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BRIEF COMMUNICATION

Multiple effects of inhibition of mitochondrial alternative oxidase pathway on photosynthetic apparatus in *Rumex* K-1 leaves

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Abstract

The effects of inhibition of mitochondrial alternative oxidase (AOX) respiratory pathway on photosynthetic apparatus in *Rumex* K-1 leaves were studied. Under high irradiance, the inhibition of AOX pathway caused over-reduction of photosystem (PS) 2 acceptor side, a decrease in the energy transfer in the PS 2 units, damage of donor side of PS 2 and decrease in pool size of electron acceptors. The inhibition of AOX pathway also decreased photosynthetic performance index (PI_{ABS}), actual photochemical efficiency (Φ_{PS2}), photochemical quenching (qP) and photosynthetic O_2 evolution rate. The results demonstrate that mitochondrial AOX pathway plays a vital role in photoprotection of photosynthetic apparatus.

Additional key words: chlorophyll *a* fluorescence, high irradiance, JIP-test, photosystem 2.

Excess radiation results in accumulation of reducing equivalents generated by photochemical reaction and accelerate generation of reactive oxygen species (ROS), leading to photoinhibition (Asada 2000, Zhang *et al.* 2010). However, many plants cannot escape from exposure to high irradiance. Therefore, they evolved different defense mechanisms (Niyogi 1999, Asada 2000), such as thermal dissipation of excess energy (Müller *et al*. 2001, Zhang *et al.* 2009, Hu *et al.* 2010), cyclic electron flow around photosystem (PS) 1 (Johnson 2005, Rumeau *et al*. 2007) and water-water cycle (Asada 1999). Though such intra-chloroplast defense systems have been studied extensively, little is known about the extra-chloroplast defense systems (Yoshida *et al*. 2007).

For the last two decades, it has been shown that excess reducing equivalents generated in chloroplasts could be transported to mitochondria and oxidized by respiratory electron transport chain (Raghavendra and Padmasree 2003, Yoshida *et al.* 2006, 2007). The electron transport in mitochondria of higher plant uses two different pathways, the cytochrome oxidase (COX) pathway and the alternative oxidase (AOX) pathway (Raghavendra and Padmasree 2003). The COX pathway generates the proton gradient to accomplish most of the ATP production in mitochondria, whereas AOX pathway is non-phosphorylating and it can oxidize the 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). A few studies have demonstrated that AOX pathway functions as a sink for excess reducing equivalents generated by photosynthesis (Yoshida *et al.* 2006, 2007). Therefore, it has been speculated that the AOX pathway might have a particular role in protecting plants from photoinhibition, but direct evidence is still limited.

To assess the possible role of AOX pathway in protecting plants against photoinhibition, salicylhydroxamic acid (SHAM), a well-known inhibitor of mitochondrial AOX pathway, was used to inhibit the AOX pathway in the present study. Chlorophyll *a* fluorescence transient (OJIP transient) combined with JIP-test (Strasser and Strasser 1995, Strasser *et al.* 2000, 2004, Singh-Rawal *et al.* 2010) as well as photosynthetic O_2 evolution rate were used to quantify the multiple effects of the inhibition of AOX pathway on photosynthetic machinery.

Rumex K-1 plants (*Rumex patientia* × *R. tianschaious*) were grown from seeds under a 14-h photoperiod, high irradiance of 800 μmol m^{-2} s^{-1} and day/night temperature

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Abbreviations: AOX - alternative oxidase; COX - cytochrome oxidase; PS - photosystem; Q_A - primary quinone electron acceptor of PS 2; ROS - reactive oxygen species; SHAM - salicylhydroxamic acid.

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of 22/18 °C in a pots containing soil. Leaf discs (0.5 cm^2) were punched from fully expanded leaves of 4-week-old plants and infiltrated with 0 (control), 0.2, 0.6 or 1.0 mM SHAM solution for 2 h in the dark at room temperature. Then the discs were exposed to irradiance of 800 μmol m^{-2} s^{-1} for 1 h at room temperature. During this period, the excess of reducing equivalents generated by photosynthesis would be transported to mitochondria *via* shuttle machineries, and oxidized by respiratory electron transport chain (Raghavendra and Padmasree 2003, Yoshida *et al.* 2006, 2007).

Actual photochemical efficiency of PS 2 (Φ_{PS2}) and photochemical quenching coefficient (qP) of leaf discs were measured with a pulse modulated fluorometer (*FMS-2*, *Hansatech Instruments*, Norfolk, UK) according to Chen *et al.* (2007). A *Chlorolab-2* liquid-phase oxygen electrode system (*Hansatech Instruments*) was used to measure photosynthetic O_2 evolution rate of leaf discs according to Jia *et al.* (2010). Chlorophyll *a* fluorescence transients of leaf discs before or after high irradiance treatment were measured with a *Handy PEA* fluorometer (*Hansatech Instruments*). All the measurements were done with 15 min dark-adapted leaf discs at room temperature. The relative variable fluorescence kinetics (W_{OP}, W_{OK}, W_{OJ}, W_{OI} and W_{IP}) was obtained by normalizing the fluorescence transients according to the equations of JIP-test (Strasser *et al.* 2000, 2004, Yusuf *et al.* 2010). Difference kinetics between the control and other treated samples were calculated as: $\Delta W = W - W_{cont.}$ where W was the relative variable fluorescence (Yusuf *et al.* 2010). The photosynthetic performance index PI_{ABS} as well as its individual partial components, the fraction of reaction center per chlorophyll of the antennae (RC/ABS), the contribution of the light reactions for primary photochemistry ($\varphi_{Po}/(1-\varphi_{Po})$) and the contribution of electron transport beyond Q_A ($\psi_O/(1-\psi_O)$), were also calculated according to the equations of JIP-test, where RC, ABS, φ_{Po} and ψ_O were the reactive centre, the light energy for absorption, the maximal quantum yield of primary photochemistry and the excitation efficiency for electron transport beyond Q_A respectively (Strasser *et al.* 2000, 2004).

The fact that treatments with 0, 0.2, 0.6 or 1.0 mM SHAM had no effects on $\Phi_{PS\ 2}$ and qP in isolated intact *Rumex* K-1 chloroplasts (data not shown) together with the fact that SHAM treatments did not change OJIP

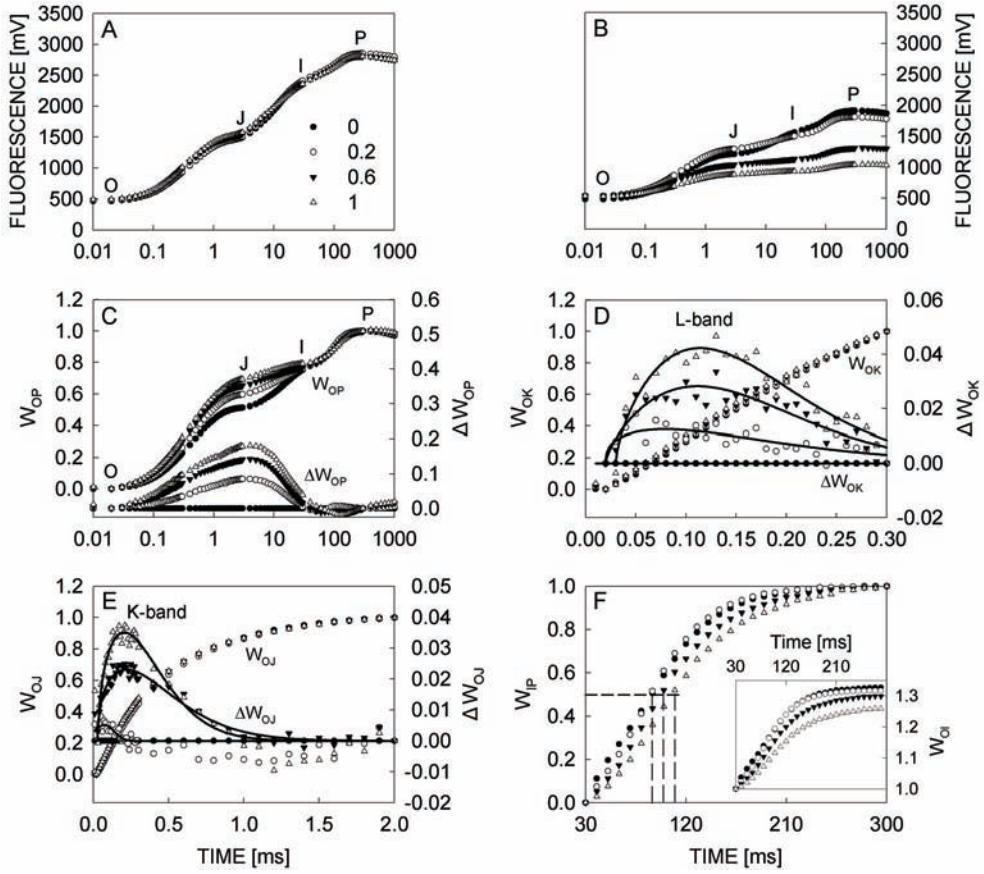

Fig. 1. Chlorophyll *a* fluorescence (OJIP) transients in *Rumex* K-1 leaves treated with 0, 0.2, 0.6 or 1 mM SHAM in the dark (*A*) or under light (*B*). The normalization of the fluorescence transients under high irradiance between the phases of OP (*C*), OK (*D*), OJ (*E*), IP (*F*) and OI (the insert in *F*). The corresponding difference kinetics between SHAM-treated leaves and control leaves ($\Delta W = W - W_{cont.}$) were also depicted. Each transient represents the average of eight samples. The difference kinetics ΔW_{OJ} and ΔW_{OK} reveal the K-band (at about 300 μs) and L-band (at about 150 μs) respectively.

transients in *Rumex* K-1 leaves in the dark (Fig. 1*A*) demonstrate that the concentrations of SHAM used in this study were sufficiently low to avoid any possible side effects on photosynthetic machinery, which was also previously documented by Raghavendra and Padmasree (2003) and Yoshida *et al.* (2006). After treated with SHAM under high irradiance, the fluorescence in *Rumex* K-1 leaves decreased gradually with the increase of SHAM concentration (Fig. 1*B*), suggesting that inhibition of AOX pathway significantly influenced the performance of photosynthetic machinery under high irradiance.

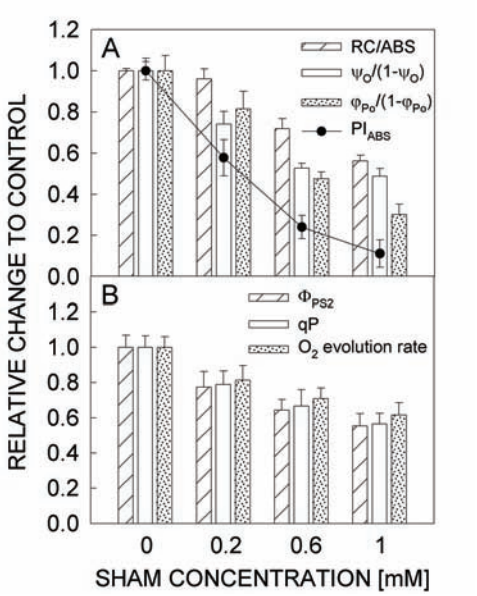

Fig. 2. The performance index (PI_{ABS}) as well as its individual partial components RC/ABS, $\varphi_{Po}/(1-\varphi_{Po})$ and $\psi_O/(1-\psi_O)$, (*A*), Φ_{PS2}, qP and photosynthetic O_2 evolution rate (*B*) in the presence of 0 (control), 0.2, 0.6 or 1 mM SHAM in *Rumex* K-1 leaves under the light. All values represent the average of eight replicates. The control value was taken as 1, whereas the others in treatments were taken as the percentage of the control. In control leaves, PI_{ABS}, RC/ABS, $\varphi_{Po}/(1-\varphi_{Po})$, $\psi_O/(1-\psi_O)$, Φ_{PSII}, qP and photosynthetic O_2 evolution rate were 1.23 ± 0.056, 0.40 ± 0.004, 3.23 ± 0.241, 0.94 ± 0.057, 0.32 ± 0.022, 0.65 ± 0.042 and 6.18 ± 0.369 μmol(O_2) m^{-2} s^{-1} respectively.

In order to unravel the detailed influence of inhibition of AOX pathway on PS 2 behaviour, we analyzed the changes in the phases of OP, OK, OJ, OI and IP in the relative variable fluorescence kinetics by normalizing the OJIP transients (Fig. 1*C*-*F*).

It was observed that fluorescence transients normalized between O and P were changed greatly by SHAM treatments. And, the most distinct peak in the relative variable fluorescence kinetics was at J step that increased with the increase of SHAM concentration (Fig. 1*C*), which indicates that the ratio of Q_A^-/Q_A increased (Strasser and Strasser 1995, Li *et al.* 2009) under high irradiance when AOX pathway was inhibited. Obviously the inhibition of AOX pathway resulted in over-reduction of PS 2 acceptor side under high irradiance.

Meanwhile, the L-bands (Fig. 1*D*) and K-bands (Fig. 1*E*) were significantly enhanced by the SHAM treatments under high irradiance. The higher L-band (Fig. 1*D*) indicates a lower energetic connectivity of the PS 2 units, which means that the PS 2 units were less grouped or less energy was being exchanged between independent PS 2 units. A lower energetic connectivity results in a worse utilization of the excitation energy and a lower stability of the system (Strasser *et al*. 2004, Yusuf *et al.* 2010). The enhanced K-band (Fig. 1*E*) indicates an increase in damage on donor side of PS 2 (Strasser *et al*. 2000, 2004, Li *et al*. 2009, Jia *et al*. 2010).

The half-times in I-P phase was extended by the increase in SHAM concentration under irradiance (Fig. 1*F*), which indicates the lower reduction of the end electron acceptors (*e.g*. $NADP^+$; Yusuf *et al.* 2010). The maximal amplitude of fluorescence in I-P phase decreased in leaves treated with 0.6 and 1.0 mM SHAM (the insert in Fig. 1*F*), suggesting that the inhibition of AOX pathway reduced the pool size of the end electron acceptors (Yusuf *et al.* 2010).

The performance index PI_{ABS} is closely related to the ability of energy conservation and the activity of photosynthetic apparatus (Strasser *et al.* 2000, 2004, Yusuf *et al.* 2010). Under strong irradiance, the PI_{ABS} decreased by 42, 76 and 89 % in leaves treated with 0.2, 0.6 and 1 mM SHAM, respectively (Fig. 2*A*), indicating that inhibition of AOX pathway significantly decreased the ability of energy conservation and the activity of photosynthetic apparatus. With the increase of SHAM concentration in the treatment, RC/ABS, $\varphi_{Po}/(1-\varphi_{Po})$ and $\psi_O/(1-\psi_O)$ all decreased (Fig. 2*A*). In addition, the fact that SHAM treatments significantly restricted Φ_{PS2}, qP and photosynthetic O_2 evolution rate (Fig. 2*B*) further supports the suggestion that inhibition of AOX pathway decreased the capacity of photosynthetic machinery.

It has been reported that mitochondrial AOX pathway functions as a sink for excess reducing equivalents generated by photosynthesis (Yoshida *et al.* 2006, 2007). Therefore, the inhibition of AOX pathway in *Rumex* K-1 leaves limited the dissipation of excess reducing equivalents *via* this pathway and caused rapid accumulation of NADPH in the chloroplast, leading to over-reduction of PS 1 acceptor side. The over-reduction of PS 1 acceptor side would further inhibit the electron transport from Q_A to PS 1, resulting in over-accumulation of Q_A^-, which is proved by the fact that J step in the OJIP transient remarkably increased when AOX pathway was inhibited (Fig. 1*C*). The over-accumulation of Q_A^- would inevitably limit electron transport from PS 2 reaction centers to the acceptor side of PS 2, leading to excess excitation pressure. It has been known that the excess excitation pressure is a key reason to cause PS 2 photoinhibition (Osmond *et al.* 1997). Our study demonstrates that the inhibition of AOX pathway significantly inactivated PS 2 reaction centers indicated by the increase of L-band (Fig. 1*D*) and damaged PS 2 donor side indicated by the increase of K-band (Fig. 1*E*).

All the influence on the above mentioned processes by inhibition of AOX pathway finally decreased the activity of photosynthetic machinery, which is supported by the fact that the performance index, photosynthetic O_2 evolution rate and PS 2 photochemical efficiency significant decreased in *Rumex* K-1 leaves when AOX pathway was inhibited (Fig. 2).

In conclusion, the inhibition of AOX pathway significantly decreased the activity of photosynthetic machinery, leading to photoinhibition under high irradiance. Therefore, mitochondrial AOX pathway plays a vital role in protection of photosynthetic apparatus.

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