Cooperation of xanthophyll cycle with water-water cycle in the protection of photosystems 1 and 2 against inactivation during chilling stress under low irradiance

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Abstract

The xanthophyll cycle and the water-water cycle had different functional significance in chilling-sensitive sweet pepper upon exposure to chilling temperature (4 °C) under low irradiance (100 μmol m^{-2} s^{-1}) for 6 h. During chilling stress, effects of non-photochemical quenching (NPQ) on photosystem 2 (PS2) in dithiothreitol (DTT) fed leaves remained distinguishable from that of the water-water cycle in diethyldithiocarbamate (DDTC) fed leaves. In DTT-fed leaves, NPQ decreased greatly accompanied by visible inhibition of the de-epoxidized ratio of the xanthophyll cycle, and maximum photochemical efficiency of PS2 (F_v/F_m) decreased markedly. Thus the xanthophyll cycle-dependent NPQ could protect PS2 through energy dissipation under chilling stress. However, NPQ had a slighter effect on photosystem 1 (PS1) in DTT-fed leaves than in DDTC-fed leaves, whereas effects of the water-water cycle on PS1 remained distinguishable from that of NPQ. Inhibiting superoxide dismutase (SOD) activity increased the accumulation of $O^{\cdot-}_2$, the oxidation level of P700 ($P700^+$) decreased markedly relative to the control and DTT-fed leaves. Both F_v/F_m and NPQ changed little in DDTC-fed leaves accompanied by little change of (A+Z)/(V+A+Z). This is the active oxygen species inducing PS1 photoinhibition in sweet pepper. The water-water cycle can be interrupted easily at chilling temperature. We propose that during chilling stress under low irradiance, the xanthophyll cycle-dependent NPQ has the main function to protect PS2, whereas the water-water cycle is not only the pathway to dissipate energy but also the dominant factor causing PS1 chilling-sensitivity in sweet pepper.

Additional key words: *Capsicum*; chlorophyll fluorescence; irradiance; temperature.

Introduction

Chilling temperatures increase the sensitivity of plants to photoinhibition due primarily to restricted photosynthetic energy utilization that results from low temperature-imposed limitations on enzymes involved in carbon metabolism (Krause 1994, Huner *et al.* 1998). Our studies showed that chilling stress under low irradiance could cause the decrease of photosynthesis and photoinhibition of both photosystems, PS2 and PS1 (Li *et al.* 2003, 2004b). The maximal photochemical efficiency of PS2 (F_v/F_m) and the oxidation level of P700 are often used to reflect the extent of photoinhibition of PS2 and PS1, respectively. Some energy dissipation mechanisms alleviate photoinhibition (van Kooten and Snel 1990, Sonoike *et al.* 1997). Photosynthetic organisms trying to remain competitive have sought out the delicate balance between efficient light-harvesting under limited irradiance and regulated energy dissipation under excess irradiance. One of the protective mechanisms is thermal energy dissipation in the light-harvesting antenna complexes of PS2 measured as non-photochemical quenching (NPQ), which protects the photosynthetic machinery from inactivation and damage caused by excess irradiance (Horton *et al.*

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Abbreviations: A – antheraxanthin; DDTC – diethyldithiocarbamate; DTT – dithiothreitol; F_m – maximum yield of fluorescence after dark adaptation; F_m' – maximum yield of fluorescence in light-acclimated leaves; F_0 – initial fluorescence; F_v/F_m – maximum photochemical efficiency of PS2 with all reaction centres open; FM – fresh mass; NPQ – non-photochemical quenching; P700 – reaction centre chlorophyll of PS1; PFD – photon flux density; PS1 – photosystem 1; PS2 – photosystem 2; SOD – superoxide dismutase (EC 1.15.1.1); V – violaxanthin; Z – zeaxanthin.
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1994). At chilling temperatures under irradiance, NPQ may be an efficient pathway to alleviate photoinhibition (Xu *et al.* 1999, Liu *et al*. 2001, Li *et al*. 2003, 2004a). The xanthophyll cycle mediating the dissipation process contributes to increased stability of the photosynthetic apparatus under excess irradiance at low temperatures (Xu *et al.* 1999).

Another important mechanism is the chloroplast water-water cycle, which is referred to the electrons from water generated in PS2 reducing atmospheric O_2 to water in PS1 without a net change of O_2. It is an effective mechanism under environmental stress (Asada 1999). However, the water-water cycle can be interrupted easily under stress, especially at chilling temperature (Jakob and Heber 1996, Sonoike 1996, Sonoike *et al.* 1997, Li *et al.* 2003, 2004b). During chilling stress, active oxygen species are produced through the cycle because enzyme activity in the Calvin cycle is slowed down, and the $NADP^+$ supplement to accept electron from the electron transport chain is restricted, leading to excess energy absorption by O_2, and active oxygen species are attributed to photoinhibition of PS1 (Sonoike 1996, Sonoike *et al.* 1997, Li *et al*. 2003, 2004b). Using thylakoids or intact chloroplasts of spinach, Jakob and Heber (1996) found that PS1 photoinhibition occurred concomitant with the accumulation of active oxygen species. Sonoike *et al*. (1997) proposed that active oxygen species are also related to the degradation of the psaB protein.

The xanthophyll cycle may transform harmlessly the excitation energy into heat and thereby prevent the formation of damaging active oxygen species. In this way it protects the photosynthetic apparatus against photodamage (Demmig-Adams and Adams 1996, Huner *et al*. 1998, Müller *et al*. 2001, Li *et al.* 2003, 2004b). The roles of the xanthophyll cycle and the water-water cycle have been discussed in relation to different chilling-sensitive plants (Li *et al*. 2003). However, the cooperation of xanthophyll cycle with water-water cycle in the protection of PS1/PS2 photoinhibition against inactivation in one plant seems unclear. Photoinhibition has often been studied under violent conditions, which usually cause severe photoinhibition of PS2 and thus cover up PS1 responses and neglect PS1 photoinhibition (Powles 1984). Even recent studies on PS1 photoinhibition *in vitro* or *in vivo* seldom mention the protection by the xanthophyll cycle (Li *et al.* 2003). In the present study, the chilling-sensitive sweet pepper was exposed to mild stress conditions at 4 °C under a weak irradiance of 100 μmol m^{-2} s^{-1} photon flux density (PFD). Under such conditions, PS2 was inhibited slightly and PS1 photoinhibition was reflected in an absorbance change at 820 nm.

Materials and methods

Plants and chilling treatments: One chilling-sensitive line of sweet pepper (*Capsicum annuum* L. line 156) was used in the experiment. Seeds were first germinated between moistened filter paper at 25 °C for 3 d. Sprouted burgeons were then planted into plastic pots (one plant per pot) filled with sterilized soil and grown at 25–30/15–20 °C (day/night) under 14 h of irradiation (300–400 μmol m^{-2} s^{-1} PFD) in a greenhouse. Functional leaves from plants were used in the experiment. With the adaxial side face-up, the detached leaves with petiole dipped into water were put in the growth chamber at 4 °C for 6 h. Fluorescent lamps provided 100 μmol m^{-2} s^{-1} PFD; this irradiance is in the range of photosynthetically active radiation.

Chlorophyll *a* fluorescence: The photochemical efficiency of PS2 expressed as the ratio of variable fluorescence (F_v) to maximum yield of fluorescence (F_m) was measured with a portable fluorometer (*FMS2*, *Hansatech*, England) according to the protocol described by van Kooten and Snel (1990). Non-photochemical quenching (NPQ) was calculated according to Schreiber *et al.* (1994). NPQ was estimated as $NPQ = F_m/ F_m' - 1$, where F_m was measured after dark adaptation for more than 2 h at room temperature prior to chilling treatments, and F_m' is the maximum yield of fluorescence in light-acclimated leaves.

820 nm absorbance: Oxidation and reduction of P700 was measured at 820 nm with a *PAM* fluorometer using an emitter-detector unit *ED800T* (*Walz*, Effeltrich, Germany) (Endo *et al*. 1999).

Determination of SOD activities and $O_2^{\cdot-}$ measurement: 0.5 g fresh mass (FM) of leaves without midrib were thoroughly ground with a cold mortar and pestle in an ice bath. The grinding medium was 4 cm^3 of 0.05 M phosphate buffer (pH 7.8) plus homogenizing glass beads. The homogenate was centrifuged at 300×*g* for 15 min at 0–4 °C. The supernatant, hereafter referred to as crude SOD extract, was used for determination.

SOD assay described by Giannopolitis and Ries (1977) was modified. The reaction mixture was composed of 13 mM methionine, 75 μM NBT, 10 μM EDTA-Na_2, and 2 μM riboflavin, and the appropriate volume of extract (in the blanks, 0.05 M phosphate buffer of pH 7.8 instead of extract). Distilled H_2O was added to bring the final volume to 3 cm^3. The mixtures were irradiated in glass tubes selected for uniform thickness and colour. Identical solutions that were not irradiated served as blanks. The absorbance at 560 nm of the reaction mixture was determined using a UV-visible spectrophotometer (*UV-1601*, *Shimadzu*. Japan) at room temperature.

The assay for $O_2^{\cdot-}$ was as described by Wang and Luo (1990). Fresh leaves without midrib were thoroughly ground with a cold mortar and pestle in an ice bath. The

grinding medium was 0.05 M phosphate buffer (pH 7.8) plus homogenizing glass beads. The homogenate was centrifuged at 5 000×*g* for 10 min at 0–4 °C. The supernatant with phosphate buffer (pH 7.8) and 10 mM hydroxylammonium chloride was incubated at 25 °C for 20 min, then 17 mM *p*-aminobenzene sulfonic acid and 7 mM α-naphthylamine were added, and the mixture was incubated at 25 °C for 20 min. Finally, ethyl ether was added into the mixture that was centrifuged at 1 500×*g* for 5 min. The water phase was used to determine the absorbance at 530 nm. The $O^{\cdot-}_2$ generation was calculated per g fresh mass of leaves.

Determination of soluble protein: 0.2 cm^3 of crude SOD extract was added to 2.8 cm^3 of Coomassie brilliant blue *G-250* to react for 2 min. Then the absorbance of the reaction mixture was determined at 595 nm.

Pigment analysis: Leaf discs were immersed in liquid N_2 immediately after F_v/F_m determination and stored at –80 °C until use. Photosynthetic pigments were extracted with 0.5 g of ice-cold acetone and quantified by reversed-phase high performance liquid chromatography (HPLC) based on a method described by Li *et al.* (2003).

Chemical feeding: DTT was used as a special inhibitor of violaxanthin de-epoxidase (Xu *et al.* 1999), which is one of the key enzymes in the xanthophyll cycle. Leaves were cut at the end of the leaf stalk and placed in vials containing 10 mM DTT or water for the control. Then, the leaves were exposed to 50 µmol(photon) m^{-2} s^{-1} at 25 °C for 4 h. Diethyldithiocarbamate (DDTC) was used in the experiments to specially inhibit activity of SOD, which is one of the key enzymes in the water-water cycle. According to Miszalski (1991), 2 h before the first measurement the leaves were sprayed with DDTC dissolved in 0.04 mM phosphate buffer (pH 7.8) (the final concentration was 1 %) and the control leaves were sprayed with the buffer alone.

Results

The xanthophyll cycle in the protection of PS1/PS2 photoinhibition: Effects of DTT on changes in NPQ and the de-epoxidized ratio of the xanthophyll cycle in chilling-sensitive sweet pepper leaves during 6 h chilling stress under low irradiance are shown in Fig. 1. NPQ decreased markedly in DTT-fed leaves relative to that of the control during stress (Fig. 1*A*). Similarly, the de-epoxidized ratio of the xanthophyll cycle, (A+Z)/(V+A+Z), also decreased (Fig. 1*B*). At the end of stress, (A+Z)/(V+A+Z) was about 61.3 % in the control and 33.2 % in DTT-fed leaves, respectively. NPQ is dependent on the de-epoxidized xanthophyll cycle components Z and A (Demmig-Adams and Adams 1996).

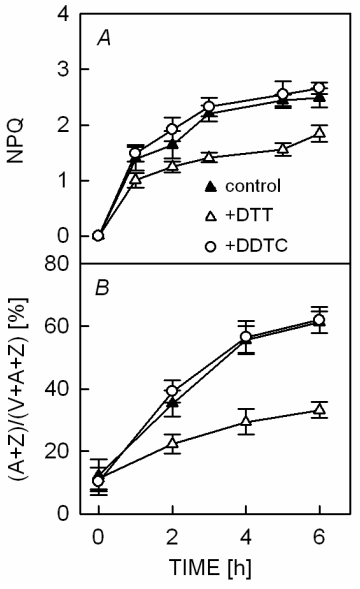

Fig. 1. Development of non-photochemical quenching (NPQ) (*A*) and the xanthophyll cycle conversion state (A+Z)/(V+A+Z) (*B*) in chilling-sensitive sweet pepper leaves at chilling temperature (4 °C) under low irradiance (100 µmol m^{-2} s^{-1} PFD). Means±SD of 5 (*A*) or 3 (*B*) measurements on separate leaves.

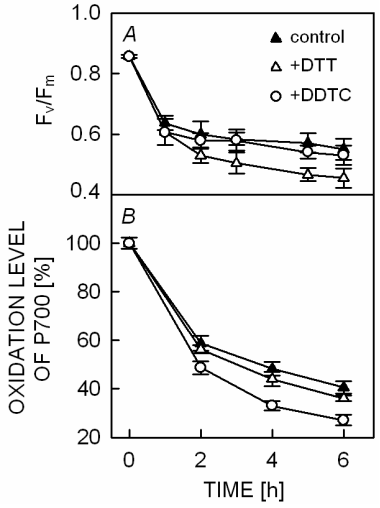

Fig. 2. Responses of maximum quantum efficiency of PS2 (F_v/F_m) (*A*) and $P700^+$ (*B*) in chilling-sensitive sweet pepper leaves at chilling temperature (4 °C) under low irradiance (100 µmol m^{-2} s^{-1} PFD). Means±SD of 5 (*A*) or 3 (*B*) measurements on separate leaves.

To study the effects of the xanthophyll cycle on protection to photosystems, we monitored the changes in F_v/F_m and the oxidation level of P700 ($P700^+$) in DTT-fed leaves (Fig. 2). F_v/F_m and $P700^+$ are used to reflect photoinhibition of PS2 and PS1, respectively. F_v/F_m decreased markedly during 6 h of chilling stress relative to the

control (Fig. 2*A*), and F_v/F_m decreased by 35.6 and 46.8 % in the control and DTT-fed leaves at the end of chilling stress, respectively. Inhibition of formation of Z and A aggravated photoinhibition of PS2. However, feeding DTT had slighter effect on PS1 than feeding DDTC. $P700^+$ decreased slightly in DTT-fed leaves during chilling stress under low irradiance relative to that in DDTC-fed leaves (Fig. 2*B*).

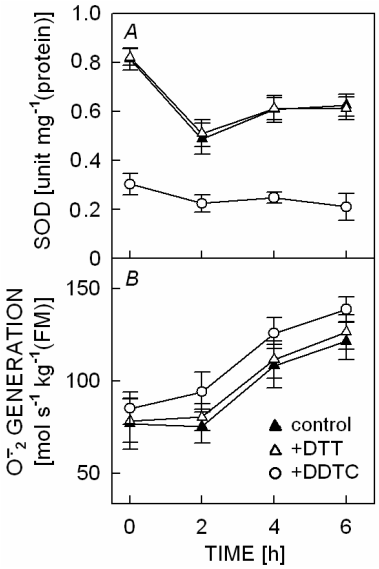

Fig. 3. Development of SOD activity (*A*) and the photogeneration of $O^{\cdot-}_2$ (*B*) in chilling-sensitive sweet pepper leaves at chilling temperature (4 °C) under low irradiance (100 μmol m^{-2} s^{-1} PFD). Means±SD of 3 measurements on separate leaves.

Cooperation of xanthophyll cycle with water-water cycle during chilling stress: Chilling temperature inhibits carbon metabolism (Krause 1994, Huner *et al.* 1998, Li *et al.* 2004b), thus more electrons might transfer to O_2 during chilling and active oxygen species accumulated in sweet pepper leaves (Fig. 3). $O^{\cdot-}_2$ is the primary product when electrons are transferred to O_2. In DTT-fed leaves, the accumulation of $O^{\cdot-}_2$ induced no visible increase (Fig. 3*B*) and SOD activity did not change (Fig. 3*A*) relative to that of the control during chilling under low irradiance.

To study the effects of water-water cycle on PS1 photoinhibition, changes in SOD activity and accumulation of active oxygen species were determined in sweet pepper leaves (Fig. 3). Feeding with DDTC, SOD activity decreased markedly relative to the control (Fig. 3*A*), and the generation of $O^{\cdot-}_2$ increased evidently (Fig. 3*B*) during chilling. However, DDTC had different effects than DTT on NPQ and the xanthophyll cycle. The de-epoxidized ratio of the xanthophyll cycle and NPQ changed little in DDTC-fed leaves during 6 h chilling stress (Fig. 1).

The water-water cycle in the protection of PS1/PS2 photoinhibition: In DDTC-fed leaves, F_v/F_m changed little relative to the control during chilling (Fig. 2*A*). Thus the water-water cycle had little effect on PS2 reaction centres in sweet pepper leaves at 4 ºC and 100 μmol m^{-2} s^{-1} PFD measured for 6 h. However, $P700^+$ decreased markedly in DDTC-fed leaves relative to the control and DTT-fed leaves at the end of stress (Fig. 2*B*). At the end of chilling, $P700^+$ decreased by 60.1 % in the control and by 72.9 % in DDTC-fed leaves, respectively. It implies that the active oxygen species caused the decrease of $P700^+$ (Fig. 2*B*).

Discussion

The roles of NPQ and xanthophyll cycle: The xanthophyll cycle-dependent NPQ is a very important mechanism to protect PS2 under excess irradiance, *e.g.* at chilling temperatures (Demmig-Adams and Adams 1996, Xu *et al.* 1999, Liu *et al.* 2001, Li *et al.* 2003, 2004a). When leaves were fed with DTT, the formation of Z and A and energy dissipation by NPQ decreased markedly (Fig. 1) and photoinhibition of PS2 in sweet pepper leaves became more severe than those in the control and DDTC-fed leaves (Fig. 2*A*). The decrease of the de-epoxidized ratio of the xanthophyll cycle induced a slight change of $P700^+$ in DTT-fed leaves during 6 h of chilling (Fig. 2*B*). In DTT-fed leaves, the surplus energy might aggravate photoinhibition of PS2 protecting PS1 from further photoinhibition (Tjus *et al.* 1998, Li *et al.* 2003).

When chilling-sensitive sweet pepper was exposed to 4 °C under 100 μmol m^{-2} s^{-1} PFD for 6 h, the xanthophyll cycle-dependent energy dissipation protected PS2 reaction centres. The xanthophyll cycle components exist in PS1 segments of cotton and maize leaves, where V is converted to Z (Thayer and Björkman 1992). The decrease of $P700^+$ in DTT-fed leaves confirms our previous results (Li *et al.* 2004a). When the xanthophyll cycle was inhibited, the reducing state of Q_A became more severe even if the decrease of F_v/F_m limited the electron transport to PS1. Thus, stroma components were over-reduced, and the decrease of $P700^+$ was induced (Li *et al.* 2004a). There are several possibilities relative to the slight effect of the xanthophyll cycle on PS1 in sweet pepper during chilling under low irradiance (Fig. 2*B*): first, the xanthophyll cycle does not exist in PS1 of sweet pepper leaves. Second, the xanthophyll cycle exists in PS1, but its protection of PS1 is so weak that it could not be reflected fully by inhibiting with DTT under such conditions. Third, the xanthophyll cycle can protect PS1 efficiently, but PS2 photoinbition protects PS1 from further photoinhibition (Tjus *et al.* 1998), since photoinhibition is more severe in PS2 in DTT-fed leaves

than in the control (Fig. 2*A*).

The roles of the water-water cycle: On one hand, plants can use O_2 as terminal electron acceptor in both photorespiration and Mehler reaction to protect the chloroplasts from photodamage (Osmond and Grace 1995, Li *et al*. 2004b). Asada (1999) proposed that water-water cycle is an effective mechanism under stress. On the other hand, the production of active oxygen species might be related to more electrons transported to Mehler reaction accompanied by the inhibition of the Calvin cycle and photorespiration during chilling stress (Li *et al.* 2003, 2004b). Thus, the water-water cycle played double role at 4 °C under 100 μmol m^{-2} s^{-1} PFD during a short-term stress. Despite the activity of SOD involved in the water-water cycle kept higher relative to that in DDTC-fed leaves, the accumulation of $O^{\cdot-}_2$ in the control and DTT-fed leaves showed that activity of SOD decreased upon exposure to chilling temperature under low irradiance (Fig. 3). Inhibiting the water-water cycle by DDTC, the decrease of $P700^+$ (Fig. 2*B*) followed the increase of $O^{\cdot-}_2$ (Fig. 3*B*). However, inhibiting SOD activity had little influence on energy dissipation in PS2. In DDTC-fed leaves during chilling stress, NPQ and the ratio of (A+Z)/(A+Z+V) did not evidently increase (Fig. 1). The more severe photoinhibition of PS1 relative to PS2 was attributed to the increase of $O^{\cdot-}_2$ in DDTC-fed leaves (Fig. 3*B*). Thus the water-water cycle is important for PS1 at chilling temperature under low irradiance. The water-water cycle not only dissipates excess energy, but also induces photoinhibition of PS1 under chilling.

We conclude that during the 6-h stress under 100 μmol m^{-2} s^{-1} at 4 °C, the main function of the xanthophyll cycle-dependent NPQ was to protect PS2. However, the water-water cycle is not only the pathway to dissipate excess energy but also the dominant factor of PS1 chilling-sensitivity in sweet pepper.

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