

Effects of cold-hardening on chilling-induced photoinhibition of photosynthesis and on xanthophyll cycle pigments in sweet pepper

Peng LIU^{*}, Qing-wei MENG^{**}, Qi ZOU^{**,+}, Shi-jie ZHAO^{**}, and Qing-zhong LIU^{*}

*Shandong Institute of Pomology, Tai'an, Shandong, 271000, P.R. China**

*Department of Plant Science, Shandong Agricultural University, Tai'an, Shandong, 271018, P.R. China***

Abstract

Two cultivars of *Capsicum annuum* L. were acclimated for 5 d at sub-optimal temperature (14 °C) and irradiance of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. This cold-hardening resulted in some reduction in the extent of photoinhibition during an 8 h exposure to high irradiance at 4 °C. Obvious differences were observed between non-hardened leaves (NHL) and cold-hardened leaves (CHL) in the recovery under low irradiance at room temperature. The CHL of both cultivars recovered faster than NHL, especially during the initial fast phase of recovery. Compared with NHL, the total content of carotenoids (Cars), based on chlorophyll, Chl (*a+b*), and the proportions of xanthophyll cycle pigments referred to total Cars increased in CHL, mainly due to an increase of violaxanthin (V) + antheraxanthin (A) + zeaxanthin (Z) content per mol Chl (*a+b*). Faster development and a higher non-photochemical quenching (NPQ) of Chl fluorescence, related to a stronger depoxidation of the larger xanthophyll cycle pool in NHL, could act as a major defence mechanism to reduce the formation of reactive oxygen species during severe chilling. This is suggested by higher content of Z or Z+A in photoinhibition as well as by its rapid decline during the initial fast phase of recovery. In contrast to the chilling-sensitive cv. 0004, the chilling-tolerant cv. 1141 did more easily acclimate its photosynthetic apparatus to low temperatures.

Additional key words: acclimation; antheraxanthin; *Capsicum annuum*; chlorophyll fluorescence; neoxanthin; violaxanthin; zeaxanthin.

Introduction

Some crops, *e.g.*, maize, rice, cotton, cucumber, and tomato, are commonly chilling-sensitive owing to their tropical and subtropical origin. When the temperature drops slightly below the growth optima, the rate of photosynthesis decreases, and the critical chilling stress temperature leads to a marked reduction of photosynthesis (Wise and Naylor 1987). The photoinhibition occurring in low temperature was termed chilling-induced photoinhibition or low temperature photoinhibition. Severe chilling-induced photoinhibition can cause photodamage (Hodgson *et al.* 1987). During the long-term course of evolution, plants developed a series of adaptive mechanisms reducing the photodamage to a minimum. Much interest was in the processes of thermal energy dissipation, which protect the photosynthetic machinery from inactivation and damage caused by excess irradiance (Horton *et al.* 1994). The xanthophyll-cycle mediated

dissipation process has received the most attention. As an important protective system, the xanthophyll cycle contributes to increased stability of the photosynthetic apparatus under excessive irradiance at low temperatures (Demmig-Adams and Adams 1992).

Some plants acclimated at low temperature are more resistant to severe chilling stress. Acclimation to low temperature results in increased cold hardiness, and short-term cold-hardening can also reduce the susceptibility of leaves to photoinhibition under low temperatures (Takahama and Nishimura 1975, Somersalo and Krause 1990, Hurry and Huner 1992, Xu *et al.* 2000). Cold hardening has a strong effect on the performance of photosynthetic apparatus, which is manifested by a decrease in photosynthetic capacity and efficiency (Nie *et al.* 1992). The decline in photosynthetic capacity is accompanied by lower activities of certain enzymes of the Benson-Calvin

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⁺ Author for correspondence; fax: +80 538 8226399, e-mail: zouqi@sdau.edu.cn

Abbreviations: A, antheraxanthin; CHL, cold-hardened leaves; F_m , F_m' , maximum fluorescence in dark-adapted and light-adapted leaves; F_v , variable fluorescence in dark-adapted leaves; NHL, non-hardened leaves; NPQ, non-photochemical quenching; PS, photosystem; V, violaxanthin; Z, zeaxanthin.

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cycle (Stamp 1987), whereas the depression of photosynthetic efficiency may be based on a zeaxanthin-related quenching of absorbed excitation at photosystem 2 (Fryer *et al.* 1995). The effects of cold hardening on membrane system and membrane protective system were studied by Wang *et al.* (1995), Shao *et al.* (1999), *etc.* However, few investigations were made on the mechanism by which

Materials and methods

Plants: Two cultivars of sweet pepper (*Capsicum annuum* L.) were used in the experiments: chilling-sensitive cv. 0004 and chilling-tolerant cv. 1141. As sweet pepper is generally chilling-sensitive, there are no fully chilling-tolerant cultivars. The description of genotypes as chilling-tolerant and -sensitive should be understood in the sense that within the general susceptibility of *C. annuum* to low temperature the cv. 1141 is much better able to cope with low temperature than the cv. 0004. Seeds were first germinated between moistened filter paper at 25 °C for 3 d. Sprouted burgeons were then planted into 13.5 cm-plastic pots (one plant per pot) filled with sterilised soil and grown in a greenhouse at 25/20 °C and 14/10 h (day/night) regime. When the fifth leaf was fully developed, the plants were used for experiment.

Cold-hardening treatment: Plants were separated into two groups, grown for 5 d in growth chambers maintaining an irradiance of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (12 h photoperiod) at either 25 °C (non-hardened) or 14 °C (cold-hardened).

Photoinhibition and recovery treatments: To induce photoinhibition, leaf discs (12 mm diameter) punched from the first fully expanded leaves were irradiated by 1 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 4 °C. During irradiation, leaf discs

Results

Non-photochemical quenching (NPQ): The dissipation of excess absorbed photons as heat was monitored by measuring the development of NPQ at 4 °C under high irradiance (1 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) during the first 20 min of the treatment (Fig. 1). The increase in NPQ was faster in CHL than in NHL of both cultivars. After 4 min, NPQ values were already significantly higher in both cvs. 0004 and 1141 by 1.5- ($p < 0.01$) and 1.7-fold ($p < 0.01$), respectively. In contrast, steady-state NPQ values were 25 and 33 % higher in CHL of cvs. 0004 and 1141, respectively. Irrespective of the growth temperature, NPQ development was faster in 1141 than in 0004.

Photoinhibition of photosystem 2 (PS2) and subsequent recovery: The F_v/F_m values in CHL were lower

cold-hardening increases the resistance to photoinhibition. Our aim was to examine the effects of cold-hardening on the susceptibility of leaves to photoinhibition at 4 °C and its recovery at 20 °C and on the composition of photosynthetic pigments in two *Capsicum annuum* L. cultivars with different sensitivity to low temperatures.

floated on a water layer cooled in a cuvette to 4 °C by water circulating from a cooling bath. To prevent heating of the samples, radiation was filtered through a flowing water layer of 10 cm. For subsequent recovery after 8-h photoinhibition treatment, leaf discs were placed on a water layer of about 20 °C and irradiated with 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Chl *a* fluorescence: Susceptibility of two sweet pepper cultivars to photoinhibition was analysed according to the time-dependent loss of photosynthetic efficiency judged from changes in the ratio of variable *versus* maximum Chl *a* fluorescence (F_v/F_m). This was determined during photoinhibition treatment and recovery periods after 30 min dark adaptation at room temperature with an *FMS2* fluorometer (Hansatech, UK). Non-photochemical quenching (NPQ) was expressed as $(F_m - F_{m'})/F_m$ (van Kooten and Snel 1990).

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 1 cm^3 of ice-cold acetone and quantified by reversed-phase high performance liquid chromatography (HPLC) based on a method described by Zhao *et al.* (1995).

than those in NHL definitely, though not enormously, suggesting that some extent of photoinhibition had occurred in CHL before high-irradiance treatment at chilling temperature (Fig. 2). This is consistent with the results in the cold-acclimated maize leaves (Greer and Hardacre 1989). Exposure to high irradiance at 4 °C resulted in a rapid and similar drop in F_v/F_m values in NHL of both cultivars. The difference in the course of photoinhibition between NHL and CHL was relatively small at the beginning of treatment and became apparent after about 2 h. The decline in F_v/F_m was slower in CHL than in NHL in both cultivars, indicating that cold acclimation resulted in some reduction of the extent of photoinhibition (Fig. 2A,C). More obvious were the differences between NHL and CHL in the recovery observed in low irradiance at

room temperature (Fig. 2B,D). In both cultivars, recovery of F_v/F_m was more complete in CHL than in NHL. Also, both leaf types exhibited a faster initial phase of recovery. Besides, the slope of recovery during the first 2 h showed that F_v/F_m in CHL recovered faster than in NHL.

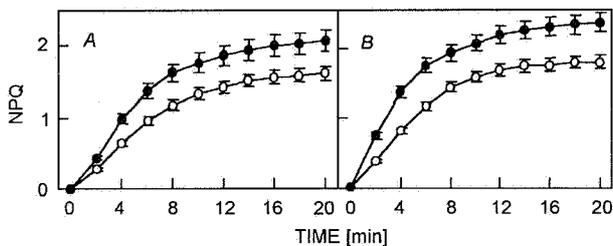


Fig. 1. Development of non-photochemical quenching (NPQ) at high irradiance treatment ($1\ 200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) at $4\ ^\circ\text{C}$ in NHL (\circ) and CHL (\bullet) of cvs. 0004 (A) and 1141 (B). Means \pm SD of four replicates. The values of untreated controls were zero and remained constant during treatment.

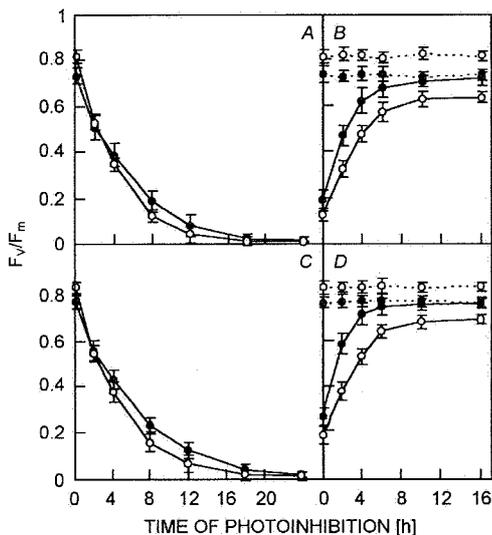


Fig. 2. Responses of maximum quantum efficiency of photosystem 2 photochemistry (F_v/F_m) to high irradiance treatment ($1\ 200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) at $4\ ^\circ\text{C}$ (A, C) and its recovery under low irradiance ($20\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) at $20\ ^\circ\text{C}$ (B, D) in NHL (\circ) and CHL (\bullet) of cvs. 0004 (A, B) and 1141 (C, D). The leaf discs were subjected to photoinhibition treatment for 8 h prior to recovery treatment. In B and D, the F_v/F_m ratios of untreated controls are indicated by broken lines. Means \pm SD; (A, C) $n = 6$; (B, D) $n = 4$.

Pigment composition: At optimal growth temperature, the composition of photosynthetic pigments showed less difference between two cultivars. Cold hardening caused substantial changes in pigments in both species (Table 1). Calculated on a Chl basis, the total Car content increased by 20–30%. Most of this increase was due to a higher pool of xanthophyll cycle pigments (V+A+Z). The increase in V+A+Z content per mol Chl ($a+b$) in CHL was

65 and 48% in cvs. 1141 and 0004, respectively. In addition, contents of β -carotene and lutein slightly increased, whereas the neoxanthin content was unaffected by the growth temperature in both cultivars when related to the Chl content. The Z content per mol Chl ($a+b$) was negligible in NHL but increased much after cold hardening. These changes indicate that the leaves of sweet pepper were capable to acclimate to low temperature and enhance their cold tolerance. Both Chl ($a+b$) and Chl a/b values in CHL were reduced only little (a not significant difference), probably because the cold hardening was not too long.

Table 1. Composition of chloroplast pigments in NHL and CHL of *Capsicum annuum* L. cv. 0004 ($n = 7$, for Chl $n = 6$) and cv. 1141 ($n = 6$, for Chl $n = 4$). Means \pm SD. The values observed in CHL were compared with NHL by Student's t -test. Carotenoid (Car) contents [$\text{mmol mol}^{-1}(\text{Chl } a+b)$], Chl contents [$\mu\text{mol m}^{-2}$]. NS, not significant; * $p \leq 0.05$; ** $p \leq 0.01$.

Parameter	Cultivar	NHL	CHL
Total Cars	0004	347.0 ± 23.0	$416.0 \pm 37.0^{**}$
	1141	365.0 ± 20.0	$475.0 \pm 48.0^{**}$
β -Carotene	0004	107.6 ± 12.1	$122.7 \pm 14.2^{\text{NS}}$
	1141	117.2 ± 16.8	$140.0 \pm 14.5^*$
Lutein	0004	132.7 ± 17.0	$153.5 \pm 10.3^*$
	1141	134.0 ± 10.4	$162.4 \pm 12.1^*$
Neoxanthin	0004	42.0 ± 4.0	$43.7 \pm 8.2^{\text{NS}}$
	1141	45.6 ± 3.4	$48.5 \pm 4.5^{\text{NS}}$
V+A+Z	0004	64.7 ± 7.5	$95.8 \pm 11.6^{**}$
	1141	68.2 ± 7.1	$112.5 \pm 13.2^{**}$
V	0004	58.3 ± 4.9	$56.6 \pm 5.8^{\text{NS}}$
	1141	60.7 ± 6.4	$55.0 \pm 6.0^{\text{NS}}$
A	0004	3.9 ± 1.6	$12.3 \pm 3.1^{**}$
	1141	6.7 ± 1.2	$18.5 \pm 2.8^{**}$
Z	0004	1.5 ± 0.4	$27.2 \pm 6.3^{**}$
	1141	0.8 ± 0.6	$38.9 \pm 5.7^{**}$
Chl ($a+b$)	0004	388.0 ± 64.0	$360.0 \pm 47.0^{\text{NS}}$
	1141	382.0 ± 41.0	$365.0 \pm 36.0^{\text{NS}}$
Chl a/b	0004	2.71 ± 0.76	$2.52 \pm 0.35^{\text{NS}}$
	1141	2.65 ± 0.29	$2.42 \pm 0.27^{\text{NS}}$

The Car/Chl ($a+b$) ratio and the proportion of xanthophyll cycle pigments for both cultivars (Fig. 3) showed that besides the pool size of total Cars also the ratio (V+A+Z)/Car (Fig. 3B) was higher in CHL than in NHL. The increase in Car/Chl ratio resulted largely from a strong increase in V+A+Z (Fig. 3C). Both cultivars contained Z and A after cold-hardening treatment (Fig. 4A,B), whereas in NHL, the contents of Z and A were negligible (Table 1). In addition, the proportions of Z and A were higher in cv. 1141 than in cv. 0004.

Xanthophyll cycle activity: The response of xanthophyll cycle to 10-h irradiation with $1\ 200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ at $4\ ^\circ\text{C}$ gives a measure of maximal de-epoxidation (Fig. 5A,C). CHL exhibited substantially faster de-epoxidation kine-

tics during high irradiance treatment at chilling temperature, as shown for Z+A formation and the de-epoxidation state in both cultivars. In both cultivars, the ratio

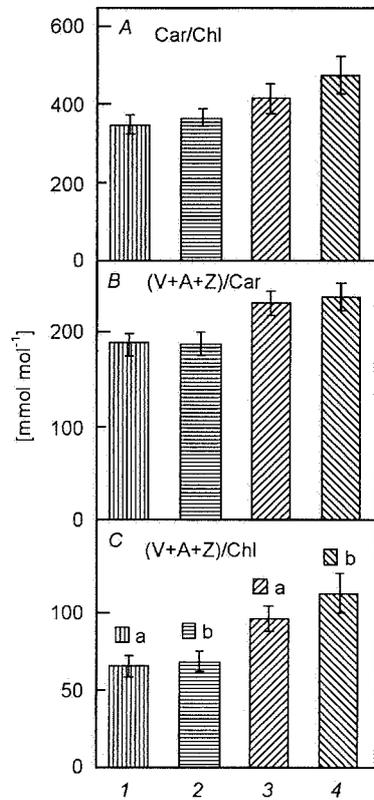


Fig. 3. Comparison of contents of total carotenoids and total xanthophyll cycle pigments (V+A+Z) in NHL (1, 2) and CHL (3, 4) of cvs. 0004 (a) and 1141 (b). Means \pm SD of four replicates.

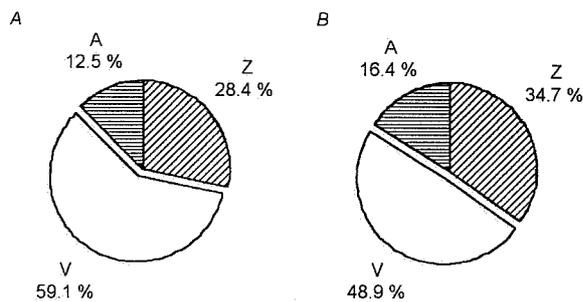


Fig. 4. Comparison of the xanthophyll cycle pigments in CHL of cvs. 0004 (A) and 1141 (B) [mol-% of total xanthophyll cycle pigments (V+A+Z)]. Means of six leaf samples are given for cvs. 0004 and 1141. SE [mol-%] were ± 3.8 and ± 4.2 for V, ± 3.1 and ± 2.8 for A, and ± 6.3 and ± 5.7 for Z, respectively.

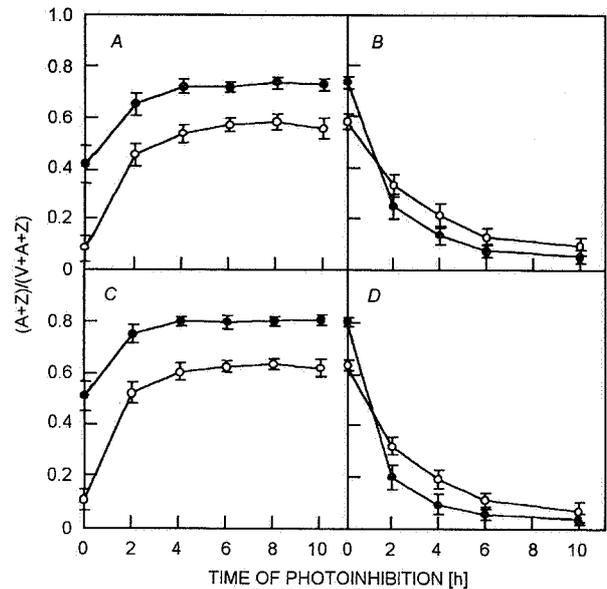


Fig. 5. Responses of the xanthophyll cycle conversion state (A+Z)/(V+A+Z) to high irradiance treatment ($1\ 200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) at 4 °C (A, C) and its recovery (B, D) under low irradiance ($20\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$) at 20 °C in NHL (\circ) and CHL (\bullet) of cvs. 0004 (A, B) and 1141 (C, D). The leaf discs were subjected to photoinhibition treatment for 8 h prior to recovery treatment. Means \pm SD; (A, C) $n = 5$; (B, D) $n = 3$.

(A+Z)/(V+A+Z) rapidly increased during the first 2 h and reached slightly higher values in CHL (0.7-0.8) than in NHL (0.55-0.6). This is consistent with the changes of NPQ during the chilling treatment.

The initial fast phase of recovery from photoinhibition is associated with the reactivation of the inhibited PS2, which is not correlated with the turnover of protein (Leitsch *et al.* 1994), but with the epoxidation of Z via the xanthophyll cycle (Thiele and Krause 1994). On transfer to recovery conditions a fast epoxidation occurred concomitant with the fast recovery phase in both leaf types, regardless of growth temperature. In CHL of both cultivars, the de-epoxidation rates were slower in the course of photoinhibition and the recovery of epoxidation was more complete than in NHL (cf. Fig. 2B,D). However, the rates of epoxidation, analysed during the first 2 h of recovery, were relatively lower than that of de-epoxidation, and the former could not reach its highest values within a long time, probably due to the depression of synthesis or activities of epoxidase. Under recovery conditions, the untreated controls reached the maximum of epoxidation within the first 2 h (values not shown), exhibiting a much faster recovery than those subjected to high irradiance.

Discussion

Cold-hardening and chloroplast pigments: At present, there is no agreement whether cold hardening in the leaves of herbaceous plants can induce cold resistance. In spinach and winter rye, resistance to chilling-induced photoinhibition was not achieved by cold-hardening, but only by growth under low temperature (Öquist and Huner 1991, Boese and Huner 1992). In contrast, cold hardening did increase the resistance to photoinhibition in chilling-sensitive maize (Haldimann *et al.* 1996, Leipner *et al.* 1997). Several long-term acclimation responses were also found during cold-hardening. We observed that the increase in total Car content per mol Chl (*a+b*) increased not due to the decrease in Chl content, but due to a higher pool of V+A+Z (Table 1, Fig. 4). These changes indicate that the cold hardening had caused chilling stress, and the leaves were capable of acclimation responses.

After cold hardening in both cultivars, contents of lutein and β -carotene showed a slight increase, whereas the neoxanthin content remained constant when related to the Chl content (Table 1). Enlarged pools of lutein and β -carotene associated with high-irradiance stress were reported for a number of plant species (Krol *et al.* 1995, Jung and Steffen 1997). The possible roles of lutein and β -carotene are not yet understood in detail but they might be related to the general antioxidative function of Cars in scavenging triplet Chl and singlet oxygen (Siefermann-Harms and Angerhofer 1998). Niyogi *et al.* (1997) suggest that lutein plays a role in the q_E mechanism of non-photochemical fluorescence quenching.

Photoinhibition, recovery, and xanthophyll cycle: Photoinhibition of photosynthesis occurs at least in two phases (Thiele *et al.* 1996). The first phase may be associated with down-regulation of photosynthesis induced by an increase in the non-radiative energy dissipation within the photosynthetic apparatus. In the second phase, the PS2 reaction centre is probably damaged. The two phases of photoinhibition are also reflected in the kinetics of the increase in photochemical efficiency of PS2 during recovery, which often exhibits two distinct phases: fast phase and slow phase (Krause 1994).

Under high-irradiance stress, the decrease in F_v/F_m can be induced by D1-protein inactivation. On the other hand, high contents of Z present in CHL under such condition may also cause a sustained reduction of F_v/F_m (Demmig-Adams and Adams 1993, Gilmore and Yamamoto 1993). In our study the synthesis of Z occurred rapidly following exposure to photoinhibitory conditions and the measured relationships between Z content and

changes in the F_v/F_m were strong during both photoinhibition and recovery. Nevertheless, the Z content in the leaves subjected to photoinhibition reached a maximum within the first few hours. And F_v/F_m continued to decline in the period when the Z content was constant. Therefore, the presence of Z might not prevent destruction and loss of the D1-protein in CHL, and Z-mediated processes are not completely effective even in the early phases of exposure.

Under our experimental conditions, the effect of Z probably diminished the difference in the decline of F_v/F_m between CHL and NHL but led to fast recovery in the former. The high contents of (Z+A) in CHL and the fast decline of amounts of these xanthophylls during the fast phase of recovery under low irradiance support this view. Li *et al.* (1996) observed that the chilling-induced photoinhibition was more significant in CHL of cucumber seedlings than in NHL, but F_v/F_m recovered more quickly in the former. The cold tolerance was expressed during recovery. It, therefore, appears that the decrease of F_v/F_m is not solely the result of damage of PS2 caused by photoinhibition under chilling but is due to the protective response of the photosynthetic apparatus to the stress. So, it may not be the extent of decrease but the recovery competence and values of F_v/F_m after treatment that mirror the resistance to photodamage (Somersalo and Krause 1990).

In both leaf types, V and A were de-epoxidised to Z during strong irradiation in the cold (Fig. 5). CHL contained Z even before severe chilling stress, whereas the xanthophyll cycle pool in NHL was completely epoxidised. In CHL the xanthophyll cycle pool could be almost fully de-epoxidised during the treatment, and the (A+Z)/(V+A+Z) was higher in contrast to NHL. CHL might develop a higher transthylakoid ΔpH during severe chilling due, for example, to a higher rate of Mehler reaction. Also, CHL may have contained a larger amount of photoconvertible V (Pfundel and Bilger 1994).

In CHL, the faster development and higher value of NPQ might be a result of the greater amount of Z and of the higher de-epoxidation state of the xanthophyll cycle. A correlation between NPQ and Z content is probable (Demmig-Adams and Adams 1996). We assume that faster development and a higher value of NPQ, related to a stronger de-epoxidation of the larger xanthophyll cycle pool in NHL, could act as a major defence mechanism to reduce the formation of reactive oxygen species during severe chilling.

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