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Temperature and light dependence of photosynthetic activities in wheat seedlings grown in the presence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]

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Abstract

Photosynthetic electron transfer was studied in thylakoids isolated from control and DCMU-grown wheat (*Triticum aestivum* L.) seedlings. When exposed to high temperature (HT) and high iradiance (HI), thylakoids showed large variations in the photosynthetic electron transport activities and thylakoid membrane proteins. A drastic reduction in the rate of whole electron transport chain ($H_2O \rightarrow MV$) was envisaged in control thylakoids when exposed to HT and HI. Such reduction was mainly due to the loss of photosystem 2, PS2 ($H_2O \rightarrow DCBQ$) activity. The thylakoids isolated from seedlings grown in the presence of DCMU showed greater resistance to HT and HI treatment. The artificial exogenous electron donors $MnCl_2$, DPC, and NH_2OH failed to restore the HI induced loss of PS2 activity in both control and DCMU thylakoids. In contrast, addition of DPC and NH_2OH significantly restored the HT induced loss of PS2 activity in control thylakoids and partially in DCMU thylakoids. Similar results were obtained when F_v/F_m was evaluated by chlorophyll fluorescence measurements. The marked loss of PS2 activity in control thylakoids was evidently due to the loss of 33, 23, and 17 kDa extrinsic polypeptides and 28-25 kDa LHCP polypeptides.

Additional key words: chlorophyll fluorescence; donor side; electron transport; photosystem; thylakoid membrane proteins; *Triticum*.

Introduction

When leaves or isolated thylakoids are gently heated or exposed to high temperature (HT) for a short period, their photosynthetic apparatus shows characteristic temperature-dependent damage. The observation that HT decreases the quantum yield of CO_2 fixation suggests damage in the primary processes of photosynthesis taking place in thylakoid membranes. Investigations with isolated membrane systems have shown that heat treatment of thylakoids primarily inactivates the photochemical reactions in thermolabile species. The photosystem 2 (PS2), the water-splitting complex, and the photophosphorylation reactions are the most sensitive systems of the membrane bound photosynthetic apparatus (Berry *et al.* 1975, Mohanty *et al.* 1987). Inactivation also occurs at temperature significantly below those associated with thermal denaturation of stromal enzyme (Santarius 1973, Mohanty *et al.* 1987) indicating that the primary site of damage is probably associated with components of the photosynthetic system located in the thylakoid membranes. PS2 mediated electron transport is particularly susceptible to heat stress (Santarius 1975) that was originally attributed to damage to the O_2 evolving complex. By contrast, PS1 mediated electron transport is heat stable (Pearcy *et al.* 1977, Armond *et al.* 1978). Heat stress also leads to modifications of thylakoid membranes such as disappearance of grana structure (Gounaris *et al.* 1984, Ivanov *et al.* 1987), vesiculation of thylakoid membranes (Gounaris *et al.* 1984), and physical separation of light-harvesting chlorophyll protein LHCP2 from the PS2 complex (Armond *et al.* 1980).

Photosynthetically active radiation drives the photochemical reactions of PS1and PS2 in O_2-evolving photo-

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Abbreviations: Chl – chlorophyll; DCBQ – 2,6-dichloro-*p*-benzoquinone; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenol indophenol; DPC – diphenyl carbazide; DTT – dithiothreitol; F_0 – minimal fluorescence; F_m – maximum fluorescence; HI – high irradiance; HT – high temperature; kDa – kilodalton; LHCP – light-harvesting chlorophyll protein; MV – methyl viologen; PAR – photosynthetically active radiation; PS – photosystem; SDS-PAGE – sodium dodecylsulphate-polyacrylamide gel electrophoresis.
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synthetic organisms. Nevertheless, excessive radiant energy inhibits photosynthesis. This phenomenon is termed photoinhibition (Critchley 1988, Long *et al.* 1994). The photoinhibition occurs at thylakoid level, particularly at PS2 (Cleland and Critchley 1985, Eckert *et al.* 1991). Strong irradiation induces primary photoinhibition associated with the degradation of D1 protein (Richter *et al.* 1990), inactivation of PS2 reaction centres (Cleland and Critchley 1985, Arntz and Trebst 1986), and probably with some modification of the LHCP complex (Bradbury and Baker 1986). Structural and functional reorganisation of thylakoid membranes in thermal or high irradiance stressed thylakoids cause partial thylakoid pigment bleaching (Williams *et al.* 1986, Waloszek *et al.* 1992, Siwiak *et al.* 1994) which is particularly pronounced at the early stage of thylakoids biogenesis (Waloszek *et al.* 1992).

A number of environmental conditions elicit photosystem stoichiometry adjustments and changes of the chlorophyll (Chl) antenna size of the photosystems in the thylakoid membrane chloroplasts (Riethman and Sherman 1988). The ability of photosynthetic organisms to respond to sub-lethal doses of herbicide is of great practical as well as fundamental importance. Triazine and urea type herbicides block photosynthetic electron transport by occupying the plastoquinone-binding site of D1. Chloroplasts respond to sublethal concentration of herbicides (Mannan and Bose 1985, Bose *et al.* 1992, Zer and Ohad 1995, Komenda and Masojídek 1998, Komenda *et al.* 2000). DCMU (diuron) is a potent member of the urea class and atrazine is a potent and widely used triazine. In addition to having nearly identical physiological effects, these herbicides competitively bind to the same site on the thylakoid membrane (Tischer and Strotmann 1977). Invariably, herbicides cause changes in the structure and composition of thylakoids, including greater grana size and increased content of unsaturated lipids (Bose *et al.* 1992). Concomitantly, a lower Chl *a/b* ratio and lower β-carotene/xanthophyll ratio has been reported (Nauš and Melis 1992). Growing plants in the presence of DCMU altered the pigment composition to a minimum extent but produced significant changes in the photosynthetic electron transport reactions (Kulandaivelu and Annamalainathan 1991, Komenda *et al.* 2000).

We have attempted to elucidate the effect of relatively high temperature (HT) and/or high irradiance (HI) on photosynthetic electron transport and thylakoid proteins in wheat seedlings treated with DCMU and also to assess whether the herbicide treatment alters the light harvesting and photon energy utilisation by the photosystems.

Materials and methods

Plants and DCMU treatment: Wheat (*Triticum aestivum* L.) seedlings were grown on three layers of coarse filter paper in a glass petri dish at 25 °C under "white fluorescent light" (1 600 μmol m^{-2} s^{-1}) provided by a bank of cool day fluorescent lamps under a 14/10 h light/dark regime. 20 cm^3 of 100 μM DCMU was added initially. Thereafter, only water was added periodically.

Isolation of thylakoids: Leaves from 12 d-old wheat seedlings were homogenised in ice cold buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, and 400 mM sucrose. Chloroplasts were pelleted by centrifuging for 5 min at 8 000×*g*. After osmotic shock in the previous buffer, but lacking sucrose, lysed chloroplasts were pelleted and re-suspended in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, and 100 mM sucrose. Chl concentration was determined spectrophotometrically following the method of Arnon (1949).

HT treatment: Thylakoids were suspended in a medium containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, and 400 mM sucrose at 500 g(Chl) m^{-3} and incubated in a water bath at 40 °C for 15 min. Samples were drawn at known time intervals for photosynthetic measurements.

HI treatment: Thylakoids were suspended in a medium containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10 mM NaCl, and 400 mM sucrose at 500 g(Chl) m^{-3} and were irradiated with "white light" (2 000 μmol m^{-2} s^{-1}) for 30 min at constant temperature at 25 °C. Samples were drawn at known time intervals for photosynthetic measurements.

Combined HT+HI treatment: The temperature pretreated (40 °C for 15 min) thylakoids suspension was magnetically stirred at 25 °C and irradiated with focussed "white light" of 2 000 μmol m^{-2} s^{-1} PAR for 30 min. The samples were used only for analysis of thylakoid proteins.

PS2 activity: Oxygen evolution was measured following the method of Noorudeen and Kulandaivelu (1982) with a Clark-type electrode (*Hansatech*) fitted with a circulating water jacket at 27 °C. Actinic radiation from a slide projector placed on the side of the electrode chamber was filtered through 9.5 cm of water. The irradiance was 1 100 μmol m^{-2} s^{-1} at the surface of the water bath cell. Thylakoids were suspended at 10 g(Chl) m^{-3} in the assay medium containing 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$, 5 mM NH_4Cl, and 100 mM sucrose supplemented with 500 μM DCBQ.

Rate of DCPIP photoreduction was determined by following the decrease in absorbance at 590 nm using a *Hitachi 557* spectrophotometer. The reaction mixture

($3\ cm^3$) contained 20 mM Tris-HCl, pH 7.5, 5 mm $MgCl_2$, 10 mM NaCl, 100 mM sucrose, 100 μm DCPIP, and thylakoid membranes equivalent to 20 μg Chl. Wherever mentioned, the concentrations of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively.

Modulated Chl fluorescence of isolated thylakoid membranes at room temperature was measured with *PAM 2000* fluorimeter (*H. Walz*, Effeltrich, Germany) (cf. the review of Roháček and Barták 1999). Measurements were done in 0.7 cm^3 of reaction mixture containing 50 mm Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 100 mM sucrose, and thylakoid membranes equivalent to 10 μg Chl. The integrated measuring irradiance (480 nm) was 0.15 μmol $m^{-2}\ s^{-1}$ with red actinic radiation (650 nm) of 100 μmol $m^{-2}\ s^{-1}$.

SDS-PAGE: Thylakoids were separated using the discontinuous polyacrylamide gel system of Laemmli (1951), with the following modifications. Gels consisted of a 10-18 % linear gradient of polyacrylamide containing 4 M urea. Samples were solubilised at 20 °C for 5 min in 2 % (m/v) sodium dodecyl sulphate (SDS) and 60 mM dithiothreitol with 8 % sucrose using SDS-Chl ratio of 20 : 1. Electrophoresis was performed at 20 °C with constant current of 5 mA. Gels were stained in methanol/acetic acid/water (4 : 1 : 5, v/v/v) containing 0.1 % (m/v) Coomassie Brilliant Blue R and de-stained in methanol/acetic acid/water (4 : 1 : 5, v/v/v). Protein content was estimated by the method of Lowry *et al.* (1951).

Results

Changes in total Chl: When wheat seedlings were grown under absence (control) or presence of DCMU for 12 d, the content of total Chl was lower in the leaves of DCMU seedlings. The total Chl content expressed on a fresh matter basis in control and DCMU was 1.946 and 1.128 g kg^{-1}, respectively. The Chl *a/b* ratio was also lower in DCMU-treated seedlings. The Chl *a/b* ratio of control and DCMU-treated seedlings was 2.9 and 2.4, respectively. DCMU did not change the growth parameters such as plant height, leaf area, leaf fresh and dry masses (values not shown). Thylakoids were isolated from 12-d-old control and DCMU grown seedlings for further experiments.

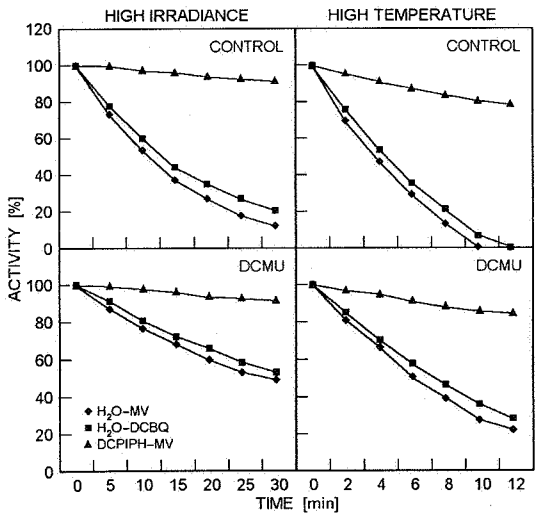

Fig. 1. Effect of HT and HI on electron transport activities in thylakoids isolated from wheat seedlings grown in the presence or absence of DCMU. The 100 % values are [mmol(O_2) kg^{-1}(Chl) s^{-1}]: H_2O → MV 36, 24; H_2O → DCBQ 45, 30; $DCPIPH_2$ → MV 50, 31, for control and DCMU thylakoids, respectively. Means of 3 experiments. All values are significant at ±5 % level.

Changes in photosynthetic activities: Effects of HT and HI treatments on photosynthetic electron transport were studied using isolated thylakoids from control and DCMU-treated seedlings (Fig. 1). PS1 activity was less sensitive to both HT and HI treatment. In contrast to this, a complete inactivation of PS2 electron transport activity was found in control thylakoids when exposed to 12 min of HT treatment, while in DCMU thylakoids a 20 % activity was observed even after 12 min treatment.

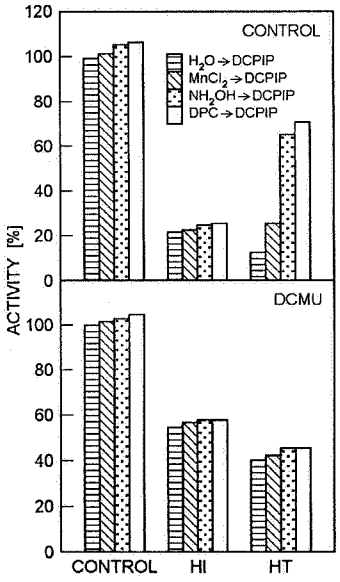

Fig. 2. Effect of various exogenous electron donors on photosystem 2 (PS2) activity in thylakoid membranes isolated from control and DCMU treated leaves. Thylakoids were treated with HT for 8 min and HI for 30 min. The 100 % values are [mmol(O_2) kg^{-1}(Chl) s^{-1}]: H_2O → DCPIP 49, 30; $MnCl_2$ → DCPIP 50, 31; DPC → DCPIP 52, 33; NH_2OH → DCPIP 51, 32, for control and DCMU thylakoids, respectively. Means of 3 experiments. All values are significant at ±5 % level.

A similar trend was also observed in whole chain electron transport activity. In thylakoids exposed to HI, the loss of whole chain and PS2 mediated electron transport rate was slower in the DCMU thylakoids than in the control thylakoids. After 30 min of HI treatment, 78 and 44 % losses of PS2 activity were observed in control and DCMU thylakoids, respectively.

Changes in DCPIP photoreduction: To locate the possible site(s) of inhibition in the PS2 reaction, DCPIP photoreduction supported by using various artificial electron donors was followed in control and DCMU thylakoids. Wydrzynski and Govindjee (1975) showed that $MnCl_2$, NH_2OH, DPC, and HQ could donate electrons to the intermediates Z_1 and Z_2 of the PS2 reaction. Fig. 2 shows the electron transport activity of PS2 in the presence and absence of three of the above compounds. In control thylakoids, the PS2 activity at the end of 8 min HT treatment showed as much as 88 % loss of the activity, when water served as electron donor. A similar trend was also found in the system $MnCl_2$ → DCPIP. A significantly loss in activity of PS2 was also found using NH_2OH and DPC as electron donor in control thylakoids. The loss of activity and differences among the individual electron donors were much smaller in DCMU thylakoids than in control thylakoids. Upon the HI treatment in both control and DCMU thylakoids, artificial exogenous electron donors did not restore the loss of PS2 activity (Fig. 2).

These results agree with the measurements obtained by modulated Chl fluorescence using various exogenous electron donors in control and DCMU thylakoids (Fig. 3). The addition of DPC and NH_2OH to 8 min-HT-treated control thylakoids induced an increase of variable fluorescence (F_v) (Fig. 3). On the other hand, HT treatment

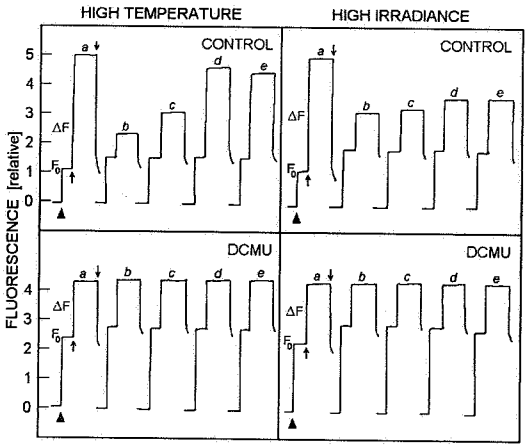

Fig. 3. Room temperature Chl fluorescence induction curves from thylakoid membranes of control and DCMU treated leaves. *a* (0 min) and *b* (8 min HT, 30 min HL), without donors; *c*, $MnCl_2$; *d*, NH_2OH; *e*, DPC. The Chl concentration was 10 g m^{-3}. Switching on the measuring radiation (480 nm, 0.15 μmol m^{-2} s^{-1}) and actinic radiation (650 nm, 100 μmol m^{-2} s^{-1}) on ↑ and off ↓, respectively.

Table 1. Changes in the relative levels of fluorescence emitted as minimal fluorescence (F_0), variable fluorescence (F_v), and the ratio of variable to maximum fluorescence (F_v/F_m) in thylakoid membranes isolated from control and DCMU-treated leaves with or without electron donors. Concentrations of $MnCl_2$, DPC, and NH_2OH were 5.0, 0.5, and 5.0 mM, respectively. The integrated measuring irradiance (480 nm) was 0.15 μmol m^{-2} s^{-1}, with red actinic radiation (650 nm) the irradiance was 100 μmol m^{-2} s^{-1}.

Treatment		F_0	F_v	F_v/F_m
HT	control, 0 min	1.20±0.01	4.00±0.11	0.76±0.03
	8 min	1.60±0.03	0.80±0.01	0.33±0.02
	$MnCl_2$	1.60±0.04	1.60±0.02	1.60±0.02
	DPC	1.60±0.03	3.10±0.05	0.66±0.01
	NH_2OH	1.60±0.02	3.00±0.05	0.65±0.02
	DCMU, 0 min	2.40±0.05	2.00±0.06	0.45±0.03
	8 min	2.90±0.08	1.60±0.03	0.36±0.01
	$MnCl_2$	2.90±0.10	1.60±0.04	0.36±0.01
	DPC	2.90±0.09	1.60±0.02	0.36±0.02
	NH_2OH	2.90±0.11	1.60±0.03	0.36±0.02
HI	control, 0 min	1.20±0.05	4.00±0.11	0.76±0.01
	30 min	2.00±0.06	1.30±0.02	0.40±0.01
	$MnCl_2$	2.00±0.05	1.50±0.02	0.43±0.02
	DPC	2.00±0.04	1.80±0.06	0.47±0.02
	NH_2OH	2.00±0.05	1.80±0.09	0.47±0.01
	DCMU, 0 min	2.40±0.09	2.00±0.06	0.45±0.02
	30 min	3.10±0.10	1.40±0.05	0.31 ±0.01
	$MnCl_2$	3.10±0.08	1.40±0.03	0.31±0.02
	DPC	3.10±0.09	1.40±0.04	0.31±0.02
	NH_2OH	3.10±0.10	1.40±0.03	0.31±0.01

significantly increased F_0 without much change in F_v by the addition of electron donors in DCMU thylakoids (Fig. 3). In thylakoids treated with HI, the F_v/F_m ratio did not increase by the addition of various electron donors both in control and DCMU thylakoids (Table 1).

Changes in thylakoid membrane proteins: Since the changes in photosynthetic electron transport activities could be caused primarily by the changes or reorganisation of thylakoid components, the polypeptide composition of control and DCMU thylakoids treated with or without HT, HI, and HT+HI were analysed by SDS-PAGE. Significant changes in thylakoid polypeptides were observed when the HT time was increased. After a 12 min-treatment, the contents of 47, 43, 33, 28-25, 23, and 17 kDa polypeptides decreased considerably more in control thylakoids than in DCMU thylakoids (Fig. 4). Polypeptides of 28-25 kDa in DCMU thylakoids were more stable even after a 12 min-HT treatment. Exposure to HI did not produce any loss of 47, 43, 33, 28-25, 23, and 17 kDa polypeptides in both control and DCMU thylakoids. In contrast to this, a drastic reduction of several polypeptides was observed both in control and DCMU thylakoids when incubated after pre-treatment with HT and subsequently with HI (Fig. 4).

Discussion

The present results indicate that exposure of control and DCMU thylakoids to HI and HT treatments produces differential loss of photosynthetic activity in that the former thylakoids are more sensitive than the later. Many studies on HT and HI on photosynthetic light reactions have indicated that PS1 activity was less affected by HT and HI than PS2 (Emmett and Walker 1973, Berry *et al.* 1975, Powles 1984, Yordanov 1992). After 12 min of HT treatment considerable inhibition of PS2 activity occurred in control thylakoids, while DCMU thylakoids were more resistant. A similar trend was also was observed both in control and DCMU thylakoids when exposed to HI.

The drastic loss of PS2 activity in control thylakoids indicates that the primary target site of HT and HI was PS2. HT and HI primarily attack the reaction centre components of PS2 (Ohad *et al.* 1990, Prášil *et al.* 1992) and the water-oxidising complex (Berry and Björkman 1980, Nedunchezhian and Kulandaivelu 1993). Thylakoids isolated from the DCMU-grown seedlings showed greater resistance to HT and HI than control thylakoids. Any change in the reaction centre proteins may alter the electron transport components and subsequently change the sensitivity to HT and HI treatment. Thus, the observed HT and HI resistance of DCMU thylakoids may be due to: (*1*) modification of herbicide binding proteins of PS2 reaction centre, and/or (*2*) the partial protection offered by herbicide that confers resistance to subsequent HT treatment.

DCMU through its effects on membrane fatty acid composition (values not shown) could bring about alteration in the effects of HT and HI on photosynthetic electron transport. Our observation that the HT and HI dependence of uncoupled irradiance-saturated rate of PS1 electron transport ($DCPIPH_2 \rightarrow MV$) was not affected, whereas the whole chain electron transport was altered as a result of DCMU treatment indicates that the DCMU-

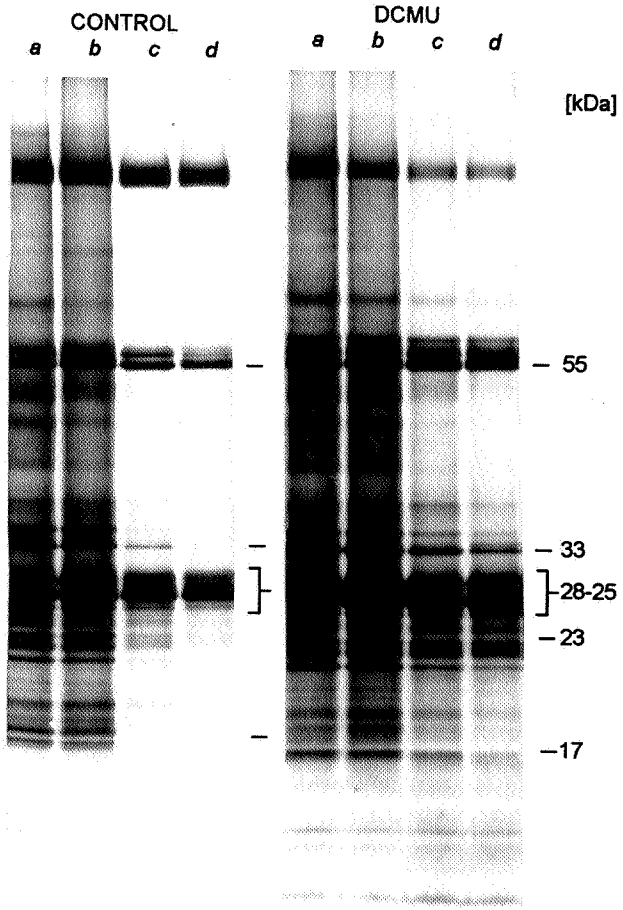

Fig. 4. SDS-PAGE polypeptide profiles of thylakoids isolated from control and DCMU treated leaves. Gel lanes were loaded with equal amounts of proteins (100 μg). *a*, 0 min (without treatment); *b*, 30 min HI; *c*, 12 min HT; *d*, 12 min HT + 30 min HI.

induced changes occur on the acceptor side of PS2.

Further experiments with artificial electron donors showed that DPC donates electrons directly to the reaction centre and NH_2OH to a site between Z_1 and Z_2 (Wydrzynski and Govindjee 1975). DPC and NH_2OH restored the HT-induced loss of PS2 activity markedly in control thylakoids, and partially in DCMU thylakoids. In contrast to this, HI treatment did not restore the loss of PS2 activity in both control and DCMU thylakoids. The sensitivity of photosynthetic electron transport to HT and HI treatments was analysed in thylakoids isolated from control and DCMU-treated seedlings. In thylakoids isolated from DCMU seedlings, HT and HI were less effective in inactivation of electron transfer. Moreover, when DCMU was added to thylakoids isolated from control seedlings, such inhibition was not observed (values not shown). This suggests alterations in structural organisation of thylakoid membranes resulting from growing the plants in the presence of DCMU. A similar observation was made in wheat seedlings grown at different temperature in the presence of *BASF 13.338* (Mannan and Bose 1986).

These results were also confirmed by measurement of modulated Chl fluorescence. After addition of DPC and NH_2OH to HT-treated control thylakoids, a marked increase in F_v and a marginal one in F_0 were observed. This indicates that the site of HT is on the oxidising side of PS2, prior to the NH_2OH donation side and perhaps close to or after the DPC donation side. In contrast to this, an increase of F_0 without any change in F_v was observed when exogenous electron donors were added to DCMU thylakoids.

The most probable explanation for the inactivation of PS2 electron transport activity is that the related protein(s) is(are) exposed at the thylakoid surface (Seidler 1994). PS2 activity showed a drastic reduction in control thylakoids during HT treatment. Such reduction was associated with a major decrease in the amounts of 33, 28-25, 23, and 17 kDa polypeptides. The extrinsic proteins of 33, 23, and 17 kDa participate in photosynthetic O_2 evolution (Åkerlund and Jansson 1981, Kuwabara *et al.* 1985). Andersson *et al.* (1985) showed by manipulations of pH or ionic strength of the medium without the use of detergents that these three extrinsic polypeptides could be detached from the inner thylakoid surface. The binding of 23 and 17 kDa polypeptides involves only electrostatic force while the binding of the 33 kDa polypeptide also includes hydrogen bonds and hydrophobic interactions. The incubation of both types of thylakoids at HT and combined treatments might have led to the breakage of electrostatic force. Thereby a marked loss in the contents of 33, 23, and 17 kDa polypeptides was observed in control thylakoids, while in DCMU thylakoids the loss was much less. The loss of these polypeptides could also be one of the reasons for the significant loss of O_2 evolution capacity in control thylakoids.

When the control and DCMU thylakoids were treated with HT, not only extrinsic proteins were lost but there also was a marked loss of the 47, 43, and 28-25 kDa polypeptides in control thylakoids which may show a greater disruption of the PS2 complex by HT treatment. Light-harvesting complexes are important in light absorption, thylakoid stacking, and distribution of energy transfer. Damage to these complexes has multiple effects on the photosynthetic system. In control thylakoids the light-harvesting complex (28-25 kDa) is markedly reduced by HT treatment. This is also one of the reasons for marked loss of O_2 evolution capacity in control thylakoids. In contrast, DCMU thylakoid content of 28-25 kDa polypeptides was stable even after 15 min of HT treatment.

We conclude that DCMU-grown thylakoids are more resistant to HT and HI treatment than control thylakoids. This is due to: (*1*) the electron transport PS2 activity decrease during HT and HI treatment is smaller in DCMU-grown thylakoids than in control thylakoids, (*2*) the recovery of PS2 activity by artificial exogenous donors DPC and NH_2OH is larger in control thylakoids than in DCMU thylakoids, and (*3*) the contents of 33, 28-25, 23, and 17 kDa polypeptides were significantly lower in control thylakoids than in DCMU-grown thylakoids. The HT and HI treatments inactivate the donor and acceptor sides of PS2 in control thylakoids, respectively. In contrast, combined treatment inactivates only at the acceptor side of PS2 and partially protects the donor side in wheat thylakoids when grown in the presence of DCMU.

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