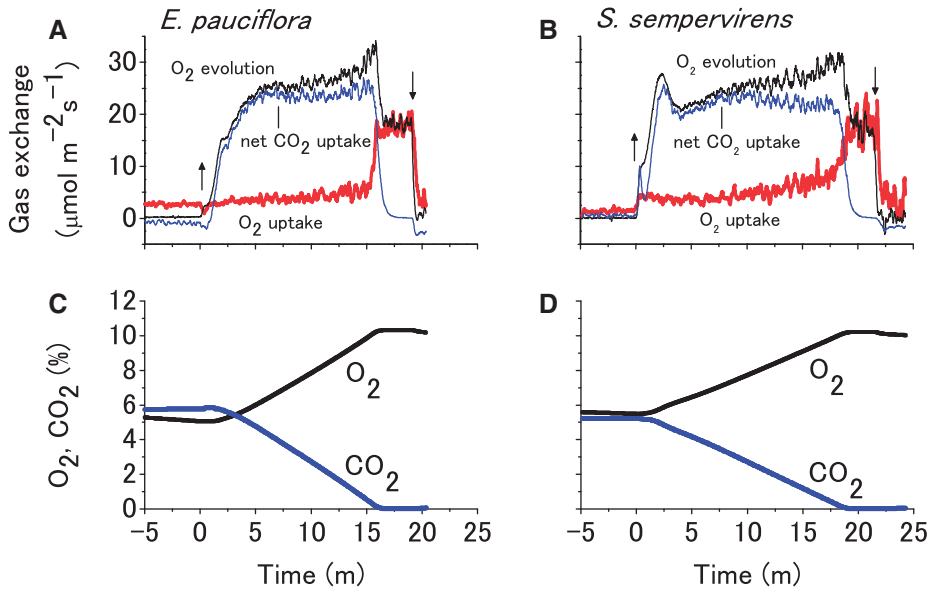

Fig. 3 Mass spectrometric measurements of rates of leaf gas exchange. (A, B) Net CO_2 uptake (blue), gross O_2 evolution (black) and gross O_2 uptake (red) of *Eucalyptus pauciflora* (A) and *Sequoia sempervirens* (B) as a function of illumination time. Data are results of 10 s moving averages. Leaf discs were in a closed chamber coupled to a mass spectrometer. Actinic light (1,000 μmol photons m^{-2} s^{-1}) was provided as indicated by arrows. (C, D) CO_2 (blue) and O_2 (black) concentrations in a leaf chamber for *Eucalyptus* (C) and *Sequoia* (D).

decrease of O_2 evolution due to down-regulation of PSII. The O_2 uptake rate dropped transiently upon illumination in *E. pauciflora* (**Fig. 3A**), but this is largely due to an artifact caused by heat from the light source.

On the other hand, in *Sequoia sempervirens* (a gymnosperm), while the gross O_2 uptake rate increased as in *E. pauciflora* (with decreasing CO_2 uptake and O_2 evolution), it also increased steeply immediately after the light was turned on at high CO_2 (**Fig. 3B**). We focused on this change directly after illumination as an indicator of O_2 photoreduction capacity, and the ratio of the rate of gross O_2 uptake immediately after onset of illumination to the rate in the dark was measured for a number of gymnosperm and angiosperm species. The ratio was about 3-fold higher in the gymnosperms than in the angiosperms (Welch's *t*-test, $P < 0.05$) (**Table 2**), suggesting the occurrence of significantly higher O_2 photoreduction in the gymnosperms. The relative magnitude of the O_2 photoreduction (**Table 2**), i.e. the ratio of the rate of light-stimulated O_2 uptake (O_2 uptake after light on minus dark respiration) to that of maximum O_2 evolution during the time course of the experiments shown in **Fig. 3**, was around 10% of the rate of total electron flow in the thylakoid in the gymnosperms studied and 0–2% of that in the angiosperms.

Factors affecting $t_{1/2}$ and t_1

In considering the data presented here, the differences induced by an alternative O_2 acceptor can probably be explained equally well by the action of a PSI-mediated Mehler reaction or the activity of an intersystem PTOX reaction. The first drains electrons from PSI directly, while the latter diverts electrons away from reaching PSI. Both reactions lead to the photoreduction of O_2, oxidation of the intersystem electron pool and oxidation of P700.

To analyze the involvement of PTOX in the rapid P700/PC oxidation in gymnosperms, we vacuum-infiltrated leaves from three gymnosperms belonging to different families (*Calocedrus formosana*, *Keteleeria davidiana* var. *formosana* and *Torreya nucifera*) with 1 mM *n*-PG, incubated them at 4°C for 24 h in the dark (see Discussion) and then measured P700/PC oxidation at room temperature. In the presence of *n*-PG, t_1 was larger than in the control (untreated) in the three gymnosperms (**Table 3**), but the t_1 values in the presence of *n*-PG were still smaller than the values in the angiosperms (>20 s, **Fig. 2D**). These results suggest that alternative electron flow to O_2 mediated by PTOX contributed partly to the rapid P700/PC oxidation in the gymnosperms.

To confirm that the reduced rate of P700/PC oxidation in **Table 3** was due to increased electron flow to PSI after PTOX had been inhibited, we simultaneously added 1 mM *n*-PG and 60 μM DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone), an inhibitor of PQH_2 reoxidation by the Cyt b_6f complex. As expected, the inhibition of P700/PC oxidation by *n*-PG was removed completely (**Table 3**), supporting the above idea that *n*-PG increased electron flow to PSI while DBMIB decreased it.

To estimate the O_2 affinity of the reaction involved in electron drain from the electron transport chain, $t_{1/2}$ and t_1 were determined at varying O_2 concentrations in the three gymnosperms (**Fig. 4**). In *C. formosana*, *K. davidiana* and *T. nucifera*, both parameters were unchanged between 10% and 21% O_2, but increased sharply below about 5% (or 10%) O_2. This result

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Table 2 Relative magnitude of the O_2 photoreduction

Taxon	Scientific name	O_2 uptake after light on/dark respiration	(Light-dependent O_2 uptake)/maximum oxygen evolution
Gymnosperms	*Cycas revoluta*	2.78 ± 0.46	0.09 ± 0.01
	Phyllocladus aspleniifolis	2.08 ± 0.15	0.10 ± 0.01
	Picea abies	2.15 ± 0.67	0.07 ± 0.02
	Pinus thunbergiana	3.27 ± 0.49	0.13 ± 0.01
	Sequoia sempervirens	3.75 ± 0.42	0.13 ± 0.02
Angiosperms	*Branchychiton rupestris*	1.30 ± 0.15	0.02 ± 0.01
	Eucalyptus pauciflora	1.11 ± 0.04	0.01 ± 0.00
	Hirschfeldia icana	1.25 ± 0.17	0.01 ± 0.01
	Manihot esculenta	1.10 ± 0.11	0.01 ± 0.01
	Nicotiana tabacum	1.10 ± 0.14	0.00 ± 0.00

The ratios of the rate of gross O_2 uptake after onset of illumination to that in the dark, and of the rate of light-stimulated O_2 uptake to that of maximum O_2 evolution were determined from the experimental time course shown in **Fig. 3** performed with each of the species.
Data are means ± SD (n = 3–5). Experimental conditions as in **Fig. 3**.

Table 3 Effects of inhibitors *n*-PG and DBMIB on P700 and PC oxidation kinetics

	t_1 (s)		
Conditions	*C. formosana*	*K. davidiana*	*T. nucifera*
Control	1.53 ± 0.39a	1.92 ± 0.25a	1.94 ± 0.25a
n-PG	13.59 ± 7.36b	4.96 ± 2.20b	2.80 ± 0.19b
n-PG + DBMIB	2.53 ± 0.49a	1.95 ± 0.11a	1.32 ± 0.06c

Data are means ± SD of the time required for full oxidation of P700 and PC (t_1).
Leaves from the three gymnosperms were treated with 0.6% ethanol (Control), 1 mM *n*-PG or 1 mM *n*-PG plus 60 μM DBMIB for 24 h at 4°C in darkness, and t_1 was measured at 23°C (see text).
Letters indicate significance of differences between data in each plant (ANOVA, $P < 0.05$) (n = 5). Experimental conditions were as in **Fig. 2**.

suggests that the O_2-related alternative electron flow in gymnosperms is saturated by about 5% O_2.

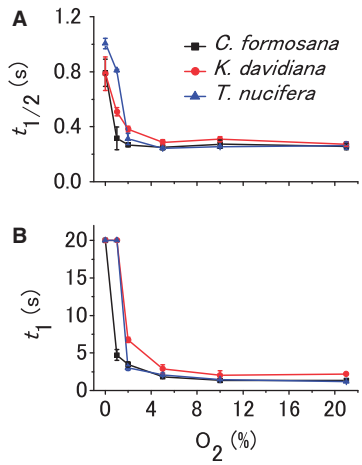

Fig. 4 O_2 dependence of $t_{1/2}$ and t_1. $t_{1/2}$ (A) and t_1 (B) as a function of O_2 concentration in air. Measurements were done as in **Figs. 1** and **2** in leaves of *Calocedrus formosana* (black), *Keteleeria davidiana* var. formosana (red) and *Torreya nucifera* (blue) in air with 400 p.p.m. CO_2 and the indicated concentration of O_2.

Discussion

A convenient method to analyze electron drain from the electron transport chain

In this study, we focused on the decay kinetics of Chl fluorescence excited by a single saturating light pulse. This method allowed us to take a quick survey of the capacity of electron flow supported by alternative electron acceptors such as the Mehler reaction in various plant species. A rationale of the method is straight forward: a decay of Chl fluorescence reflects electron flow to downstream acceptors in the electron transport chain. Bukhov et al. (2001) analyzed the decay kinetics in barley under the conditions where a leaf is dark adapted and electron flow to the Calvin cycle is not possible. They showed that there are four kinetically distinct components for the decay, and that two phases with a $t_{1/2}$ of 60–80 ms (intermediate component) and 650–680 ms (slow component) relate to limitation of electron flow to downstream acceptors (since these components disappeared in the presence of methylviologen, producing a single fast decay with a $t_{1/2}$ of 6–8 ms). The decay component that we focused on is considered to correspond to a combination of the intermediate and slow components. For comparison, a very slow decay phase with a lifetime of, for example, 10–20 s, has been attributed to inactive PSII centers with long-lived Q_A^-.

The intermediate component ($t_{1/2}$ of 60–80 ms) has been ascribed to a restriction of electron flow from Q_A^- to the PQ pool or to charge recombination within PSII (between Q_A^- and Yz or Tyr161 of the D1 polypeptide), while the slow component ($t_{1/2}$ of 650–680 ms) has been ascribed to the recombination between Q_A^- and an intermediate on the oxidizing side of PSII (the S_2 state of the water-splitting complex) (Dekker et al. 1984,

Cao and Govindjee 1990, Bukhov et al. 2001). That is, a principal reason for making these decay components is in the limitation of electron flow from Q_A^- to the PQ pool, which in turn induces the various types of recombination within PSII. Therefore, the finding that $t_{1/2}$ was greater in the angiosperms than in the gymnosperms is thought to be related to a restriction of electron flow from Q_A^- to the PQ pool. In the gymnosperms, this restriction is thought to be relieved as a result of electron flow to oxygen as an acceptor.

Evidence from phylogenetic analysis of DNA sequences showed that *Amborella trichopoda* is sister to all other extant angiosperms, meaning that this plant is the earliest or most primitive extant angiosperm (Qiu et al. 1999, APG III 2009). Because *Amborella* is an endangered plant and difficult to obtain, we used water lilies (*Nymphaea*) and star anise (*Illicium floridanum*), belonging to the second and third earliest diverging lineages, respectively, and being similarly considered to be basal angiosperms with *Amborella*. In these plants, the fluorescence decay was slower ($t_{1/2}$ = 0.555–1.197 s, Nos. 1–4 in **Supplementary Table S2**) than in the gymnosperms, and the A_{830} rise either was not detected (except for the initial small one) or was suppressed strongly in the 20 s induction period (Nos. 1–4 in **Supplementary Table S2**). The same was true in other primitive angiosperms belonging to the Magnoliales and Laurales (Nos. 5–11). Likewise, the cycads are assumed to be the most basal group of gymnosperms (Chaw et al. 2000, Palmer et al. 2004). In *Cycas revoluta*, the fluorescence decay and A_{830} rise were rapid, as in the other gymnosperms ($t_{1/2}$ = 0.28 s, t_1 = 1.40 s; No. 32 in **Supplementary Table S1**). These results support a distinct difference in the fluorescence decay and the A_{830} rise, i.e. electron drain from the electron transport chain, between angiosperms and gymnosperms (**Figs. 1**, **2**).

Possible factors affecting O_2-dependent electron flow in gymnosperms

A rapid electron outflow, as we observed here, has been reported previously by measuring Chl fluorescence and A_{820} changes during induction, but this conclusion was based on comparison between several *Pinus* and *Pisum sativum*, and the cause was attributed to fast activation of FNR (Schansker et al. 2008). Although we do not have data to refute their conclusion, our data, i.e. the effects of O_2 removal on $t_{1/2}$ and t_1 (**Figs. 1**, **2**, **Table 1**) and *n*-PG on t_1 (**Table 3**), cannot be explained by FNR.

The promotion of O_2 uptake by illumination (**Fig. 3**, **Table 2**) implies that the potential for the Mehler reaction and electron flow to oxygen mediated by PTOX were high in the gymnosperms, as detailed below. The measurements were conducted after dark adaptation which should inactivate FNR and key enzymes involved in CO_2 assimilation. In addition, to induce the O_2-dependent electron flow sufficiently, the measurements were started in 5–6% O_2 on the basis of the O_2 dependency of $t_{1/2}$ and t_1 (**Fig. 4**). Further, to suppress photorespiration, which also causes O_2 uptake and is difficult to differentiate from the Mehler reaction and PTOX reaction, 5–6% CO_2 was used. The presence of inhibitory effects of such a high CO_2 was always checked by comparing CO_2 uptake rates under the conditions with those obtained at lower CO_2 concentrations. From the experimental conditions, we consider that the increase of O_2 uptake immediately after turning on the light was not due to photorespiration.

With regard to O_2 uptake activity of leaves during photosynthesis, O_2 reduction mediated by PTOX may deserve similar consideration to the Mehler reaction, as judged from the recent findings on the involvement of PTOX in electron transport in the thylakoid (Cournac et al. 2000, Joët et al. 2002, Josse et al. 2003, Streb et al. 2005, Rosso et al. 2006, Stepien and Johnson 2009). It was previously shown that, in Arabidopsis overexpressing PTOX (Joët et al. 2002), fluorescence decay after application of a saturating pulse was faster than in the wild type. We confirmed that the fluorescence decay was slower in the Arabidopsis *im* mutant lacking PTOX than in the wild type (data not shown). In addition, in the three gymnosperms, *n*-PG delayed P700/PC oxidation (**Table 3**). These results suggest that electron flow to O_2 mediated by PTOX can be a factor for the electron drain from PSII.

Normally, the effects of a photosynthesis inhibitor can be seen within a few hours, e.g. for *n*-PG after 1 h in tobacco leaf disks (Joët et al. 2002), and after 2.5 h in a suspension of green algal symbiotes from lichen (Ilík et al. 2006). However, the retardation of P700/PC oxidation required 24 h (**Table 3**). This would be at least partly due to poor penetration of the chemical into cells, attributable to the thick cell walls and the large cell size, as the appearance of the (hard and thick) leaves suggests. Considering the long incubation periods, the incubation of leaves with chemicals was done at 4°C, which was intended to suppress respiration. At room temperature (23°C), t_1 and also F_o of Chl fluorescence became significantly larger even in untreated leaves (data not shown), implying the induction of (chloro-)respiratory reduction of the PQ pool, which disturbed the analyses of redox changes in the electron transport chain. In contrast, incubation at 4°C almost suppressed those effects on t_1 (**Table 3**) and F_o (data not shown).

n-PG delayed P700 and PC oxidation in the gymnosperms (**Table 3**), but the P700/PC oxidation was still faster than that in the angiosperms in the absence of *n*-PG (t_1 of >20 s, **Fig. 2D**). This incomplete inhibition of P700/PC oxidation by *n*-PG suggests that the presence of another type of O_2-related reaction seems to be more important than PTOX in the gymnosperms.

The O_2 dependencies of $t_{1/2}$ and t_1 in the three gymnosperms suggest that the oxygen-related reaction requires 5–10% O_2 for saturation (K_m of roughly 2% O_2) (**Fig. 4**). This is not consistent with the high affinity for O_2 of mitochondrial respiration (about 0.25% O_2 for saturation) (Forti and Caldiroli 2005). Also, it is inconsistent with that of photorespiration, which saturates above 80% O_2 (K_m of 30–40% O_2) (Osmond 1981). However, the moderate affinity shown (**Fig. 4**) is close to that measured for the Mehler reaction (K_m of 5–10% O_2) (Heber and French 1968, Osmond 1981, Asada 2000).

The intermediate affinity for O_2 of the Mehler reaction, i.e. between that of mitochondrial respiration and photorespiration, has been recognized as one of the important characteristics for this reaction (Ort and Baker 2002).

On the other hand, the existence of PTOX has not necessarily been assumed in studies investigating the Mehler reaction thus far. However, it has been suggested in a *Chlamydomonas* mutant lacking Cyt b_6f, in which PTOX can be analyzed separately, that PTOX has a lower affinity for oxygen than that of mitochondrial respiration and that the affinity appeared to be similar to that of the Mehler reaction (>2% O_2 for saturation) (Bennoun 2001). This means that these two factors (Meher and PTOX) are difficult to differentiate. We conclude that, although small, the O_2-dependent electron flow with the moderate O_2 affinity (**Fig. 4**) would also include electron flow mediated by PTOX.

The Mehler and PTOX reactions in gymnosperms

The importance of oxygen as an electron acceptor has been repeatedly proposed (Radmer and Kok 1976). Especially in dark-adapted leaves, O_2 may be the only electron acceptor available. In this condition, the Mehler reaction has been considered to play a role as a trigger of photosynthesis (Ziem-Hanck and Heber 1980, Kobayashi and Heber 1994, Makino et al. 2002). That is, electron flow into the water–water cycle (i.e. the flow associated with O_2 reduction and recovery of ascorbate) contributes to the generation of a pH gradient across the thylakoid membrane (Schreiber and Neubauer 1990). The pH gradient drives the synthesis of ATP, which is then used to activate RubisCO. Indeed, as shown in *Sequoia* (**Fig. 3**), induction of CO_2 uptake was rapid, exhibiting a transient steep rise upon illumination. However, this activity was insensitive to glycolaldehyde (10 mM) which suppressed subsequent steady-state CO_2 uptake completely (**Supplementary Fig. S1**). This means that the steep rise of CO_2 uptake on illumination might not be due to activation of RubisCO and thus cannot be seen as evidence for the trigger of photosynthesis, although we do not know the actual cause of it at present. However, it should be noted that glycolaldehyde (10 mM) did not inhibit O_2 uptake (**Supplementary Fig. S1**), confirming that this activity is not due to photorespiration.

In a dark-adapted leaf, electron flow in the thylakoid is limited at the reducing side of PSI, owing to inactivation of FNR and enzymes of the Calvin cycle. Following illumination, the limiting site shifts from the acceptor to the donor side of PSI, and is normally explained by the operation of the so-called 'photosynthetic control at Cyt b_6f', which suppresses the electron flow to PSI by increasing the counter-pressure to proton release during PQH_2 oxidation at Cyt b_6f (Harbinson and Hedley 1993, Laisk et al. 2005, Tsuyama and Kobayashi 2009). This mechanism is consistent with the slow electron drain from PSII and P700/PC oxidation in the angiosperms (**Figs. 1**, **2**). However, the A_{830} rise in the gymnosperms was too rapid to be explained by the photosynthetic control, since it requires the accumulation of protons in the lumen and thus takes more time to initiate. Thus, we assume that electron drain from PSI in the Mehler reaction (but not the coupled proton accumulation) is important in the gymnosperms for the rapid P700/PC oxidation. On the other hand, unlike the Mehler reaction, electron flow depending on PTOX appears not to generate a ΔpH across the membrane (i.e. non-electrogenic), because PTOX orientates toward the stromal side of the thylakoid membrane (Lennon et al. 2003, McDonald et al. 2010), meaning that PTOX would help the rapid P700/PC oxidation simply by draining electrons from the PQ pool. Therefore, we propose (i) that gymnosperms have a distinct mechanism to shift the limiting site of electron flow during induction and (ii) that the mechanism depends on electron flow due to the Mehler and PTOX reactions.

Driever and Baker (2011) showed in French bean that the rate of the Mehler reaction corresponded to 4.4% of the maximum rate of total electron flow in the thylakoids, from mass spectrometry experiments conducted under excess light and at the CO_2 compensation point in the absence of photorespiration (3% O_2). This rate is intermediate compared with the rates observed in the angiosperms and gymnosperms (**Table 2**), but relatively close to those in the angiosperms (0–2% of total). The occurrence of the Mehler reaction was previously studied in transgenic tobacco lines with an antisense gene directed against the mRNA of the small subunit of RubisCO (Ruuska et al. 2000). A reduced activity of the Calvin cycle can be expected to promote the Mehler reaction. However, in the transgenic with RubisCO of 10% of the wild-type level, a rate of O_2 uptake during induction (at 970 μmol photons m^{-2} s^{-1}, 1–2% CO_2 and 20% O_2) was similar to that in the dark. This result was interpreted to suggest a low or virtually no potential for the Mehler reaction. Our results agree with the conclusion and imply that it is common to angiosperms (**Table 2**, **Figs. 1**, **2**).

The data from the ferns were similar to those from the gymnosperms (**Fig. 2**, **Supplementary Table S1**), suggesting a high potential for the O_2-dependent electron flow, i.e. the Mehler reaction and that mediated by PTOX. The Mehler reaction is also large in algae and cyanobacteria (Asada 2000, Badger et al. 2000). Therefore, angiosperms may be an exception in this property (i.e. a potential for the O_2-dependent electron flow). On the other hand, Miyake et al. (2012) suggested that during induction the Mehler reaction induces cyclic electron flow around PSI (and that within PSII) by preventing over-reduction of the electron transport chain. Therefore, the magnitude itself (of the O_2-dependent electron flow) may not necessarily be important for regulation of photosynthesis.

However, at present, it is difficult to discuss the magnitude or potential for the Mehler reaction and that by PTOX in a systematic manner. In several lichen species with the green alga *Trebouxia* as a symbiont, P700 oxidation was rapid, as in the gymnosperms in our study, and was ascribed to rapid electron outflow from PSI due to the Mehler reaction or the activation of FNR (Ilík et al. 2006). In a marine cyanobacterium,

Synechococcus WH8102, P700 oxidation was also fast (i.e. a gymnosperm type), although a contribution of chlororespiratory electron flow mediated by PTOX to the rapid P700 oxidation was suggested (Bailey et al. 2008). In the cyanobacterium *Anacystis nidulans*, P700 oxidation, being O_2 dependent in agreement with our results (i.e. a gymnosperm type), was not increased rapidly (an angiosperm type) (Maxwell and Biggins 1977). However, the fluorescence decay technique will be difficult to apply in species such as algae and cyanobacteria which have significant intersystem electron donors such as the thylakoid NDH complex and a shared respiratory electron transport chain.

As a general tendency of conifers (a main component of gymnosperm genera), many of them grow in the mid- to high-latitude regions of the Northern Hemisphere where severe climatic conditions such as chilling temperatures are often experienced. As a result, conifers may be required to be more flexible than angiosperms to control photosynthesis according to surrounding environmental conditions. On the other hand, the Mehler reaction is thought to be responsible for the extra production of ATP through generation of the proton gradient across thylakoid membranes (Schreiber and Neubauer 1990). Also, ROS generated in the Mehler reaction can induce the down-regulation of PSII through suppression of the repair of photodamaged PSII (Murata et al. 2007, Takahashi and Badger 2011). Do these functions relate to the ecophysiological trait of conifers? In this study, P700 oxidation was shown to be rapid in gymnosperms, which means avoidance of over-reduction of the electron transport chain, and thus can be seen as an advantageous control. Although more research is needed to clarify the mechanism of this seemingly adaptive behavior, a clear separation lies in the potential for O_2 photoreduction (probably due to both the Mehler and PTOX reactions) between gymnosperms and angiosperms.

Conclusion

We conclude that gymnosperms have a higher capacity for O_2-dependent electron flow (~10% of the maximum electron flow) than angiosperms. This difference can be ascribed to differences due to the Mehler reaction and electron flow mediated by PTOX. The former can enhance electron flow at the reducing side of PSI, and the latter can divert electron flow away from PSI, thereby enabling rapid electron outflow from PSII together with the rapid oxidation of P700 and PC.

Materials and Methods

Plant material

Gymnosperms (35 plants, 33 species, 23 genera, 11 families) (**Supplementary Table S1**) were selected so as to encompass its four constituent divisions, the cycads, *Ginkgo*, the Gnetales and the conifers (Palmer et al. 2004). Among the conifers, the largest group in the gymnosperms, we used at least one species from each of the seven families placed in the taxon (Nos. 1–31, **Supplementary Table S1**) (Stefanović et al. 1998). Angiosperms (71 plants, 68 species, 51 genera, 37 families) (**Supplementary Table S2**) were selected so as to cover the three groups that characterize its phylogenic relationships, i.e. the primitive angiosperms (Nos. 1–11), the monocots (Nos. 12–14) and the eudicots (Nos. 15–71) (APG III 2009).

Most plants were obtained on the campus of Kyushu University, and some from university forests (Fukuoka and Miyazaki, Japan). *Illicium*, *Nymphaea*, *Gnetum* and *Welwitschia* were purchased from a breeder; the latter three were adapted to glasshouse conditions (30°C, 70% relative humidity; Biotron Institute, Kyushu University) and *Illicium* was adapted to field conditions. For mass spectrometry experiments (**Fig. 3**, **Table 2**), plants were adapted to glasshouse conditions (25°C, 70% relative humidity; The Australian National University), except for *Picea abies* and *Hirschfeldia icana* which were grown in outside conditions in Canberra, Australia, in August and September.

Chl fluorescence and P700 absorbance

Modulated Chl fluorescence and leaf absorbance (A_{830}) were measured as described (Klughammer and Schreiber 1994, Tsuyama and Kobayashi 2009): the former with a mini-PAM fluorometer and the latter with a PAM-101 fluorometer equipped with a dual-wavelength (810/860 nm) emitter–detector unit (ED-P700 DW, H. Walz). Fluorescence F_o was induced by red-modulated measuring light from light-emitting diodes (LEDs; <0.5 μmol photons m^{-2} s^{-1}), and F_m by saturating light pulse from a halogen lamp (~1 s, 5,500 μmol photons m^{-2} s^{-1}; KL-1500, Schott). The actinic light (1,000 or 1,600 μmol photons m^{-2} s^{-1}) was provided by a halogen lamp. To obtain air with the desired O_2 and CO_2 concentration, pure O_2, CO_2 and N_2 were mixed in a gas blender with mass flow controllers (GB-3C, KOFLOC, Japan). Leaves were dark-adapted for at least 30 min, usually for more than an hour, before use. The A_{830} signal includes small (~10%) contributions of PC and Fd (Harbinson and Hedley 1989, Oja et al. 2003), but we considered A_{830} as reflecting the redox state of P700 and PC unless otherwise noted.

Membrane inlet mass spectrometry

O_2 and CO_2 exchange were measured using a closed leaf chamber attached to a mass spectrometer via a membrane inlet (Isoprime-EA, GV Instruments) (Ruuska et al. 2000). Leaf discs (approximately 1 cm^2) were taken from dark-adapted leaves. When using conifers, about 10 pieces of needle were appropriate for each experiment. The disc or the pieces of conifer needles were placed in the chamber, containing known volumes of $^{18}O_2$ and pure CO_2, for about 10 min to allow O_2 (mass 32 and 36) and CO_2 (mass 44) signals to stabilize. Subsequently, actinic light was turned on and photosynthesis was allowed to proceed until CO_2 in the chamber was depleted. The chamber was thermostated to 25°C. Rates were calculated on a projected area basis. A level of approximately 5% O_2 was used to minimize

photorespiration and maximize the signal to noise resolution on the O_2 mass signals. CO_2 was used at 5% to suppress photorespiration.

Treatment of inhibitor of electron transport

The 1 mM *n*-PG (3,4,5-trihydroxy benzoic acid *n*-propyl ester) and 60 μM DBMIB were vacuum infiltrated into leaves (thorough pinholes) and the leaves were left in the solution for 24 h in darkness at 4°C (see text) (Joët et al. 2002). To prepare stock solution, we dissolved the inhibitors first in ethanol (plus dimethylsulfoxide for DBMIB). The final ethanol concentration was ≤0.6%. Control solution contained solvent only. The DBMIB concentration (60 μM) was higher than that normally used in in vitro experiments (Joët et al. 2002), but this was necessary to have an observable effect on electron flow in vivo.

Statistics

The statistical significance ($P < 0.05$) of differences between data was analyzed by Welch's *t*-test or (one-way or two-way) analysis of variance (ANOVA), followed by Scheffé's *F*-test of differences between group means ($P < 0.05$) in Excel software (Microsoft) with add-in Statcel 3 software (OMS).

Supplementary data

Supplementary data are available at PCP online.

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