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

H.S. JIA[*,**], Y.Q. HAN[***,+], and D.Q. LI[**,++]

Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China[*]
College of Life Sciences, Shandong Agricultural University, Shandong Tai'an 271018, China[**]
Acta Botanica Sinica Editorial Office, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China[***]
Department of Biology,Liaocheng University,Liaocheng 252059, China[+]

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 (H_2O_2), superoxide (O_2^-), hydroxyl radical ($\cdot$OH), and singlet oxygen ($^1O_2$), were determined. Leaf net photosynthetic rate (P_N) 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 H_2O_2 production was found as a result of photorespiration rise. If photorespiration was inhibited by sodium bisulfite ($NaHSO_3$), 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 (H_2O_2 and O_2^-), 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 CO_2 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 (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot$OH) and singlet oxygen ($^1O_2$). 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, Scandalios 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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regarded as the result of photosyn-thetic 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 photoinhibition with the help of dissipating excess photon energy.

Since the destructive and constructive elements of

AOS metabolism affect photoinhibition, we speculate that there exists a close relationship between AOS metabolism and photoinhibition. 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 NaHSO$_3$), 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 %, CO$_2$ concentration 350±5 cm^3 m^{-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 (P_N), intercellular CO$_2$ concentration (C_i), stomatal limitation (L_s), and Chl fluorescence parameters, steady-state fluorescence (F_s), and maximal fluorescence (F_m') 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 (F_v/F_m). The PS2 non-cyclic electron transport (*P*-rate) was calculated according to the formula *P*-rate = $(F_m' - F_s)/F_m' \times$ PPFD (Genty *et al.* 1989). Leaf carboxylation efficiency (CE) was reflected by the linear correlation coefficient of P_N response to C_i below 150 cm^3 m^{-3}.

Active oxygen species measurement: Hydrogen peroxide (H_2O_2) 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) titanyl sulphate and concentrated NH$_4$OH solution, the peroxide-titanium complex was precipitated. This was dissolved in 15 cm^3 of 2 M H$_2$SO$_4$, making the final volume of 20 cm^3 with cold water. The absorbance was read at 415 nm against water blank. The H$_2$O$_2$ content was calculated from a standard curve prepared in a similar way.

Assay of superoxide (O_2^-) was made according to Schneider and Schlegel (1981) with modifications. O_2^- 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 O_2^-, 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 sulphanilic 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 O_2^- content was calculated from a nitrite standard curve prepared in a similar way.

The singlet oxygen ($^1O_2$) generation was determined by monitoring N,N-dimethyl-*p*-nitrosoaniline (RNO) reactions using a spectrophotometric method as described by Chakraborty and Tripathy (1992). Histidine was used as a trap for $^1O_2$. 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 lipoid. 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_s increased at slight drought
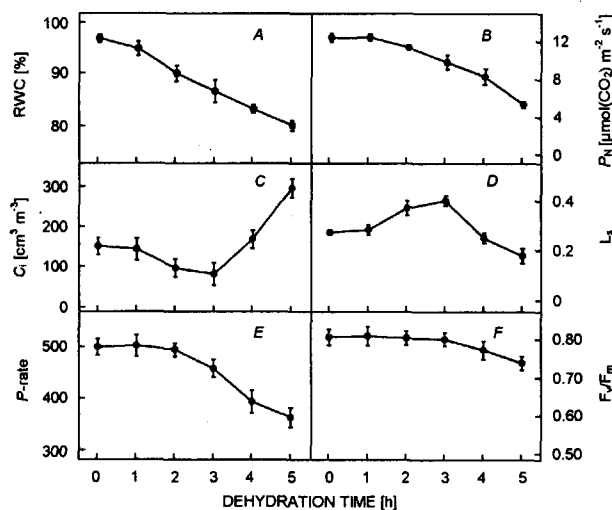

Fig. 1. Development of (A) relative water content (RWC), (B) net photosynthetic rate (P_N), (C) intercellular CO_2 concentration (C_i), (D) stomatal limitation (L_s), (E) PS2 non-cyclic electron transport (P-rate), and (F) PS2 maximal photochemical activity (F_v/F_m) in detached apple leaves during dehydration under irradiance of 100 μmol m^{-2} s^{-1}, 28±2 °C, and relative humidity of 75±5 %. After a given time of dehydration, leaves were irradiated at 800 μmol m^{-2} s^{-1} for 10 min and then all the measurements were carried out except an additional 20 min dark time being applied in the F_v/F_m test. Means ($n = 4$) with standard deviations.

and dropped at moderate drought (Fig. 1D), which was contrary to C_i changes (Fig. 1C). Decrease in P_N was attributed to stomatal and non-stomatal limitation during the slight and moderate drought according to the definition of Farquhar and Sharkey (1982). Dehydration also

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.

Photoreaction 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_2O_2, O_2^-, $^1O_2$, and ·OH were produced in detached apple leaves (Fig. 3) following the decrease in RWC. H_2O_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 $^1O_2$ and ·OH had a noticeable rise at 5-h dehydration (Fig. 3C,D). Change of H_2O_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_2O_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 photorespiration at slight drought and upon Mehler reaction at moderate drought occurred in irradiated detached

apple leaves, which directly caused the rise in H_2O_2 and O_2^- production. When $NaHSO_3$ inhibited photorespiration, both the Rfp increase and P-rate decrease were accelerated at slight drought (Fig. 4A,B). Especially at 2-h
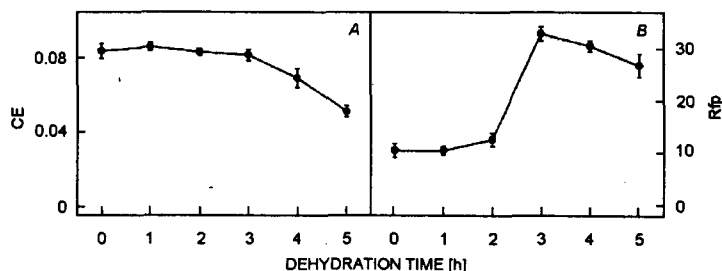

Fig. 2. Changes of (A) carboxylation efficiency (CE) and (B) rate of post-irradiation transient increase in chlorophyll fluorescence (Rfp) in detached apple leaves during dehydration. The experimental process was the same as in Fig. 1. Means ($n = 4$) with standard deviations.
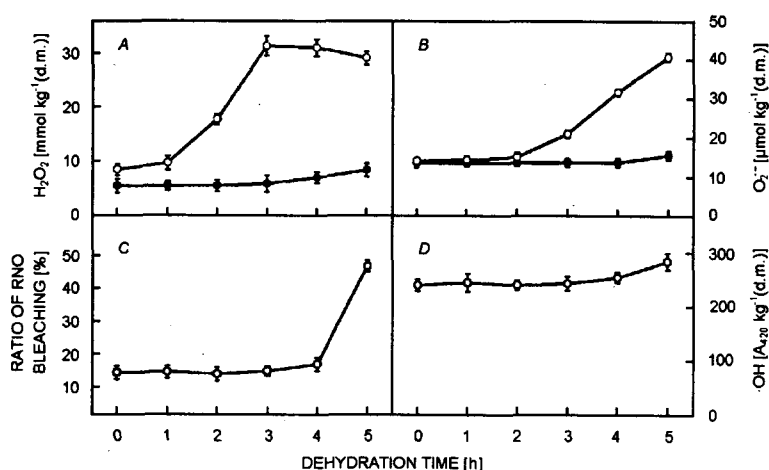

Fig. 3. Changes in AOS production of (A) hydrogen peroxide (H_2O_2), (B) superoxide (O_2^-), (C) singlet oxygen ($^1O_2$), and (D) hydroxyl radical (·OH) under irradiance of 800 (o) or 400 (●) μmol m^{-2} s^{-1} PPFD in detached apple leaves during dehydration. Production of $^1O_2$ and ·OH was reflected by the ratio of RNO bleaching in the absorbance at 420 nm, respectively. Leaves were irradiated at 800 or 400 μmol m^{-2} s^{-1} for 10 min after dehydration for a given time. Means ($n = 4$) with standard deviations.

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 photorespiration 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 photorespiration 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 photoinhibition through its photooxidation on photosynthetic apparatus (Sakaki *et al.* 1983). The AOS production and its relation to photoinhibition in detached apple leaves were investigated under irradiation. We found that changes in AOS production, H_2O_2
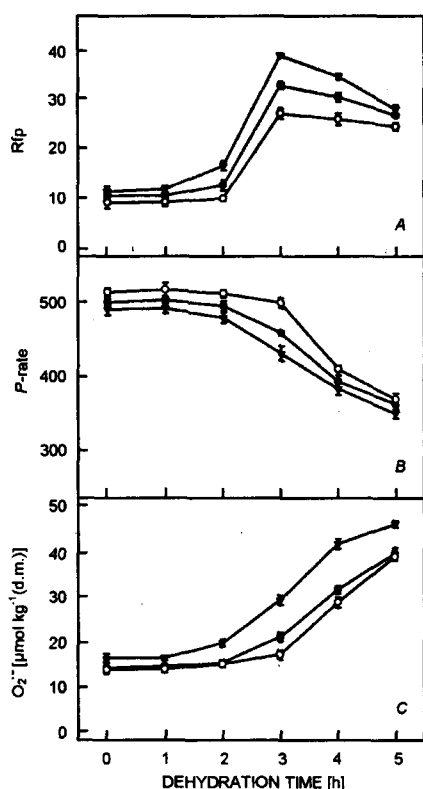
Fig. 4. Changes in (A) Rfp, (B) P-rate, and (C) production of O_2^- in detached apple leaves pre-treated with Gly (o) or . NaHSO$_3$ (▼), and control (●) during dehydration. The chemical treatments are described in Materials and methods, and the experimental process is the same as in Fig. 1. Means ($n = 4$) with standard deviations.
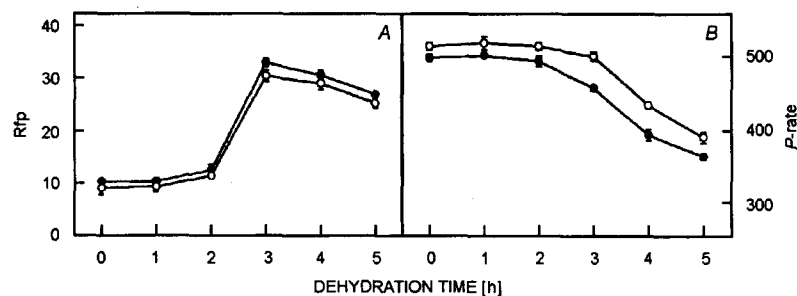
and O_2^- appeared prior to photoinhibition in detached apple leaves during dehydration (Figs. 1 and 3), but this AOS production could be effectively postponed by lowering irradiance (Fig. 3). Hence AOS production was originally induced by irradiation in detached apple leaves during dehydration but was 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 photorespiration 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_N 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 photorespiration, the excess assimilatory power could be metabolised and only a negligible ascent of Rfp was found at 2-h dehydration (Fig. 2B). If NaHSO$_3$ inhibited photorespiration, 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 photorespiration 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.

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.

With the dehydration time passing, photosynthetic CO_2 assimilation was increasingly blocked by water deficiency (Fig. 1B). Though P_N 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 photorespiration even with the help of Mehler reaction, and photoinhibition occurred unavoidably (Fig. 1E). At 3-h dehydration, increase either in photorespiration 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 photorespiration 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 photorespiration under moderate drought in detached apple leaves.

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