Elevated Temperature Treatment Induced Alteration in Thylakoid Membrane Organization and Energy Distribution between the Two Photosystems in *Pisum sativum*[#]

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Two-week-old pea (*Pisum sativum* var. Arkal) plants were subjected to elevated temperature (38 °C/42 °C) in dark for 14–15 h. The effect of heat treatment on light- induced phosphorylation of LHCII and LHCII migration in the thylakoid membranes were investigated. The heat treatment did cause a substantial (more than two fold) increase in the extent of LHCII phosphorylation as compared to the control. Upon separation of appressed and non-appressed thylakoid fractions by digitonin treatment, the heat-treated samples showed a decrease in LHCII-related polypeptides from the grana stack (appressed region) over the control. Further, a small increase in the intensity of these (LHCII-related) bands was detected in stromal thylakoid fraction (non-appressed membranes). This suggests an enhanced extent of migration of phosphorylated LHCII from appressed to non-appressed regions due to *in vivo* heat treatment of pea plants. We also isolated the LHCII from control and heat treated (42 °C) pea seedlings. Analysis of CD spectra revealed a 5–6 nm blue shift in the 638 nm negative peak in heat treated samples suggesting alteration in the organization of Chl *b* in the LHCII macro-aggregates. These results suggest that *in vivo* heat stress not only alters the extent of migration of LHCII to stromal region, but also affects the light harvesting mechanism by LHCII associated with the grana region.

Introduction

High temperature causes changes in the structural organization of thylakoid membranes affecting excitation energy distribution as the well as rates of photochemical reactions. Thus, high temperature causes alterations not only in the physiochemical properties but also the functional organization of the thylakoid membranes (Pastenes and Horton, 1996). Heat stress has been reported to affect various photosynthetic processes including electron flow on the donor side of PSII (Nash *et al.*, 1985; Enami *et al.*, 1994) and at the acceptor side of PSII (Bukhov *et al.*, 1990; Cao and Govindjee, 1990), inactivation of enzymes like ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) and ferredoxin-NADP reductase (FNR), photophosphorylation (Berry and Bjørkman, 1980; Havaux, 1993; Bukhov and Mohanty, 1999) as well as the energy distribution between the two photosystems (Anderson and Andersson, 1988; Sane *et al.*, 1984).

In almost all algae and higher plants, the absorption of light at any particular wavelength results in unbalanced excitation of the two photosystems, PSII and PSI in the thylakoid membranes. Since the electron flow from water to reduce NADP which is needed for CO_2 fixation requires the operation of PSI and PSII in series, both the photosystems must operate at appropriate rates in order to achieve optimal photosynthetic efficiency (Fork and Satoh, 1986). Reversible state transitions occurring over a time scale of minutes provide a mechanism whereby a balanced excitation of the two photosystems is achieved (Murata, 1969). State 1 refers to greater excitation of PSI by light predominantly absorbed by PSI. State 1 results in an increase in absorption cross-section of PSII and/or retardation of energy transfer from PSII to PSI (no spillover). Conversely, state 2 refers to a greater excitation of

Abbreviations: β-ME, β-mercaptoethanol; CD, circular dichroism; FW, fresh weight; LHCII, light harvesting chlorophyll protein complex of PSII; PQ, plastoquinone; SDS, sodium dodecyl sulphate.

[#] Dedicated to Professor Govindjee on his birthday.

PSI, by the wavelengths predominantly absorbed by PSII, and results in an increase in absorption cross-section of PSI and/or increased energy transfer from PSII to PSI (spillover).

In spillover, energy transfer proceeds in a thermodynamically favored direction from PSII to PSI and not vice versa. By contrast, an alteration in cross-section of a particular photosystem involves movement of antenna pigment-proteins themselves (Fork and Satoh, 1986). A number of experiments by Bennett *et al.* (1980 a,b) demonstrated that a protein kinase and a protein phosphatase bound to thylakoid-membranes to be involved in phosphorylation/dephosphorylation of pigment-protein complexes that resulted in the lateral migration of a fraction of the antenna of PSII from appressed region to non-appressed regions of thylakoids. This movement results in a decrease in the effective antenna size of PSII. The kinase responsible for the phosphorylation is not yet identified, although it is suggested that it is located in the core of PSII or in contact with Cyt b_6/f complex (Nilsson *et al.*, 1997). Further, it has been demonstrated that the key factor controlling kinase activation is the oxidation/reduction state of plastoquinone (PQ) which functions as an intersystem electron carrier (Allen *et al.*, 1981). Recently, it has been suggested that the LHCII kinase is in direct contact with Cyt b_6/f complex and as long as the PQ oxidation site on Cyt b_6/f complex is occupied with plastoquinol, the protein kinase remains active and catalyzes the phosphorylation of LHCII (Vener *et al.*, 1997). Further, *in vitro* phosphorylation of isolated thylakoid membranes may not represent the *in-vivo* phosphorylation capacity of LHCII proteins, since it has been recently shown that LHCII phosphorylation is regulated *in vivo* by a mechanism present in chloroplasts but absent in isolated thylakoid membranes (Rintamaki *et al.*, 1997; Carlberg *et al.*, 1999). In light, a faster electron transfer by PSII than by PSI results in reduction of the PQ pool of electron carriers, which leads to kinase activation and addition of negatively charged phosphate groups to LHCII complex, thus causing dissociation of LHCII due to repulsive electrostatic forces. Consequently, LHCII-P complex is found in the non-appressed thylakoid regions where it acts as light harvesting antenna for photosystem I (Anderson and Boardman, 1966; Allen *et al.*, 1981; Larsson *et al.*, 1987).

It has also been demonstrated that elevated temperature treatment of isolated photosynthetic membranes results in de-coupling of peripheral antenna of PSII (LHCII) and its diffusion towards stromal thylakoid membranes (Anderson and Andersson, 1988; Sundby *et al.*, 1986; Timmerhaus and Weis, 1990). However, most of these studies involved heat treatment *in vitro* with isolated chloroplasts and thylakoid membranes. Joshi *et al.* (1995), studied effects of high temperature stress on state 1–state 2 transitions in pathos leaves (in situ). They incubated the leaf segments at 42 °C or 47 °C for 5 min in dark and subsequently probed the energy distribution between PSII and PSI by Chl *a* fluorescence at room temperature. The authors concluded that mild heat stress altered the state shift but the severe heat stress (47 °C) totally abolished transition of state 1 to state 2, although many of the affected PSII units still evolved oxygen. However, these studies involved indirect assessment of heat stress on state transitions but did not prove that phosphorylation and associated lateral migration of LHCII complex actually occurred.

Material and Methods

Growth conditions and heat treatment

Seeds of *Pisum sativum* (var. Arkal) were surface sterilized with 0.1% $HgCl_2$ for 10 min and rinsed with distilled water. The pea plants were grown on mineral nutrient medium (Vani *et al.*, 1996) under cool fluorescent light (30–35 $\mu E\ m^{-2}\ s^{-1}$) (Philips, India) at 85% relative humidity. Two week old plants were subjected to high temperature (38 °C or 42 °C) for 14–15 h in dark and subsequently used for various experiments. Plants grown at 25 °C were taken as control. Chlorophyll was estimated according to (Porra *et al.*, 1989) using dimethyl formamide as solvent or according to (Arnon, 1949) using 80% acetone as solvent.

Phosphorylation of thylakoids with γ-[^{32}P] labeled ATP

C-type chloroplasts (thylakoid membranes) were isolated according to the procedure described in (Nakatani and Barber, 1977). For phosphorylation of LHCII with γ-[^{32}P] ATP, the thylakoid membranes equivalent to 0.4 mg Chl/ml were

incubated with 0.4 μl γ-[^{32}P] ATP and 10 mM NaF (as phosphatase inhibitor) for 20 min in light or in dark separately. Subsequently the samples were prepared for electrophoresis by mixing with sample buffer containing 2% SDS, 62.5 mM Tris [hydroxymethyl] aminomethane–HCl (pH 6.8), 2.5% β-ME, 10% glycerol and bromophenol blue and heated for 3 min. The samples were loaded on a 12.5% denaturing polyacrylamide gel and electrophoresis was carried out according to Laemmli (1970). Gels were dried after the run and the extent of γ-[^{32}P] incorporation in LHCII (28–30 KDa) was analyzed by auto-radiography.

Isolation of appressed and non appressed thylakoid membranes

Isolation of appressed and non appressed thylakoid membranes was performed by digitonin fractionation (0.4% digitonin) as described in (Anderson and Boardman,1966) with some modifications. Thylakoids were prepared according to Nakatani and Barber (1977) and then treated at chlorophyll concentration equivalent to 0.4 mg/ml with digitonin, 0.4% final conc. for 1 h. The undigested thylakoids were pelleted at 5000 × *g*. The supernatant consisting of digested thylakoids was then centrifuged at 42,000 × *g*. The pellet so formed contained appressed membranes and was washed twice. The non-appressed membranes present in the supernatant were pelleted at 1,40,000 × *g*. All steps were carried out at 4 °C.

Urea SDS-PAGE

Granal and stromal thylakoid polypeptides as well as isolated LHCII polypeptides were resolved by Urea, SDS-PAGE. The samples were prepared by mixing the thylakoid membranes or LHCII in sample buffer containing 6 M urea. Gels were made according to (Laemmli, 1970). Samples equivalent to 15 μg Chl were loaded per lane. Polypeptides were resolved on a 7–15% acrylamide gradient separating gel containing 2 M urea. Gels were run at a constant voltage and the polypeptides were detected by Coomassie Brilliant Blue R-250 staining.

Isolation and spectral characterization of LHCII

Isolation of LHCII from control and heat treated pea plants was performed according to Burke *et al.* (1978) with some minor modifications. Pea leaves were homogenized in Na-tricine buffer, and the slurry was filtered through four layers of Mira cloth (Calbiochem, USA). The filtrate was centrifuged at 1000 × *g*. The pellet formed after centrifugation was suspended in 5 mM EDTA + 0.1 M sorbitol and centrifuged at 10,000 × *g* for 10 min. The pellet was re-suspended in distilled water at 0.8 mg Chl/ml and treated with 0.7% Triton X-100 for 30 min and centrifuged at 42,000 × *g*. The supernatant was loaded on a 0.1–1.0 M sucrose gradient and centrifuged at 1,00,000 × *g* for 15 h. A deep red fluorescent band on the top of the gradient contained LHCII and was taken with the help of a syringe. Room temperature absorption spectra of the isolated LHCII macro-aggregates were recorded on Shimadzu UV-160 spectrophotometer with samples equivalent to 10 μg Chl ml^{-1}. Circular dichroism (CD) spectra of isolated LHCII from control and heat treated plants were measured in the visible range from 350 nm to 700 nm and UV-range (190–250 nm) using a Jasco J-720 CD spectrometer. The spectra were recorded from 350–670 nm, at 0.5 nm step resolution, with sensitivity 50 mdeg, band width of 1.0 nm and a scan speed of 50 nm/min. The samples were analyzed on equal chlorophyll (50 μg/ml) in the visible region and on equal protein (50 μg/ml) basis in the UV region.

Results and Discussion

Effect of exposure of two week old pea plants to 42 °C (for 15 h in dark) on pigment content (Chl and carotenoids) was studied. Results are presented in Table I. No significant alteration in pigment content was observed in 42 °C treated plants as compared to the controls (25 °C) (Table I). The total chlorophyll content decreased from 3.0 mg per *g* F W in control to 2.5 mg per *g* F W in 42 °C treated pea plants indicating only a marginal loss of about 15–16% in total chlorophyll. Among Chl *a* and *b*, Chl *a* recorded a 20% loss whereas Chl *b* recorded only 9% loss. As a consequence, Chl *a/b* ratio decreased from 3.0 in control to 2.5 in treated samples (16–17% loss). This is a clear evidence for a marginal decline in the light harvesting capacity of the photosystems in the heat treated plants or in other words, loss in antenna pigment molecules. Carotenoid content

Table I. Estimation of the photosynthetic pigments in control and heat-treated pea plants. Photosynthetic pigments were estimated in the leaf samples from two week old pea plants (control and heat treated, 42 °C for 15 h) according to Arnon (1949) using 80% acetone as the solvent. Data represent mean of three independent experiments ± SE.

Temp.	Chl *a* [mg/g FW]	Chl *b* [mg/g FW]	Chl Total [mg/ gFW]	% change	Chl a/b	Carotenoids [mg/g FW]	% Change
25^0C	2.23 ±0.18	0.77 ±0.05	3.0	100	3.0 ±0.1	0.336 ±0.009	100
42^0C	1.78 ±0.14	0.7 ±0.06	2.5	84	2.5 ±0.01	0.3	90

however, did not show any change in heat treated plants.

In order to study the effect of *in vivo* heat treatment on the state changes of the thylakoid membranes, the extent of light-induced phosphorylation of LHCII in thylakoids isolated from control and heat-stressed pea plants was monitored with $\gamma[^{32}P]$ labeled ATP (Fig. 1). Lane 1 & 3 show the extent of $\gamma[^{32}P]$ labeled ATP incorporation in LHCII of thylakoid membranes isolated from control and heat treated pea leaves respectively. Lane 2 & 4 show the extent of LHCII phosphorylation in the thylakoid samples isolated from control and heat treated samples in the dark. We observed a significant enhancement in the extent of phosphorylation of LHCII in heat treated samples

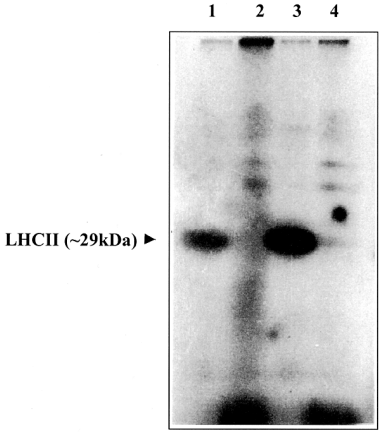

Fig. 1. Autoradiograph showing the light induced phosphorylation of LHCII using $\gamma[^{32}P]$ labeled ATP in the thylakoids isolated from control (25 °C) and heat-treated pea plants. Lanes 1–4 indicate control samples in light, control in dark , 42 °C treated samples in light and 42 °C treated samples in dark respectively. The LHCII band is indicated by an arrow.

(more than two fold as seen in the Fig. 1) as compared to the controls, although some proteins other than LHCII also seemed to get phosphorylated in heat treated samples. Further, as expected, phosphorylation was observed only in samples incubated in light. Our results suggest that *in vivo* heat treatment of the plants leads to an inhibition of electron transfer at various sites which include the plastoquinol (PQH_2) oxidation site at Cyt b_6/f complex. We assume that the oxidation site on Cyt b_6/f complex remains occupied by plastoquinol resulting the enhanced activation of LHCII kinase in heat treated, isolated thylakoid sample compare to control. It is possible that heat stress might also affect the activities of enzymes involved in phosphorylation/de-phosphorylation of LHCII. In either case, an enhanced level of phosphorylation-induced migration of LHCII from appressed to non-appressed thylakoid membranes would occur due to heat stress.

Hence, we have made an attempt to look for migration of some of the LHCII polypeptides from appressed to non-appressed thylakoid membranes in the heat treated plants. The Chl *a/b* ratios of appressed thylakoid membranes from control plants was 2.3 and 42 °C-treated plants recorded 2.4. The non-appressed thylakoid membranes from control plants recorded a Chl *a/b* ratio of 6.25 which decreased to 5.5 in heat treated plants. Figure 2 shows the SDS PAGE analysis of the appressed and non-appressed membranes from the control and heat treated pea plants. The polypeptide profiles of appressed and non-appressed membranes clearly indicate that the preparations are free from cross contamination. We also observed a loss in the polypeptides ranging 28–30 kDa of the appressed membranes isolated from heat treated plants (38 °C and 42 °C as

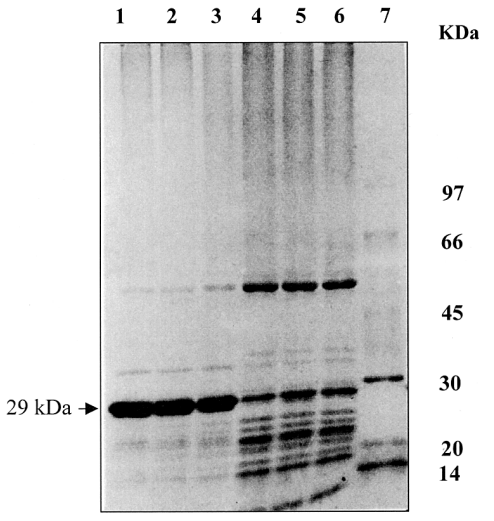

Fig. 2. SDS PAGE showing the polypeptide profile of appressed (lanes 1–3), and non- appressed, thylakoid membranes (lanes 4–6) from control and heat-treated pea plants. Lanes 1–3 represent temperatures 25 °C, 38 °C and 42 °C respectively and lanes 4–6 represent 25 °C, 38 °C and 42 °C respectively. Lane 7 represents the molecular weight marker proteins. LHCII polypeptide band is shown by an arrow. Thylakoids equivalent to 15 μg Chl was loaded per lane on a 7–15% acrylamide gradient.

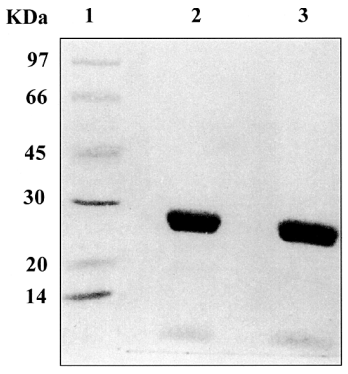

Fig. 3. SDS-UREA PAGE analysis of the LHCII isolated from control and heat-treated pea plants. Lane 1 represents the molecular weight marker proteins. Lanes 2 and 3 represent LHCII macro-aggregates from 25 °C and 42 °C treated pea plants respectively. Samples equivalent to 15 μg Chl was loaded per lane on a 7–15% acrylamide gradient.

shown by arrows) as compared to the control. This decline is more prominent in 42 °C heat treated samples (~ 13% decrease) as compared to 38 °C treated plants (~ 7% decrease). There is a simultaneous increase in the same molecular weight protein bands (LHCII) in non-appressed membranes isolated from heat treated pea plants. There was about 10% and 17% increase in the LHCII of thylakoids isolated from 38 °C and 42 °C heat treated samples respectively as compared to control. This indicates migration of LHCII Polypeptides after phosphorylation to the PSI where they woud function as light harvesting machinery of PSI. Such a migration has been reported earlier by several groups under *in vitro* heat treatment (Bennet, 1980b; Andersson and Styring, 1991). We therefore demonstrated that thylakoids under heat stress exist in state 2 (increased absorption cross section of PSI) and hence, state 1–state 2 transition is enhanced.

Although isolation of LHCII complex has been performed by several groups over past three decades, no reports are available on isolation of this complex *under in vitro* or *in vivo* heat stress conditions. We succeeded in the isolation of LHCII macro-aggregates from both control and heat treated plants to electrophoretic homogeneity. LHCII macroaggregates was isolated on 0.1 to 1.0 M sucrose gradient from control and heat treated plants. PSI holocomplex (PSI_{110}) was also simultaneously separated at 1.0 M sucrose. The Chl *a/b* ratio of LHCII was found to be 1.3 in both control and heat treated samples. Figure 3 shows the polypeptide profile of the LHCII complex isolated from control and heat treated plants. We suggest that *in vivo* heat treatment of pea plants does not cause any significant alteration in the polypeptide composition of LHCII complex. The LHCII complex constitutes about 56% of the total Chl and its efficiency for conversion of light energy to chemical energy depends on the macromolecular organization of the pigment and protein molecules within the complex. Therefore, to see *in vivo* heat stress induced alterations in the pigment-pigment/pigment-protein interactions if any, and macromolecular organization of LHCII complex, we analyzed the spectral characteristics of isolated LHCII macro-aggregates.

Room temperature absorption spectra of LHCII macro-aggregates isolated from control (25 °C) and heat stressed (42 °C. 15 h) pea plants were recorded from 400 nm to 750 nm and normalized at 750 nm. The control samples showed absorbance peaks at 437 and 476 nm in the blue region and at

653 and 674 nm in the red region of the spectrum. The absorption spectrum of control samples was almost identical to one recorded by Burke *et al.* (1978) in pea plants (data omitted). The absorption spectra of LHCII complex isolated from heat treated plants was similar to the control samples except for 1.5 nm red shift in the Soret peaks and the short wavelength red absorption band (data not shown).

Circular dichroism (CD) spectrum of a pigment containing protein complex in the visible range reflects the chromophore-chromophore and chromophore-protein interactions (Haworth *et al.,* 1983). Since LHCII is a chlorophyll (also carotenoid) protein complex, we performed CD analysis of the complex under unstressed and heat stressed conditions. The CD spectrum of the protein sample in the UV region describes the α-helical content of the protein (Campbell and Dwek, 1984). The α-helical content did not alter in the heat stressed samples compared to the controls (data not shown). Figure 4 shows that CD spectra (in the visible region) of LHCII macro-aggregates isolated from control and heat treated plants. Control samples exhibited major negative signals at 638 nm, 461 nm and 493 nm (which may correspond to psi band, polymerization or salt induced bands as described in (Simidjiev *et al.,* 1998). The major positive signals were recorded at 654 nm and 403 nm. The negative optical transitions at 637 nm, 493 nm and 463 nm are thought to be linked to Chl *b* in the LHCII complex (Haworth *et al.,* 1983). The CD spectrum of LHCII macro-aggregates from heat stressed plants recorded almost identical positive and negative signals except for the negative peak at 638 nm, which showed a 5–6 nm blue shift (632–633 nm) in 42 °C treated

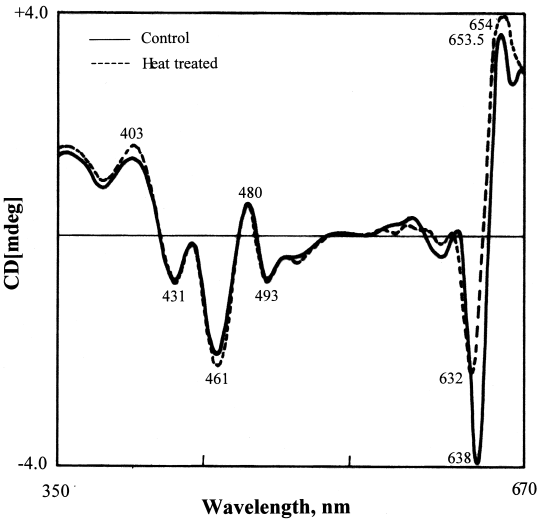

Fig. 4. CD spectral analysis of LHCII isolated from control (25 °C) and heat treated pea plants in the visible region of the spectrum (solid line—control) (broken line ---- heat treated).

samples. Such a blue shift may indicate an alteration in the internal environment of LHCII aggregates and may involve alteration in orientation of the pigment molecules (Chl *b*) in heat stressed samples. We therefore suggest that *in vivo* exposure of plants to high temperature did affect pigment–protein/pigment–pigment interactions of the major light harvesting complex, LHCII, which needs an intensive investigation.

Acknowledgments

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