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Determination of the Rate of Photoreduction of O_2 in the Water-Water Cycle in Watermelon Leaves and Enhancement of the Rate by Limitation of Photosynthesis

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A study was performed to determine how the electron fluxes for the photosynthetic carbon reduction (PCR) and the photorespiratory carbon oxidation (PCO) cycles affect the photoreduction of O_2 at PSI, which is the limiting step in the water-water cycle. Simultaneous measurements were made of CO_2-gas exchange, transpiration and quantum yield of PSII [Φ(PSII)] using leaves of watermelon (*Citrullus lanatus*). The total electron flux in PSII [Je(PSII)], as estimated from Φ(PSII), was always larger than the total electron flux required for the PCR and PCO cycles at various partial pressures of CO_2 and O_2 and 1,100 μmol photons m^{-2} s^{-1}. This observation suggested the existence of an alternative electron flux (Ja). Ja was divided into O_2-dependent [Ja(O_2-depend)] and O_2-independent [Ja(O_2-independ)] components. The magnitude of half Ja(O_2-depend), 7.5 to 9.5 μmol e^- m^{-2} s^{-1}, and its apparent K_m for O_2, about 8.0 kPa, could be accounted for by the photoreduction of O_2 at PSI either mediated by ferredoxin or catalyzed by monodehydroascorbate reductase. The results indicated that Ja(O_2-depend) was driven by the water-water cycle. A decrease in the intercellular partial pressure of CO_2 from 23 to 5.0 Pa at 21 kPa O_2 enhanced Ja(O_2-depend) by a factor of 1.3. Saturation of the activities of both the PCR and PCO cycles by increasing the photon flux density induced Ja. These results indicate the electron flux in PSII that exceeds the flux required for the PCR and PCO cycles induces the photoreduction of O_2 in the water-water cycle.

Key words: Alternative electron flow — Mehler reaction — Photoreduction of O_2 — Photorespiration — Watermelon (*Citrullus lanatus* sp.) — Water-water cycle.

On the reducing side of PSI in thylakoids, O_2 is univalently photoreduced to O_2^-. From in vitro experiments, it has been suggested that at least three components of chloroplasts mediate the photoreduction of O_2 (Asada 1999): the Fe/S-center of PSI (Asada et al. 1974); ferredoxin (Fd) (Furbank and Badger 1983), Fd-NADP oxidoreductase (FNR) (Goetze and Carpentier 1994) and monodehydroascorbate radical reductase (MDAR) (Miyake et al. 1998).

Superoxide radicals that are photoproduced at PSI are disproportionated to H_2O_2 and O_2 by superoxide dismutase (SOD). The H_2O_2 that accumulates in the stroma oxidatively inactivates the enzymes in the photosynthetic carbon reduction (PCR) cycle, namely, NADP-glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase and phosphoribulokinase (Kaiser 1976, 1979). In addition, H_2O_2 generates a hydroxyl radical through the Fenton reaction if transition metals are present (Asada 1996). The hydroxyl radical oxidatively damages RuBisCO (Ishida et al. 1997, 1998) and glutamine synthetase (Palatnik et al. 1999). To prevent such damages, H_2O_2 must be scavenged as rapidly as it is produced.

The water-water cycle scavenges O_2^- and H_2O_2 (Asada 1999). In this cycle, O_2^- produced at PSI disproportionates to O_2 and H_2O_2 in a reaction catalyzed by SOD and then APX reduces the H_2O_2 to water with ascorbate as the electron donor. The monodehydroascorbate radical (MDA) produced in the latter reaction is reduced to AsA either by Fd or by NADPH in a reaction catalyzed by MDAR. The sequential reactions of the water-water cycle are as follows:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^- \qquad \text{(PSII)},$$

Abbreviations: A, net CO_2 assimilation rate; APX, ascorbate peroxidase; AsA, ascorbate; Ci, the intercellular partial pressure of CO_2; Φ(PSII), the quantum yield of electron transport in PSII at a steady state, defined as $(F'_m - F_s)/F'_m$ or $(\Delta F/F'_m)$; Fd, ferredoxin; F_m, maximum fluorescence yield, as determined with dark-adapted leaves; F'_m, maximum variable fluorescence yield; FNR, Fd-NADP oxidoreductase; gm, mesophyll conductance to CO_2; Γ^*, the partial pressure of CO_2 at which the rate of carboxylation equals the rate of photorespiratory evolution of CO_2; IRGA, an infrared gas analyzer; Ja, alternative electron flux in PSII; Ja(O_2-depend), Ja that depends on pO_2; Ja(O_2-independ), Ja that does not depend on pO_2; Je(PSII), the electron flux in PSII; Je(PCR), the electron flux to the PCR cycle; Je(PCO), the electron flux to the PCO cycle; Je(PCR+PCO), Je(PCR)+Je(PCO); MDA, monodehydroascorbate radical; MDAR, MDA reductase; PCO cycle, photorespiratory carbon oxidation cycle; pCO_2, ambient partial pressure of CO_2; PCR cycle, photosynthetic carbon reduction cycle; PFD, photosynthetically active photon flux density; pO_2, ambient partial pressure of O_2; Rd, rate of day respiration; SOD, superoxide dismutase; *Vc*, rate of carboxylation of RuBP; *Vo*, rate of oxygenation of RuBP; Sr, relative specificity of RuBisCO; RuBisCO, RuBP carboxylase/oxygenase; RuBP, ribulose-1,5-bisphosphate.

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$2e^- + 2O_2 \rightarrow 2O_2^-$ (PSI),
$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (SOD),
$H_2O_2 + 2AsA \rightarrow 2H_2O + 2MDA$ (APX),
$2MDA + 2e^- + 2H^+ \rightarrow 2AsA$ (Fd or MDAR).

Apart from the disadvantages associated with the photoreduction of O_2 at PSI for photosynthesis, the photoreduction of O_2 is important for protection of PSII from photoinhibition. Exposure of leaves to irradiances at which the oxidation of plastoquinone limits the photosynthetic linear electron transport renders PSII inactive as a result of the degradation of the D1 protein of the PSII complex. The fragmentation of the D1 protein is triggered by attack of the singlet oxygen ($^1O_2$) that is produced in the reaction of triplet-excited P680 with O_2 (Hideg et al. 1994a, b; Mishra et al. 1994). The photoreduction of O_2 at PSI results in the oxidation of plastoquinone (Osmond and Grace 1995) and lowers the quantum yield of PSII (Schreiber et al. 1995) by generating an electron flux in the water-water cycle (Asada 1999). The electron transport driven by the water-water cycle produces a pH gradient across the thylakoid membrane, which reduces the quantum yield of PSII, as detected by monitorinig the non-photochemical quenching of Chl fluorescence. This phenomenon in turn suppresses the harmful excitation of P680 that leads to the production of $^1O_2$. In other words, the photoreduction of O_2 at PSI can be good or bad for a plant, depending on the environmental conditions (Polle 1996).

The rate of photoreduction of O_2 at PSI in attached leaves is important for an understanding of the physiological significance of this reaction. The rate of photoreduction of O_2 in vivo has been reported (Wu et al. 1991, Brestic et al. 1995, Lovelock and Winter 1996, Kingston-Smith et al. 1997, Fryer et al. 1998). The cited authors estimated the rate from the effects of various partial pressures of O_2 (pO_2) on the quantum yield of PSII [Φ(PSII)]. A decrease in pO_2 from 21.0 to about 2.0 kPa O_2 lowers Φ(PSII), as estimated from quenching analysis of Chl fluorescence. The remaining Φ(PSII) at 2.0 kPa O_2 is assumed to be related to the photoreduction of O_2 at PSI since the rate of the reaction catalyzed by washed thylakoids reaches a maximum at 2.0 kPa O_2 (Heber and French 1968, Takahashi and Asada 1982, Miyake et al. 1998) and the oxygenase reaction of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which limits the turnover of the photorespiratory carbon oxidation (PCO) cycle has an apparent K_m for O_2 of about 40.0 kPa (von Cammerer et al. 1994). It remains to be determined, however, whether or not a decrease in pO_2 from 21 to 2.0 kPa O_2 suppresses the photoreduction of O_2 that is catalyzed by a mediator with a low affinity for O_2.

We can also ask whether MDAR and Fd are involved in the photoreduction of O_2 to O_2^- in vivo. The photoreduction of O_2 that is mediated by these components does not reach a maximum rate at 2.0 kPa O_2, and the respective K_m value for O_2 are about half the partial pressure of O_2 in the atmosphere. The rate of photoreduction of O_2 by these enzymes is, furthermore, higher than that by washed thylakoids (Furbank and Badger 1983, Miyake et al. 1998). Laisk and Loreto (1996) showed that, in an intact leaf, the electron flux in PSII [Je(PSII)] exceeds the total electron flux required for both the PCR and the PCO cycles. The excess flux depended on pO_2 and reached a maximum at 21.0 kPa O_2. It might have been due to the photoreduction of O_2 at PSI mediated by Fd or by MDAR. Furthermore, the rate of uptake of O_2 that was not dependent on the PCO cycle was reported to be about 40 μmol electrons $m^{-2}\,s^{-1}$, as determined by monitoring the uptake of $^{18}O_2$ by leaf disks (Biehler and Fock 1996). This rate can not be accounted for by the rate of photoreduction of O_2 that is catalyzed by washed thylakoids. Identification of the mediators in vivo of the photoreduction of O_2 at PSI should be supported by their respective biochemical characteristics. It is also great interest to determine how the activities of the PCR and PCO cycles affect the rate of the photoreduction of O_2 at PSI.

We determined the rate of photoreduction of O_2 at PSI in watermelon leaves. We assumed that the photoreduction of O_2 at PSI is associated with the excess electron flux, namely, the alternative electron flux (Ja). We detected Ja in watermelon leaves and studied the effects of pO_2 on Ja. Ja could be divided into O_2-dependent [Ja(O_2-depend)] and O_2-independent [Ja(O_2-independ)] fluxes. Ja(O_2-depend) was never saturated at 2.0 kPa O_2. Moreover, it exhibited similar affinity for O_2 in the Fd- and in the MDAR-dependent photoreduction of O_2 at PSI. Finally, we showed that suppression of the activities of both the PCR cycle and the PCO cycle induced the photoreduction of O_2 at PSI.

Materials and Methods

Growth conditions—Wild-type watermelon (*Citrullus lanatus* sp.; Tottori Horticultural Center.; International Watermelon Seed Bank #101117-1) was grown from seed under standard air-equilibrated conditions with 35°C/25°C, light/dark cycles, 40% relative humidity, 1,000 μmol $m^{-2}\,s^{-1}$ photon flux density and a 16-h light/8-h dark period. Seeds were sown in a 0.5 $(dm)^3$ pot contained commercial peat-based compost. Plants were watered daily and fertilized (Hyponex 8-12-6; Hyponex Japan, Osaka, Japan) twice a week. All measurements were made using the fourth fully expanded leaf three weeks after sowing. Measurements of photosynthetic parameters and collection of leaves were initiated 7 h after the start of the light period.

Measurements of photosynthetic parameters—The rates of CO_2 exchange and transpiration of a leaf attached to a plant were determined with an open gas-exchange system that was equipped with a temperature-controlled chamber, as described by Makino et al. (1985), with some modifications. The rate of photosynthetic gas exchange was measured over an area of 0.785 cm^2 of attached

leaf. Leaf temperature was measured with a copper-constantan probe (EH-100-03; Chino, Tokyo) and adjusted to 30.0±0.5°C. Gas with the indicated mixture of pure O_2 and CO_2 was prepared by mixing 99.99% (v/v) N_2, 20.1% (v/v) O_2 in 79.9% (v/v) N_2, and 1% (v/v) CO_2 in 99% (v/v) N_2 using a mass-flow controller (Kofloc model GB-3C; Kojima Instruments Corp., Kyoto, Japan). The mixture of gases was saturated with water vapor at 18± 0.1°C, which corresponded to 20.454 kPa. Half of the flow of gas was passed through the leaf chamber and the other half by-passed the chamber to serve as a reference. The flow rate of gas through the chamber was measured with a flow meter (Kofloc model RK 1400; Kojima Instruments) and it varied from 0.75 to 0.78 liter min^{-1}, depending on the rate of gas exchange by the leaf. Boundary-layer conductance was greater than 4.5 mol H_2O m^{-2} s^{-1}. The partial pressure of CO_2 (pCO_2) supplied to the leaf chamber was measured in an absolute mode with an infrared gas analyzer (IRGA; LI-6252; Li-COR, Lincoln, NE, U.S.A.) and the pCO_2 passing through the leaf chamber was measured in differential mode with an IRGA (LI-6262; Li-COR). Each IRGA was calibrated before experiments with the standard mixture of gases. The ambient partial pressure of O_2 (pO_2) before the mixture of gases was introduced into the leaf chamber was measured in an absolute mode with an oxygen analyzer (S-3A/DOX; AEI Technologies, Pittsburgh, PA, U.S.A.). The partial water pressure of the gas stream after passage through the leaf chamber was measured in the differential mode with an IRGA (LI-6262; Li-COR) that had been calibrated in the differential mode with standard mixtures of water vapor, produced by a dew-point generator (LI-610; Li-COR), prior to experiments. Actinic illumination was provided by a halogen lamp (KL-1500; Walz, Effeltrich, Germany). It entered the leaf chamber at an angle of 90° through glass-fiber optics that were linked to the pulse-amplitude-modulated (PAM) chlorophyll fluorometer that is described below. The PFD was measured with a quantum sensor (LI-189; Li-COR). All measurements of photosynthetic parameters were made when steady rates of CO_2 and H_2O exchange had been established under the above-described conditions. The rate of CO_2 exchange in darkness (dark respiration) was measured at a pCO_2 of 36 Pa and 21 kPa O_2.

Measurments of the CO_2-assimilation rate as a function of the intercellular pressure of CO_2 (Ci) were made with single leaves by decreasing pCO_2 successively from about 36.0 to 5.0 Pa. The pO_2 in the mixture of gases was varied between 21.0 and 1.7 kPa. Measurements were performed six times on the each leaf. Gas-exchange parameters were calculated as described by von Caemmerer and Farquhar (1981).

Fluorescence measurements—The Chl fluorescence originating from PSII in attached leaves was measured in a temperature-controlled chamber (30°C) with a cellophane window that allowed simultaneous measurements of gas exchange and Chl fluorescence. Fluorescence was measured with a PAM Chl fluorometer (PAM-101; Walz). The leaf was illuminated with a low-intensity, modulated measuring beam at a PFD of less than 0.5 μmol m^{-2} s^{-1} and also with actinic light through the fiber-optic probe that was positioned at an angle of 90° to the window of the chamber (diameter, 1 cm). The Chl fluorescence emitted from the leaf upon excitation with the measuring beam was detected through the same fiber-optic probe. The steady-state fluorescence yield (F_s) was monitored continuously and 900-ms pulses of saturating light (PFD, 5,000 μmol m^{-2} s^{-1}) were supplied at intervals of about 300 s for determination of maximum variable fluorescence (F'_m). The protocol for measurements of fluorescence was similar to that described by Genty et al. (1989) but measurements were made on attached leaves. The terminology used in this study of fluorescence corresponds to that recommended by van Kooten and Snel (1990). The relative quantum yield of PSII [Φ(PSII)] at the steady state is defined as $(F'_m-F_s)/F'_m$ or $\Delta F/F'_m$, as proposed by Genty et al. (1989).

Estimation of the rate of electron transport through PSII in leaves—The rate of electron transport through PSII [Je(PSII)] was measured as described by Harley et al. (1992). According to Genty et al. (1989), Je(PSII) is equal to $\alpha \times \Phi(PSII) \times PFD$ or $\alpha \times \Delta F/F'_m \times PFD$, where α is a constant that depends on the molar ratio of PSII to PSI in the thylakoid membranes and the efficiency of absorption of light by the leaf. The value of α was calculated from the rate of the PCR cycle and the fluorescence yield under non-photorespiratory conditions (40 Pa CO_2/1.9 kPa O_2). The net CO_2 assimilation rate (A) can be expressed as $A = Vc - 0.5 \times Vo - Rd$, where Rd (rate of day respiration) is the rate of evolution of CO_2 due to processes other than the PCO cycle, and Vc and Vo are the rates of carboxylation and oxygenation of RuBP, respectively, by RuBisCO (von Caemmerer and Farquhar 1981). Rd was estimated from curves of A versus Ci obtained at various PFDs, as described by Brooks and Farquhar (1985). Under non-photorespiratory conditions, Vc is equivalent to A+Rd and Je(PSII) is equal to $4 \times Vc$ or $4 \times (A+Rd)$. Then, α is equal to $4 \times (A+Rd)/[PFD \times (\Delta F/F'_m)]$. A value of α of 0.31± 0.02 (n=3) was calculated for the watermelon leaves used in the present study.

Calculations of CO_2/O_2 specificity of RuBisCO, mesophyll conductance (g_m) and the partial pressure of CO_2 at the carboxylation site of RuBisCO (Cc)—Mesophyll conductance to CO_2 (g_m) was calculated from $g_m = A/\{Ci - [\Gamma^* \times (Je(PSII) + 8 \times (A+Rd))/(Je(PSII) - 4 \times (A+Rd))]\}$ as described by Harley et al. (1992). In this calculation, we assumed that Rd was not dependent on Ci and we determined Rd by the method described above. Je(PSII) was the rate of the total linear electron flow that was calculated from fluorescence and gas exchange measurements under non-photorespiratory conditions. Γ^*, the partial pressure of CO_2 at which the rate of carboxylation of RuBP equals the rate of photorespiratory evolution of CO_2, was determined as described by Brooks and Farquhar (1985). Mesophyll conductance in watermelon leaves was calculated to be 0.82±0.11 mol m^{-2} s^{-1} (n=4) from the values of A, Ci, and Je(PSII) determined for each pCO_2. The partial pressure of CO_2 at the carboxylation site, Cc, was calculated from $Cc = Ci - (A/g_m)$ as described by Harley et al.(1992). Over the range of values of pCO_2 used in this study, Cc was almost the same as Ci. Therefore, we used Ci instead of Cc in our calculations. The value of Γ^* was measured as described by Brooks and Farquhar (1985) from CO_2-response curves obtained at low pCO_2; 10.1, 21 and 40 kPa O_2; and three PFDs of light below 350 μmol m^{-2} s^{-1} at a leaf temperature of 30±0.5°C. According to the model proposed by von Caemmerer et al. (1981), CO_2/O_2 relative specificity of RuBisCO, Sr, can be expressed as $Oc/(2 \times \Gamma^*)$, where Oc is the partial pressure of O_2 at the oxygenation site of RuBisCO. In this study, Oc was assumed to be equal to pO_2. The relative specificity in watermelon leaves was determined to be 75±2.8 (n=3) at a leaf temperature of 30± 0.5°C.

Estimation of the rate of alternative electron flow—The net CO_2 assimilation rate can be expressed as $A = Vc - 0.5 \times Vo - Rd$, as described above. The rate of oxygenation by RuBisCO can be expressed as $Vo = (Vc \times pO_2)/(Sr \times Ci)$ (von Caemmerer and Farquhar 1981). Thus, the rate of carboxylation by RuBisCO can be expressed as follows:

$$A = Vc - (Vc \times pO_2)/(2 \times Sr \times Ci) - Rd$$
$$= Vc \times [1 - pO_2/(2 \times Sr \times Ci)] - Rd.$$

Therefore,

$$Vc = (A + Rd)/[1 - pO_2/(2 \times Sr \times Ci)].$$

Under atmospheric conditions, both the PCR and the PCO cycle are limited by the RuBisCO-catalyzed reactions. Moreover, the electron fluxes in the two cycles can be expressed as $4 \times Vc$ and $4 \times Vo$, respectively (Krall and Edwards 1992). Thus, the total flux of electrons forwarded to the two cycles, Je(PCR+PCO), is equal to $4 \times Vc + 4 \times Vo$. An alternative flux, Ja, due to electrons that are not used by the PCR and/or PCO cycles in the total electron flux driven by PSII can be estimated from Je(PSII) − Je(PCR+PCO).

Results

Effects of decreases in pO_2 and Ci on Je(PSII) and the net CO_2 assimilation rate—To analyze the effects of pO_2 and Ci on the electron flux of the alternative electron flow (Ja) in watermelon leaves, we determined the PFD that saturated the net CO_2 assimilation rate. At a PFD of around 1,000 μmol m^{-2} s^{-1}, the net CO_2 assimilation rate nearly reached a plateau value and the maximum rate was about 20 μmol CO_2 m^{-2} s^{-1} at Ci of 23 Pa and 21 kPa O_2 (Fig. 1). In subsequent experiments, we employed a PFD of 1,100 μmol photon m^{-2} s^{-1}.

Next, we measured Je(PSII) and the net CO_2 assimilation rate at various values of Ci and pO_2 at 1,100 μmol m^{-2} s^{-1} PFD. With a decrease in Ci to 5.0 Pa, Je(PSII) at 21 kPa O_2 decreased to 60% of that at a Ci of 22 Pa (Fig. 2A). Je(PSII) also decreased with the lowering of pO_2 from 21 to 1.7 kPa over the range of values of Ci examined. The decrease in Ci at 21 kPa O_2 caused the net CO_2 assimilation rate to decrease to close to zero (Fig. 2B).

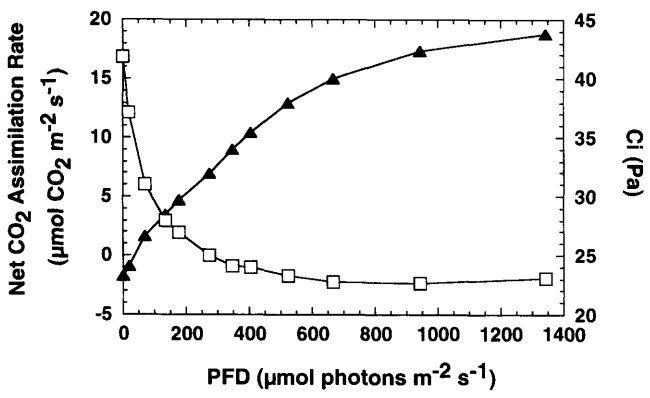

Fig. 1 Effects of PFD on the net CO_2 assimilation rate (▲) and the intercellular partial pressure of CO_2 (Ci, □) in a watermelon leaf. Measurements of the net CO_2 assimilation rate were made at partial pressures of ambient CO_2 and O_2 (pCO_2 and pO_2, respectively) of 36 Pa and 21 kPa, respectively.

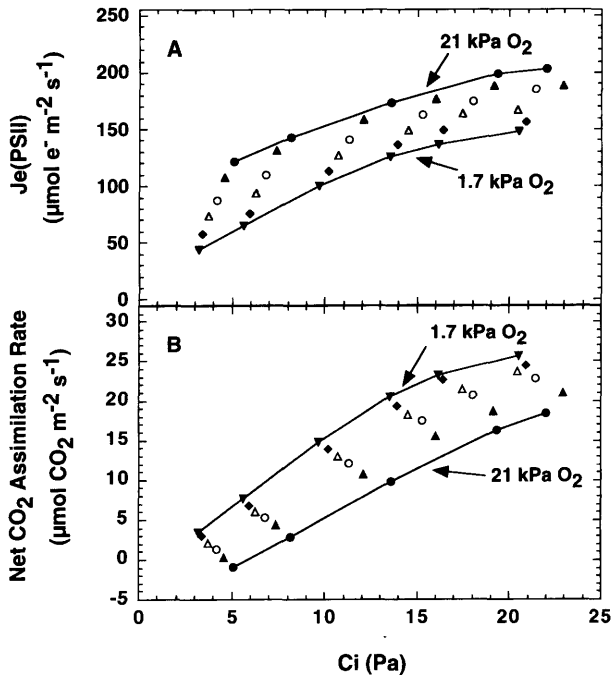

Fig. 2 Influence of pO_2 on the electron flux of PSII [Je(PSII)] (A) and the net CO_2 assimilation rate (B) as a function of Ci. Je(PSII) and the net CO_2 assimilation rate were measured as described in Materials and Methods, with the exception that pO_2 and pCO_2 were varied at a PFD of 1,100 μmol m^{-2} s^{-1}. Je(PSII) and the net CO_2 assimilation rate are plotted against Ci at several values of pO_2 (●, 21 kPa O_2; ▲, 15.1; ○, 10.1; △, 7.1; ◆, 4.1; ▼, 1.7). The lines are drawn through data points obtained at 21 and 1.7 kPa pO_2.

In contrast to Je(PSII), the net CO_2 assimilation rate increased with the decrease in pO_2 from 21 to 1.7 kPa.

Effects of decreases in pO_2 and Ci on the turnover rates of the PCR and PCO cycles and the electron flux of the alternative electron flow—We calculated the rates of carboxylation (*Vc*) and oxygenation (*Vo*) of RuBP from the net CO_2 assimilation rate, obtained as described above, and then we calculated the turnover rates of the PCR and PCO cycles [Je(PCR) and Je(PCO), respectively] and Ja using Je(PSII), as described in Materials and Methods. We plotted Je(PCR), Je(PCO) and Ja against Ci at several values of pO_2 (Fig. 3).

The decrease in Ci from 23 to 5.0 Pa caused Je(PCR) to decrease by 80% over the range of values of pO_2 examined (Fig. 3A). By contrast, as pO_2 was decreased from 21 to 1.7 kPa, Je(PCR) increased (Fig. 3A). However, the decrease in pO_2 to 1.7 kPa caused Je(PCO) to decrease to 7% of that at 21.0 kPa O_2 over the range of values of Ci examined (Fig. 3B). By contrast, as we decreased Ci at 21.0 kPa O_2, Je(PCO) increased gradually and reached a peak at 10 Pa Ci (Fig. 3B). No increase in Je(PCO) at values of Ci below 10 Pa could be detected.

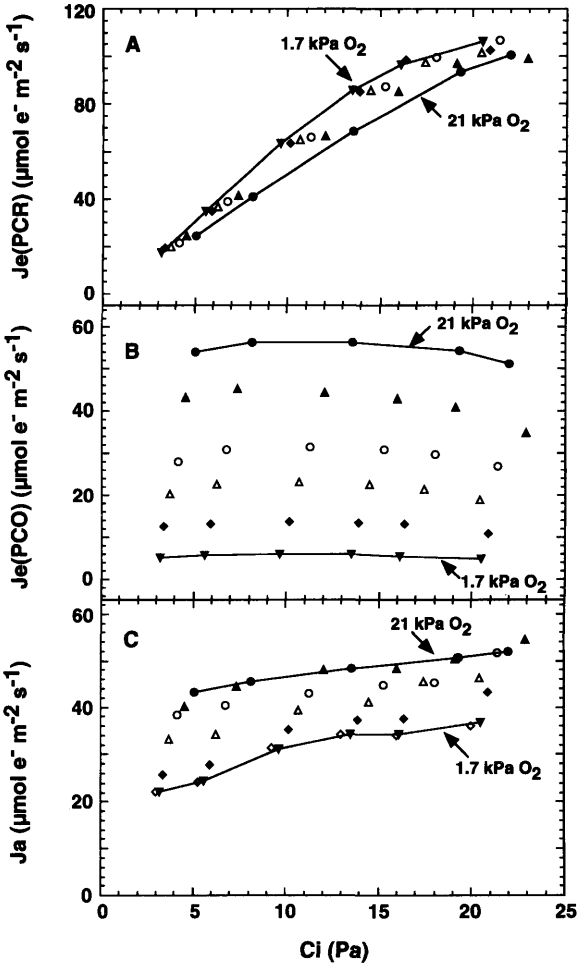

Fig. 3 Effects of pO_2 on Je(PCR) (A), Je(PCO) (B) and Ja (C) as a function of Ci. Je(PCR), Je(PCO) and Ja were calculated from the data in Figs. 2A and 2B, as described in Materials and Methods. Je(PCR), Je(PCO) and Ja are plotted against Ci at several values of pO_2 (●, 21 kPa O_2; ▲, 15.1; ○, 10.1; △, 7.1; ◆, 4.1; ▼, 1.7). The lines are drawn through the data points obtained at 21 and 1.7 kPa O_2. The data for Ja at 0 kPa O_2 (◇) are plotted in (C).

Ja fell by 20% at 21 kPa O_2 as Ci was decreased from 23 to 5.0 Pa Ci. In the range of values of Ci examined, the decrease in pO_2 from 21.0 to 1.7 kPa caused Ja to decline. The extent of the decline in Ja increased as Ci decreased. At a Ci close to 5.0 Pa, Ja decreased by 50% with a decrease in pO_2 to 1.7 kPa. In addition to the Ja that was influenced by pO_2, there was another Ja that appeared to be independent of pO_2 (Fig. 3C). Ja at 0 kPa O_2 was the same as Ja at 1.7 kPa O_2 over the range of values of Ci examined (Fig. 3C). This O_2-independent Ja, which has not yet been defined, accounted for about 70% of the total Ja at a Ci of 20 Pa and decreased to about 50% of the total Ja at a Ci of 5.0 Pa.

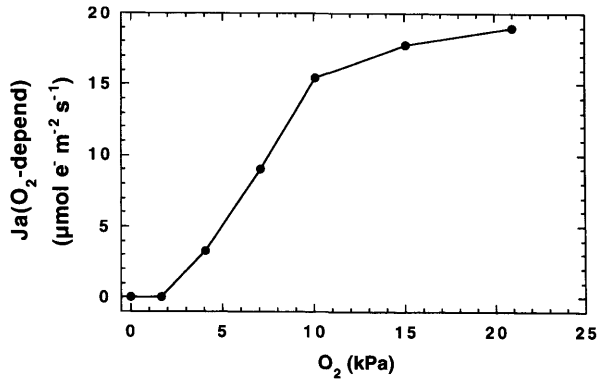

Fig. 4 Effects of pO_2 on Ja(O_2-depend) at a Ci of 5.0 Pa and a PFD of 1,100 μmol m^{-2} s^{-1}. The numerical values of Ja(O_2-depend) were defined as the values of Ja that depended on pO_2 and corresponded to the difference between Ja of 1.7 and at 21 kPa pO_2 in Fig. 3C. Ja(O_2-depend) at a Ci of 5.0 Pa and several values of pO_2 was calculated and is plotted against pO_2.

Dependencies of the O_2-dependent Ja on pO_2 and Ci—Ja can be divided into O_2-dependent and independent fluxes [Ja(O_2-depend) and Ja(O_2-independ), respectively]. We studied the dependencies of Ja(O_2-depend) on both pO_2 and Ci. Ja(O_2-depend) at 5.0 Pa Ci can be calculated from the difference between Ja at 21.0 and Ja at 1.7 kPa O_2. Ja(O_2-depend) at 5.0 Pa Ci declined with decreases in pO_2 and the apparent K_m of Ja(O_2-depend) for pO_2 was about 8.0 kPa (Fig. 4).

We calculated Ja(O_2-depend) at 21.0 kPa O_2 at several values of Ci from the data in Figure 3C and plotted the values against Ci (Fig. 5). The decrease in Ci from 20 to 5.0 Pa caused a 1.3-fold increase in Ja(O_2-depend).

Relationships among Je(PCR), Je(PCO) and Ja(O_2-depend) at several values of Ci—As described above, Je(PSII) consists of Je(PCR), Je(PCO), Ja(O_2-depend) and

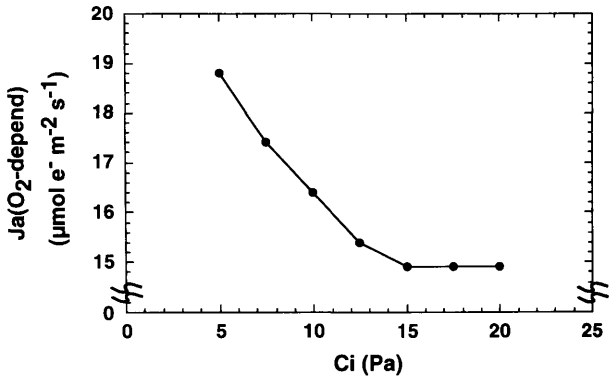

Fig. 5 Effects of Ci on Ja(O_2-depend) at 21 kPa O_2 at a PFD of 1,100 μmol m^{-2} s^{-1}. Ja(O_2-depend) at 21 kPa pO_2 and several values of Ci was determined from the data in Fig. 3C and is plotted against Ci.

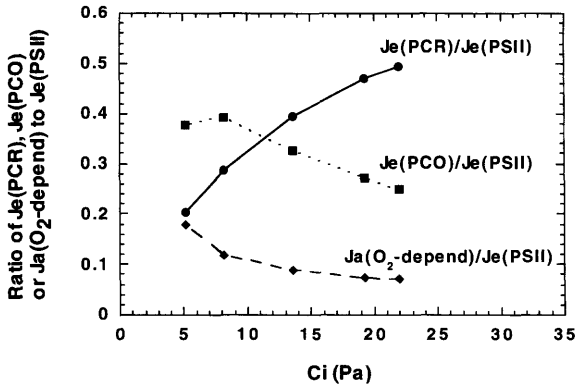

Fig. 6 Effects of Ci on Je(PCR)/Je(PSII), Je(PCO)/Je(PSII) and Ja(O_2-depend)/Je(PSII) at 21 kPa O_2 and a PFD of 1,100 μmol m^{-2} s^{-1}. Each ratio of Je(PCR) (●), Je(PCO) (■) and Ja(O_2-depend) (◆) to Je(PSII) at 21 kPa pO_2 and several values of Ci was calculated from the data in Fig. 2A, 3A, 3B and 3C, as described in Materials and Methods. Ratios are plotted against Ci.

Ja(O_2-indepen d). To analyze the way in which Ja(O_2-depend) was affected by Je(PCR) and Je(PCO), we calculated the ratio of each electron flux to Je(PSII) at 21.0 kPa O_2 and at several values of Ci using the data in Figures 2A, 3A, 3B and 3C. Each set of ratios was then plotted against Ci (Fig. 6). The suppression of the PCO cycle by increases in Ci caused Ja(O_2-depend) to decrease and the turnover rate of the PCR cycle to increase. At a Ci of 20 Pa, Je(PCR) was the predominant component of the electron flux in PSII and accounted for about 50% of Je(PSII). The ratio of Je(PCR) to Je(PSII) fell by 60% as Ci was decreased to 5.0 Pa (Fig. 6). By contrast, decreases in Ci caused the O_2-dependent electron flux of both Ja(O_2-depend) and Je(PCO) to increase. The results for Ja(O_2-depend)/Je(PSII) led us to expect further increases at values of Ci below 5.0 Pa, where the increase in Je(PCO)/Je(PSII) reached a plateau and Je(PCO) was steady at about 40% of Je(PSII) (Fig. 6). In the range of values of Ci examined, Ja(O_2-depend)/Je(PSII) was consistently lower than Je(PCO)/Je(PSII), as shown in Figure 6.

Discussion

Identification in vivo of Ja(O_2-depend) as the electron flux of the water-water cycle—The water-water cycle has two sites for electron flow: one for the photoreduction of O_2 at PSI and the other for the regeneration of AsA from oxidized AsA. The photoreduction of MDA follows the photoreduction of O_2 at PSI and the steady-state flux in the former reaction must be the same as that in the latter. The apparent half-time of the various reactions mediated by SOD, APX, Fd and MDAR in the water-water cycle ranges from nanoseconds to microseconds. It is three to four order of magnitude lower than the half time of the photoreduction of O_2 mediated by Fd or MDAR (Asada 1996, Miyake et al. 1998). This difference suggests that the electron flux of the water-water cycle should be limited by the rate of the photoreduction of O_2 at PSI. However, it must be emphasized that all these results were obtained when reactions were analyzed in vitro. The magnitude of the electron flux in the water-water cycle in vivo, and the physiological nature of the cycle have not previously been evaluated, and these were the issues that we examined in detail in the present study. By relating the dependence of Ja on pO_2, as well as the magnitude of Ja with the biochemical characteristics of the O_2^--producing mediators, we propose that Ja(O_2-depend) represents the electron flux of the water-water cycle, as follows.

The affinity of Ja(O_2-depend) for O_2 closely reflected values of the K_m for O_2 in the photoreduction of O_2 by MDAR and Fd in thylakoids. The MDAR-catalyzed photoreduction of O_2 has a K_m (O_2) of 80 μM (Miyake et al. 1998), which corresponds to about 7 kPa O_2. The Fd-mediated reaction has a K_m (O_2) greater than 60 μM (about 5 kPa O_2) at a concentration of 1 mM of Fd in the stroma (Furbank and Badger 1983, Robinson 1988). By contrast, the rate of production of O_2^- by washed thylakoids reaches a plateau at only 2.0 kPa O_2 (Heber and French 1968, Takahashi and Asada 1982, Miyake et al. 1998). Thus, the MDAR- and/or Fd-mediated transfer of electrons to O_2 accounts for Ja(O_2-depend). From the stoichiometry of the water-water cycle, the rate of the photoreduction of O_2 at PSI corresponds to half of Ja(O_2-depend). Under light-saturated conditions, MDAR and Fd can produce O_2^- at a maximum rate of about 300 μmol O_2^- (mg Chl)$^{-1}$ h^{-1} (Furbank and Badger 1983, Miyake et al. 1998). The corresponding rate of production of O_2^- by washed thylakoids is 10–30 μmol O_2^- (mg Chl)$^{-1}$ h^{-1} (Asada et al. 1974, Miyake et al. 1998). In watermelon leaves, the maximum value of Ja(O_2-depend) was calculated to be about 120 μmol e^- (mg Chl)$^{-1}$ h^{-1}, for a Chl content of 60 μg cm^{-2} leaf area. In view of the stoichiometry of the water-water cycle, the maximum value of Ja(O_2-depend) corresponds to a rate of production of O_2^- at PSI of about 60 μmol O_2^- (mg Chl)$^{-1}$ h^{-1}. Thus, the rate of production of O_2^- mediated by MDAR and/or Fd accounts for the magnitude of Ja(O_2-depend) in watermelon leaves. These considerations strongly suggest that Ja(O_2-depend) represents the electron flux in the water-water cycle. Although FNR photoreduces O_2 at a high rate, similar to that of the photoreduction of O_2 by MDAR (Miyake et al. 1998), the possibility that FNR catalyzes this reaction can be excluded for the following reason. In intact chloroplasts, all of the FNR binds to the thylakoids. It is only when chloroplasts are exposed to oxidative stress that FNR is released from thylakoids (Palatnik et al. 1997). Intact thylakoids require free FNR for effective photoreduction of O_2 (Miyake et al. 1998), an observation that indicates that thylakoid-bound FNR can

not accept electrons directly from PSI.

In the leaves of wild-type watermelon that we studied, Ja(O_2-depend) ranged from 15 to 20 μmol e^- m^{-2} s^{-1}. This value was similar to the Ja(O_2-depend) of leaves of another watermelon cultivar and of tobacco (data not shown). Rates of photoreduction of O_2, 50% of Ja(O_2-depend), were consistent with those of the uptake of $^{18}O_2$ that is independent of the PCO cycle (Biehler and Fock 1996). The rates were below 40 μmol e^- m^{-2} s^{-1}. The rate of photoreduction of O_2 at PSI, as determined from the analysis of $^{18}O_2$ uptake can be accounted for by reactions mediated by Fd or MDAR. These observations confirm the validity of our method for measuring the rate in vivo of the photoreduction of O_2 in the water-water cycle.

Potato leaves do not show any evidence of the photoreduction of O_2, even at low Ci (Tourneux and Peltier 1995). By contrast, exposure to water-deficit conditions stimulates the uptake of $^{18}O_2$ in wheat leaves (Biehler and Fock 1996) and increases the relative electron flux at PSII to that required for both the PCR and PCO cycles (Cheeseman et al. 1997). Thus, Ja(O_2-depend) can respond to environmental stress for dissipation of excess photoenergy. The biochemical mechanism for the regulation of Ja(O_2-depend) is of interest with respect to our limited understanding of the physiological significance of the water-water cycle and it remains to be clarified.

The result that the photoreduction of O_2 at PSI did not occur at 1.7 kPa O_2 (Fig. 4) contradicts previous results, as described in the Introduction (Wu et al. 1991, Brestic et al. 1995, Lovelock and Winter 1996, Kingston-Smith et al. 1997). The cited authors determined the electron flux of the photoreduction of O_2 at PSI at 2.0 kPa O_2, to suppress the PCO cycle. Thus, they were unable to detect the photoreduction of O_2 to O_2^- mediated by Fd or MDAR, which both have a lower affinity for O_2. The estimation of the photoreduction of O_2 at PSI in vivo at 2.0 kPa O_2 leads to an underestimation or,rather, provides an estimate of Ja(O_2-independ), as we have shown in the present study. Thus, for determination of the rate of photoreduction of O_2 at PSI, it is necessary to evaluate the dependence of Ja on pO_2.

Ja(O_2-independ) decreased from 36 to 22 μmol e^- m^{-2} s^{-1} as Ci was decreased from 20.5 to 3 Pa, while the net CO_2 assimilation rate decreased from 25 to 4 μmol m^{-2} s^{-1} (Fig. 2B, 3C). A candidate for the other alternative, O_2-independent electron flow is the assimilation of nitrate by the electrons produced at the photosystems of thylakoid membranes. However, the rate of reduction of one molecule of nitrate corresponds to about 10% of the rate of fixation of one molecule of CO_2 during photosynthesis in soybean mesophyll cells(Robinson 1986). The observed high ratio of Ja(O_2-independ) to the net CO_2 assimilation rate at lower values of Ci did not reflect this ratio of assimilation rates of carbon and nitrogen. The biochemical events and the nature of Ja(O_2-independ) will be the focus of future studies.

Comparison of Ja(O_2-depend)/Je(PSII) with Je(PCR)/Je(PSII) and Je(PCO)/Je(PSII)—In the present study, we established a method that allowed us distinguish between the electron flux in the water-water cycle and the fluxes in the PCR and PCO cycles in leaf tissue. From our results, we were able to compare the effects of Ci on the relative extents of these electron fluxes in the overall electron flux at PSII (Fig. 6). Our efforts revealed the way in which the rate of photoreduction of O_2 at PSI is affected by the turnover of the PCR and the PCO cycles.

A decrease in Ci suppressed the electron flux required for the PCR cycle but enhanced that for the PCO cycle. The decreased turnover rate of the PCR cycle can be explained by the kinetics of reactions catalyzed by RuBisCO. At atmospheric pO_2 and lower values of Ci, the carboxylase reaction of RuBisCO limits the turnover of the PCR cycle (Farquhar et al. 1980, Krall and Edwards 1992). By contrast, the suppression of the carboxylase reaction of RuBisCO by a decrease in Ci stimulates the turnover of the PCO cycle since the oxygenation of RuBP by RuBisCO competes with its carboxylation (Sharkey 1988). Our results show that, at a Ci of around 10 Pa, the PCO cycle-dependent flux of electrons reaches a maximum value and then decreases as Ci falls further. This decline in the turnover in the PCO cycle might be due to the inactivation of RuBisCO. The activation ratio of RuBisCO was reported to fall to 50% of its original value upon a decrease in Ci from 10 to 5.0 Pa in leaves of *Raphanus sativus* (von Caemmerer and Edmondson 1986) but this issue requires further analysis (Perchorowitz et al. 1982, Anwaruzzaman et al. 1995).

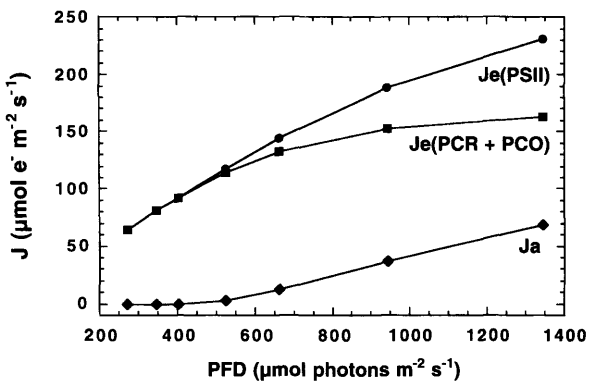

Fig. 7 Saturation of Je(PCR+PCO) enhances Ja with increasing PFD. Je(PSII), Je(PCR + PCO) and Ja were calculated from the data in Fig. 1, as described in Materials and Methods, with the exception that all rates of day respiration at each data point were assumed to be the same as that determined at a PFD of 1,100 μmol m^{-2} s^{-1} over the range of PFDs that we used. The values of Je(PSII) (●), Je(PCR+PCO) (■)and Ja (◆) are plotted against PFD.

Decreases in Ci stimulated the photoreduction of O_2 in the water-water cycle. Increases in Ja(O_2-depend) were accompanied by suppression of the PCR cycle and stimulation of the PCO cycle (Fig. 6). For example, under the conditions where a decrease in Ci lowered Je(PCR) and caused Je(PCO) to increase, Ja(O_2-depend) was enhanced (Fig. 3A, B, 5, 6). Furthermore, as the electron flux from PSII exceeded the fluxes required for both the PCR and PCO cycles, Ja was induced (Fig. 7). In other words, Ja was detectable only above photon flux densities at which Je(PCR+PCO) was saturated and the turnover of the PCR and the PCO cycles limited the utilization of photoenergy. These results indicate that, at high PFD, when there are too many electrons for both the PCR and PCO cycles, and at low Ci, when the activity of RuBisCO is suppressed and the use of NADPH by the PCR and PCO cycles is limited, the pressure of electrons at PSI increases and the probability of donation of electrons to O_2 by Fd and MDAR increases, as reflected by the induction of Ja. This phenomenon leads to the production of O_2^- or the dissipation of the energy of excess photons through the water-water cycle in chloroplasts (Asada 1999).

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