Mitochondrial alternative oxidase pathway protects plants against photoinhibition by alleviating inhibition of the repair of photodamaged PSII through preventing formation of reactive oxygen species in Rumex K-1 leaves

Li-Tao Zhang, Zi-Shan Zhang, Hui-Yuan Gao*, Zhong-Cai Xue, Cheng Yang, Xiang-Long Meng and Qing-Wei Meng

State Key Laboratory of Crop Biology, Shandong Key Laboratory of Crop Biology, Shandong Agricultural University, Tai’an 271018, Shandong, China

The purpose of this study was to explore how the mitochondrial AOX (alternative oxidase) pathway alleviates photoinhibition in Rumex K-1 leaves. Inhibition of the AOX pathway decreased the initial activity of NADP-malate dehydrogenase (EC 1.1.1.82, NADP-MDH) and the pool size of photosynthetic end electron acceptors, resulting in an over-reduction of the photosystem I (PSI) acceptor side. The over-reduction of the PSI acceptor side further inhibited electron transport from the photosystem II (PSII) reaction centers to the PSI acceptor side as indicated by an increase in VJ (the relative variable fluorescence at J-step), causing an imbalance between photosynthetic light absorption and energy utilization per active reaction center (RC) under high light, which led to the over-excitation of the PSII reaction centers. The over-reduction of the PSI acceptor side and the over-excitation of the PSII reaction centers enhanced the accumulation of reactive oxygen species (ROS), which inhibited the repair of the photodamaged PSII. However, the inhibition of the AOX pathway did not change the level of photoinhibition under high light in the presence of the chloroplast D1 protein synthesis inhibitor chloramphenicol, indicating that the inhibition of the AOX pathway did not accelerate the photodamage to PSII directly. All these results suggest that the AOX pathway plays an important role in the protection of plants against photoinhibition by minimizing the inhibition of the repair of the photodamaged PSII through preventing the over-production of ROS.

Abbreviations – $\phi_{Eo}$, the quantum yield of electron transport; $\phi_{P0}$, the maximum quantum yield for primary photochemistry; $\Phi_{PSII}$, photosystem II actual photochemical efficiency; $\psi_{0}$, the efficiency for electron transport; ABS/RC, light absorption per active reaction center; AOX, alternative oxidase; APX, ascorbate peroxidase; CAT, catalase; CM, chloramphenicol; COX, cytochrome oxidase; DAB, 3, 3-diaminobenzidine; DTT, dithiothreitol; ET0/RC, the electron transport per active reaction center; $F_F$, the fluorescence at $J$-step; $F_0$, $F_m$, initial and maximum fluorescence; $F''_0$, $F''_m$, the minimal and maximum fluorescence in the light-adapted state; $F_s$, the steady-state fluorescence; $F_t$, the fluorescence at any time $t$; HSD, honestly significant difference; LED, light-emitting diode; LSD, least significant difference; NADP/NADPH, oxidized/reduced form of nicotinamide-adenine dinucleotide phosphate; NADP-MDH, NADP-malate dehydrogenase; NBT, nitrotetrazolium blue chloride; nPG, n-propyl gallate; OAA, oxaloacetate; PFD, photon flux density; PSI, photosystem I; PSII, photosystem II; QA, primary quinone electron acceptor of PS II; $q_p$, photochemical quenching; RC, active reaction center; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid; SOD, superoxide dismutase; TR0/RC, trapping of excitation energy per active reaction center; $V_J$, the relative variable fluorescence at the J-step.
Introduction

Excess light energy damages the photosynthetic machinery during photosynthesis, causing photoinhibition (Murata et al. 2007, Takahashi and Murata 2008). It has been demonstrated that photodamage is attributable to light absorbed directly by manganese in the oxygen-evolving complex (Nishiyama et al. 2006, Tyystjärvi 2008). Subsequent to photodamage to the oxygen-evolving complex, the reaction center of photosystem II (PSII) is damaged by light absorbed by the photosynthetic pigments (Hakala et al. 2005, Ohnishi et al. 2005). The damaged PSII is rapidly and effectively repaired by the replacement of the damaged PSII proteins with newly synthesized proteins, primarily the D1 protein (Aro et al. 2005, Mattoo and Edelman 1987, Nishiyama et al. 2011). Thus, photoinhibition occurs only under conditions in which the rate of photodamage exceeds the rate of its repair (Murata et al. 2007, Nishiyama et al. 2011, Takahashi and Murata 2008). To prevent photoinhibition, plants have evolved mechanisms to suppress photodamage to PSII and/or to minimize the inhibition of the repair of the photodamaged PSII. It has been demonstrated that the photosynthetic pathway and antioxidant systems protect plants by minimizing the inhibition of the repair of the photodamaged PSII (Nishiyama et al. 2001, Takahashi et al. 2007), and chloroplast avoidance movement can help to prevent photoinhibition by directly suppressing the photodamage to PSII (Kasahara et al. 2002). Furthermore, Takahashi et al. (2009) suggested that photosynthetic cyclic electron flow can protect plants against photoinhibition by both preventing photodamage to PSII and minimizing the inhibition of the repair of the photodamaged PSII.

Recent publications have also suggested that the mitochondrial alternative oxidase (AOX) pathway plays an important role in protecting photosynthetic machinery from photoinhibition (Florez-Sarasa et al. 2011, Noguchi and Yoshida 2008, Raghavendra and Padmasree 2003). The AOX pathway is an alternative electron transport pathway in addition to the cytochrome oxidase (COX) pathway in the mitochondria. This pathway diverts electrons from the ubiquinone pool and reduces oxygen to water without any proton translocation or ATP synthesis (Rasmusson et al. 2009, Van Aken et al. 2009). Apparently, the AOX pathway is non-phosphorylating and can oxidize reducing equivalents efficiently without being restricted by the proton gradient across the mitochondrial inner membrane or the cellular ATP/ADP ratio (Yoshida et al. 2007). Several recent studies have demonstrated that the inhibition of the AOX pathway leads to decreases in photosynthetic rate in leaves (Yoshida et al. 2006) and protoplasts (Dinakar et al. 2010a, 2010b, Padmasree and Raghavendra 1999a, 1999c). It has been shown that the AOX pathway is induced to protect the photosynthetic apparatus from photoinhibition in Arabidopsis, comprising the cyclic electron flow around photosystem I (PSI) (Yoshida et al. 2007). However, it remains to be elucidated whether the photoprotection of the mitochondrial AOX pathway is correlated with the suppression of photodamage to PSII and/or minimizing the inhibition of the repair of the photodamaged PSII.

The accumulation of ROS induced by excess light energy has been suggested to play an important role during photoinhibition (Murata et al. 2007, Takahashi and Murata 2008). A recent study, using plant material lacking AOX1a, demonstrated that the AOX pathway plays a central role in reducing the accumulation of ROS generated by photosynthesis in plants (Giraud et al. 2008). However, the generation site of ROS in chloroplasts and the role of ROS accumulation during photoinhibition conditions because of the inhibition of AOX pathway have not yet been clarified.

The aim of this study was to explore the role of ROS accumulation resulting from the inhibition of the AOX pathway during photoinhibition and to clarify whether the photoprotection of the mitochondrial AOX pathway is correlated with the suppression photodamage to PSII and/or with minimizing the inhibition of the repair of the photodamaged PSII. In addition, using a chlorophyll fluorescence transient combined with the JIP-test (Jia et al. 2010, Kalachanis and Manetas 2010, Mathur et al. 2011, Oukarroum et al. 2009, Strasser et al. 2000, 2004), we studied photosynthetic behaviors (including the energy fluxes of absorption, energy trapping and electron transport) to analyze the generation site of ROS in chloroplasts under high light when the AOX pathway is inhibited.

Materials and methods

Materials and treatments

Rumex K-1 plants (Rumex patientia × Rumex tianschanus) were grown from seeds under a 14-h photoperiod at 22/18°C (day/night) in a pot containing soil. The plants were thinned to one plant per pot 2 weeks after sowing. Nutrients and water were supplied sufficiently throughout the study to avoid any potential nutrient and drought stresses. The photon flux density (PFD) during growth was approximately 800 μmol m⁻² s⁻¹. Fully expanded leaves of 4-week-old plants were used in the experiments.

Intact chloroplasts were isolated according to the protocol of Bartoli et al. (2005). Leaves were ground in buffer containing 50 mM HEPES-KOH (pH 7.5),
330 mM sorbitol, 2 mM Na₂-EDTA, 1 mM MgCl₂, 5 mM ascorbic acid and 0.05% (w/v) BSA using a hand-held homogenizer. The homogenate was filtered through a 20 μm pore size nylon mesh and centrifuged at 3000 g for 5 min. The pellet was suspended in 1 mM KCN and 20 mM SHAM were used to inhibit respiratory measurements were taken. The inhibitors leaf discs were incubated in the dark for 10 min before Yoshida et al. (2006) and Zhang et al. (2010). The capacity of 1 mM salicylhydroxamic acid (SHAM) or n-propyl gallate (nPG). The isolated chloroplasts were 75% intact according to the ferricyanide-dependent O₂ evolution (Walker, 1988), which was measured with a Chlorolab-2 oxygen electrode (Hansatech Instruments, Norfolk, UK).

Leaf discs (0.5 cm²) were punched from fully expanded leaves and infiltrated with 0 (control) or 1 mM SHAM or nPG solutions, respectively, for 2 h in the dark at room temperature. Then, the discs were exposed to high light measuring 800 μmol m⁻² s⁻¹ for 1 h at room temperature.

Capacity of AOX pathway measurement

The respiratory rate in the leaf discs treated with high light was measured using an Oxytherm oxygen electrode (Hansatech Instruments, Norfolk, UK) at 25°C according to Yoshida et al. (2006) and Zhang et al. (2010). The leaf discs were incubated in the dark for 10 min before respiratory measurements were taken. The inhibitors 1 mM KCN and 20 mM SHAM were used to inhibit the COX and AOX pathways, respectively. The AOX pathway capacity was defined as the O₂ uptake rate in the presence of KCN that was sensitive to SHAM.

Enzyme assays

Enzymes were extracted from the leaves according to Dutilleul et al. (2003). Leaf discs (7.5 cm²) were ground in liquid nitrogen and extracted in 50 mM HEPES-KOH (pH 7.5) buffer containing 10 mM MgCl₂, 1 mM Na₂-EDTA, 5 mM dithiothreitol (DTT), a protease inhibitor tablet, 5% (w/v) insoluble polyvinylpyrrolidone and 0.05% (v/v) Triton X-100. After centrifugation for 5 min at 14 000 g, the enzyme activity in the supernatant was measured with an UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). The initial activity of NADP-malate dehydrogenase (EC 1.11.1.6, CAT) was measured according to the method of Mishra et al. (1993).

Measurements of chlorophyll a fluorescence transient

Chlorophyll a fluorescence (OJIP) transients before and after the high light treatment were measured with a Handy PEA fluorometer (Hansatech Instruments, Norfolk, UK). The transients were induced by red light measuring approximately 3000 μmol m⁻² s⁻¹ provided by an array of three light-emitting diodes (LEDs, peak 650 nm). All measurements were performed with 15-min dark-adapted leaf discs at room temperature.

OJIP transients were analyzed by the IIP-test (Appenroth et al. 2001, Oukarroum et al. 2009, Strasser and Strasser 1995, Strasser et al. 2000, Yusuf et al. 2010), using the following original data: (1) the fluorescence intensity at 20 μs, considered to be F₀, when all PSII reaction centers were open; (2) the maximal fluorescence intensity, Fₘ (F₀, at about 300 ms), assuming that the excitation intensity was high enough to close all of the PSII RCs; and (3) the fluorescence intensities at 2 ms (I-step, F₁) and 30 ms (I-step, F₁). The following parameters were used for the quantification of PSII behavior referring to time 0:

1. The chlorophyll a fluorescence transient from I to P after double normalization between the F₀ and F₁ points, W₁₀ = (F₁ − F₀)/(F₁ − F₀), where F₁ is the fluorescence intensity at any time.
2. The relative variable fluorescence at J-step (VJ), VJ = (F₁ − F₀)/(F₁ − F₀).
3. The specific energy fluxes (per RC) for absorption (ABS/RC), trapping (TR₀/RC) and electron transport (ET₀/RC).
4. The flux ratios or yields, i.e. the maximal quantum yield of primary photochemistry (ϕₚ = TR₀/ABS = F₀/Fₘ), the efficiency with which a trapped exciton can move an electron into the electron transport chain further than QÅ (the excitation efficiency for electron transport beyond QÅ), Ψ₀ = ET₀/TR₀ and the quantum yield of electron transport (ϕET₀ = ET₀/ABS).

Measurements of modulated chlorophyll fluorescence parameters

The modulated chlorophyll fluorescence parameters Ψ₂₆ (PSII actual photochemical efficiency) and qP
(photochemical quenching coefficient) of intact Rumex K-1 chloroplasts were measured with a FMS-2 pulse-modulated fluorometer (Hansatech Instruments, Norfolk, UK) integrated with a Chlorolab-2 oxygen electrode (Hansatech Instruments, Norfolk, UK). The actinic light at 800 μmol m⁻² s⁻¹ was offered by the light source. Following the onset of actinic light irradiation, the $F_S$, $F'_m$ and $F_o$ were recorded when the chlorophyll fluorescence attained steady-state levels; $\Phi_{PSII} = 1 - F_o/F'_m$ and $qP = (F'_m - F_S)/(F'_m - F_o)$ (Chen et al. 2007).

Measurement of P700 redox state

The redox state of P700 was measured according to Schansker et al. (2003) and Kim et al. (2001) using a PEA Senior fluorometer (Hansatech Instruments, Norfolk, UK). Red light, measuring 800 μmol m⁻² s⁻¹, was produced by an array of four 650-nm LEDs (peak, 650 nm), and the modulated (33.3 kHz) far-red measuring light has a wavelength of 820 nm was provided by an OD820 LED (Opto Diode Corp., Newbury Park, USA). Upon irradiation with a red pulse (800 μmol m⁻² s⁻¹), the transmission at 820 nm in leaves increases gradually, which is mainly caused by the reduction of P700⁺.

Histochemical detection of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂)

Leaf discs (1.3 cm²) treated with 0 (control) or 1 mM SHAM or nPG were vacuum-infiltrated with 0.1 mg ml⁻¹ nitrotetrazolium blue chloride (NBT) or 1 mg ml⁻¹ 3,3-diaminobenzidine (DAB, pH 3.8) and incubated under high light (800 μmol m⁻² s⁻¹) conditions for 1 h. After these treatments, leaf discs were decolorized by immersion in boiling ethanol (96%) for 10 min. After cooling, the leaf discs were extracted at room temperature with fresh ethanol and photographed (Jabs et al. 1996, Thordal-Christensen et al. 1997, Zeng et al. 2010).

Statistical analysis

Least significant difference (LSD) was used to analyze differences between the measurements and Tukey’s honestly significant difference (HSD) was used for the multiple comparison using SPSS 16.

Results

SHAM has been widely used to inhibit the AOX pathway (Bartoli et al. 2005, Padmasree and Raghavendra 1999a, 1999b, 1999c). However, to avoid its side effects and ensure the sufficient inhibition of the AOX pathway, we compared the effects of SHAM with another AOX inhibitor, nPG (Yoshida et al. 2006), in this study. The results showed that the two different inhibitors have similar effects on the inhibition of the AOX pathway (data not shown). In addition, there were no differences in photosynthetic behaviors between leaves treated with SHAM (Figs 3, 5–7) and those treated with nPG (Figs S2–S5). Therefore, we only discussed the effects of the SHAM treatment in the text, and those caused by the nPG treatment are presented in the Supporting Information.

Given that some components of the photosynthetic electron transport chain in chloroplasts are similar to those in the respiratory chain of mitochondria, we examined whether the concentrations of SHAM and nPG used in this study had direct effects on the photosynthetic electron transport chain. The results showed that the treatments with 1 mM SHAM or 1 mM nPG had no direct effects on the actual PSI photochemical efficiencies ($\Phi_{PSII}$) and photochemical quenching coefficients ($qP$) in intact chloroplasts isolated from Rumex K-1 leaves (Figs 1 and S1), suggesting that the concentrations of SHAM and nPG used in this study had no direct effects on photosynthetic behaviors. Therefore, all of the effects caused by the SHAM and nPG treatments were actually because of the inhibition of the mitochondrial AOX pathway.

To determine a working concentration of the inhibitor for this study, we examined the effects of the inhibitor on the capacities of the AOX pathways in Rumex K-1 leaves in the dark or in the high light. After the high light treatment, the AOX pathway capacities in the control leaves
NADP-MDH is the key enzyme of the malate-OAA shuttle that is the major machinery for the transport of excess reducing equivalents from chloroplasts to mitochondria for oxidation by the respiratory electron transport chain (Noguchi and Yoshida 2008, Raghavendra and Padmasree 2003, Scheibe 2004). The initial activity of NADP-MDH increased by approximately 181% after high light treatment in the control leaves. The SHAM treatment did not alter the initial activity of NADP-MDH in the dark, indicating that the 1 mM SHAM treatment had no direct effect on NADP-MDH. However, the 1 mM SHAM treatment decreased the initial activity of NADP-MDH by approximately 31% under high light in Rumex K-1 leaves (Fig. 2B), suggesting that SHAM-treated leaves exported the reducing equivalents from chloroplasts at lower rates.

The chlorophyll a transient has become one of the most popular tools in photosynthetic research (Jia et al. 2010, Kalachanis and Manetas 2010, Mathur et al. 2011, Oukarroum et al. 2009). The shape of the OJIP transient is very sensitive to environmental stresses. Strasser and Strasser (1995) developed a procedure to quantify the OJIP transient, known as the JIP-test. With this test, it is possible to calculate several phenomenological and biophysical expressions of the photosynthetic electron transport chain (Strasser and Strasser 1995, Strasser et al. 2000, 2004). The JIP-test is thus a powerful tool for the in vivo investigation of photosynthetic behaviors, including the energy fluxes of absorption, trapping and electron transport (Strasser et al. 2000, 2004). In the present study, we used the chlorophyll a fluorescence transient (OJIP) to detect changes in photosynthetic behaviors when the AOX pathway was inhibited. In the dark, the SHAM treatment did not alter the shape of the OJIP transient (Fig. 3A). However, it decreased the fluorescence intensities significantly in both the control and SHAM-treated leaves.

increased significantly (Fig. 2A). The 1 mM SHAM treatment inhibited 59% and 55% of the AOX pathway capacities in the leaves in the dark and high light, respectively.
leaves under high light, and the extent of decrease was much greater in the SHAM-treated leaves than in the control leaves (Fig. 3B). These results indicate that the inhibition of the AOX pathway significantly influenced photosynthetic behaviors.

The extent of the reduction of P700 was more pronounced in the SHAM-treated leaves than the control leaves (Fig. 4A). According to the JIP-test, the maximal amplitude of the OJIP transient in the I–P phase after normalization between the O and I phases decreased significantly when the AOX pathway was inhibited by 1 mM SHAM under high light (Fig. 4B), suggesting that the pool size of the PSI end electron acceptors decreased (Kalachanis and Manetas 2010, Yusuf et al. 2010). The relative variable fluorescence at J-step ($V_J$) represents the subsequent kinetic bottlenecks of the electron transport chain, resulting in the momentary maximum accumulations of $Q_A^-$ (Strasser and Strasser 1995, Li et al. 2009). In this study, the SHAM treatment increased the $V_J$ significantly compared with the control leaves after high light treatment (Fig. 5). Additionally, compared with the control leaves, the SHAM-treatment increased the light absorption per active reaction center (ABS/RC, the average antenna size) and trapping of excitation energy per active reaction center (TR$_0$/RC), and it decreased the efficiency of electron transport ($\phi_E$), the quantum yield of electron transport ($\phi_E$), and electron transport per active reaction center (ET$_0$/RC) after the high light treatment (Fig. 5), suggesting that the balance between photosynthetic light absorption and energy utilization was disturbed when the AOX pathway was inhibited.

To determine the oxidative stress that the Rumex K-1 leaves suffered when the AOX pathway was inhibited under high light, we detected the in situ accumulation of $O_2^-$ and $H_2O_2$ using the NBT and DAB staining procedures, respectively. They showed that the inhibition of the AOX pathway enhanced the accumulation of ROS ($O_2^-$ and $H_2O_2$) significantly under high light in Rumex K-1 leaves (Fig. 6A). The SHAM
Production of $O_2^-$ and $H_2O_2$ (A) and activities of SOD, APX and CAT (B) in Rumex K-1 leaves treated with 0 (control) or 1 mM SHAM under high light. Leaf discs (1.3 cm$^2$) treated with 0 (control) or 1 mM SHAM were vacuum-infiltrated with 0.1 mg ml$^{-1}$ NBT or 1 mg ml$^{-1}$ DAB (pH 3.8) and incubated under high light (800 μmol m$^{-2}$ s$^{-1}$) conditions for 1 h. Representative images from five independent experiments are shown in the figure. Means ± SE of three replicates are presented. Different letters indicate significant difference between the SHAM treatment and control at $P < 0.05$, LSD.

Furthermore, compared with control leaves, the SHAM treatment significantly accelerated the decrease in the maximum quantum yield for primary photochemistry ($\phi_{PSI}$), the excitation efficiency for electron transport beyond QA ($\Psi_0$) and the quantum yield for electron transport ($\phi_{E0}$) under high light in the absence of the chloroplast D1 protein synthesis inhibitor chloramphenicol (CM) in Rumex K-1 leaves (Fig. 7A, C, E). However, in the presence of CM, the SHAM treatment had no significant effects on the levels of $\phi_{PSI}$, $\Psi_0$ and $\phi_{E0}$ under high light (Fig. 7B, D, F).

**Discussion**

Under high light, excess reducing equivalents generated in chloroplasts can be transported to other organelles through the malate-OAA shuttle and oxidized in metabolic pathways (Noguchi and Yoshida 2008, Raghavendra and Padmasree 2003, Scheibe 2004). NADP-MDH is the key enzyme in the malate-OAA shuttle (Noguchi and Yoshida 2008, Scheibe 2004). It is noteworthy that the initial activity of NADP-MDH noticeably increased in Rumex K-1 leaves under high light (Fig. 2B), indicating that the malate-OAA shuttle was activated to transport excess reducing equivalents generated in the chloroplasts to mitochondria. The equivalents transported from the chloroplasts can be oxidized by the respiratory electron transport chain (Noguchi and Yoshida 2008, Yoshida et al. 2007). It was suggested that, in this situation, the mitochondrial non-phosphorylating electron transport pathway, the AOX pathway, would play a role in the dissipation of chloroplast-derived reducing equivalents (Dinakar et al. 2010b, Yoshida et al. 2007, 2008). The fact that the AOX pathway capacity in the leaves of Rumex K-1 significantly increased (Fig. 2A) under high light support this suggestion.

The fact that the treatments of intact chloroplasts isolated from Rumex K-1 leaves with 1 mM SHAM and nPG had no effects on $\phi_{PSI}$ and $q_P$ (Figs 1 and S1), together with the fact that neither the SHAM nor the nPG treatments altered the OJIP transients in the dark (Figs 3A and S2A), demonstrate that the 1 mM SHAM and nPG used in this study had no direct effects on photosynthetic behaviors, which was also demonstrated by Padmasree and Raghavendra (1999a), Bartoli et al. (2005) and Yoshida et al. (2006) in their studies, using other plant materials. Therefore, the observed effects of the SHAM and nPG treatments on the photosynthetic apparatus under high light in this study were because of the inhibition of the mitochondrial AOX pathway. It was reported that when the reducing equivalents accumulated in the mitochondria, the activity of the malate-OAA shuttle would be reduced by a feedback mechanism (Padmasree and Raghavendra 2001). Thus, it is reasonable to consider that, under high light, the significant decrease in the initial activity of NADP-MDH caused by the inhibition of the AOX pathway (Fig. 2B) was because of the accumulation of reducing equivalents in the mitochondria because of the consumption of reducing equivalents through the AOX pathway. The significant decrease in the initial activity of NADP-MDH would inevitably limit the export of reducing equivalents from the chloroplasts to mitochondria, resulting in the accumulation of reducing equivalents in the chloroplast stroma.

Furthermore, the SHAM and nPG treatments markedly altered the OJIP transient under high light (Figs 3B and S2B), suggesting that the inhibition of the AOX pathway significantly influenced the performance of the photosynthetic machinery. The observation that P700 was more reduced in SHAM-treated leaves under high light (Fig. 4A) indicates that the inhibition of the AOX pathway caused an over-reduction of the PSI acceptor side under high light because of the accumulation of...
excess reducing equivalents in the chloroplasts. The fact that the maximal amplitude in the I–P phase of the OJIP transient decreased obviously in the SHAM-treated leaves under high light (Fig. 4B) indicates a decrease in the pool size of the PSI end electron acceptors (Kalachanis and Manetas 2010, Yusuf et al. 2010), further supporting the above suggestion. Moreover, the fact that the relative variable fluorescence at J-step (V_j) increased significantly in both the SHAM- and nPG-treated leaves compared with the control leaves (Figs 5 and S3) suggests that the ratio of Q_A^-/Q_A increased (Li et al. 2009, Strasser and Strasser 1995) under high light when the AOX pathway was inhibited. Thus, the inhibition of the AOX pathway decreased the electron transport capacity at the acceptor side of PSII, resulting in an over-reduction of the PSII acceptor side. In addition, the electron transport per active reaction center (ET0/RC) decreased, but the light absorption per active reaction center (ABS/RC) and trapping of excitation energy per active reaction center (TR0/RC) increased, and the excitation efficiency for electron transport beyond Q_A (φ_E0) and the quantum yield for electron transport (φ_E0) decreased significantly in the SHAM- and nPG-treated leaves (Figs 5 and S3), indicating that the balance between photosynthetic light absorption and energy utilization was disturbed by the over-reduction of the PSII acceptor side because of the inhibition of the AOX pathway under high light. The imbalance between photosynthetic light absorption and energy utilization will inevitably lead to the over-excitation of the PSII reaction centers (Vass 2011).

Under photoinhibitory condition, there are two major sites of ROS generation in chloroplasts: the end of photosynthetic electron transport chain (acceptor side

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**Fig. 7.** Photosynthetic parameters derived from the JIP-test analysis of chlorophyll a fluorescence transients in Rumex K-1 leaves treated with 0 (control) or 1 mM SHAM in the absence (A, C, E) or presence (B, D, F) of 1 mM CM. Leaf discs (0.5 cm²) treated with 0 (control) or 1 mM SHAM were vacuum-infiltrated with 0 or 1 mM CM in the dark and then exposed to high light at 800 μmol m⁻² s⁻¹ for 1, 2 and 3 h. Means ± SE of eight replicates are presented. Different letters indicate significant difference between the SHAM treatment and control at P < 0.05, LSD.
of PSI and the PSII reaction centers (Bartoli et al. 2005, Niyogi 1999, 2000, Vass 2011). An over-reduction of the PSI acceptor side and over-excitation of PSII reaction centers because of the inhibition of the AOX pathway would inevitably enhance the generation of ROS under high light. Our observation that the accumulation of ROS (O$_2^-$ and H$_2$O$_2$) in leaves was increased by the SHAM and nPG treatments (Figs 6A and S4) supports the above suggestion. The observation that the activity of the ROS scavenging system (SOD, APX and CAT) increased in SHAM-treated leaves under high light (Fig. 6B) indicates that the over-accumulation of ROS was not caused by the influence of the SHAM treatment on the activity of ROS scavenging system directly but by the over-reduction of the photosynthetic electron transport chain.

The SHAM and nPG treatments caused more severe photoinhibition as they accelerated decreases in $\phi_{P_0}$ (Figs 7A and 5A), $\Psi_0$ (Figs 7C and 5C) and $\phi_{Eo}$(Figs 7E and 5E). How did the inhibition of the AOX pathway accelerate photoinhibition? Some studies have demonstrated that the accumulation of ROS induced by excess light energy do not accelerate photodamage to PSII directly but inhibits the repair of the photodamaged PSII at the step of the $de$ novo synthesis of the D1 protein (Murata et al. 2007, Nishiyama et al. 2006, Takahashi and Murata 2008). However, Krieger-Liszkay et al. (2011) recently reported that O$_2^-$ generated in PSI can damage PSII directly. Therefore, the over-accumulation of ROS due to the inhibition of the AOX pathway under high light (Figs 6A and S4) either damaged PSII directly or inhibited the repair of the photodamaged PSII. To distinguish the effect of the inhibition of the AOX pathway on the photodamage of PSII and the inhibition of the repair of the photodamaged PSII, we used CM, an inhibitor of D1 protein synthesis (Takahashi et al. 2009), to inhibit the repair of the photodamaged PSII in this study. We observed that SHAM and nPG treatments did not accelerate photoinhibition in the presence of CM, indicated by decreases in $\phi_{P_0}$ (Figs 7B and 5B), $\Psi_0$ (Figs 7D and 5D) and $\phi_{Eo}$(Figs 7F and 5F), but the treatments did accelerate photoinhibition in the absence of CM, indicating that the AOX pathway protected plant leaves against photoinhibition, mainly by alleviating the inhibition of the repair of the photodamaged PSII rather than by preventing photodamage to PSII directly.

In conclusion, the inhibition of the AOX pathway resulted in the loss of a sink of excess reducing equivalents and accelerated the accumulation of NADPH in chloroplasts, leading to the over-reduction of the PSI acceptor side and over-excitation of the PSII reaction centers to induce the over-production of ROS in chloroplasts under high light. The over-production of ROS inhibited the repair of the photodamaged PSII (Fig. 8). Therefore, the mitochondrial AOX pathway plays an important role in the protection of plants against photoinhibition by alleviating the inhibition of the repair of the photodamaged PSII through preventing the formation of ROS.

Fig. 8. Schemes of the possible phenomena in the cell when AOX is inhibited under high light. AOX, alternative oxidase; OAA, oxaloacetate; Pre-D1, precursor to D1 protein; PSI/I, photosystem II/I; ROS, reactive oxygen species.
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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

- **Fig. S1.** Photosystem II actual photochemical efficiencies (\( \Phi_{\text{PSII}} \)) and photochemical quenching coefficients (\( q_P \)) in isolated Rumex K-1 chloroplasts in the absence (control) or presence of 1 mM nPG.
- **Fig. S2.** Changes of chlorophyll a fluorescence transients in Rumex K-1 leaves treated with 0 (control) or 1 mM nPG in the dark (A) or in the high light (B).
- **Fig. S3.** Photosynthetic parameters derived from the JIP-test analysis of chlorophyll a fluorescence transients shown in Fig. S2B.
- **Fig. S4.** Production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in Rumex K-1 leaves treated with 0 (control) or 1 mM nPG under high light.
- **Fig. S5.** Photosynthetic parameters derived from the JIP-test analysis of chlorophyll a fluorescence transients in Rumex K-1 leaves treated with 0 (control) or 1 mM nPG in the absence (A, C, E) or presence (B, D, F) of 1 mM CM.

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