Photoinhibition and active oxygen species production in detached apple leaves during dehydration

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Abstract

In the course of dehydration, the gas exchange and chlorophyll (Chl) fluorescence were measured under irradiance of 800 µmol m⁻² s⁻¹ in detached apple leaves, and the production of active oxygen species (AOS), hydrogen peroxide (H₂O₂), superoxide (O₂⁻), hydroxyl radical (·OH), and singlet oxygen (¹O₂), were determined. Leaf net photosynthetic rate (Pn) was limited by stomatal and non-stomatal factors at slight (2–3 h dehydration) and moderate (4–5 h dehydration) water deficiency, respectively. Photoinhibition occurred after 3-h dehydration, which was defined by the decrease of photosystem 2 (PS2) non-cyclic electron transport (P-rate). After 2-h dehydration, an obvious rise in H₂O₂ production was found as a result of photorespiration rise. If photorespiration was inhibited by sodium bisulfite (NaHSO₃), the rate of post-irradiation transient increase in Chl fluorescence (Rfp) was enhanced in parallel with a slight decline in P-rate and with an increase in Mehler reaction. At 3-h dehydration, leaf P-rate decrease could be blocked by glycine (Gly) or methyl viologen (MV) pre-treatment, and MV was more effective than Gly at moderate drought time. AOS (H₂O₂ and O₂⁻), prior to photoinhibition produced from photorespiration and Mehler reaction in detached apple leaves at slight water deficiency, were important in dissipating photon energy which was excess to the demand of CO₂ assimilation. So photoinhibition could be effectively prevented by the way of AOS production.

Additional key words: chlorophyll fluorescence; gas exchange; irradiance; Malus pumila; Mehler reaction; photorespiration; photosystem 2 non-cyclic electron transport.

Introduction

In normal metabolism, plants generate active oxygen species (AOS), including superoxide (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radicals (·OH) and singlet oxygen (¹O₂). If not readily removed, a prolonged AOS exposure could cause lipid peroxidation, DNA damage, and protein denaturation, leading to cell injury or possible death (Elstner 1991, Scandalias 1993). Many stresses including drought, low temperatures, and high irradiance result in the enhancement of AOS production in plants (Wise and Naylor 1987, Levine et al. 1994, Chandra et al. 1997).

The response of plants to a variable and stressful environment has long been the focus of ecophysiology because it limits plant distribution and productivity. After decades of research, mitochondria and chloroplasts have been viewed as two major sources of AOS in plant cells under stress (Wise and Naylor 1987, Price et al. 1989, Elstner 1991, Asada 1994, Krause 1994), and thus the most progressive understanding of AOS is the constructive function in plants under stress (Neunenschwander et al. 1995, Foyer and Noctor 2000). Photoinhibition was

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Abbreviations: AOS – active oxygen species; CE – carboxylation efficiency; Chl – chlorophyll; Ci – intercellular CO₂ concentration; DMSO – dimethyl sulfoxide; F₅ and Fₐ – steady-state fluorescence and maximal fluorescence in light-adapted state; F₅ and Fₐ – variable fluorescence and maximal fluorescence in dark-adapted state; Gly – glycine; H₂O₂ – hydrogen peroxide; Ls – stomatal limitation; MSA – methane sulfonic acid; MV – methyl viologen; O₂⁻ – superoxide; ·OH – hydroxyl radicals; ¹O₂ – singlet oxygen; Pn – net photosynthetic rate; P-rate – PS2 non-cyclic electron transport; PS2 – photosystem 2; Rfp – rate of post irradiation transient increase in Chl fluorescence; RH – relative humidity; RNO – N,N-dimethyl-p-nitrosoaniline; RWC – relative water content.
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regarded as the result of photosynthetic apparatus damage caused by AOS in chloroplasts and thylakoid membranes at its early research stage (Björkman 1987), while Long et al. (1994) stated that the AOS metabolism can protect plants from photo inhibition with the help of dissipating excess photon energy.

Since the destructive and constructive elements of AOS metabolism affect photo inhibition, we speculate that there exists a close relationship between AOS metabolism and photo inhibition. In order to ascertain whether at water deficiency actually the former induces the latter or vice versa, the production of AOS and occurrence of photoinhibition were adopted to investigate their relationship during dehydration in detached apple leaves.

Materials and methods

Plants and treatments: For experiments we used three or four fully developed leaves on the medium part of trunk of two-year-old apple trees (Malus pumila Mill. cv. Tengmu No. 1) grown in field. The young trees with no branches were about 1.0 m high. Petioles of the detached leaves were soaked in water and leaves were pre-irradiated for 1.0 h at an irradiance of 400 μmol m⁻² s⁻¹. Then water was removed and the leaves were exposed to air temperature of 28±2 °C, 75±5 % relative humidity (RH), and irradiance of 100 μmol m⁻² s⁻¹ to imitate drought stress. At a given time after dehydration, leaves were exposed to saturating irradiance of 800 μmol m⁻² s⁻¹ for 10 min and then all the experimental tests were carried out. The exogenous chemical substances were fed via transpiration stream in the course of pre-irradiation, with the aim to promote photorespiration (5 mM NaHSO₃), inhibit photorespiration (2 mM Gly), promote Mehler reaction (0.5 mM MV), and assay hydroxyl radicals (0.6 mM DMSO), respectively.

Leaf relative water content (RWC) was calculated from the ratio (fresh mass − dry mass)/(water-saturated mass − dry mass). The water-saturated mass was measured after floating the leaves in water for 6 h. Dry mass was determined after drying the leaves at 70–80 °C for 24 h.

Gas exchange and chlorophyll (Chl) fluorescence measurement: A gas exchange system CIRAS-1 (PP-Systems, U.K.) and a modulated fluorescence monitor system FMS-2 (Hansatech, U.K.) were used for gas exchange and Chl fluorescence measurements, respectively. The leaf, after a given time dehydration at irradiance of 100 μmol m⁻² s⁻¹, was clamped into the cuvette of CIRAS-1 which was connected with FMS-2. The cuvette condition was controlled by CIRAS-1 at air temperature 28±2 °C, RH 75±5 %, CO₂ concentration 350±5 cm⁻³, and irradiance of 800 μmol m⁻² s⁻¹. After 10-min irradiation of the leaf in cuvette, gas exchange parameters such as net photosynthetic rate (Pₙ), intercellular CO₂ concentration (Cᵢ), stomatal limitation (Lₛ), and Chl fluorescence parameters, steady-state fluorescence (Fₛ), and maximal fluorescence (Fₘ) in light-adapted state, were recorded by a computer. Then radiation was switched off and the rate of post-irradiation transient increase in Chl fluorescence (Rᶠp) was measured according to Mi et al. (1995). When the Rᶠp test was finished, the cuvette was covered with a black cloth and the leaf darkened for 20 min to measure photosystem 2 (PS2) maximal photochemical activity (Fₚ/Fₘ). The PS2 non-cyclic electron transport (P-rate) was calculated according to the formula P-rate = (Fₘ’ − Fₛ)/Fₘ’ × PPFD (Genty et al. 1989). Leaf carboxylation efficiency (CE) was reflected by the linear correlation coefficient of Fₚ response to Cᵢ below 150 cm⁻³.

Active oxygen species measurement: Hydrogen peroxide (H₂O₂) was extracted and estimated according to Mukherjee and Choudhuri (1983). Isolation was made from 5 g of leaf tissue in ice-cold acetone. By addition of 5 % (m/v) tannyl sulphate and concentrated NH₄OH solution, the peroxide-titanium complex was precipitated. This was dissolved in 15 cm⁻³ of 2 M H₂SO₄, making the final volume of 20 cm⁻³ with cold water. The absorbance was read at 415 nm against water blank. The H₂O₂ content was calculated from a standard curve prepared in a similar way.

Assay of superoxide (O₂⁻) was made according to Schneider and Schlegel (1981) with modifications. O₂⁻ was isolated from 5 g of leaves tissue with 6 cm⁻³ of 65 mM sodium phosphate buffer (pH 7.8). Extract of 1 cm⁻³ was supplemented with 1 cm⁻³ of 1 mM hydroxylamine hydrochloride. In the presence of O₂⁻, hydroxylamine was oxidised to nitrite. The formation of nitrite was determined by adding 1 cm⁻³ each of 7 mM α-naphthylamine and 17 mM sulphamic acid solutions to 1 cm⁻³ of the reaction mixture. The components were mixed and left at room temperature for 20 min, 2 cm⁻³ n-butanol was used to extract, and absorbance was read at 530 nm from the upper layer solution. The O₂⁻ content was calculated from a nitrite standard curve prepared in a similar way.

The singlet oxygen (¹O₂) generation was determined by monitoring N,N-dimethyl-p-nitrossoaniline (RNO) reactions using a spectrophotometric method as described by Chakraborty and Tripathy (1992). Histidine was used as a trap for ¹O₂. Chloroplasts [100 μg(Chl) cm⁻³] were incubated with RNO (300 g m⁻³) and 10 mM histidine. After irradiation at 800 μmol m⁻² s⁻¹ for 30 min, the incubation solution was centrifuged and the supernatant was read for absorbance at 440 nm. Singlet oxygen production was reflected by the absorbance ratio of RNO mixed with sample to that mixed with no sample.

Hydroxyl radical (•OH) was measured by spectrophotometry according to Steiner and Babbs (1990).
About 2 g of leaves, pre-fed with 0.6 mM DMSO, were harvested after dehydration and irradiation, and thereafter were frozen with liquid N$_2$, ground with a mortar and pestle, and the resulted powder was extracted with 10 cm$^3$ distilled water, then centrifuged to remove solid substances. Sulfinic acid (MSA) could be produced upon DMSO oxidation by OH. Most interfering anions are much less hydrophilic than MSA itself. To remove a portion of such interfering species, an equal volume of toluene/n-butanol (3:1) was added into the supernatant to dispel disturbance from lipid. The lower aqueous phase was transferred with a Pasteur pipette to a test tube containing 2 cm$^3$ of 15 mM fast blue BB salt (freshly prepared and kept in the dark). Ten minutes were allowed for product development at room temperature in the dark. Then 3 cm$^3$ of toluene/n-butanol (3:1) was added and mixed thoroughly with the aqueous phase for 60 s in a Vortex mixer. The lower phase, containing non-reacted diazonium salt, was removed by aspiration and discarded. The toluene/n-butanol phase was washed with 5 cm$^3$ of n-butanol-saturated water to remove the remaining non-reacted diazonium salt. The tubes were centrifuged at 500×g for 3 min, and the upper phase was transferred to a cuvette. 1 cm$^3$ of pyridine/glacial acetic acid (95:5) was added to stabilise the colour and the absorbance was determined at 420 nm against a black carried by water through the same procedure.

**Results**

RWC decreased in detached apple leaves (Fig. 1A). Dehydration for 2–3 h and 4–5 h could cause RWC lowering by 8–10 and 10–20% which was defined as slight and moderate water stress, respectively (Hsiao 1973). Leaf $P_N$ decreased continuously in the whole course of dehydration (Fig. 1B), while $L_n$ increased at slight drought decreased the photochemical activity in detached leaves (Fig. 1E,F). $P$-rate and $F_{v}/F_{m}$ began to drop at 3-h and 4-h dehydration, respectively. Though $F_{v}/F_{m}$ was commonly used to define photoinhibition, we recently proposed that $F_{v}/F_{m}$ is not the right index when photoinhibition occurs with no damage of PS2 reaction centres, and $P$-rate should be used for correct defining photoinhibition (Jia and Li 2002). Thus, drought-induced photoinhibition occurred in detached apple leaves at 3-h dehydration. Under slight drought, the carboxylation efficiency (CE) was nearly not changed, but it obviously decreased when leaf was subjected to moderate drought (Fig. 2A). This suggests that the capacity for CO$_2$ assimilation was not affected by slight drought but done by moderate drought.

Photorespiration and dark reaction are coupled in photosynthesis because the former provides assimilatory power to the latter for CO$_2$ assimilation and the latter supplies ADP and NADP to the former for assimilatory power regeneration. Decrease in assimilatory power demanded in dark reaction can cause accumulation of ATP and NADPH in leaves under irradiation, which can be reflected by the rate of transient post-irradiation increase in Chl fluorescence (Rfp) (Mi et al. 1995). Plenty of assimilatory power accumulated in detached apple leaves was reflected by apparent rise of Rfp at 3-h dehydration (Fig. 2B).

Active oxygen species (AOS), H$_2$O$_2$, O$_2^-$, $^1$O$_2$, and OH were produced in detached apple leaves (Fig. 3) following the decrease in RWC. H$_2$O$_2$ content increased by 109.3% at 2-h dehydration (Fig. 3A). O$_2^-$ began to increase at the 3-h dehydration with 42.8% (Fig. 3B), and $^1$O$_2$ and OH had a noticeable rise at 5-h dehydration (Fig. 3C,D). Change of H$_2$O$_2$ production was prior to photoinhibition occurrence, but O$_2^-$ generation appeared simultaneously.

Photoinhibition usually originates from the excess photons trapped by leaf. When irradiance was lowered, H$_2$O$_2$ and O$_2^-$ production was effectively delayed (Fig. 3A, B), which showed that the initial AOS production was not the direct result of water deficiency but the
result of leaf trapping of excess photons. We showed previously (Jia and Li 2001) that strong metabolism upon photosynthesis at slight drought and upon Mehler reaction at moderate drought occurred in irradiated detached apple leaves, which directly caused the rise in H$_2$O$_2$ and O$_2^-$ production. When NaHSO$_3$ inhibited photosynthesis, both the Rfp increase and P-rate decrease were accelerated at slight drought (Fig. 4A,B). Especially at 2-h dehydration, the P-rate decreased negligibly, but Rfp and O$_2^-$ increased markedly in leaf with NaHSO$_3$ treatment, which differed in control leaves with little changes in Rfp and O$_2^-$ (Fig. 4). These facts demonstrated that if photosynthesis was blocked, the assimilatory power could accumulate and Mehler reaction increased in detached apple leaves during the 2-h dehydration period. When leaves were treated with Gly, increase of Rfp and O$_2^-$ was weaker (Fig. 4). P-rate was still kept unchanged in Gly treated leaves in contrast to control at 3-h dehydration, and photoinhibition was effectively avoided if the photosynthesis was enhanced enough even though photosynthetic CO$_2$ assimilation was lowered to some extent. In addition, MV treatment accelerating Mehler reaction could effectively block the P-rate decrease at moderate drought stage in detached apple leaves and avoid photoinhibition at 3-h dehydration (Fig. 5B), but it had little influence on Rfp (Fig. 5A).

**Discussion**

Under drought stress many metabolic processes can produce AOS, and the electron transport in mitochondria and chloroplast is the main source of AOS in plants (Martin *et al*. 1997, Bowler and Fluhr 2000, Chen and Li 2001). Research has proved that AOS act upon leaf photoinhibition at least in two aspects. One is to dissipate excess photons for leaves against photoinhibition (Asada 1999), another is to induce photooxidation through its photooxidation on photosynthetic apparatus (Sakaki *et al*. 1983). The AOS production and its relation to photooxidation in detached apple leaves were investigated under irradiation. We found that changes in AOS production, H$_2$O$_2$
and $O_2^-$ appeared prior to photoinhibition in detached apple leaves during dehydration (Figs. 1 and 3), but this AOS production could be effectively postponed by lowering irradiance (Fig. 3). Hence AOS production was originally induced by irradiation in detached apple leaves during dehydration but was not the direct result of water deficiency.

The enhancement of $H_2O_2$ production in detached apple leaves initiated at 2-h dehydration, which was proved to result from photosuppression by our previous work (Jia and Li 2001), but CE and $P_{-}$ rate did not change at this stage (Figs. 2A and 1E). This suggested that water deficiency did not affect leaf capacity for both radiant energy transformation and assimilatory power formation. Decrease of $P_{-}$ indicated that the demand of assimilatory power in photosynthetic CO$_2$ assimilation dropped (Fig. 1B). So the excess of assimilatory power, relative to demand of CO$_2$ assimilation, appeared in detached apple leaves. Because of increase in photosuppression, the excess assimilatory power could be metabolized and only a negligible ascent of Rfp was found at 2-h dehydration (Fig. 2B). If NaH$_2$SO$_4$ inhibited photosuppression, Rfp and $O_2^-$ increased obviously (Fig. 4A, C). These facts revealed that when photosynthetic CO$_2$ assimilation was limited by closure of stomata at 2-h dehydration, the enhancement of photosuppression could supply more electron acceptors for assimilatory power regeneration, the potential photon surplus relatively to photoreaction could be prevented, and thus photoinhibition was effectively avoided. In addition, the potential photon surplus could also be prevented by Mehler reaction. So $P_{-}$ rate was faintly affected and there was no noticeable photoinhibition.

With the dehydration time passing, photosynthetic CO$_2$ assimilation was increasingly blocked by water deficiency (Fig. 1B). Though $P_{-}$ was still limited mainly by closure of stomata at 3-h dehydration, the excess photosynthetic electron in contrast to that used for CO$_2$ assimilation was not wholly consumed by photosuppression even with the help of Mehler reaction, and photoinhibition occurred unavoidably (Fig. 1E). At 3-h dehydration, increase either in photosuppression or in Mehler reaction could promote the excess energy of the leaf trapped to dissipate, which was useful to prevent photoinhibition (Figs. 4 and 5). Under moderate drought, CE decreased noticeably in detached apple leaves and photosynthesis was seriously limited by non-stomatal factors. Increase in photosuppression had little effect on $P_{-}$ rate (Fig. 4B), but Mehler reaction rate increased by MV treatment could obviously improve $P_{-}$ rate (Fig. 5B), showing that Mehler reaction played a more important role in excess photon energy dissipation than photosuppression under moderate drought in detached apple leaves.

Fig. 4. Changes in (A) Rfp, (B) $P_{-}$ rate, and (C) production of $O_2^-$ in detached apple leaves pre-treated with Gly (o) or NaH$_2$SO$_4$ (v), and control (●) during dehydration. The chemical treatments are described in Materials and methods, and the experimental process is the same as in Fig. 1. Means (n = 4) with standard deviations.

Fig. 5. Changes of (A) Rfp and (B) $P_{-}$ rate in detached apple leaves pre-treated without MV (●) and with MV (o) during dehydration. The MV treatment is explained in Materials and methods, and the experimental process is the same as in Fig. 1. Means (n = 4) with standard deviations.
References


