Senescence-Related Changes in the Subcomplex Arrangement of the Major Light-Harvesting Chlorophyll *a/b*-Protein Complex of Photosystem II (LHCII) as Influenced by Cytokinin

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The major light-harvesting chlorophyll *a/b*-protein complex of photosystem II (LHCII) from fresh barley leaves could be resolved by non-denaturing IEF into five trimeric subcomplexes designated 1–5 in order of decrasing pI value. IEF-based analysis of PSII particles isolated from leaves in which the processes of senescence were induced by detachement and dark-incubation in the presence of water for 0–8 days let us reveal that substantial rearrangements of LHCII organization took place throughout the course of senescence comprising a step-wise decline in relative abundance of subcomplexes 1–3 (down to 0–58% of the initial abundance during 8 days of aging) and an increase in the relative abundance of the subcomplexes 4 and 5. Using SDS-PAGE and immunoblot analysis it was shown that the rearrangements were linked to the changes in the relative levels of LHCII apoproteins i.e. 26.7 and 25.6 kDa ones. The changes comprised the preferential disappearance of the 26.7 kDa polypeptide and an enrichment of 25.6 kDa one and most probably reflect the heterogeneity among LHCII apoproteins concerning their stability under the conditions of chl loss. Kinetin was able to repress the senescence-related rearrangements in LHCII subcomplex organization at late stages of aging (5–8 days) by preventing over this time period the disappearance of 26.7 kDa polypeptide and the enrichement of 25.6 kDa one.

Introduction

The major light-harvesting chlorophyll *a/b*-protein complex of photosystem II, indicated as LHCII by a large number of research groups, is the most abundant energetic antenna in chloroplasts, standing for more than 50% chl inside the thylakoid membrane (Chitnis and Thornber, 1988). LHCII is believed to be a complicated supramolecular structure comprised of peripheral and inner subpopulations i.e. molecular subcomplexes exhibiting different behaviour during state I – state II transitions and apparently having different arrangement around PSII center complexes (Staehelin and Arntzen, 1983; Larsson *et al.*, 1987). The subpopulations most probably correspond to distinct trimeric LHCII subcomplexes separated and characterized biochemically by some research groups in recent years (Bassi *et al.*, 1988; Spangfort and Andersson, 1989; Ruban *et al.*, 1994; Jackowski and Przymusiñski, 1995).

It was shown that PSII antenna apparatus can be regulated in response to environmental stimuli including long-term changes in irradiance intensity (Lichtenthaler *et al.*, 1982; Leong and Andersson, 1986), light quality (Kim *et al.*, 1993), elevated leaf temperatures (Sundby and Andersson, 1985), developmental and mutational factors (Morrissey *et al.*, 1989; Greene *et al.*, 1988) as well as diurnal cycle (Busheva *et al.*, 1991; Liker and Garab, 1995). The modulations which accompany acclimations to light intensity were found to be mainly due to changes in the peripheral subpopulation of LHCII (Larsson *et al.*, 1987). The involvment of LHCII peripheral pool in such modulations comprised a specific increase of this subpopulation at low-light irradiation and specific decrease under high-light conditions (Maenpaa and Andersson,

Abbreviations: CBB G-250, Coomassie Brilliant Blue G-250; chl, chlorophyll; DM, *n*-dodecyl-β-D-maltoside; IEF, isoelectric focusing; LHCII, the major light-harvesting chlorophyll *a/b*-protein complex of photosystem II; pI, isoelectric point; PSII, photosystem II; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TCA-trichloroacetic acid.

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1989). The utilization of chlb-less and chlb-deficient mutants of soybean allowed addressing also the question of adjustment of PSII antenna apparatus during plant development under stable environmental conditions. It was suggested that discrete LHCII peripheral-containing subcomplexes were added to developing PSII holocomplex as its maturation proceeded (Morrissey *et al.*, 1989) while the size of LHCII – inner was constant at all stages of biogenesis of the thylakoid membrane. On the contrary, no data are available concerning the behaviour of individual LHCII subcomplexes as pigment-protein entities during leaf senescence though senescence-related loss of chl, carotenoids and LHCII apoproteins (Young *et al.*, 1991; Jackowski *at al.*, 1991) as well as the decline in the expression of LHCII apoproteins (Bate *et al.*, 1991) indicate the substantial decomposition of LHCII holocomplex.

Senescence-related decomposition of LHCII holocomplex could be envisaged as happening either by a sequential disassembly of discrete subcomplexes or by a simultaneous disassembly of all LHCII components. It was therefore the primary objective of the present work to discriminate between these two pathways by the analysis of disappearance/maintenance kinetics of individual LHCII subcomplexes and apoproteins. In addition, the fates of the subcomplexes and their apoproteins were examined in the presence of cytokinin to shed some more light on the mode of antisenescence operation of this hormone (Wozny *et al.*, 1977; Jackowski *et al.*, 1991). To reach these goals we exploited detached, dark-incubated barley leaves as a model system to study the processes of foliar senescence (Srivastava and Ware, 1965).

Materials and Methods

Plant material

Barley (*Hordeum vulgare L.*) seeds were germinated in moist lignin in complete darkness for 3 days followed by 7 days of illumination with continuous white light of 75 μE m^{-2} s^{-1}. The primary leaves of the plants were detached, placed with their bases in water or 300 μM kinetin solution and allowed to senesce in darkness for 0,2,5 and 8 days at 25 C°.

Isolation of PSII and LHCII

PSII particles were isolated from the primary leaves according to the method of Berthold *et al.* (1981) with the modification described by Dunahay *et al.* (1984).

Triton X-100: chl ratio of 4.5 : 1 was used to solubilize thylakoid membranes. LHCII was isolated from PSII particles essentially as described earlier (Jackowski and Kluck, 1994). PSII and LHCII samples were stored in 10% glycerol in dry ice.

The resolution of LHCII subcomplexes

PSII particles and LHCII samples were solubilized and resolved by non-denaturing IEF according to Jackowski and Przymusiñski (1995) except that focusing was performed for 7–8h at voltage increasing in 50–480V range. 3%DM final concentration (instead of 1%) was applied to obtain a surfactant extract of PSII samples isolated from the leaves incubated in darkness for 8 days.

SDS-PAGE and immunoblotting

The polypeptides of LHCII subcomplexes were precipitated by adding 5 volumes of 80% acetone and the pellets were dissolved, analysed by SDS-PAGE and the gels stained as described by Jackowski and Przymusiñski (1995). For immunoblot analysis electrophoretically separated thylakoid membrane samples were electrotransferred to nitrocellulose filters using transfer apparatus with buffer of Towbin *et al.* (1979) containing 0.1% SDS. To estimate transfer efficiency the blots were temporarily stained with 0.2% Ponceau S in 3% TCA. The filters were then immunostained using specific polyclonal antibodies raised in rabbits against carnation LHCII apoproteins according to the protocol of Legocka *et al.* (1990) except that the colour developmment of immunoblots was performed applying biotinylated secondary antibodies in conjunction with streptavidin-peroxidase complex.

Relative quantitation of IEF-gels and immunoblots

To perform a relative quantitation of immunostained bands of LHCII apoproteins nitrocellulose filters scanned at 600 nm in a reflectance mode applying Shimadzu CS-9000 Flying Spot Dual-

Wavelength Scanner with on-board integration system. To determine a relative content of non-stained LHCII subcomplexes non-denaturing IEF gels were scanned at 650 and 675 nm in a transmission mode and the relative areas under the bands of individual subcomplexes were calculated according to Genge *at al.* (1974).

Other methods

Chl was assayed according to the method of Arnon (1949).

Results

LHCII from barley thylakoid membrane can be resolved by a vertical-bed, non-denaturing IEF into five trimeric subcomplexes covering pH range of 4.02–4.24, labelled 1–5 in order of decreasing pI value (Fig. 1), as described in a paper companion to this one (Jackowski, 1996). As a first step in examining whether foliar senescence is accompanied by rearrangements of subcomplex organization of LHCII we tried to isolate LHCII from water – and kinetin – treated leaves which had been kept in darkness for 2,5 and 8 days. We were not able, however, to release LHCII by K^+-induced aggregation of n-heptyl-thioglucoside-treated PSII particles from senescent leaves, the approach found to be successfull in the case of LHCII isolation from fresh leaves.

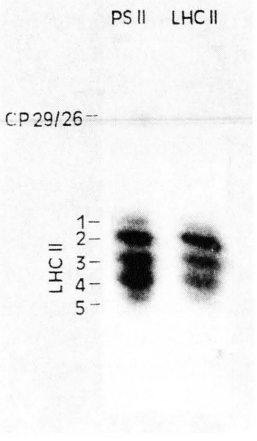

Fig. 1. Non-denaturing IEF of LHCII and PSII isolated from fresh leaves. 50 μg chl of LHCII or PSII was resolved in a 0,5mm-thin analytical, vertical 7% polyacrylamide gel containing 0.75% DM (pH 3.5–5.0). The symbols of trimeric LHCII subcomplexes and their measured pI values are indicated. The gel was not stained.

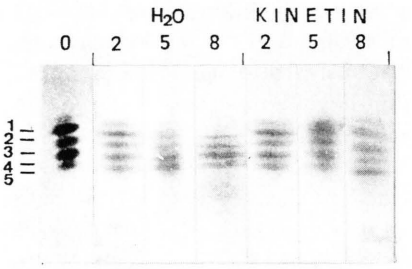

Fig. 2. Non-denaturing IEF of PSII isolated from detached, dark-incubated leaves. 20 μg chl of PSII from the leaves dark-incubated for 2,5 and 8 days in the presence of water or kinetin were resolved as described in Figure 1. The gel was not stained.

To obviate this difficulty we used PSII particles which could be isolated from senescing leaves as easily as from fresh ones. When the particles, isolated from fresh leaves, were separated by non-denaturing IEF they yielded precisely the same pattern of LHCII subcomplexes as in the case of IEF-based separation of purified LHCII (Fig. 1). In order to reveal senescence – related rearrangements in subcomplex organization of LHCII we performed a quantification of ratios at which the subcomplexes were associated with one another at various stages of senescence. For this purpose we analysed by non-denaturing IEF a series of PSII samples prepared from leaves which were dark-incubated for 2,5 and 8 days in the presence of water or kinetin (Fig. 2). The estimation of the stoichiometry of LHCII subcomplexes in PSII samples was carried out by measuring the distribution of chl among pigmented, IEF-resolved bands using an integrating densitometry. The results are shown in Figure 3. It can easily be noticed that, with advancing senescence, the relative amount of the subcomplex 1 gradually declines to the point where it entirely disappears in 8 days-old leaves. Relative amounts of the subcomplexes 2 and 3 also decline considerably as senescence progresses although these subcomplexes are still clearly detectable in 8 days-old leaves, comprising 37.3 and 57.8%, respectively, of their relative levels found in fresh leaves. By contrast, the subcomplexes 4 and 5 account for gradually increasing proportion of LHCII – associated chl over the whole senescence period so that in 8 days-old leaves 36.2%

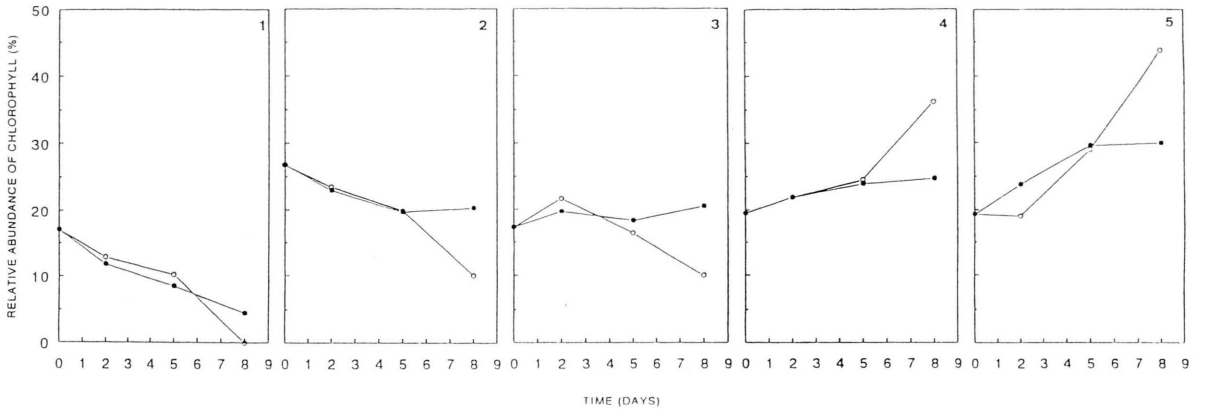

Fig. 3. Time-course of changes in relative abundancies of LHCII subcomplexes in detached, dark-incubated leaves. PSII particles isolated from water-treated (o) or kinetin -treated (•) leaves were resolved by non-denaturing IEF and nonstained IEF gels were scanned at 650 and 675 nm. The relative abundancies of LHCII subcomplexes were determined as percentages of integrated chl peaks and plotted against the time of dark-incubation. 1,2,3,4,5 – plots for LHCII subcomplexes labelled 1,2,3,4 and 5, respectively.

and 43.8% of LHCII – associated chl was attributable to the subcomplexes 4 and 5, respectively, as compared to 19.5 and 19.3% in fresh leaves. Our results show that at early stages of senescence (0–5 days) there is little or no differences in the pattern of changes in relative abundance of the subcomplexes between water-and kinetin-treated leaves. At later stages (5–8 days), however, the relative amounts of the subcomplexes 2–5 remain almost unchanged in kintin-treated leaves while the changes in the arrangement of subcomplexes in water-treated leaves still proceed. The relative abundance of the subcomplex 1 decrease between days 5 and 8 in both groups of leaves although at the day 8 the subcomplex remains measurable in kinetin-treated leaves while in water-treated ones it is completely missing.

There are indications that the limited availability of chl, e.g. in the case of chlb-less mutants or intermittent light-grown plants, influences the accumulation of individual LHCII apoproteins differently, resulting in altered stoichiometric ratios of the apoproteins (Dreyfuss and Thornber 1994; Król *et al.* 1995). As the leaves' senescence is also accompanied by a large decrease in chl content we have attempted at determining – by SDS-PAGE and immunoblot analysis – if the senescence-related modulations of relative amounts of LHCII subcomplexes from barley arise from heterogeneity of maintenance/disappearance ratio of individual apoproteins. To do this samples of thylakoid membrane polypeptides representing individual stages of senescence were subjected to SDS-PAGE, transferred to nitrocellulese and probed with monospecific anti-LHCPII antibodies (Jackowski *et al.* 1991). The relative intensity of staining of individual LHCII apoproteins in each lane of immunoblots (Fig. 4) was measured by an integrating densitometry yielding the values given in Fig. 5A. Namely, in water-treated leaves a relative amonut of 26.7 kDa apoprotein gradually declines with age so that at the day 8 it is about 18% less abundant than in fresh leaves whereas 25.6 kDa apoprotein is enriched appropriately over the same time period. At early stages of senescence (0–5 days) the pattern of changes in 26.7/25.6 kDa

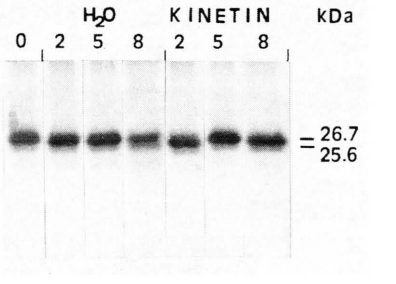

Fig. 4. Immunoblot analysis of thylakoid membranes from detached, dark-incubated leaves. 10 μg chl of thylakoid membranes from the leaves dark-incubated for 2,5 and 8 days in the presence of water or kinetin was subjected to SDS-PAGE, transferred to nitrocellulose and probed with polyclonal antibody raised against carnation LHCII apoprotein.

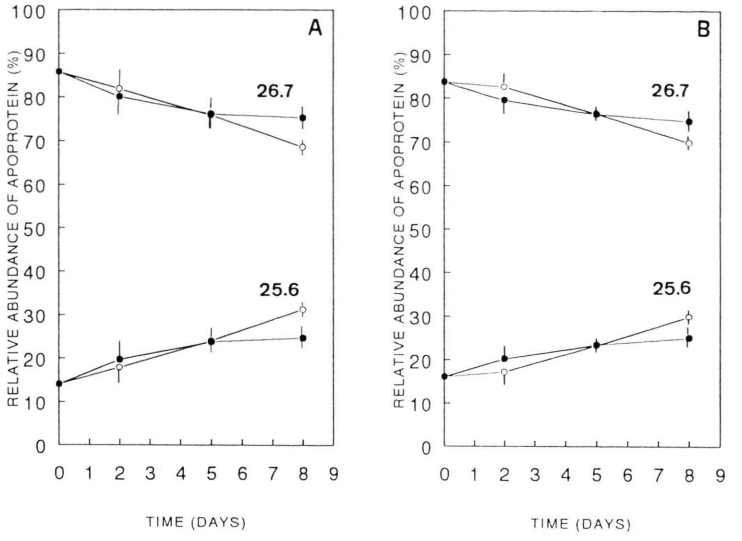

Fig. 5. Time-course of changes in relative abundancies of LHCII apoproteins in detached, dark-incubated leaves. (A) The abundancies were directly measured by an integrating densitometry of immunoblots as in Figure 4. (B) The abundancies were calculated taking into account the apoproteins ratios measured for the subcomplexes 1–5 in fresh leaves and the relative amounts of the subcomplexes measured after 2,5 and 8 days. The mesaured and calculated abudancies were plotted against the time of dark-incubation. Leaves were dark-incubated in the presence of water (o) or kinetin (•). Each point is the mean ± s.e. of at least 3 determinations obtained from 2–3 separate experiments.

ratio in kinetin-treated leaves is very similar to that found for water-treated ones. As senescence progresses (5–8 days), however, the relative contributions of the apoproteins do not change further in kinetin-treated leaves whereas in water-treated ones 26.7 kDa apoprotein becomes still less abundant than by the day 5. As the subcomplexes 1 and 2 contain single 26.7 kDa polypeptide while the subcomplexes 3–5 can be resolved into 26.7 and 25.6 kDa polypeptides associated at ratios 2.54:1, 2.20:1 and 2,16:1, respectively (Fig. 6), it is evident that the decline in relative amounts of LHCII subcomplexes 1–3 is related to the preferential disappearance of 26.7 kDa apoprotein whereas the increase in the relative amount of the subcomplexes 4–5 is linked to the preferential maintenance of 25.6 kDa one. The observations

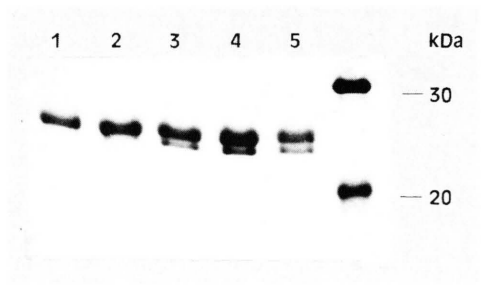

Fig. 6. SDS-PAGE analysis of polypeptide composition of LHCII subcomplexes.4 μg chl of the subcomplexes 1–5 isolated from fresh leaves were resolved and stained by CBB G-250.

demonstrate that kinetin is able to influence the pathway of LHCII rearrangements by preventing, at late stages of aging, the loss of subcomplexes 1–3 and enrichment in subcomplexes 4–5, both events being a consequence of the repression of the changes in relative abundancies of LHCII apoproteins (Fig. 4 and 5A). Apart from measuring by integrating densitometry of immunoblots the relative abundance of LHCII apoproteins from 2,5 and 8 days-old, water-and kinetin-treated leaves might be calculated taking into the account the 26.7/25.6 kDa ratios determined for the subcomplexes 1–5 isolated from fresh leaves and the proportion of subcomplexes measured at different stages of senescence. The calculation yields the values presented in Fig. 5B. It is clear that calculated (IEF) and measured (immunoblot analysis) values of the 26.7/25.6 kDa ratios are very similar, an observation corroborating the reliability of the received data.

Discussion

The senescence of leaf is the final stage of its ontogenesis comprising a syndrom of controlled, deteriorative metabolic processes leading to the leaf's death. The most conspicuous metabolic changes constituting the senescence syndrom is an increase in the activity of enzymes catabolising proteins (Thimann, 1980) and chl (Thomas *et al.*, 1989). In the case of photosynthetic membranes

the senescence-related metabolic changes bring about, among others, a large decrase in the content of pigments (Young *et al.,* 1991) and pigment-binding proteins, including LHCII apoproteins (Roberts *et al.,* 1987; Jackowski *et al.,* 1991; Okada *et al.,* 1992). Since leaf senescence is accompanied by significant decline in the expression of LHCII apoproteins (Bate *et al.,* 1991), the prevailing majority of LHCII particles found in senescing leaves should be ones which temporarily survive the decomposition events rather than the products of an assembly of diminishing pool of pigments with de novo synthesized apoproteins.

It is not clear whether the decomposition of LHCII starts from the disappearance of pigments or apoproteins. There are, however, reasons to believe that breakdown of chl may come first. Using a mutant of meadow fescue (*Festuca pratensis*) in which senescence-related chl breakdown was disabled, Hilditch *et al.* (1989) have shown that the apoproteins of LHCII were not degraded during senescence although the mutant leaf tissues had the same proteolytic activities as the wild ones. As LHCII apopproteins are known to be turned down rapidly under the conditions of chl-deficiency (Bennett, 1981; Bellemare *et al.,* 1982) it appears that chl catabolism is a requirement for breakdown of LHCII apoproteins. The decomposition of LHCII holocomplex may occur either by a sequential breakdown of chl and polypeptide components of discrete trimeric subcomplexes or via a simultaneous catabolism of the components of all the subcomplexes. To discriminate between sequential and simultaneous pathways we sought to analyze the arrangement of LHCII subcomplexes at different stages of leaf senescence. If the decomposition followed a sequential pathway one should expect senescence-related changes in subcomplex organization of LHCII reflecting its progressive truncation. Unexpectedly, the isolation of LHCII from leaves by K+-induced aggregation of n-heptylthioglucoside-solubilized PSII particles proved not to be possible though the same protocol worked efficiently with LHCII from fresh leaves.The basis for the inability of LHCII from senescent leaves to be aggregated is not clear but a likely explanation is that aggregation-promoting, N-terminal domain of LHCII apoprotein is cleft off due to dark incubation-related enhancement of anti-LHCII proteolytic activities, recently discovered by Anastassiou and Argyroudi-Akoyunoglou (1995). As IEF-based separation of PSII and LHCII samples from fresh leaves gave precisely the same stoichiometries of LHCII subcomplexes we studied the arrangement of the subcomplexes in senescing leaves by resolving their PSII particles.

The results presented in this paper demonstrate that the decomposition of barley LHCII follows sequential pathway as the advancement of senescence processes was accompanied by considerable rearrangements in organization of LHCII comprising gradual decline (within 0–8 days of senescence) in the relative amount of the subcomplexes 1–3 and an increase in the relative abundance of the subcomplexes 4 and 5. Ordered, senescence-dependent changes in relative amounts of LHCII subcomplexes may, however, be an artefact reflecting a preferential loss of defined subcomplexes during preparation of labile PSII particles from yellowing leaves. If this were the case one would expect PSI-enriched supernatants, obtained after pelleting of PSII particles, to be heavily contaminated by LHCII subcomplexes associated at different ratios, depending on the stage of senescence. We have found, however, that at all stages of senescence PSI-enriched supernatants contained very small, roughly equimolar amounts of LHCII subcomplexes (data not shown).

We have shown that the preferential maintenance of the subcomplexes 4 and 5 reflects the preferential maintenance of 25.6 kDa LHCII appoprotein. As senescence is accompanied by a substantial decline in the the expression of LHCII apoproteins (Bate *et al.*, 1991) it is inferred that preferential maintenance of barley 25.6 kDa apoprotein reflects its higher resistance towards proteases operating under the conditions of limited chl availability. In fact, it was shown recently that there is a heterogeneity among LHCII apoproteins concerning their resistance to thylakoid-bound proteolytic activity (Lindahl *et al.,* 1995). The preferential maintenance of 25.6 kDa polypeptide would thus imply that this polypeptide is situated more proximally with respect to PSII reaction center core than 26.7 kDa one as it is reasonable to suggest that the proteolysis of LHCII components proceeds, for mechanistic reasons, from PSII core-distant to PSII core-proximal ones (Harrison and Melis, 1992). The pattern of mainte-

nance/disappearance of individual LHCII apoproteins during senescence of barley leaves may, however, be alternatively attributed to the heterogeneity relative to the affinity for stabilizing chl which is present in limiting amounts. If this is true, the observed preferential maintenance of 25.6 kDa polypeptide would correlate to the higher binding constant of chl to this apoprotein than to 26.7 kDa one. Besides, these two possibilities need not be mutually excluding i.e. the 25.6 kDa polypeptide may both reside in closer proximity to PSII reaction center core and have a higher affinity for chl than 26.7 kDa one. In fact, there are indications that the apoproteins of reaction centre core have higher affinity for chl with respect to LHCII apoproteins since they receive priority in the hierarchy of maintenance under the conditions of limited chl availability induced by transfer to darkness of the plants with immature photosynthesis machinery (Argyroudi-Akoyunoglou *et al.*, 1982; Tzinas *et al,*. 1987). On the other hand, however, it has been repeatedly observed that natural and dark-induced senescence is accompanied by a decrease in chla/b ratio and it may suggest that LHCII apoproteins are more resistant towards breakdown than the polypeptides residing more adjacent to PSII reaction center core (Kura Hotta *et al.*, 1987; Jenkins *et al.*, 1981). Detailed, topographical studies on PSII holocomplex arrangement are thus needed to elucidate relative location of barley LHCII apoproteins within PSII structure .

Our results indicate that kinetin is able to prevent, at later stages of senescence i.e. within 5–8 days of dark-incubation, the changes in relative abundancies of LHCII apoproteins and, consequently, to restrain the rearrangements at the level of LHCII subcomplexes that would have taken place under control conditions. Since kinetin was shown to prevent the extensive loss of chl occuring in detached, barley leaves between 5 and 10 days of dark-incubation (Jackowski *et al.*, 1991) it would be reasonable to assume that the hormone influences the pathway of LHCII rearrangements through its effect on chl catabolism. However, the effects on the expression of LHCII apoproteins similar to that described for senescing *Lemna gibba* (Tobin and Turkaly, 1982) cannot be excluded at present.

Kinetin was not able to prevent significant changes in LHCII organization that occur during the first 5 days of dark-incubation. The earliest of these changes may reflect dark adaptations rather than senescence events (Malik, 1987).

Acknowledgments

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