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Modification of the Photosystem II light-harvesting chlorophyll *a/b* protein complex in maize during chill-induced photoinhibition

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The changes in the functional and molecular organization of the light-harvesting chlorophyll *a/b* protein complex (LHC II), associated with Photosystem II (PS II), are examined when maize leaves are exposed to high light at 5°C for 6 h. Chlorophyll-fluorescence kinetic analyses of thylakoids isolated from mesophyll cells of stressed and control leaves demonstrate that the stress produces a reduction in energy transfer from LHC II to PS II. The polypeptide complements of thylakoids isolated from stressed leaves showed the accumulation of a 31 kDa polypeptide; no other changes in thylakoid polypeptides were observed between control and stress leaves. The 31 kDa polypeptide was a component of purified LHC II from stressed leaves and was immunologically related to the LHC II polypeptides. Differences in fluorescence emission spectra at 77 K of purified LHC II from control and stressed leaves suggested that the stress induces a perturbation of the environment of the chlorophyll species. The appearance of the 31 kDa polypeptide correlated with a modification of LHC II and its functional association with PS II. It is suggested that this polypeptide is a precursor of LHC II polypeptides and is inserted into the membrane prior to processing.

Introduction

Maize leaves when exposed to high light at low temperatures suffer damage to the photosynthetic apparatus that results in a large decrease in the quantum yield of carbon assimilation [1]. Concomitant with this change are a decrease in the quantum yield of PS II primary photochemistry and a modification of the fluorescence spectral characteristics of the thylakoids with an enhancement of the emissions at wavelengths above 700 nm being observed relative to the PS II and LHC II emission bands [2]. Recent studies have demonstrated that thylakoids isolated from mesophyll cells of the stressed maize leaves cannot perform State 1–State 2 transitions (Bradbury, M., Baker, N.R. and Hayden, D.B., unpublished data). Since there is no significant loss of chlorophyll from the stressed leaves [3] such a perturbation of thylakoid photochemical activities may be due to organizational changes in the light-harvesting apparatus. In this study modifications to the functional and molecular organization of LHC II in the mesophyll cells of maize leaves exposed to a photon flux density of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 6 h at 5°C are examined. It is demonstrated that this stress induces a decrease in excitation energy transfer

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Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; LHC II, light-harvesting chlorophyll *a/b* protein associated with Photosystem II; PS II, Photosystem II.

from LHC II to PS II and modifies the polypeptide complement and low-temperature fluorescence characteristics of LHC II isolated from the leaves. These stress-induced changes in LHC II correlate with the accumulation of a 31 kDa polypeptide in LHC II.

Materials and Methods

Maize plants (*Zea mays* L. cv. LG11) were grown in John Innes potting compost No. 2 in a growth cabinet (Conviron S10H) at 25°C under a photon flux density of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a 16 h photoperiod. After 10 days plants were transferred to a cold room at 5°C and irradiated with a photon flux density of 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 6 h. Plants treated in this way are referred to subsequently as chilled. Non-treated control plants were maintained in the growth conditions specified above until use.

Primary leaves were discarded and thylakoids prepared from mesophyll cell chloroplasts as described previously [4]. Such thylakoid preparations are free of bundle sheath chloroplast and thylakoid contamination [4].

Measurements and analyses of the kinetics of 685 nm fluorescence emission from thylakoids in the presence of DCMU on excitation with 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of 450 nm radiation were performed as previously described [5].

Polypeptides were separated using the discontinuous polyacrylamide gel system of Laemmli [6] with the following modifications. Gels consisted of a 10–18% gradient of polyacrylamide containing 4 M urea. Samples were solubilized at 20°C for 5 min in 2% (w/v) sodium dodecyl sulphate (SDS) and 60 mM dithiothreitol at a SDS/chlorophyll weight ratio of 20:1. Electrophoresis was performed at 20°C with a constant current of 5 mA. Gels were stained in methanol/acetic acid/water (4:1:5, v/v/v) containing 0.1% (w/v) Coomassie brilliant blue R and destained in methanol/acetic acid/water (8:3:29, v/v/v).

LHC II was isolated and purified by detergent fractionation, density gradient centrifugation and salt precipitation of thylakoid membranes using the method of Steinback et al. [7]. Three successive washes in low salt medium were required to produce thylakoid membranes amenable to detergent fractionation. After washing, cation-depleted membranes were resuspended in distilled water to a concentration of 1 mg chlorophyll per cm^3 and 1% (w/v) Triton X-100 was added to give a final concentration of 0.5 mg chlorophyll and 0.5% detergent. Fluorescence emission spectra from isolated LHC II preparations containing 10 μg chlorophyll per cm^3 and frozen to 77 K were measured as previously described [8] using 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of 440 and 470 nm excitation (half-band width, 10 nm). Absorption spectra of isolated LHC II were measured on samples containing 10 μg chlorophyll per cm^3 using a Cary 210 spectrophotometer. Total chlorophyll, chlorophyll *a* and chlorophyll *b* contents of LHC II preparations were determined according to the method of Arnon [9]. The purity of the LHC II preparations from control and chilled leaves was judged by a number of criteria. Chlorophyll *a*/*b* ratios of the preparations were approx. 1.2, consistent with previously reported values for purified LHC II [7,10,11]. Absorption spectra of the purified LHC II complexes from controlled and chilled leaves are shown in Fig. 1; it is evident that there is little difference between the two preparations and that the spectra are similar to those previously published [10,12].

Immunoblotting of fractionated polypeptides was carried out as described by Montano and Lane [13]. Polypeptides were transferred onto nitrocellulose paper for 16 h at 80 mA. The blot was incubated first for 8 h in a 1:2000 dilution of

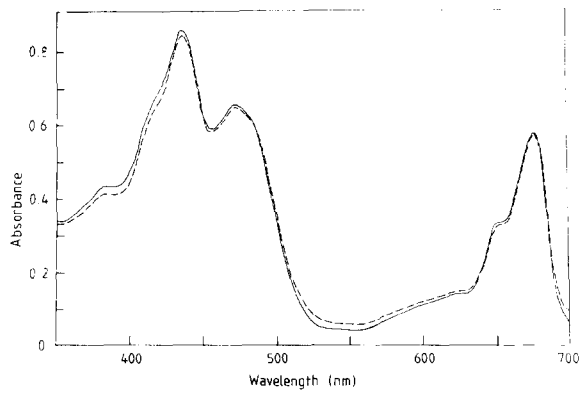

Fig. 1. Absorption spectra at 20°C of purified LHC II isolated from thylakoids of control (———) and chilled (— — —) leaves.

antibodies specific to LHC II [14], then overnight in a 1 : 100 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin. The blot was then developed with a 4-chloro-1-naphthol substrate solution. Full details of the preparation of the LHC II antibodies and their specificity have been reported previously [14].

Results

Fluorescence induction curves of thylakoids isolated from control and chilled leaves in the presence and absence of 5 mM Mg^{2+} are shown in Fig. 2, together with the semilogarithmic plots of the area growth of these curves. The values of the fluorescence parameters F_0, F_m, F_v/F_m and β_{max} associated with these induction characteristics are given in Table I. The first-order plot for the control thylakoids in the presence of 5 mM Mg^{2+} demonstrates the well-established biphasic nature of the induction kinetics. The rapid phase of the kinetics has been attributed to PS II_α centres [15,16], which have been shown to be PS II complexes energetically associated with LHC II [5]. By depleting the thylakoids of divalent cations, which effectively disconnects LHC II from PS II [17–20], only the α phase of the kinetics is modified and not the slow β phase [5,18,21]. The relative contributions of the α and β phases to the induction of variable fluorescence can be quantitated using the parameter β_{max}, which is determined from the intercept obtained on the y-axis on extrapolation of the slow β phase of the semilogarithmic plot of the area growth [15,19]. Chilled thylakoids show a reduced contribution of the α phase, and consequently an increased β_{max} value, and also reduced F_0, F_m and F_v/F_m values compared to control thylakoids (Fig. 2, Table I). As expected, removal

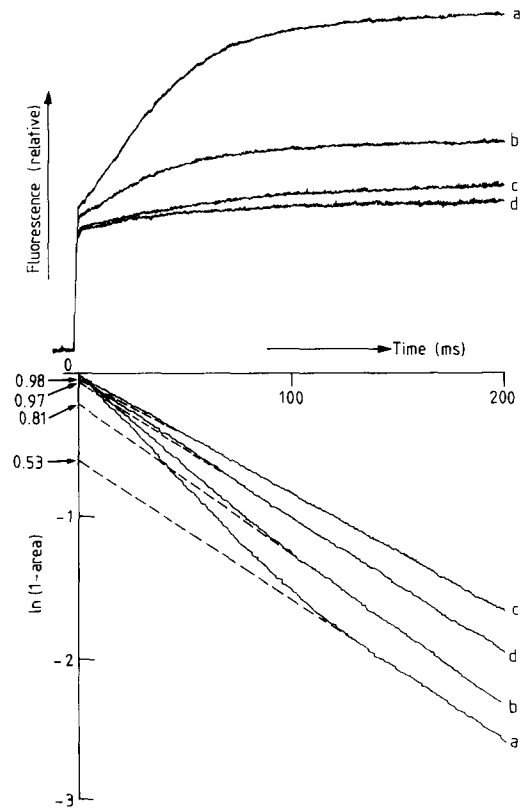

Fig. 2. Kinetics of 685 nm fluorescence emission from DCMU-poisoned thylakoids isolated from control (a and c) and chilled (b and d) leaves in the presence (a and b) and absence (c and d) of 5 mM Mg^{2+}. First-order analyses of the kinetics of the area growth over the respective fluorescence induction curves are shown below.

TABLE I

FLUORESCENCE INDUCTION CHARACTERISTICS OF THYLAKOIDS ISOLATED FROM CONTROL AND CHILLED LEAVES

Fluorescence parameters F_0, F_m, F_v/F_m and β_{max} in the presence and absence of 5 mM Mg^{2+} are given for DCMU-treated thylakoids isolated from control and chilled leaves. Data are the means of five replicates and standard errors are given in parentheses.

Thylakoids	Mg^{2+} (mM)	F_0	F_m	F_v/F_m	β_{max}
Control	5	50 (1)	115 (2)	0.57 (0.01)	0.52 (0.01)
	0	40 (1)	56 (2)	0.29 (0.04)	0.99 (0.02)
Chilled	5	44 (1)	70 (3)	0.39 (0.02)	0.80 (0.01)
	0	40 (2)	50 (2)	0.18 (0.01)	0.97 (0.02)

of Mg^{2+} from control thylakoids also induced an increase in β_{max} and decreases in F_0, F_m and F_v/F_m (Table I). The increase in β_{max} induced by Mg^{2+} depletion was greater than that produced by the stress treatment on the leaves; however, on removal of Mg^{2+} from chilled thylakoids a similar β_{max} value was obtained to that found for control thylakoids (Table I). These data imply that the stress treatment on the leaves perturbs excitation energy transfer between LHC II and PS II, but not as extensively as Mg^{2+} depletion.

Examination of the thylakoid polypeptides by polyacrylamide gel electrophoresis demonstrated the appearance of a 31 kDa polypeptide in the chilled thylakoids; no other polypeptide differences between the chilled and control thylakoids were apparent (Fig. 3A). Polypeptide profiles of the LHC II isolated from chilled, but not control leaves show the presence of the 31 kDa species (Fig. 3B). The polypeptide complement of control LHC II preparation contains only species between 22–29 kDa (Fig. 3B). Although reports vary as to the number and size of LHC II polypeptides, it is well established that they are in this molecular size range [7,11,22,23]. The major LHC II polypeptides are synthesized as soluble precursors of molecular weight 30–32 kDa, which are then processed and incorporated into the thylakoid membrane [24–28]. The polypeptides of thylakoids and purified LHC II complexes from control and chilled leaves, after fractionation by electrophoresis, were transferred to nitrocellulose sheets and challenged with antibodies specific to LHC II. The immunoblot of the control thylakoid polypeptides (Fig. 4, lane a) shows that only the polypeptides of LHC II react with the LHC II antibodies and demonstrates the specificity of the antibodies. Fig. 4 illustrates that the constituent polypeptides of both control and chilled LHC II are all immunologically related.

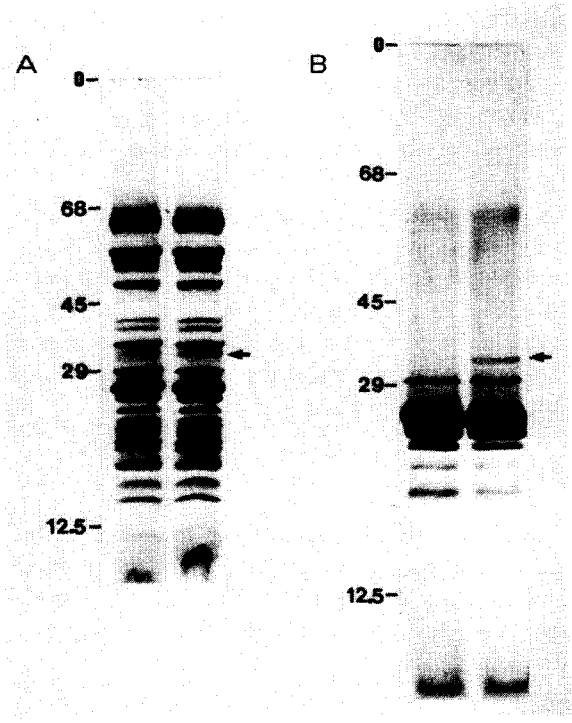

Fig. 3. Polypeptide profiles of thylakoids (A) and purified LHC II (B) isolated from control (left lane) and chilled (right lane) leaves. 10 μg chlorophyll was loaded onto each lane. The numbers to the left of the gels correspond to markers of known M_r expressed in kilodaltons. The origins of the gels are indicated (O). Electrophoresis of LHC II polypeptides was performed on longer gels than in A to improve resolution. The arrows mark the position of the 31 kDa polypeptide.

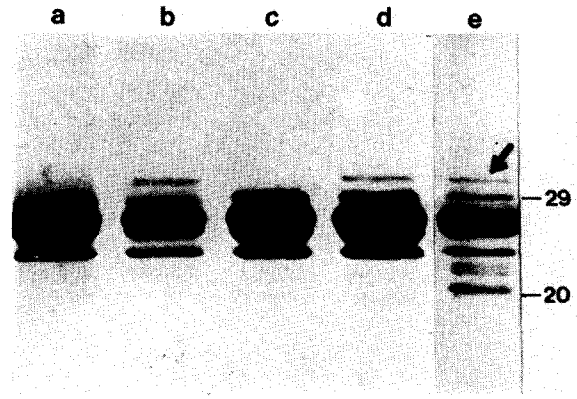

Fig. 4. Immunoblot analyses of LHC II polypeptides. Samples of thylakoids (a and b) and purified LHC II (c and d) from control (a and c) and chilled (b and d) leaves, containing 10 μg chlorophyll, were electrophoretically separated into constituent polypeptides and transferred to nitrocellulose paper. Antibody incubations and blot development are described in the text. The polypeptide profile of the purified LHC II from chilled leaves, used for the immunoblot of lane d, is shown in lane e. The numbers to the right of the gel correspond to markers of known M_r expressed in kilodaltons. The consituent polypeptides of LHC II of control and chilled leaves are immunologically related, as is the 31 kDa polypeptide (arrow) present in the chilled LHC II.

The 31 kDa polypeptide found in the thylakoids and purified LHC II preparations from chilled leaves reacts immunologically with the LHC II antibody (Fig. 4) suggesting strongly that this species is a precursor form of the LHC II polypeptides.

Fluorescence emission spectra at 77 K of control and chilled purified LHC II excited with 440 and 470 nm radiation are shown in Fig. 5. The spectrum of control LHC II, excited with 440 nm radiation, exhibited a maximum at 685 nm and shoulders at 695 and 740 nm. On excitation at 470 nm an enhancement of emissions at 695 and 740 nm relative to that at 685 nm occurred, producing a maximum at 695 nm. These data imply that chlorophyll *b* preferentially contributes to the 695 and 740 nm emission bands, whereas chlorophyll *a* preferentially contributes to the 685 nm emission. The emission spectrum of purified LHC II from chilled leaves excited at 440 nm was markedly different from that of control LHC II (Fig. 5); the 685 nm emission band was severely reduced and maxima were observed at 700 and 725 nm. Excitation at 470 nm produced an increase in the emissions from the chilled LHC II above 710 nm relative to the 700 nm peak. Clearly there has been a major modification of the energetics of LHC II during chilling. A specific chromophore or set of chromophores within the complex must be modified by the chill treatment. It would appear that emissions preferentially produced from excitation of chlorophyll *b* are enhanced in the LHC II isolated from the stressed leaves compared to the controls. The possibility that chill-induced aggregation of the LHC II may produce such changes should not be ignored, since the absorption of chilled complexes in the 510–600 nm region is greater than that observed for control LHC II (Fig. 1).

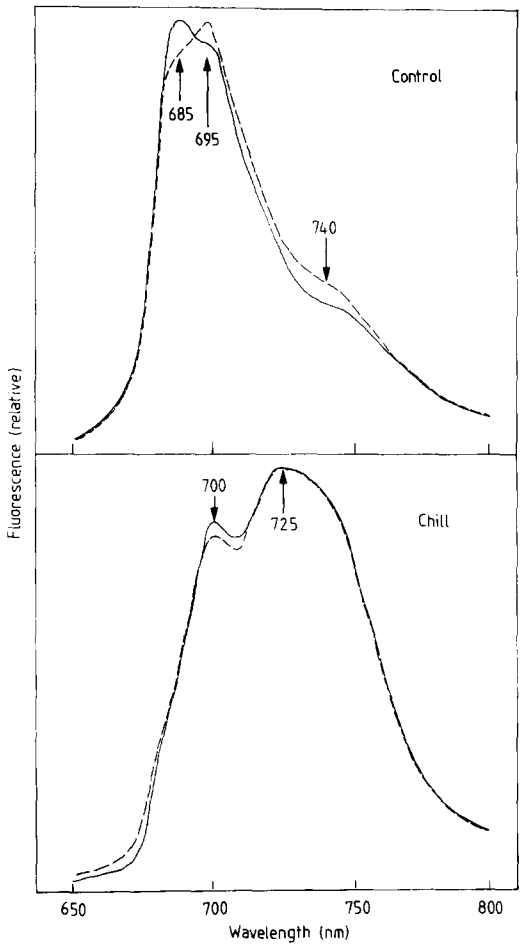

Fig. 5. Fluorescence emission spectra at 77 K of purified LHC II isolated from thylakoids of control and chilled leaves on excitation with 440 (———) and 470 (— — —) nm radiation.

It should be emphasized that the effects reported above are observed only when maize leaves are exposed simultaneously to both low temperature (5°C) and high light (1,500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 6 h. Low temperature or high light treatments alone over this time period do not elicit this response.

Discussion

Chill-induced photoinhibition in maize leaves produces changes in the structure and function of LHC II. The accumulation of a 31 kDa polypeptide in the LHC II complex could be responsible for such changes. The absorption properties of isolated LHC II and the chlorophyll extracts of isolated LHC II from chilled and control leaves were similar showing that changes in the function of chilled LHC II were not due to any loss of total chlorophyll or differences in the ratio of chlorophyll *a*:chlorophyll *b*. Differences in the fluorescence emission spectra at 77 K of chilled and control LHC II could be accounted for by stress-induced modifications to the energetic interactions

between chlorophylls in the LHC II complex. The contribution of chlorophyll *b* to the emission spectrum was enhanced in the chilled LHC II suggesting changes in excitation transfer from chlorophyll *b* to chlorophyll *a* in the complex. This may have serious consequences for the transfer of energy from the LHC II complex to PS II. Analyses of the kinetics of fluorescence emission from thylakoids demonstrated that the contribution of LHC II to the photochemistry of PS II is considerably reduced in chilled thylakoids. This may arise due to modifications in energy transfer within the chilled LHC II complexes. Alternatively, a detachment of the LHC II from PS II would account for such an observation. Both of these possibilities would be compatible with the observed decrease in the quantum efficiency of Photosystem II primary photochemistry, i.e., Q reduction, and may occur concomitantly. The inability of the chilled thylakoids to perform a State 1–State 2 transition (M. Bradbury, N.R. Baker and D.B. Hayden, unpublished data) would support a stress-induced detachment of the LHC II from PS II. Such perturbation of PS II photochemistry in stressed leaves would have major implications for the quantum efficiency of non-cyclic electron transport, ATP synthesis and carbon assimilation. Such modifications of the LHC II complex and its functional organization within the thylakoid may occur in many photoinhibitory situations. Inhibition of energy transfer from LHC II to PS II would reduce the exciton density at PS II reaction centres and may serve to minimise photodamage to reaction centres and electron-transport components. However, there is no evidence to suggest that this actually confers a selective advantage to plants under photoinhibitory conditions.

It is well established that the major polypeptides of LHC II are synthesized in the cytoplasm in the form of a pair of 30-32 kDa soluble precursors; these precursors are imported into the chloroplast and it has been presumed that they are processed prior to insertion into the thylakoid membrane [24–29]. The 31 kDa polypeptide that appears in thylakoids isolated from chilled leaves is similar in size to the LHC II precursors. This polypeptide is a component of isolated and purified LHC II from chilled thylakoids and is immunologically related to the polypeptides of LHC II. Thus, both the molecular size and immunological properties of this 31 kDa polypeptide are consistent with its being a precursor of LHC II polypeptides. Whilst it has been shown that in pea and barley two LHC II polypeptide precursors can exist in the chloroplast that differ slightly in size [24–26,28], we were only able to detect one polypeptide in the 31–32 kDa range in the LHC II of chilled maize even using a higher-resolution 2-dimensional gel electrophoretic fractionation system. Since this stress-induced 31 kDa polypeptide is clearly tightly associated with the LHC II complex, it must be argued that it has been inserted into the thylakoid membrane. If indeed this polypeptide is a precursor of LHC II polypeptides, its insertion into LHC II is not consistent with the suggestion that the precursor must be processed prior to assembly into the LHC II complex. Alternatively, it has been suggested that LHC II polypeptide precursors are processed only after their assembly in the membrane [25,30]. Recently, it has been demonstrated that an in vitro synthesized precursor from *Lemna gibba* can be imported into barley plastids and incorporated into LHC II of the thylakoids, also the precursor as well as the processed form can be detected in thylakoids of greening maize plastids in a similar in vitro system [30]. Information may reside in the chain extension of the precursor not only for import, but also for specific localization in the thylakoid as well. Thus, a two-step processing mechanism may exist. The data we have presented suggest strongly that this is in fact the case and that the second processing step has been impaired in maize leaves by the photoinhibitory stress at low temperatures. These data may be explained by an inactivation at low temperatures of the protease necessary for the final processing step of the precursor. This system would appear to offer an excellent opportunity to help resolve the sequence of events in the processing of the 31 kDa precursor of LHC II.

Acknowledgements

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