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Effect of irradiance on the thermal stability of thylakoid membrane isolated from acclimated wheat leaves

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Abstract

Thermal stability of thylakoid membranes isolated from acclimated and non-acclimated wheat (*Triticum aestivum* L. cv. HD 2329) leaves under irradiation was studied. Damage to the photosynthetic electron transport activity was more pronounced in thylakoid membranes isolated from non-acclimated leaves as compared to thylakoid membrane isolated from acclimated wheat leaves at 35 °C. The loss of D1 protein was faster in non-acclimated thylakoid membrane as compared to acclimated thylakoid membranes at 35 °C. However, the effect of elevated temperature on the 33 kDa protein associated with oxygen evolving complex in these two types of thylakoid membranes was minimal. Trypsin digestion of the 33 kDa protein in the thylakoid membranes isolated from control and acclimated seedlings suggested that re-organisation of 33 kDa protein occurs before its release during high temperature treatment.

Additional key words: D1 protein; oxygen evolving complex; photosynthesis; proteins; temperature; *Triticum aestivum*; trypsin.

Introduction

Exposure of plants to sub-lethal temperature enhances the thermal stability of photosynthesis (Berry and Björkman 1980). However, the mechanism of enhanced thermal stability in acclimated plants is not clearly understood. It may depend on the duration of acclimation. During short-term acclimation, the protection mechanism involves the presence of soluble factor(s) in the stroma that stabilise the photosynthetic apparatus membrane (Levitt 1980). In contrast, long term acclimation involves structural changes in the thylakoid membrane and the properties are retained in the membrane (Berry and Björkman 1980).

Among other cell functions, PS2 is the component most sensitive to high temperature (Singh and Singhal 1999a). The sensitivity of PS2 to high temperature is primarily due to release of Mn and proteins associated with OEC (Nash *et al.* 1985). Therefore the enhanced thermal stability in acclimated plants may be a result of stabilisation of OEC by some factor(s). Several *in vitro* studies have shown that molecules such as sucrose, polyethylene glycol, glycerol, and glycinebetaine protect PS2 activity by stabilising the proteins involved in OEC (Volger and Santarius 1981, Venediktov and Krivo-shejeva 1983, Mamedov *et al.* 1993). However, involvement of such factors *in vivo* in thermal protection of OEC is not shown. The involvement of HSP in the protection of PS2 activity during high temperature treatment has been shown by Schroda *et al.* (1999).

Most of the studies on the damage and thermal adaptation of photosynthetic apparatus during high temperature treatment have been carried out in dark. Very little information is available on the interactive effect of temperature and irradiance on damage and thermal adaptation of photosynthetic apparatus. Photosynthetically active radiation (PAR) protects the photosynthetic apparatus during high temperature stress (Weis 1982). The protective effect of irradiation on high temperature induced damage may be due to the pumping of protons across the membranes which increases the proton concentration in the intrathylakoid space. The heat stability of OEC in isolated thylakoid membrane largely depends on pH of the medium. Increasing the pH from acidic to basic inside the thylakoid lumen drastically decreases the

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Abbreviations: AT, thylakoid membrane from heat acclimated plant; Chl, chlorophyll; DCIP, 2,6-dichlorophenol indophenol; DPC, 1,5-diphenylcarbazide; HSP, heat shock protein; NT, thylakoid isolated from control plant; OEC, oxygen evolving complex; PAGE, polyacrylamide gel electrophoresis; PAR, photosynthetically active radiation; PS2, photosystem 2; SDS, sodium dodecyl sulfate.
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stability of OEC (De las Rivas *et al.* 1992).

PAR may also destabilise PS2 during heat stress. This was suggested due to the inactivation of OEC by high temperature that leads to the production of highly oxidative radicals which can be involved in destruction of pigments and proteins (Singh and Singhal 1999b). Indeed, the formation of cross-linking products was observed in thylakoid membrane during irradiation of plants at high temperature (Schuster *et al.* 1988, Singh and Singhal 1999a). This formation takes place even at lower irradiance which otherwise does not induce photo-inhibition at ambient temperature. Several *in vitro* studies have shown the increased susceptibility of PS2 to PAR where the OEC has been inactivated (Mori *et al.* 1995).

We studied the effect of temperature in combination with PAR in thylakoid membranes isolated from non-acclimated and short-term acclimated wheat leaves.

Materials and methods

Plants: Wheat seeds (*Triticum aestivum* L. cv. HD 2329; Indian Agricultural Research Institute, New Delhi, India) were germinated on moist germination paper for 48 h in dark. The germinating seedlings were then transferred in plant growth chamber maintained at 25 °C under a 14-h photoperiod. Seedlings were irradiated with a combination of fluorescent tubes and incandescent bulbs to provide PAR of 50 μmol m^{-2} s^{-1}.

Temperature treatment: 8-d-old wheat seedlings were sequentially exposed to 30 °C for 1 h, 35 °C for 1 h, and 42 °C for 2 h, under an irradiance of 50 μmol m^{-2} s^{-1}. After the treatment, leaves were chopped with the help of scissors and used to isolated thylakoid membranes.

Thylakoid membrane isolation, temperature treatment, and electron transport assay: Thylakoid membranes from 8-d-old wheat seedlings were isolated and treated with high temperature as described in Singh and Singhal (1999a). The PS2 activity was determined spectrophotometrically following photo-reduction of DCIP in the absence or presence of DPC (Singh and Singhal 1999a).

Trypsin digestion: Thylakoid membranes (10 μg Chl) were mixed with trypsin in 50 mm^3 reaction mixture containing 50 mM NaCl and 10 mM Tris, pH 7.5, and incubated for 10 min at room temperature in dark. Subsequently, trypsin inhibitor was added and incubation was continued for another 10 min.

SDS-PAGE and Western-blotting: Proteins were analysed on SDS-PAGE as described by Laemmli (1970) in the presence of 6 M urea. Thylakoid proteins were solubilised in sample buffer containing 4 % SDS, 50 mM Tris-HCl (pH 6.8), 2 % β-mercaptoethanol, and 10 % glycerol by incubating for 40 min at 40 °C. Proteins were electrophoresed on 13 % polyacrylamide gel at constant 100 V. Proteins were either visualised by CBB-staining or were electrophoretically transferred on nitrocellulose membrane. The blotted membrane was immuno-decorated with respective antibodies as described in Singh and Singhal (1999a).

Results and discussion

In an earlier study, we found that sensitivity of PS2 to high temperature drastically increased under iradiation (Singh and Singhal 1999a). Irradiation of thylakoid membranes at elevated temperature resulted in the loss of a number of proteins with subsequent appearance of high-molecular mass cross-linking products consisting of PS2 proteins. Further study showed that loss of proteins and formation of cross-linking products results from inhibition of OEC and over-production of radicals. Since heat acclimation enhances the thermal stability of photo-synthetic activity (Berry and Björkman 1980), we studied the effect of short-term acclimation of wheat plants on the loss of thylakoid proteins during irradiation of isolated thylakoid membranes at elevated temperature.

Electron transport activity: Fig. 1*A* shows the profiles of inactivation of PS2 electron transport measured as DCIP photoreduction in the absence or presence of DPC due to irradiation of thylakoid membranes isolated from non-acclimated (NT) and acclimated (AT) plants at 35 °C. Acclimation had a distinct effect on the thermal stability of PS2 electron transport. Inhibition of electron transport activity from H_2O to DCIP was faster in NT as compared to AT. A similar profile of heat inactivation was observed in the PS2 mediated electron transport from DPC to DCIP: The short term acclimation of leaves prior to isolation of thylakoid membranes resulted in protection of photosynthetic activity and this acquired protection mechanism operated in the isolated thylakoid membranes. Besides, similar pattern of inhibition of electron transport activity measured either as H_2O to DCIP or DPC to DCIP suggested that the acquired stability of PS2 might not be

related to stabilisation of OEC in the AT. However, as described earlier, the differences observed in the stability of OEC during incubation of thylakoid membranes at high temperature in dark is lost during the irradiation (Singh and Singhal 1999a). Therefore, NT and AT were subjected to high temperature treatment in dark. The profile of inactivation of PS2 electron transport at 35 °C (Fig. 1*B*) shows that the inactivation of electron transport from H_2O to DCIP was faster in NT as compared to AT. However, the inactivation of electron transport from DPC to DCIP was similar in both types of thylakoid membrane suggesting that acclimation process has resulted in the stabilisation of OEC.

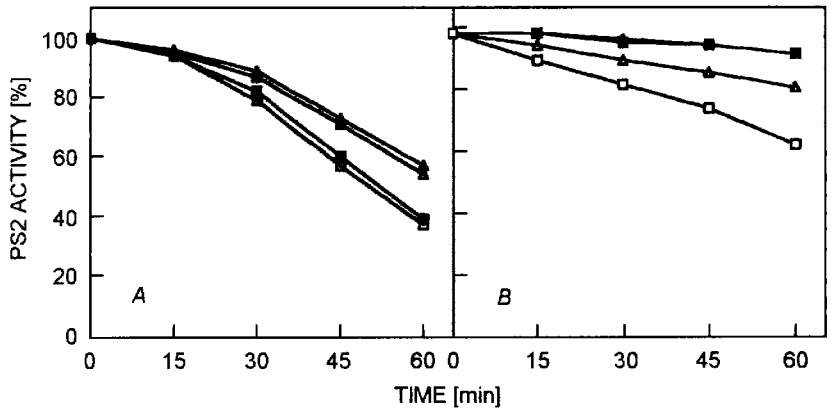

Fig. 1. Effects of high temperature acclimation on the photosystem 2 (PS2) electron transport. The isolated thylakoid membranes from acclimated (*triangles*) and non-acclimated (*squares*) leaves were treated at 35 °C for 0 to 60 min under irradiance of 200 μmol m^{-2} s^{-1} (*A*) or the treatment was done in the dark (*B*) in the absence (*open symbols*) or presence (*closed symbols*) of DPC.

D1 protein: High irradiance of leaves or isolated photosynthetic complexes at ambient temperature leads to the inhibition of electron transport through PS2 and subsequent degradation of D1, the PS2 reaction centre protein. At elevated temperature, however, the D1 protein forms a high molecular mass cross-linking product with other proteins although protease involved in D1 protein is active at high temperature (Singh and Singhal 1999a, b). Electron transport activity measurements showed an improved thermal stability in thylakoid membrane isolated from short-term acclimated leaves. We therefore determined the effect of elevated temperature on the loss of thylakoid proteins in AT and NT during irradiation at 35 °C (Fig. 2). Some proteins disappeared in both types of thylakoid membrane, but a protein band of approximately 80 kDa appeared in both of them. Its identity remains to be established. Another protein band of more than 97 kDa was found both in NT and AT in control but disappeared during high temperature treatments. Immunological determination showed a loss of D1-protein, faster in NT than in AT (Fig. 3). The loss of D1 protein is apparently due to formation of high-molecular mass cross-linking products with other PS2 proteins (Singh and Singhal 1999a). This result suggests that the enhanced thermal stability of PS2 electron transport in the AT due to acclimation process has also resulted in the protection of D1 protein.

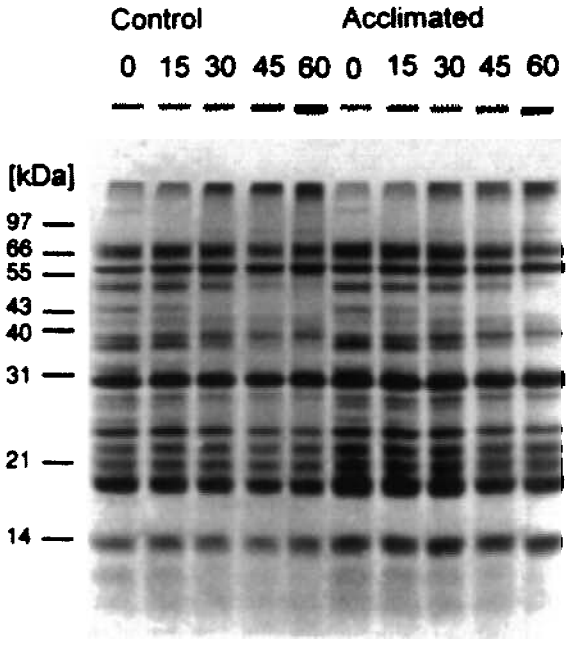

Fig. 2. Effect of high temperature acclimation on the loss of thylakoid proteins. The isolated thylakoid membranes from control and acclimated plants were treated at 35 °C for 0 to 60 min under irradiance of 200 μmol m^{-2} s^{-1}.

33 kDa protein: Heat inactivation of oxygen evolution is accompanied by the solubilisation of Mn and three extrinsic proteins (Nash *et al.* 1985, Enami *et al.* 1994). Since short-term acclimation of wheat seedlings lead to

the enhanced thermal stability of OEC (Schuster *et al.* 1988), we determined the effect of high temperature on the loss of 33 kDa protein in NT and AT. Acclimated and non-acclimated plants were exposed to various temperatures for 1 h, thylakoid membranes were isolated, and concentration of the 33 kDa protein was immunologically determined. In both types of seedling, temperature treatment had no significant effect on the level of the 33-kDa protein (Fig. 4). However, amount of the 33-kDa protein in non-acclimated seedlings slightly decreased as compared to control at 45 °C while in the acclimated seedlings there was no change.

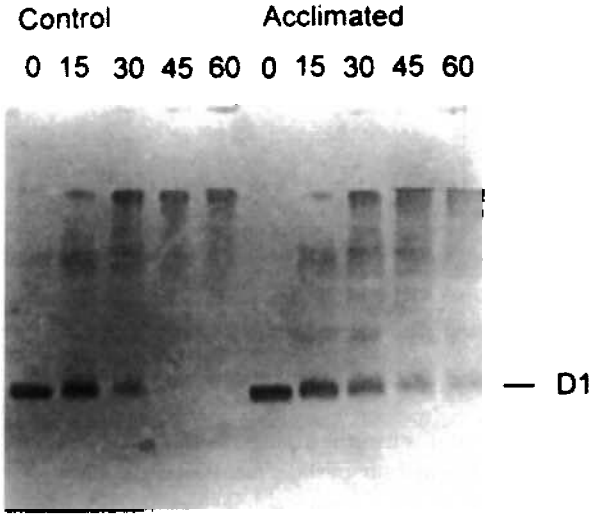

Fig. 3. Immunoblot showing effect of high temperature acclimation on D1 protein. The experimental conditions are the same as described in Fig. 2.

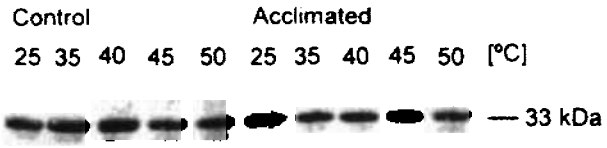

Fig. 4. Immunoblot showing effect of high temperature acclimation on the 33 kDa protein in intact seedlings. The control and acclimated seedlings were treated at 25 to 50 °C for 60 min under irradiation of 50 μmol m^{-2} s^{-1}.

Trypsin digestion of the 33-kDa protein: The 33-kDa protein has a structural role because it helps to maintain the functional conformation of Mn cluster and the efficient water splitting (Debus 1992). Therefore slight disturbances in the organisation of this protein may result in disturbances of Mn complex or even release of Mn from OEC, thereby inhibiting water splitting. Hence at least in the early stage of temperature treatment, the disturbances of organisation of the 33-kDa protein rather than by its release may lead to the inhibition of oxygen evolution. Structurally selective and functionally specific proteolytic enzymes can be used to study such changes. Treatment of thylakoid membrane with trypsin is a useful tool because the 33-kDa protein is rich in lysine and arginine residues (Wales *et al.* 1989). Most of these residues will be obscured in its native form associated with PS2 complex. However, as other shielding proteins (23 and 17 kDa) are removed or the 33-kDa protein may be re-organised due to high temperature treatment, new lysine and arginine residues may be exposed thus making it more vulnerable to trypsin. Thylakoid membranes isolated from acclimated and non-acclimated wheat seedlings, which were subjected to high temperature treatment, were treated with trypsin, fractionated on SDS-PAGE, and immunoblotted with the 33-kDa protein (Fig. 5). The concentration of intact 33-kDa protein in NT drastically decreased after 35 °C in trypsin treated thylakoid membranes (Fig. 5) while in untreated thylakoid membranes the decrease was found only after 40 °C (Fig. 4). As compared to this, the concentration of intact 33-kDa protein also decreased in AT, however, this decrease was not significant as compared to untreated thylakoid membranes. There were also marked differences in the appearance/disappearance of bands upon trypsin digestion of 33 kDa in both types of thylakoid membrane with increasing temperature. The patterns of bands observed after trypsin treatment in both

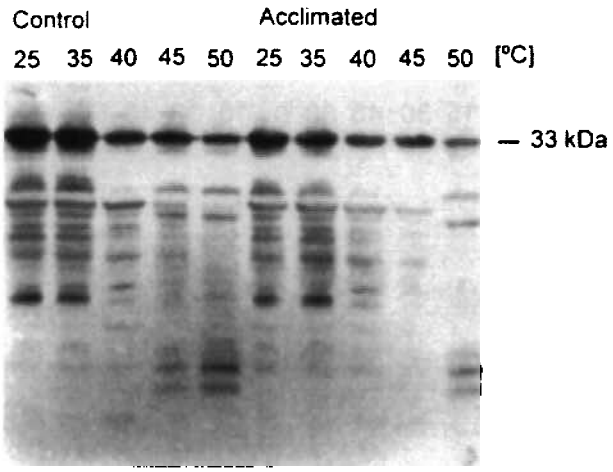

Fig. 5. Trypsin digestion of the 33 kDa protein. The experimental conditions are same as described in Fig. 4.

types of thylakoid membrane at 35 and 40 °C were similar. However, as the temperature increased, most of the bands disappeared and four prominent bands began to appear at 50 °C in both membrane types. The appearance of these bands was easily detected at 45 °C in NT but not in AT. Thus re-organisation of 33 kDa takes place before the release of 33 kDa. Since the loss of 33-kDa protein due to high temperature treatment could be detected only

at 45 °C, the concentration of intact 33-kDa protein was reduced drastically in trypsin treated thylakoid membranes at 40 °C. A short-term acclimation stabilised the 33-kDa protein and prevented the trypsin from acting similarly as in control.

In conclusion, we found that short-term acclimation leads to the protection of PS2 complex during temperature treatment under irradiation. The protection mechanism operates also in isolated thylakoid membranes and involves the enhanced stability of binding and/or structural re-organisation of the 33-kDa protein to the PS2 complex.

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