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Increase in resistance to low temperature photoinhibition following ascorbate feeding is attributable to an enhanced xanthophyll cycle activity in rice (*Oryza sativa* L.) leaves

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Abstract

The mechanistic basis for protection of exogenous ascorbate against photoinhibition at low temperature was examined in leaves of rice (*Oryza sativa* L.). Exposure of intact leaves to chilling temperature resulted in a drastic decrease in the speed of development of non-photochemical fluorescence quenching (NPQ). This was related to the low temperature-imposed restriction on the formation of the fast relaxing component of NPQ (q_f). Feeding with 20 mM ascorbate markedly increased the rate of q_f development at chilling temperature due primarily to the enhanced rate of zeaxanthin (Z) formation. On the other hand, ascorbate feeding had no influence on photosystem 2 (PS2)-driven electron flow. The reduced state of the PS2 primary electron acceptor Q_A decreased in ascorbate-fed leaves exposed to high irradiance at chilling temperature owing to the increased Z-associated thermal energy dissipation in the light-harvesting antenna system of PS2. Furthermore, ascorbate feeding increased the photosynthetic apparatus of rice leaves to resist photoinhibition at low temperature. The protective effect of exogenous ascorbate was fully accounted for by the enhanced xanthophyll cycle activity.

Additional key words: antheraxanthin; chilling; chlorophyll fluorescence; high irradiance; photochemical quenching; thermal energy dissipation; violaxanthin; zeaxanthin.

Introduction

Photoinhibition results from over-excitation of the photosynthetic apparatus (Powles 1984). It occurs when plants absorb more photons than can be utilised through photosynthesis. Chilling temperatures increase the sensitivity of plants to photoinhibition due primarily to restricted photosynthetic energy utilisation that results from low temperature-imposed limitations on enzymes involved in carbon metabolism (Krause 1994, Huner *et al.* 1998).

Plants have developed a range of mechanisms to protect their photosynthetic machinery against over-excitation (Anderson *et al.* 1997). One such protective mechanism that has received much recent attention is the thermal dissipation of the excess radiant energy in the light-harvesting antenna complexes of PS2 measured as NPQ (for reviews see Demmig-Adams and Adams 1996, Horton *et al.* 1996, Gilmore 1997). The exact mechanisms of NPQ are still under extensive investigation. Nevertheless, it has been demonstrated that a major proportion of NPQ is a result of the combined effects of the lumen acidification and deepoxidation of violaxanthin (V) into Z and antheraxanthin (A) in the xanthophyll cycle (Horton *et al.* 1996, Eskling *et al.* 1997, Gilmore 1997). A recent hypothesis emphasizes the importance of the structural flexibility of the thylakoid membrane in controlling the energy dissipation (Horton 1999). This mechanism ameliorates the excitation pressure on PS2 by converting excitation energy harmlessly into heat and

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Abbreviations: A - antheraxanthin; Asc – ascorbate; Chl - chlorophyll; DTT - dithiothreitol; ETR - *in vivo* PS2-driven electron transport rate; F_m and F_m' - maximal fluorescence with all PS2 reaction centres closed in the dark- and light-adapted state, respectively; F_s - steady-state fluorescence in light; F_v - variable fluorescence in the dark-adapted state; NPQ - non-photochemical quenching; PPFD - photosynthetic photon flux density; PS - photosystem; Q_A - the primary stable quinone electron acceptor of PS2; q_f - fast relaxing non-photochemical quenching; q_I - slowly relaxing non-photochemical quenching; q_P - photochemical quenching; V – violaxanthin; Z - zeaxanthin.
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thereby preventing the formation of damaging reactive oxygen species; it thus protects the photosynthetic apparatus against photodamage (Demmig-Adams and Adams 1996, Huner *et al.* 1998).

The control of deepoxidation of V into Z *via* intermediate A is achieved primarily through the enzyme deepoxidase (Yamamoto 1979). This enzyme is located in the lumen of thylakoids and is activated by the light-driven acidification of the thylakoid lumen (Hager 1969). The activity of the deepoxidase is also co-regulated by the concentration of ascorbate, an essential co-substrate of deepoxidase (Bratt *et al.* 1995).

Ascorbate is an important antioxidant in plant tissues. It non-enzymically scavenges hydrogen peroxide, superoxide, and lipid peroxide, and serves as a reductant for ascorbate peroxidase, an enzyme responsible for removing hydrogen peroxide generated in pseudocyclic photosynthetic electron transport (Asada 1996). The non-assimilatory linear electron flow mediated by the Mehler-peroxidase reaction cycle consumes reducing equivalents and generates the transmembrane proton gradient necessary for V deepoxidation and associated NPQ under limited CO_2-fixation (Neubauer and Yamamoto 1993). Thereby it protects the photosynthetic apparatus against photoinhibitory damage (Biehler and Fock 1996, Park *et al.* 1996).

Ascorbate increases the ability of the photosynthetic apparatus to resist photoinhibition (Asada 1996). The mechanistic basis for ascorbate protection has been related to its antioxidant activity (Richter *et al.* 1990), its action as a co-factor for violaxanthin deepoxidase (Forti *et al.* 1999), and its stimulating effect on non-assimilatory electron flow (Calatayud *et al.* 1999). Under photoinhibitory conditions, ascorbate protects the thylakoid membranes against an irreversible loss of the light-induced structural changes associated with energy dissipation (Gussakovsky *et al.* 1997, Istokovics *et al.* 1997). So far, however, there is a paucity of information about the role of ascorbate in the protection against low temperature photoinhibition. We addressed this issue in the present study with an emphasis on the mechanistic basis for such a protection.

Materials and methods

Plants and photoinhibition treatments: Rice (*Oryza sativa* L.) cv. Lemont was grown from seed under a 14-h photoperiod at 25°C. The photosynthetic photon flux density (PPFD) during growth was about 100 μmol m^{-2} s^{-1}. Fully expanded leaves of 3-week-old plants were used in all experiments.

For photoinhibition treatments, 3 cm-long leaf segments were floated on water in Petri dishes with the adaxial side face-up. They were exposed to a PPFD of 1000 μmol m^{-2} s^{-1} (a set of halogen lamps, essentially as described Xu *et al.* 1999) at 10 or 25 °C for 2 h.

Chemical feeding: Leaves were excised at the base of epicotyl and allowed to take up a solution containing 20 mM ascorbate, adjusted to pH 6.25 with KOH, or/and 3 mM dithiothreitol (DTT) through transpiration stream at about 15 μmol m^{-2} s^{-1} and 25 °C for 3 h. Control leaves were fed with water only. A fan was used to facilitate the uptake of the chemicals by increasing the leaf transpiration.

Measurement of chlorophyll (Chl) fluorescence: Chl *a* fluorescence was analysed using a pulse-amplitude modulated fluorometer (*PAM-2000, Walz*, Effeltrich, Germany). Photochemical quenching (q_P) and non-photochemical quenching (NPQ) was calculated according to Schreiber *et al.* (1994). NPQ was estimated from the Stern-Volmer equation as $(F_m - F_m')/F_m'$, where F_m' and F_m are maximum yields of fluorescence in light-acclimated or dark-adapted leaves, respectively. In this case, F_m was measured after a 20-min dark adaptation at room temperature prior to chilling. NPQ was resolved into fast relaxing (q_f) and slowly relaxing (q_l) components by extrapolation in semi-logarithmic plots of the maximum fluorescence yield *versus* time as described by Johnson *et al.* (1993). The relative PS2-driven electron transport rate (ETR) was calculated as $(1 - F_s/F_m')\times 0.5\times$ PPFD×leaf absorptance according to Genty *et al.* (1989), where F_s is the steady-state fluorescence yield, 0.5 is a factor assuming an equal distribution of absorbed photons between PS2 and PS1, and leaf absorptance is taken as 0.85. The sustained decrease in the ratio of F_v/F_m, recorded after dark adaptation for 20 min, served as an estimate of the extent of photoinhibition. The maximum variable fluorescence F_v was obtained by subtraction of the initial fluorescence (F_0) from F_m.

Pigment analysis: Leaf segments for pigment analysis were frozen in liquid nitrogen and stored until analysed. Leaf samples were powdered in liquid nitrogen with a mortar and pestle and extracted in ice-cold 100 % acetone. The extracts were centrifuged at 20 000×*g* for 5 min and the resulting supernatants were filtered through a 0.45 μm membrane filter before injection into HPLC. Photosynthetic pigments were separated and quantified essentially following the protocol of Thayer and Björkman (1990). Details of procedures for measurements with HPLC were described by Xu *et al.* (1999).

Results

The time course of changes in NPQ estimated by the Stern-Volmer equation $F_m/F_m' - 1$ in rice leaves exposed to a PPFD of 1000 μmol m^{-2} s^{-1} at two different temperatures is shown in Fig. 1*A*. As shown by Bilger and Björkman (1991), Adams *et al.* (1994), and Arvidsson *et al.* (1997), the rate of NPQ development was much slower at chilling temperature relative to that observed at warm temperature. The maximal level of NPQ reached after 2 h of treatment was, however, significantly higher at 10 than 25 °C.

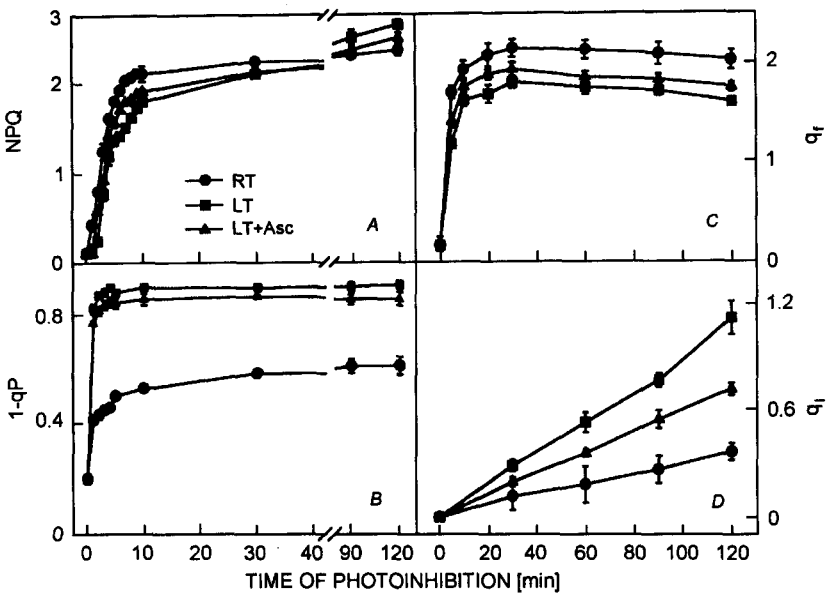

Fig. 1. The development of (*A*) non-photochemical fluorescence quenching (NPQ) and its (*C*) fast (q_f) and (*D*) slowly (q_I) relaxing components, and (*B*) changes in the reduction state of Q_A (1 - q_P), in rice leaves exposed to a PPFD of 1000 μmol m^{-2} s^{-1} at 25 °C (RT) or 10 °C (LT). The figure shows also the effects of ascorbate (Asc) feeding. To compute the NPQ, the parameter F_m for all samples was measured after dark adaptation for 20 min before high irradiation. F_m' at zero time of treatment was measured after pre-irradiation for 30 min under growth conditions. The two NPQ components were resolved by extrapolation in semi-logarithmic plots of the maximum fluorescence yield *versus* time as described by Johnson *et al.* (1993). In *B*, the values at zero time of treatment were measured after pre-irradiation for 30 min under growth conditions. For ascorbate pre-treatment, leaves were fed with 20 mM ascorbate at 15 μmol m^{-2} s^{-1} at 25 °C for 3 h. Error bars indicate the standard error (n = 3-5). Where not present, the errors were smaller than the symbol size.

To distinguish between different components of NPQ, the dark-relaxation kinetics after various periods of high irradiance were analysed. Two components of NPQ, namely q_f and q_I, were resolved following the protocol of Johnson *et al.* (1993). The parameter q_f presumably includes the contributions of both the energy-dependent quenching and quenching associated with state transitions. q_I is an estimate of the quenching that either relaxes slowly or not at all, and therefore includes components related to slowly reversible photoprotective processes and photoinhibitory damage (Krause and Weis 1991). The time course of changes in q_f and q_I is depicted in Fig. 1*C,D*. The fast relaxing component of quenching increased rapidly at 25 °C, reaching its maximum after 30 min of treatment. The speed of q_f development and maximum level of q_f were much lower at 10 than at 25 °C. On the other hand, the rate of development of q_I was markedly accelerated at chilling temperature relative to that observed at warm temperature. Clearly, at chilling temperature, the decreased rate of NPQ development in the initial stage of exposure to high irradiance at 10 °C is the result of the inhibited development of q_f. A comparison of Figs. 1*A,C*, and *D* reveals that the continued increase in NPQ after q_f reaching its maximum is attributable to the rise of q_I.

As shown in Fig. 1*A*, feeding of rice leaves with 20 mM ascorbate markedly increased the speed of NPQ development at 10 °C. This was mainly due to the ascorbate-induced increase in the formation of q_f (Fig. 1*C,D*). Because a major proportion of q_f is related to the energization of thylakoid membrane (Krause and Weis 1991), the ascorbate-induced increase in the formation of q_f may be explained by its role in the stimulation of linear electron flow and therefore in the build-up of a transmembrane proton gradient. However, an analysis of Chl fluorescence quenching revealed no appreciable effect of ascorbate on PS2-driven electron flow (Fig. 2). On the other hand, ascorbate feeding caused a significant

decrease in the state of Q_A reduction measured as the fluorescence parameter 1 - q_P (Fig. 1*B*).

The formation of deepoxidized pigments of xanthophyll cycle was strikingly reduced by low temperature (Fig. 3*A*). Remarkably, feeding of rice leaves with 20 mM ascorbate substantially increased both the rate of Z formation (Fig. 3*A*) and the rate of NPQ development (Fig. 1*A*). The maximum V to Z conversion was, however, not influenced by ascorbate feeding (Fig. 3*A*). The ascorbate concentration used in our experiments seemed to be saturated for both Z deepoxidation and NPQ formation since rising the concentration of ascorbate further produced no significant effects (values not shown). These results suggest that the increased formation of q_f after ascorbate feeding may be explained by its action as a co-substrate for deepoxidase in the xanthophyll cycle. They also show that in rice leaves exposed to chilling temperature the reduced activity of the xanthophyll cycle is, at least partially, due to the limitation of ascorbate, the co-substrate for deepoxidase. Feeding with 20 mM ascorbate also caused small increases in rates of both Z formation and NPQ development in rice leaves during the initial several minutes of high irradiation at room temperature (values not shown). In this case, however, the increased development of NPQ following ascorbate feeding was associated with an increase in the PS2-driven electron flow. A similar result was recently reported by Calatayud *et al.* (1999).

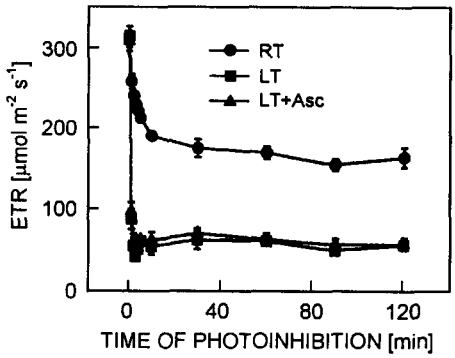

Fig. 2. Changes in the *in vivo* PS2-driven electron transport rate (ETR) in rice leaves exposed to a PPFD of 1000 μmol m^{-2} s^{-1} at 25 °C (RT) or 10 °C (LT) and effects of ascorbate (Asc) feeding. The quantum yield (1 - F_s/F_m') for calculating ETR at zero time of treatment was measured after pre-irradiation for 30 min under growth conditions. The experimental conditions were the same as in Fig. 1. Error bars indicate the standard error (n = 5).

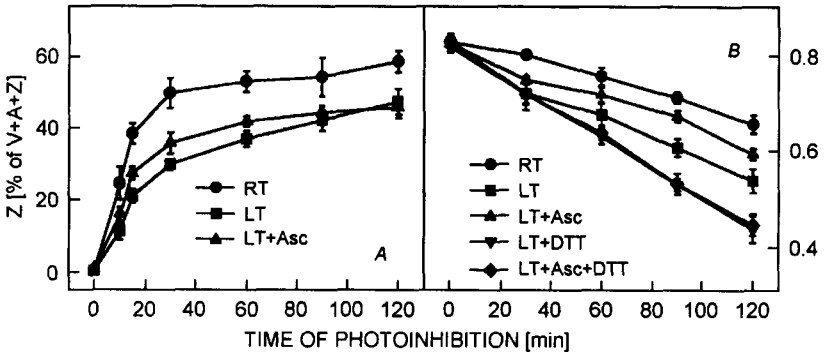

Fig. 3. (*A*) Changes in the concentration of zeaxanthin (Z) and (*B*) time course of photoinhibition as indicated by changes in the photochemical efficiency of PS2 (F_v/F_m) in rice leaves exposed to a PPFD of 1000 μmol m^{-2} s^{-1} at 25 °C (RT) or 10 °C (LT). Effects of ascorbate (Asc) and dithiothreitol (DTT) feeding are also shown. The experimental conditions were similar to those of Fig. 1. Error bars indicate the standard error (n = 3).

Since the photoprotective function of NPQ has been proven both *in vitro* and *in vivo* (Krause 1994), pre-feeding with ascorbate may increase the capacity of rice leaves to resist low temperature photoinhibition. This was verified as shown in Fig. 3*B*. The extent of photoinhibition as indicated by a sustained decrease in F_v/F_m was significantly enhanced in the presence of DTT (Fig. 3*B*), indicating an important role of the xanthophyll cycle in the protection against low temperature photoinhibition in rice leaves. The protective effect of ascorbate was almost entirely eliminated in the presence of DTT, suggesting that the effect of ascorbate depends on its action as a co-substrate for deepoxidase. If one excludes the side effects of DTT on the formation and removal of active oxygen species, this result would also imply that exogenous ascorbate, in this condition, confers no protection against low temperature photoinhibition through its action as an antioxidant.

Discussion

Our results demonstrated an important role of ascorbate in the protection of PS2 against low temperature photoinhibition in rice, a chilling-sensitive plant. The protective effect of ascorbate can be fully explained by its

function as a co-substrate for deepoxidase. The enhanced rate of V deepoxidation in ascorbate-fed leaves facilitates a more effective down-regulation of PS2 photochemistry through the increased development of the xanthophyll cycle-associated thermal energy dissipation and thus contributing to the increased resistance to photoinhibition at chilling temperature.

The protection afforded by ascorbate feeding can not be ascribed to its role in direct detoxification of reactive O_2 species (Fig. 3*B*). The possible function of ascorbate in supporting non-assimilatory linear electron flow mediated by the Mehler-ascorbate peroxidase reaction cycle could also be excluded under our experimental noc-ditions because ascorbate feeding had no significant effect on PS2-driven electron transport (Fig. 2). This is in contrast to the results obtained in lichen thalli exposed to the atmospheric pollutant SO_2 (Calatayud *et al.* 1999) and in rice leaves photoinhibited at room temperature (values not shown). Therefore, the decreased reduction state of Q_A, reflected by an increase in $1 - q_P$ in ascorbate-fed leaves exposed to high irradiance at chilling temperature (Fig. 1*B*), can be attributable to the enhanced thermal dissipation of excess energy in the light-harvesting system of PS2. The lack of stimulating effect of ascorbate on ETR may suggest either that in chilling-sensitive plant rice exposed to high irradiance at chilling temperature the non-assimilatory electron flow mediated by the Mehler-ascorbate peroxidase reaction cycle is not limited by the availability of ascorbate, or that the photosynthetic electron transport is not constrained by the availability of the electron acceptor at the reducing side of PS1. In fact, an analysis of fluorescence quenching revealed a sharp drop of PS2-driven electron flow and a rapid rise of the reduced state of Q_A (Figs. 1*B* and 2). These sudden effects may result from the low temperature-imposed thermodynamic constraints on plastoquinol re-oxidation, which is dependent upon lateral diffusion of the electron and proton carrier, plastoquinol (Haehnel 1984) as suggested by Brüggemann and Linger (1994) for another chilling-sensitive plant, tomato, exposed to chilling temperatures. Another possibility is that the chilling temperature-induced decrease in PS2-driven electron flow may be related to an impaired utilisation of reduction equivalents due to restricted carbon assimilation (Havaux 1987, Brüggemann 1992).

Fig. 3*A* shows that both the rate and degree of maximal V to Z conversion were drastically decreased at chilling temperature. Arvidsson *et al.* (1997) reported similar results for an *in vitro* system using isolated spinach thylakoids. The temperature dependence of NPQ development was ascribed to effects of chilling temperature on V deepoxidation by Brüggemann and Koroleva (1995), or more specifically, on deepoxidase responsible for the conversion of V to A and Z by Adams *et al.* (1994). Our results showed that feeding with 20 mM ascorbate increased the rate of Z formation (Fig. 3*A*), but the maximum speed in the presence of saturating amount of ascorbate at 10 °C was still lower than the rate observed at 25°C. This suggests a partial limitation of the xanthophyll cycle activity by the availability of ascorbate in chilling-sensitive rice exposed to high irradiance at chilling temperature. On the other hand, the maximum degree of V to Z conversion was not affected by ascorbate feeding (Fig. 3*A*), indicating that the availability of ascorbate is not responsible for limited V to Z conversion at chilling temperature.

The development of NPQ is controlled by the xanthophyll cycle activity as well as by the extent of lumen acidification. The enhancement of V deepoxidation in ascorbate-fed leaves resulted in an increased rate of NPQ development (Figs. 1*A* and 3*A*). This finding points out the importance of the rate of Z formation in determining NPQ in chilling-sensitive plant rice exposed to high irradiance at chilling temperature.

In conclusion, our results suggest a key role of the xanthophyll cycle-associated thermal energy dissipation in the protection against low temperature photoinhibition in chilling-sensitive plants, in agreement with several recent fingings (Leipner *et al.* 1997, Haldimann 1998, Jung *et al.* 1998, Xu *et al.* 1999). The ascorbate-induced increase in the activity of the xanthophyll cycle would allow an earlier and more effective engagement of thermal energy dissipation in photoprotection. This may be of particular importance for plants to tolerate the low temperature photoinhibition, since the xanthophyll cycle activity is strikingly suppressed by chilling temperature, and the Z formation seems to be the limiting factor for the xanthophyll cycle-associated thermal energy dissipation.

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