Inhibition of Photosystems I and II in Chilling-Sensitive and Chilling-Tolerant Plants under Light and Low-Temperature Stress

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The responses of photosystems (PS) I and II to light stress at 4 °C and 20 °C were studied in leaf discs from three chilling-sensitive plant species, *Cucumis sativus*, *Cucurbita maxima* and *Nicotiana tabacum*, and in the chilling-tolerant *Spinacia oleracea*. The chilling-sensitive plants were grown at 24 °C under 80–120 μmol photons m^{-2} s^{-1} (*Cucumis* and *Cucurbita*) or 30 μmol photons m^{-2} s^{-1} (*Nicotiana*). *Spinacia* was cultivated outdoors during winter and early spring. The P700 absorbance change around 820 nm served as a relative measure of PSI activity. The potential efficiency of PSII was determined in dark-adapted leaf discs by means of the ratio of variable to maximum chlorophyll (Chl) *a* fluorescence emission (F_V/F_M). In *Cucurbita*, *Nicotiana* and *Spinacia*, PSI was not or only slightly inhibited by 2 h illumination with 200 μmol m^{-2} s^{-1} at 4 °C or with 2000 μmol m^{-2} s^{-1} at 20 °C. In leaves of *Cucurbita* and *Nicotiana*, exposure to 2000 μmol photons m^{-2} s^{-1} at 4 °C resulted in a decline in PSI activity and potential PSII efficiency approximately to the same extent (about 50% within 2 h). In contrast, in *Cucumis*, both moderate and high light at low temperature caused a PSI inhibition that proceeded considerably faster than the decline in PSII efficiency. Such preferential photoinhibition of PSI was not observed in the other three species tested. In *Spinacia*, a lower susceptibility of PSI and PSII to photoinhibition at 4 °C was associated with a faster de-epoxidation kinetics of violaxanthin, as compared to the three chilling-sensitive species. In addition, leaves of *Spinacia* were characterized by a significantly larger pool of xanthophyll-cycle pigments and a higher content of β-carotene based on Chl *a*+*b*. When leaves of *Cucurbita* were preincubated with methylviologen, which catalyzes formation of superoxide anion radicals at the acceptor side of PSI, the decline in potential PSII efficiency under 2000 μmol photons m^{-2} s^{-1} at 20 °C and 4 °C was strongly enhanced, whereas the P700 signal was less affected. Our data demonstrate that in the species tested, PSI may be inhibited *in vivo* besides PSII under light stress, but preferential photoinhibition of PSI is not a general phenomenon in chilling-sensitive plants. At low temperatures, a reduced function of the xanthophyll cycle and of the antioxidative scavenging system might account for enhanced PSI and PSII inhibition *in vivo*.

Introduction

Photoinhibition of photosynthesis has been observed under a variety of conditions in many plant species (see e.g. Powles, 1984; Krause, 1988; Aro *et al.*, 1993; Leitsch *et al.*, 1994; Long *et al.*, 1994; Krause *et al.*, 1995; Thiele *et al.*, 1998). Photoinhibition has been defined as a slowly reversible, light-induced inhibition of photosynthesis, which comprises both damaging and regulatory protective processes. Inhibition of photosynthesis occurs when plants absorb light in excess of photosynthetic energy utilization and is enhanced by combinations of stress factors such as excessive light and chilling, heat or drought (see Powles, 1984; Krause, 1994; Huner *et al.*, 1996). It is widely accepted that PSII is a primary site of photoinhibition. Damaging and protective mechanisms related to photoinhibition of PSII have been thoroughly investigated

Abbreviations: A, antheraxanthin; AOS, active oxygen species; AsA, ascorbic acid; AP, ascorbate peroxidase; β-Car, β-carotene; Chl, chlorophyll; DHA, dehydroascorbate; F_V/F_M, ratio of maximum variable to maximum total chlorophyll *a* fluorescence yield; MV, methylviologen; PAR, photosynthetically active radiation, PSI (II), photosystem I (II); P700, reaction center chlorophyll of photosystem I; SOD, superoxide dismutase; V, violaxanthin; VDE, violaxanthin de-epoxidase; Z, zeaxanthin.

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both in chilling-sensitive and chilling-tolerant plants (see Aro *et al.*, 1993; Baker and Bowyer, 1994). In the latter, cold acclimation leads to an increased tolerance to light stress at low temperatures (see Krause, 1994). In contrast to cold-sensitive plants, cold-tolerant species, such as spinach (in the cold-acclimated state), are characterized by high pool size and turnover of the xanthophyll-cycle pigments (Hurry and Huner, 1992; Koroleva *et al.*, 1994, 1995; Thiele *et al.*, 1996; Adams and Barker, 1998) and increased activity of the antioxidative scavenger system (see Krause 1994). Furthermore, a lowered activation energy of ribulose-1,5-bisphosphate carboxylation, the key reaction of the Calvin cycle, has been reported (Huner and MacDowell, 1979; Grafflage and Krause, 1993). Fast turnover of the D1 protein in the PSII reaction center does not account for diminished photoinhibition (Hurry and Huner, 1991). Rather, in cold-acclimated plants, D1 inactivation under light stress is largely prevented (Thiele *et al.*, 1996).

It has been assumed that PSI, in general, is not or only marginally affected by excess irradiation *in vivo* (Critchley *et al.*, 1981; Somersalo and Krause, 1990). However, there is growing evidence that PSI may become inhibited under light stress, particularly at chilling temperatures (0–10 °C). Terashima *et al.* (1994, 1998) and Sonoike (1995a, b) described a preferential photoinhibition of PSI in cold-sensitive cucumber (*Cucumis sativus* L.) and common bean (*Phaseolus vulgaris* L.) when leaves were chilled under low irradiance. A similar effect was observed under high light in potato leaves (*Solanum tuberosum* L.) by Havaux and Davaud (1994). The presence of molecular oxygen (O_2) and electron flow from PSII to PSI are required for PSI photoinhibition. Inhibition of PSI could not be observed in the absence of O_2 or when electron transport from PSII to PSI was inhibited by diuron (Terashima *et al.* 1994, 1998; Havaux and Davaud, 1994; Sonoike, 1996a; Tjus *et al.*, 1998). It was concluded that active oxygen species (AOS) are involved in the inhibition of PSI.

When natural electron acceptors such as $NADP^+$ are scarce, electrons are transferred to O_2 at the acceptor side of PSI (Mehler reaction; Mehler, 1951; see Halliwell, 1991; Asada, 1994; Polle, 1995) generating superoxide anion radicals ($\cdot O_2^-$) that are converted to H_2O_2 by superoxide dismutase (SOD). By reaction of $\cdot O_2^-$ with H_2O_2 in the presence of catalytic metal ions such as Fe(III) (Haber-Weiss/Fenton reaction) or by direct reaction of H_2O_2 with Fe(II) or reduced iron-sulfur (FeS) centers, the extremely reactive ·OH radicals are formed (see Halliwell, 1991; Foyer and Harbinson, 1994). It has been proposed that ·OH damages the FeS centers (F_X, F_A/F_B) as the primary targets during photoinhibition of PSI *in vivo* and *in vitro* (Inoue *et al.*, 1986; Jakob and Heber, 1996; Sonoike, 1997; Terashima *et al.*, 1998). Destruction of the FeS centers apparently triggers proteolysis of the PSI-A/B reaction center proteins, as evidenced by detection of degradation products (Sonoike, 1996b; Sonoike *et al.*, 1997; Tjus *et al.*, 1998).

The mechanisms protecting PSI from photodamage have not been clarified. According to Havaux and Davaud (1994), Sonoike (1996a), Tjus *et al.* (1998) and Terashima *et al.* (1998), the antioxidative scavenging system seems to play a primary role in protecting PSI. It has been shown that PSI is much more sensitive to light stress in isolated thylakoid membranes than in intact leaves. This was described for chilling-sensitive as well as chilling-tolerant plants (Sonoike, 1995a). These observations demonstrate that PSI is protected by an efficiently working mechanism within the chloroplast stroma. There, the H_2O_2 formed from $\cdot O_2^-$ is detoxified by ascorbate peroxidase (AP) (Miyake *et al.*, 1993). The ascorbate (AsA) recycling reactions, in which NADPH serves as electron donor, are catalyzed by monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase. This system operates not only as a scavenger for AOS, but also regulates the electron flow from PSII to PSI by accepting electrons from PSI and generating a high transthylakoid pH gradient (Foyer *et al.*, 1994). Under these conditions, P700 accumulates in the cation radical form $P700^+$ (Harbinson and Hedley, 1993), which can dissipate excess light energy as heat (Nuijs *et al.*, 1986; Foyer *et al.*, 1994).

In addition to its scavenging function, AsA is the co-substrate of violaxanthin de-epoxidase (VDE) in the thylakoid lumen. The conversion of violaxanthin (V) to antheraxanthin (A) and zeaxanthin (Z) by VDE in the xanthophyll cycle is known as a photoprotective mechanism for PSII (Demmig-Adams, 1990; Demmig-Adams and Ad-

ams, 1992; Eskling *et al.*, 1997; Gilmore, 1997), but might also protect PSI. According to Färber *et al.* (1997), PSI contains approximately the same amount of xanthophyll-cycle pigments as does PSII. Furthermore, several authors (Thayer and Björkmann, 1992; Lee and Thornber, 1995; Färber *et al.*, 1997) reported de-epoxidation of V to Z in PSI. However, so far nothing definite is known about a protective role of the xanthophyll cycle in PSI.

To evaluate the importance of PSI photoinhibition *in vivo*, we investigated the effect of light and low-temperature stress on the activity of PSI in comparison with PSII efficiency in three chilling-sensitive plant species, cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita maxima* L.) and tobacco (*Nicotiana tabacum* L.), and in the chilling-tolerant spinach (*Spinacia oleracea* L.). Chloroplast pigments were analyzed in order to assess the activity of the xanthophyll cycle during exposure to light stress. Moreover, to test the protection by the antioxidative scavenging system, we exposed leaves to excessive light after preincubation with methylviologen (MV), which facilitates the Mehler reaction and, thus, the formation of AOS at PSI (Sandmann and Böger, 1980; Duke, 1990).

Materials and Methods

Plant material

The chilling-sensitive plants cucumber (*Cucumis sativus* L. *cv.* Mervita), pumpkin (*Cucurbita maxima* L. *cv.* Riesenmelone) and tobacco (*Nicotiana tabacum* L. *cv.* Samsun) were grown in the greenhouse. *Nicotiana* is considered as a less chilling-sensitive species compared to the two *Cucurbitaceae* (Murata, 1983). Photosynthetically active radiation (PAR) was 80–120 μmol m^{-2} s^{-1} for *Cucumis* and *Cucurbita* and about 30 μmol m^{-2} s^{-1} for *Nicotiana*. Dark/light cycles were 8/16 h in both cases. The temperature was about 24 °C and the relative humidity 70%. Mature, but not senescent leaves from 4–6 week-old plants of *Cucumis* and *Cucurbita* and leaves from 8–10 week-old *Nicotiana* plants were utilized for the experiments. Cold-acclimated spinach plants (*Spinacia oleracea* L. *cv.* Polka) were obtained by outdoor cultivation during winter and early spring in the Botanical Garden at Düsseldorf University. Temperatures were between −7 °C and 14 °C and PAR reached 1000 μmol m^{-2} s^{-1}.

Methylviologen treatment

For MV uptake through the transpiration stream, detached *Cucurbita* leaves were incubated via the petiole with a 2 mM MV solution. The incubation occurred under approximately 30 μmol m^{-2} s^{-1} white light at 24 °C in the greenhouse for 16 h. On average, 1 μmol MV (g fresh weight)$^{-1}$ was taken up. Some leaves exhibited slight Chl bleaching near the middle vein after MV incubation. For all experiments, discs were taken from unbleached regions of the leaf.

Photoinhibitory treatment

Before photoinhibitory treatment, whole plants or leaf discs (in case of MV treatment) floating on a water layer were dark-adapted for at least 1 h. The upper surface of leaf discs (1.5 cm^2) was exposed to 200 or 2000 μmol m^{-2} s^{-1} white light (measured with a quantum sensor, LI 189, LI-COR, Lincoln, NE, USA) provided by two halogen lamps (120 V, 150 W) for 2 h. During illumination, leaf discs were floating on water ventilated with air. Leaf temperature was kept at 4 °C or 20 °C measured on the lower side of the discs. At the times given in the graphs, one disc each was taken for pigment analysis and for determination of PSII and PSI activities.

Determination of potential PSII efficiency

The ratio of maximum variable to total Chl *a* fluorescence (F_V/F_M) was used as a measure of potential PSII efficiency (in the following termed 'PSII activity'). The decrease in this ratio indicates the degree of inhibition of PSII (Demmig-Adams and Björkmann, 1987; Krause and Weis, 1991). F_V/F_M ratios were determined with a portable PAM 2000 fluorometer (Walz, Effeltrich, Germany) (Schreiber *et al.*, 1986) equipped with a palmtop computer (Hewlett Packard 200LX, Corvallis, OR, USA). For data acquisition, the software DA-2000 (Walz) was used. DLC-8 aluminum leaf clips (2 cm diameter, Walz) were used for vertical positioning of the PAM fiber optics with respect to the upper leaf surface. Before recording the initial fluorescence (F_O) in weak modulated 'measuring' light,

weak far red illumination was applied for 3 s in order to fully reoxidize intersystem electron carriers between PSII and PSI. Maximum total fluorescence (F_M) was determined by a saturating pulse (0.8 s). During the saturation pulse, the modulation frequency was increased from 0.6 kHz to 20 kHz. To obtain correct F_V/F_M values, the intensity of the saturating pulse was set to assure that F_M reached a plateau line of the induction signal at least 160 ms before termination of the pulse.

Determination of PSI activity

PSI activity was determined after the F_V/F_M measurement by means of P700 absorbance changes around 820 nm. A PAM 101 fluorometer (Walz, Effeltrich, Germany) was connected with an emitter-detector unit (ED-800T; Walz) by using two arms of a four-arm fiber optics (F/4, Walz). A recorder (Servogor 320, BBC Goerz, Austria) was used for signal recording. The measuring system was further equipped with a KL-1500 lamp (Schott, Mainz, Germany) via the third arm of the fiber optics to provide actinic white light. The fourth small arm of the fiber optics was positioned at a KL-150 lamp (Schott). A filter combination of RG 659 and IF 720 (Schott) was mounted on the top of the lamp to produce far-red light. Intensity was approximately 19 W m^{-2}, uncorrected for wavelength sensitivity of the pyranometer (Li-200SA, LI-COR, Lincoln, NE, USA). Leaf discs on a wet paper tissue were enclosed in a cuvette (LSC-2, ADC Ltd., Hoddesdon, United Kingdom) and ventilated with a moistened air stream. Temperature was kept at 20 °C with a thermostate. The upper leaf surface was illuminated for 10 min with 200 μmol m^{-2} s^{-1} white light via the fiber optics through a window in the cuvette. Subsequently, the baseline representing the reduced form of P700 was recorded. Then far-red light was applied, to trigger photooxidation of P700. The signal difference (ΔA_{820}) between reduced and oxidized state of P700 (steady-state signal in far-red light) was taken as a relative measure of PSI activity (Weis and Lechtenberg, 1989; Klughammer and Schreiber, 1991). It has been reported that besides P700, plastocyanin contributes with about 35% to ΔA_{820} (Harbinson and Hedley, 1989; Klughammer and Schreiber, 1991) when P700 and its donor become oxidized. Thus, in our experiments, the ΔA_{820} signal, designated as 'PSI activity', comprises oxidation of both P700 and plastocyanin.

Chloroplast pigment analysis

For quantitative photosynthetic pigment analysis, leaf discs were plunged into liquid nitrogen and homogenized in a mortar in the presence of a small amount of Na_2CO_3. The pigments were extracted with 1.5 ml 99.5% acetone. The extract was centrifuged for 3 min at 14000 rpm (Microcentrifuge 5415 C, Eppendorf, Hamburg, Germany). The supernatant was filtered through 0.2 μm Anotrop™ microfilters (Merck, Darmstadt, Germany). Pigments were analyzed by means of High Performance Liquid Chromatography (HPLC) according to a method described by Färber *et al.* (1997).

Determination of ascorbate content

A modified method of Law *et al.* (1983) was used. The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA in acidic solution. Fe^{2+} forms a complex with 2,2-bipyridine that absorbs at 525 nm ($\varepsilon_{525\,nm}$ = 0.0087 cm^{-2} $nmol^{-1}$). Determination of total ascorbate (DHA + AsA) occurred after a reduction of DHA to AsA by 1,4-dithiothreitol.

Samples (10 leaf discs, approximately 0.2 g total fresh weight) were extracted at 4 °C with 5 ml of 5% (w/v) 5-sulfosalicylic acid in a cold mortar with quartz sand. The extract was centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was mixed with an equal volume of 150 mM KH_2PO_4 (pH 6.5). The pH of this mixture was set between 5.5 and 6.5 by adding 5 M NaOH. One half of the extract was used for total ascorbate, the other half for AsA assay; determination was done 3 times. To 400 μl sample 200 μl water (for AsA assay) or 100 μl of 10 mM 1,4-dithiothreitol (15 min incubation time) and 100 μl of 0.5% (w/v) N-ethylmaleimid (30 s incubation time) were added (for total ascorbate assay). Then 400 μl of 10% (w/v) trichloroacetic acid, 400 μl of 44% (v/v) H_3PO_4, 400 μl 4% (w/v) 2,2-bipyridine in 70% (v/v) ethanol and 200 μl 3% (w/v) $FeCl_3$ were added to each sample. Mixed samples were incubated at 30 °C for 60 min and A_{525} was recorded. References for both assays were prepared simultaneously with samples by

adding 150 mM KH_2PO_4 (pH 6.5) instead of supernatant.

Results and Discussion

PSI and PSII inhibition at 4 °C

In order to check whether chilling alone did affect PSI and PSII *in vivo*, leaf discs of the four plant species tested were kept in darkness at 4 °C and activities of PSII and PSI were determined after 2 h. Neither in the chilling-sensitive plants nor in chilling-tolerant spinach, changes in ΔA_{820} and F_V/F_M, referred to unchilled controls, could be detected (data not shown).

In leaves of *Spinacia*, almost no inhibition of both photosystems was observed after 2 h illumination with 200 μmol m^{-2} s^{-1} at 4 °C (Fig. 1A). In contrast, illumination of *Cucumis* discs resulted in a 55% inhibition of PSI, with respect to controls, whereas F_V/F_M was only decreased by 20% (Fig. 1B). These findings are in agreement with data of Terashima *et al.* (1994, 1998). However, in the two other chilling-sensitive plant species *Cucurbita* and *Nicotiana*, PSI and PSII activities were only slightly or not at all affected under these conditions (Fig. 1C, D).

Chilling-tolerant *Spinacia* was considerably less susceptible to photoinhibitory treatment under 2000 μmol photons m^{-2} s^{-1} at 4 °C (Fig. 2A) than the three chilling-sensitive species. In *Cucumis* (Fig. 2B), PSI activity declined significantly faster than activity of PSII. *Cucurbita* and *Nicotiana* were more tolerant to the strong irradiance at low temperature compared to *Cucumis* (Fig. 2C, D). In *Spinacia*, *Cucurbita* and *Nicotiana,* activities of PSI and PSII decreased approximately in parallel.

In comparison with *Spinacia* (Fig. 3A), the chilling-sensitive plant species exhibited a slow de-epoxidation kinetics of V under 2000 μmol photons m^{-2} s^{-1} at 4 °C (Fig. 3B-D). In the chilling-sensitive plants, the level of Z ranged from 12 to 20 mmol (mol Chl $a+b)^{-1}$ after 2 h high-light exposure. In *Spinacia*, a 3-fold higher content of Z was found. Furthermore, the four species differed in their pigment composition (see controls in Table I). Cold-acclimated *Spinacia* was characterized by a significantly larger amount of xanthophyll-cycle pigments, a higher content of β-carotene (β-Car) and a tendency of a lower content of lutein (based on Chl), as compared to the chilling-sensitive species. The content of neoxanthin did not differ in the four plant species investigated.

De-epoxidized xanthophylls are supposed to serve as photoprotectors in the Chl *a/b*-binding light-harvesting complexes (see, e.g. Gilmore, 1997). The high β-Car content, as seen in *Spinacia*,

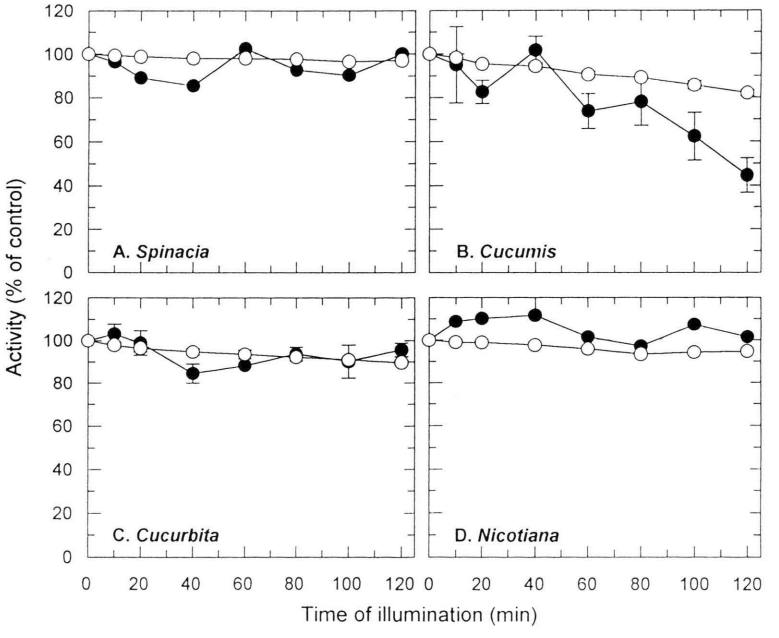

Fig. 1. A-D. Activities of PSI (P700 absorbance change around 820 nm; ΔA_{820}) and PSII (F_V/F_M ratio) in leaf discs of *Spinacia*, *Cucumis*, *Cucurbita,* and *Nicotiana* illuminated with 200 μmol m^{-2} s^{-1} at 4 °C. Mean values of two (A, D) or means ± SD of three (B, C) independent experiments are given. (SD not shown when smaller than symbols.) F_V/F_M ratios of controls: 0.814 (A), 0.810 ± 0.011 (B), 0.847 ± 0.008 (C), 0.805 (D). Symbols: ● PSI, ○ PSII.

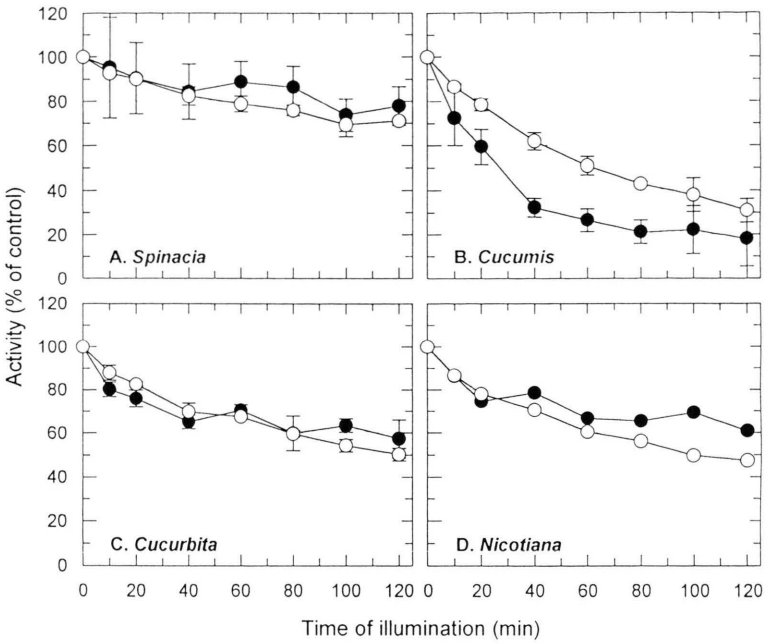

Fig. 2. A–D. Effects of photoinhibitory treatment on activities of PSI and PSII in leaf discs of *Spinacia*, *Cucumis*, *Cucurbita*, and *Nicotiana* exposed to a PAR of 2000 μmol m^{-2} s^{-1} at 4 °C. Mean values ± SD of four (A), three (B, C) or means of two (D) independent experiments are shown. F_V/F_M ratios of controls: 0.810 ± 0.007 (A), 0.807 ± 0.004 (B), 0.828 ± 0.008 (C), 0.803 (D). Symbols: ● PSI, ○ PSII.

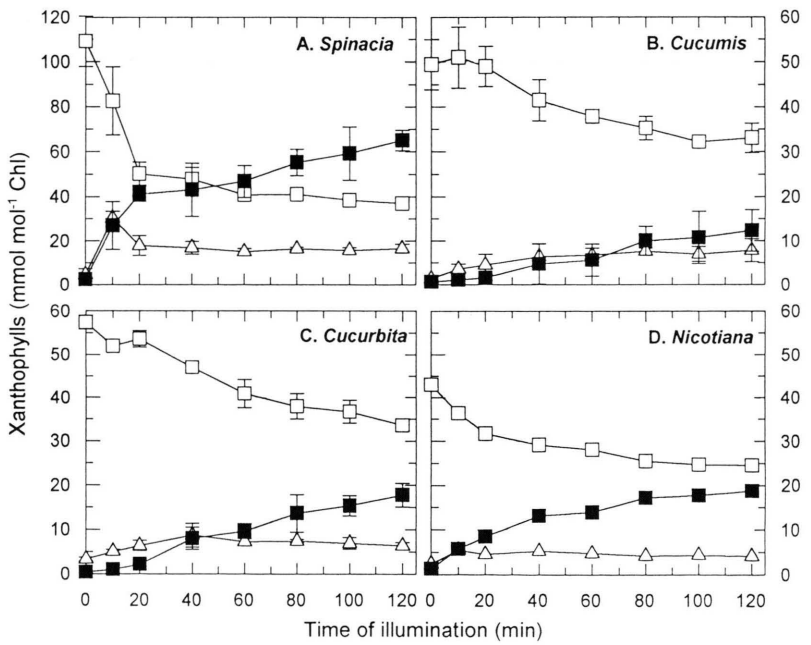

Fig. 3. A–D. Kinetics of de-epoxidation of V in leaf discs of *Spinacia*, *Cucumis*, *Cucurbita*, and *Nicotiana* exposed to a PAR of 2000 μmol m^{-2} s^{-1} at 4 °C. Mean values ± SD of three (A-C) or means of two (D) independent experiments are depicted. Because of the larger pool size of xanthophyll-cycle pigments in *Spinacia*, a scale different from B-D was choosen in A. Symbols: □ violaxanthin, △ antheraxanthin, ■ zeaxanthin.

may warrant an improved photoprotection of Chl *a*-binding core antennae (Lichtenthaler, 1981; Siefermann-Harms, 1987). In the chilling-sensitive species, a significant decrease of β-Car was observed after 2 h photoinhibitory treatment at 4 °C, referred to dark controls (Table I). In contrast, the content of β-Car did not decline significantly in chilling-tolerant *Spinacia*. As PSI contains, due to the larger core antenna, more β-Car than PSII (Yamamoto and Bassi, 1996; Färber *et al.*, 1997), the loss of β-Car might be related to the strong PSI inhibition in the three chilling-sensitive spe-

Table I. Content of chlorophyll *a+b* (Chl), total pool of xanthophyll-cycle pigments (VAZ), neoxanthin (N), lutein (L) and β-Carotene (β-Car) in chilling-tolerant (*Spinacia*) and in chilling-sensitive (*Cucumis*, *Cucurbita*, *Nicotiana*) leaves. Mean values ± SD (except for illuminated *Nicotiana* with *n*=2) of darkened controls (*Spinacia*, *n*=9; *Cucumis*, *n*=14; *Cucurbita*, *n*=11; *Nicotiana*, *n*=8), and of illuminated leaf discs at 4 °C (*Spinacia*, *n*=4; *Cucumis*, *Cucurbita*, *n*=3) and at 20 °C (*Spinacia*, *Cucurbita*, *n*=3; *Cucumis*, *n*=4) are given. PAR was 2000 μmol m^{-2} s^{-1} for 2 h.

Species	Treatment	Chl *a+b* (μmol m^{-2})	Chl *a*/Chl *b* (mol mol^{-1})	VAZ/Chl *a+b* (mmol mol^{-1})	N/Chl *a+b* (mmol mol^{-1})	L/Chl *a+b* (mmol mol^{-1})	β-Car/Chl *a+b* (mmol mol^{-1})
Spinacia	Dark	316 ± 50	3.36 ± 0.16	114 ± 12	50 ± 2	168 ± 5	151 ± 8
	Light, 4 °C	312 ± 47	3.43 ± 0.07	115 ± 8	51 ± 2	169 ± 9	138 ± 7
	Light, 20 °C	321 ± 11	3.25 ± 0.07	97 ± 10	53 ± 1	175 ± 5	151 ± 7
Cucumis	Dark	218 ± 42	3.20 ± 0.05	49 ± 7	52 ± 3	174 ± 10	136 ± 4
	Light, 4 °C	207 ± 41	3.16 ± 0.04	53 ± 6	51 ± 4	179 ± 11	116 ± 3
	Light, 20 °C	215 ± 30	3.21 ± 0.05	46 ± 2	51 ± 1	196 ± 8	125 ± 9
Cucurbita	Dark	234 ± 34	3.43 ± 0.28	59 ± 5	52 ± 7	199 ± 16	133 ± 6
	Light, 4 °C	260 ± 45	3.35 ± 0.21	58 ± 1	53 ± 1	196 ± 8	115 ± 2
	Light, 20 °C	223 ± 23	3.07 ± 0.16	55 ± 6	52 ± 4	205 ± 11	133 ± 9
Nicotiana	Dark	290 ± 23	2.96 ± 0.09	46 ± 3	51 ± 2	198 ± 8	138 ± 4
	Light, 4 °C	291	2.80	47	52	196	124
	Light, 20 °C	286	2.78	45	52	205	126

cies. Possibly, a high β-Car content, as observed in cold-acclimated *Spinacia*, provides protection for PSI under high-light stress at low temperatures. However, since the β-Car content was diminished to a similar degree during photoinhibitory treatment in the three chilling-sensitive species analyzed, the preferential inhibition of PSI in *Cucumis* cannot be explained by the loss of β-Car. In all four species, the high irradiation at 4 °C did neither cause changes in lutein and neoxanthin levels (based on Chl) nor in Chl content per leaf area unit (Table I).

Our data demonstrate that PSI is inhibited besides PSII *in vivo* at low temperature both in chilling-sensitive and chilling-tolerant plants. Sonoike (1998) proposed that in chilling-sensitive plants, PSI and not PSII is the site of photoinhibition when leaves are exposed to weak or moderate irradiation at low temperature, i.e. when the FeS centers of the PSI acceptor side are largely reduced. It has been suggested that under this condition, the formation of ·OH is enhanced by reaction of H_2O_2 with the reduced FeS centers. In high light, the PSI acceptor side is supposed to be predominantly in the oxidized state (Havaux and Davaud, 1994) due to restriction of electron transport from PSII to PSI. Consequently, in high light, PSI would be less vulnerable and PSII would become more susceptible to photoinhibition than PSI. We can support this suggestion with our data obtained with *Cucumis* (but not with *Cucurbita* and *Nicotiana*) illuminated with moderate light (Fig. 1B). However, under high-light at low temperatures in *Cucumis* (Fig. 2B) and in potato leaves (Havaux and Davaud, 1994) the decline in activity of PSI also proceeded faster than the decrease in PSII efficiency. These results and the parallel courses of PSI and PSII inhibition in *Cucurbita* and *Nicotiana* (Figs. 1C, D; 2C, D) do not support the above hypothesis of a preferential inhibition of PSI in low and of PSII in high light.

We conclude that selective photoinhibition of PSI is not a general phenomenon in chilling-sensitive plants. Obviously, light-stress responses of PSI and PSII at low temperatures differ among chilling-sensitive plant species. However, we cannot explain at present the particularly high sensitivity of *Cucumis*. Possibly, besides the chloroplast pigments, the membrane lipid composition is responsible for varying sensitivity of PSI and PSII to photoinhibition (Murata, 1983). Moreover, a strongly reduced activity of the antioxidative scavenging system at low temperatures, not tested here, might enhance the photoinhibition in *Cucumis*. A pronounced temperature effect on AP activity in leaves of *Cucumis* has been reported by Terashima *et al.* (1998).

Growth conditions are known to have an impact on the susceptibility of plants to photoinhibition. Sonoike (1995b) demonstrated that the decrease in the P700 content of common bean leaves, illuminated at 3.5 °C for 5 h, occurred considerably faster when plants were grown at low as compared to high irradiance. In our study, *Nicotiana* plants

were grown in much lower light than *Cucumis* and *Cucurbita*. In *Nicotiana*, the lower growth light resulted in larger Chl *a,b* antennae, as evidenced by significantly lower Chl *a/b* ratio (Table I) and relatively high sensitivity to light stress at 4 °C: Similar responses of PSI and PSII were observed in *Cucurbita* and *Nicotiana* (Fig. 2C, D), although the latter is known as a less chilling-sensitive species (Murata, 1983).

PSI and PSII inhibition at 20 °C

In cold-tolerant *Spinacia*, PSII was less susceptible to photoinhibition at 20 °C (as at 4 °C, see above) than in the three other species tested (Fig. 4). The time course of PSI and PSII inhibition in *Spinacia* at 20 °C (Fig. 4A) was very similar to that at 4 °C (*cf.* Fig. 2A). The insensitivity of *Spinacia* to the low temperature was obviously related to the cold-acclimation of these plants (see Somersalo and Krause, 1990). In contrast, in the chilling-sensitive species, PSI and PSII were less affected at 20 °C (Fig. 4B-D) than at 4 °C (*cf.* Fig. 2B-D). In particular, PSI activity was significantly more stable at 20 °C. In *Cucurbita* and *Nicotiana*, PSI was not stronger affected than in *Spinacia*, whereas PSI in *Cucumis* was more sensitive. Within 2 h, high light at 20 °C caused an about 50% decrease of PSI activity in parallel with PSII photoinhibition in *Cucumis*. A preferential photoinhibition of PSI was not observed at 20 °C.

Similar to high-light exposure at 4 °C, leaves of *Spinacia* (Fig. 5A) exhibited at 20 °C a considerably faster de-epoxidation of V than the three chilling-sensitive species (Fig. 5B-D). The slowest de-epoxidation was observed in *Cucumis*. All four species showed a faster de-epoxidation kinetics in high light at 20 °C (Fig. 5A-D) than at 4 °C (*cf.* Fig. 3A-D). However, the temperature effect on the xanthophyll cycle was much stronger in the chilling-sensitive plants than in *Spinacia*. In the latter, the same high Z levels were reached in 2 h at 4 °C and 20 °C, whereas in *Cucumis*, *Cucurbita* and *Nicotiana* the Z content remained considerably lower at 4 °C than at 20 °C after 2 h illumination. No significant changes both in the content of Chl and of carotenoids were caused by high-light stress at 20 °C in the four species tested (Table I).

The generally higher activity of the xanthophyll cycle in the cold-acclimated leaves of *Spinacia* (Figs. 3 and 5; see also Thiele *et al.*, 1996) and of the antioxidative scavenging system (see Krause, 1994) supposedly are important factors that reduce the susceptibility of PSII to photoinhibition both at 20 °C and 4 °C. At 20 °C, in the chilling-sensitive *Cucurbita* and *Nicotiana*, but not in *Cu-*

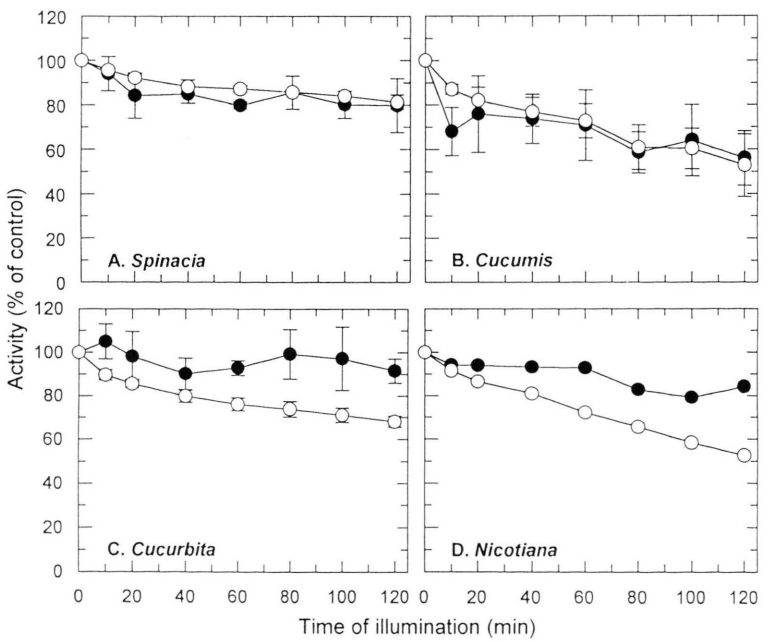

Fig. 4. A-D. Effects of irradiation with 2000 μmol m^{-2} s^{-1} at 20 °C on activities of PSI and PSII in leaf discs of *Spinacia*, *Cucumis*, *Cucurbita*, and *Nicotiana*. Mean values ± SD of three (A, C), four (B) or means of two (D) independent experiments are given. F_V/F_M ratios of controls: 0.806 ± 0.004 (A), 0.800 ± 0.003 (B), 0.833 ± 0.001 (C), 0.792 (D). Symbols: ● PSI, ○ PSII.

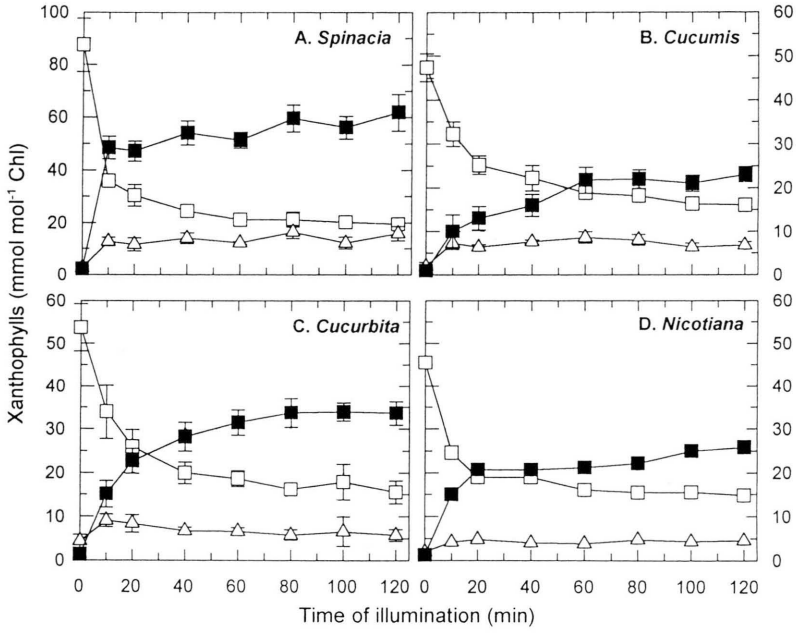

Fig. 5. A-D. Kinetics of conversion of xanthophyll-cycle pigments in leaf discs of *Spinacia*, *Cucumis*, *Cucurbita*, and *Nicotiana* illuminated with 2000 μmol m^{-2} s^{-1} at 20 °C. Mean values ± SD of three (A, C), four (B) or means of two (D) independent experiments are shown. Notice the different scale in Fig. 5A for *Spinacia*. Symbols: □ violaxanthin, △ antheraxanthin, ■ zeaxanthin.

cumis, PSI appeared to be as well protected as in *Spinacia*. The strong enhancement, particularly of PSI photoinhibition at low temperature, seen in all three chilling-sensitive species, may be related to a reduced function of the scavenger system for AOS and to the restriction of xanthophyll-cycle activity (Fig. 3B-D). Lowered activities of enzymes of the antioxidative scavenging system (except of SOD) at 5 °C, compared with 19 °C, have been reported for two *Zea* species by Jahnke *et al.* (1991). Similar to the data obtained here for *Cucumis*, Havaux and Davaud (1994) observed a preferential photoinhibition of PSI, compared to PSII, under high-light stress at 3 °C, but not at 23 °C, in leaves of potato (*Solanum tuberosum*). Potato is not considered a chilling-sensitive plant. Possibly, PSI was highly susceptible to photoinhibition at 3 °C due to lacking cold-acclimation, as the plants had been grown at warm temperatures. When in the potato leaves the SOD activity was blocked by diethyldithiocarbamate (DDC), the course of PSI inhibition at 23 °C was similar to the inhibition that occurred at 3 °C in the absence of DDC. Havaux and Davaud (1994) concluded from their data that the major cause of PSI damage at low temperature is a reduced scavenging activity for $\cdot O_2^-$, rather than an enhanced formation of AOS including singlet oxygen.

Complete inhibition of the xanthophyll cycle by dithiothreitol (DTT) in leaves of *Cucumis* and *Cucurbita* did not enhance photoinhibition of PSI at 20 °C (Barth and Krause, 1998). At first sight, this suggests that Z and A formed in the xanthophyll cycle do not protect PSI. However, one cannot exclude that the reducing power of DTT compensated for a possible loss of protection by the de-epoxidized xanthophylls.

PSI and PSII inhibition in the presence of methylviologen

Experiments with MV were carried out with leaves of *Cucurbita* to gain information on the protective role of the antioxidative scavenging system. The presence of MV during high-light exposure at 20 °C had relatively little effect on PSI activity (Fig. 6A; *cf.* Fig. 4C). Although there was a wide variation of data (possibly due to an unequal MV distribution in the leaf tissue), a dramatic decrease (about 90% within 2 h) of PSII activity could be observed. As a tendency, a similar effect on PSII was seen when MV-treated leaves were illuminated at 4 °C (Fig. 6B). These results were surprising, as the MV-catalized formation of $\cdot O_2^-$ occurs at the acceptor side of PSI. Sonoike *et al.* (1997) proposed that MV may protect PSI during

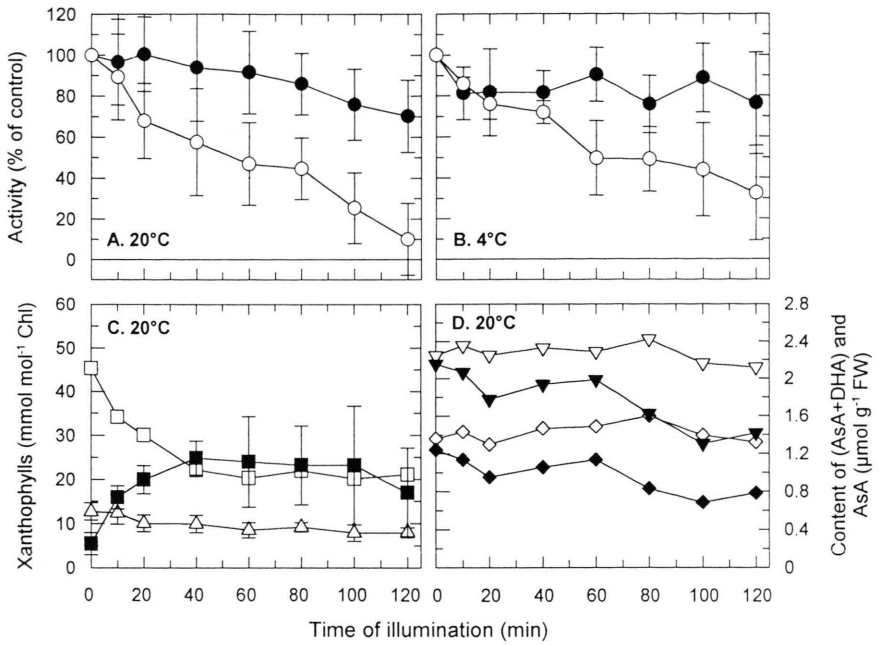

Fig. 6. A-D. Effects of methylviologen in illuminated *Cucurbita* leaves on PSI and PSII activities at 20 °C (A) and at 4 °C (B), on the xanthophyll cycle at 20 °C (C) and on the contents of dehydroascorbate + ascorbate and ascorbate at 20 °C (D). Leaves were preincubated for 16 h under irradiation with 30 µmol m^{-2} s^{-1} via the petiole with a 2 mM MV solution. After illumination of leaf discs with 2000 µmol m^{-2} s^{-1} at 20 °C (A) or 4 °C (B), activities of PSI and PSII were measured and de-epoxidation of V at 20 °C (C) was determined. Contents of DHA+AsA and AsA were measured in the presence and absence of MV during irradiation with 2000 µmol m^{-2} s^{-1} at 20 °C (D). Mean values ± SD of five (A, C), six (B) or means of two (D) independent experiments are depicted. For reasons of clarity SD of V (between 2.2 and 4.5 mmol mol^{-1} Chl) were omitted (C). F_V/F_M ratios for controls: 0.787 ± 0.020. Symbols: ● PSI, ○ PSII, □ violaxanthin, △ antheraxanthin, ■ zeaxanthin, ▽ dehydroascorbate + ascorbate and ◇ ascorbate in the absence of MV, ▼ dehydroascorbate + ascorbate and ◆ ascorbate in the presence of MV.

illumination with low or moderate light by preventing an accumulation of reduced FeS centers in PSI and, thus, suppresses formation of ·OH. Jakob and Heber (1996) have demonstrated that *in vitro* and *in vivo* ·OH formation was indeed diminished in the presence of MV. On the other hand, the enhanced formation of AOS in the presence of MV is potentially damaging, and this is indeed apparent from Figs. 6A and 6B for PSII. The low sensitivity of PSI in the presence of MV shown in Fig. 6A, B may indicate that even in high light, efficient electron acceptors such as MV that keep the FeS centers in the oxidized state provide protection.

The presence of MV also affected the xanthophyll cycle (Fig. 6C). Its activity was assessed at a PAR of 2000 µmol m^{-2} s^{-1} both at 20 °C (Fig. 6C) and at 4 °C (data not shown). In both cases, some A and Z was detected at t = 0 min. This was probably a result of preincubation with MV in low light. In the presence of MV, only relatively low Z levels which tended to decline after 100 min illumination were reached under high light at 20 °C (Fig. 6C; *cf.* Fig. 5C). This effect may result from lack of AsA, the essential co-substrate of VDE. In the presence of MV, AsA will be turned over in high rates in the AP reaction that detoxifies H_2O_2 formed from $\cdot O_2^-$ in the chloroplasts. It has been proposed that AP has a higher affinity to AsA than VDE (Neubauer and Yamamoto, 1992). AsA regeneration obviously is limited due to diversion of electrons via MV to O_2 (Jakob and Heber, 1996). Fig. 6D supports this suggestion: Both the content of DHA+AsA and AsA declined by approximately 30% within 2 h strong irradiation in the presence of MV. When MV was absent, changes in the content of DHA+AsA and AsA in leaf discs exposed to 2000 µmol photons m^{-2} s^{-1} at 20 °C did not occur (Fig. 6D). The kinetics of de-epoxidation determined at 4 °C was not af-

fected by MV indicating that the low temperature, but not AsA, was the limiting factor for xanthophyll-cycle activity (*cf.* Fig. 3C).

In summary, in the presence of MV, high light enhanced inhibition of PSII, whereas the decline in the P700 signal was less pronounced (*cf.* Fig. 4C and Fig. 6A). In addition, MV restricted xanthophyll-cycle activity at 20 °C possibly due to lowered concentration of AsA, the substrate of VDE (*cf.* Fig. 5C and Fig. 6C, D). Our data suggest that at room temperature, PSI is protected better than PSII against photoinhibition by AOS and becomes even more stabilized when the acceptor side is efficiently kept in the oxidized state, as is the case in the presence of MV. This supports the assumption that the antioxidative scavenging system is an important mechanism for protecting PSI under high-light stress.

Conclusion

Our data indicate that under light and low-temperature stress, PSI may be affected in addition to PSII both in chilling-tolerant and chilling-sensitive plants (see Figs. 1B, 2, 4). Leaves of chilling-tolerant spinach (in the cold-acclimated state) revealed a higher *in vivo* tolerance to light stress at low and room temperature (see Figs. 1A, 2A, 4A) than the three chilling-sensitive species tested. In the latter, photoinhibition of PSI and PSII was enhanced at low compared to room temperature. However, the preferential inhibition of PSI in *Cucumis* at low temperature cannot be considered a general phenomenon among chilling-sensitive plants.

PSII inhibition under high-light stress at low temperatures probably results in chilling-sensitive plants both from diminished activity of the xanthophyll cycle (*cf.* Fig. 3B-D and Fig. 5B-D) and the scavenger system for AOS. The strong effect of AOS on PSII (Fig. 6A) is demonstrated by the action of MV. PSI photoinhibition supposedly results mainly from a limited function of the antioxidative scavenging system. So far, it has not been clarified, whether the xanthophyll cycle operating in PSI contributes to protection. A limitation of electron transport, e.g. when at low temperatures rates of carbon metabolism are reduced, results in an increase of reducing power, reduction of FeS centers and enhanced formation of $\cdot O_2^-$ and other AOS at the acceptor side of PSI, as proposed by Sonoike *et al.* (1997) and Terashima *et al.* (1998) for low-light conditions. Our data demonstrate that the factors leading to PSI inhibition are not restricted to low or moderate light. Rather, in all plants tested, PSI was more severely affected in high than in low light at 4 °C (*cf.* Figs. 1, 2). Possibly, also in high light a fraction of reduced FeS centers is responsible for formation of damaging ·OH from H_2O_2. The relatively small effect of MV during strong illumination on PSI, compard to PSII (Fig. 6A, B) could be explained by enhanced oxidation of recuced of FeS centers. Overall, a highly active antioxidative scavenging system in the chloroplast appears to be essential to protect the photosynthetic apparatus against light stress at suboptimal and optimal temperatures.

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