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Chlorophyll *b* deficiency in soybean mutants. II. Thylakoid membrane development and differentiation

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The expression of chlorophyll (Chl) *b* deficiency in the y_9y_9 virescent soybean mutant depends strongly on the light intensity during plant growth. The mutant phenotype (high Chl *a*/Chl *b* ratios, low chlorophyll content per leaf area and low chlorophyll to carotenoid ratios) is expressed at moderate to high intensities, whereas at low light intensities mutant and wild-type plants are indistinguishable. Expression of the mutation appears to be developmentally controlled and is manifested at the membrane level by inhibition of assembly/incorporation of the peripheral complement of the Chl *a*/*b* light-harvesting complex II (LHC II-peripheral). Analysis of thylakoid membrane polypeptides by SDS-polyacrylamide gel electrophoresis revealed the presence of novel polypeptides of 12–15 kDa in the y_9y_9 mutants. These polypeptides were the most abundant protein in the thylakoid membrane when the mutation was fully expressed (Chl *a*/Chl *b* = 8) but were largely absent both from the mature wild-type and from the fully developed y_9y_9 chloroplasts (Chl *a*/Chl *b* = 3.5). The relative concentration of polypeptides in the 12–15 kDa region was inversely proportional to that of LHC II, suggesting a role for these novel proteins in the assembly of the LHC II and/or chloroplast development.

Introduction

The preceding article [1] described the photochemical apparatus characteristics of two Chl-*b*-deficient soybean mutants, the Clark y_9y_9 and the Clark $Y_{11}y_{11}$. In both cases, Chl *b* deficiency resulted in smaller Chl antenna size for the two photosystems, higher PS II/PS I stoichiometric ratio, and enhanced PS II_β content. A model was proposed for the development of the Chl antenna of PS II. Two distinct steps were identified. The first involved incorporation of the tightly bound complement of the LHC II (LHC II-inner) which effectively converted a PS II complex with 50 Chl *a* molecules into PS II_β (about 120 Chl $a+b$ molecules). The second step involved the further incorporation of a peripheral complement of the LHC II (LHC II-peripheral), effectively forming the mature PS II_α with 210–250 Chl $a+b$ molecules. This two-step mechanism for the formation of the PS II antenna received support from work with the Chl-*b*-less chlorina f2 mutant of barley in

Abbreviations: Chl, chlorophyll; PS, Photosystem; LHC, light-harvesting complex; PSU, photosynthetic unit.

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which both the LHC II-inner and the LHC II-peripheral were missing, resulting in the accumulation of PS II complexes containing the Chl *a* antenna associated with CP47, CP43 and CP29 only [2]. In the Chl-*b*-deficient soybean mutants, the LHC II-peripheral was largely missing from the thylakoid membrane, resulting in the accumulation of PS II_β [1].

The above observations constitute useful but preliminary information on the assembly and organization of the photosynthetic unit of PS II. The broader question of the mechanism regulating the assembly of the LHC II in the thylakoid membrane remains beyond reach. In the particular case of the Chl-*b*-deficient mutants, interesting questions to address include (a) the ability of the chloroplast to regulate the assembly of the photosynthetic unit as evidenced by the selective incorporation of the LHC II-inner and by the lack of incorporation of the LHC II-peripheral, (b) the fate of the LHC II-peripheral subunits that fail to assemble and/or incorporate in the functional PSU, (c) the targeting of individual Chl-protein subunits to the respective domain(s) of the thylakoid lamella and the regulation of the process of assembly. In the y_9y_9 Chl-*b*-deficient soybean mutant, the assembly/incorporation of the LHC II-peripheral is delayed; therefore, one might expect a pronounced turnover of unstable LHC II polypeptides [3].

The work presented in this article addresses the regulation of chloroplast development and the mechanism of assembly/incorporation of LHC II in the thylakoid membrane of y_9y_9 soybean mutants. This developmental mutant offers unique advantages for the elucidation of the above questions, mainly because of the retarded development of its photosynthetic units. At various stages during the development of the chloroplasts, we correlated the pigment and protein content in the thylakoid membrane. We report the presence of two novel polypeptides with apparent molecular masses of 15.1 and 12.4 kDa, respectively. They constituted the most abundant protein in the thylakoid membrane of developing chloroplasts but were largely absent from the membrane of mature chloroplasts. The role of the 15.1 and 12.4 kDa polypeptides in the regulation of LHC II assembly is discussed.

Materials and Methods

Wild-type soybean (Glycine max, Clark L1) and Chl-*b*-deficient Clark y_9y_9 plants were grown in vermiculite either in the greenhouse under direct sunlight (average light intensity at noon was about 1000 $\mu E \cdot m^{-2} \cdot s^{-1}$) or in a growth chamber under continuous illumination provided by a combination of fluorescent and incandescent lamps.

Leaves were harvested from plants at different stages of growth. Leaf surface area was determined from the paper weight of photocopies of leaves, made against a white background. The concentration of pigments per leaf area was determined spectrophotometrically from the acetone extract of leaf discs; Chl *a* and Chl *b* concentrations were calculated from the absorbance at 663 and 645 nm by a variation of Arnon's equations [4]. Total carotenoid concentration was determined from the absorbance at 480 nm, using an absorption coefficient of 135 $mM^{-1} \cdot cm^{-1}$ [5].

Thylakoid membranes were isolated from freshly harvested leaves as described previously [1]. The membranes were washed twice in a hypotonic buffer, resuspended in 20 mM Tricine and 10% sucrose, and denatured at 100 °C for 3 min in the presence of 2% SDS and 2% mercaptoethanol. Analysis of thylakoid membrane polypeptides was carried out by SDS polyacrylamide gel electrophoresis in a 10–17.5% (w/v) linear gradient of polyacrylamide containing 0.1% SDS using the buffer system of Chua [6,7]. Electrophoresis was carried out at a constant current (4.5 mA) for about 18 h at 4 °C. The proteins in the polyacrylamide gels were stained with Coomassie brilliant blue and scanned linearly at 580 nm for the quantitation of the density of Coomassie stain. Care was exercised to ensure the linearity between A_{580} (relative stain incorporation) and the amount of protein loaded to the gel.

Polypeptides from the polyacrylamide slab were transferred to nitrocellulose in a medium containing 25 mM Tris (pH 8.3), 150 mM glycine, 20% methanol and 0.1% SDS. For this purpose, a Hoeffer protein-transfer TE52 apparatus was used. Polypeptides on nitrocellulose were probed with polyclonal antibodies raised against isolated LHC II in a medium containing 20 mM Tris (pH 7.5)

buffer and 2% powder milk. Antibodies bound to protein were detected by the horseradish peroxidase reaction [8].

The isolation and purification of LHC II from wild-type soybean chloroplasts was performed according to the method of Burke et al. [9] as modified by Ryrie et al. [10].

Results

In the preceding article (Fig. 1 in Ref. 1), evidence was presented suggesting that the expression of the y_9y_9 Chl-*b*-deficient phenotype depends strongly on the age of the plant and also on the light intensity during plant growth. The effect of light intensity on the expression of Chl *b* deficiency is documented in Fig. 1 with 7-day-old plants grown in a growth chamber. Fig. 1 shows the Chl *a*/Chl *b* ratio of the leaves as a function of the light intensity in the growth chamber. It is observed that, over the light intensity range examined, the Chl *a*/Chl *b* ratio in the wild type soybean leaves (Fig. 1, solid circles) remained between 3.2 and 3.5, apparently independent of light intensity. Mutant plants, however, revealed a strong dependence of the Chl *a*/Chl *b* ratio as a function of light intensity (Fig. 1, open circles). Clearly, at higher light intensities the acquisition of Chl *b* is delayed, resulting in higher Chl *a*/Chl *b* ratios.

The mechanism by which light intensity regulates the acquisition of Chl *b* in the thylakoid membrane of the y_9y_9 mutant is totally unknown. An attempt was made to distinguish among two broad alternatives: (a) The sensitivity of Chl *b* to light is manifested after the completion of the assembly of the photosynthetic unit. It is possible that, due to a defect, mutant plants can afford little or no photoprotection to Chl *b*, resulting in its prompt photodestruction. This hypothesis will explain the light intensity dependence of the expression of the y_9y_9 phenotype. (b) The adverse effect of light is manifested prior to the functional association of LHC II with PS II, perhaps during or even before the assembly of the LHC II in the chloroplast. In this case, one would expect to observe the turnover of polypeptides from the LHC II that failed to assemble in the functional PSU of PS II.

To help distinguish between the two alternatives, the reversibility of Chl *b* deficiency in y_9y_9 was tested when low-light-grown plants were transferred to high-light conditions. According to alternative (a), it was reasoned, the Chl *b* deficiency in y_9y_9 should reappear when low-light-grown plants are transferred to high-light conditions, and this process should be independent of the developmental stage of the chloroplast.

Fig. 2 shows the Chl *a*/Chl *b* ratio of the primary leaves of wild-type (solid circles) and of y_9y_9 soybean plants (open circles) as a function of the plant age under different experimental conditions. When wild-type and mutant plants are grown under high-light conditions (Fig. 2, left panel) the Chl *b* deficiency is clearly expressed in

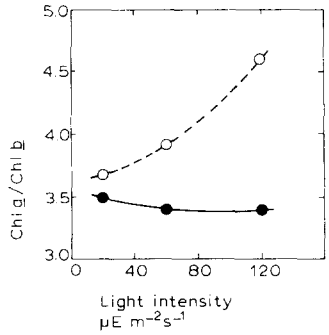

Fig. 1. Chl *a*/Chl *b* (mol/mol) ratio of primary leaves from 7-day-old wild-type (solid circles) and Clark y_9y_9 soybean mutant (open circles) as a function of the light intensity during plant growth. Plants were grown in a growth chamber under continuous illumination.

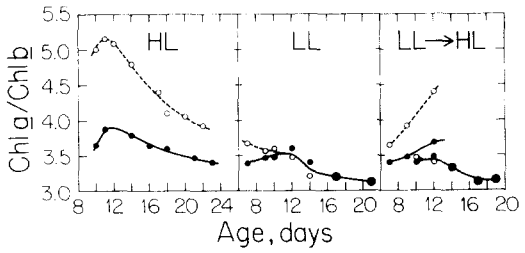

Fig. 2. Chl *a*/Chl *b* (mol/mol) ratio of wild-type (solid circles) and mutant soybean plants (open circles) as a function of plant age. Plants were grown either in a greenhouse (HL), or in a growth chamber under a 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity (LL). Low-light-grown plants were transferred from the growth chamber to the greenhouse (LL → HL) on either the 7th or the 10th day after germination.

the mutant plants (open circles) but not in the wild-type ones (solid circles). In plants grown under low-light-intensity conditions, the Chl *b* deficiency is suppressed to the point that Chl *a*/Chl *b* ratios in wild-type and mutant chloroplasts are practically indistinguishable after the 10th day of growth (Fig. 2, center panel, LL). The results produced upon a transition of low-light-grown plants to high light intensity depended strongly upon the developmental stage of the chloroplasts (age of the plants). Fig. 2 (right panel, LL → HL) shows that when 7-day-old plants were transferred from low-light to high-light conditions, the Chl *a*/Chl *b* ratio of the y_9y_9 increased with time, suggesting that the chloroplasts reverted to the Chl-*b*-deficient phenotype. A smaller increase in the Chl *a*/Chl *b* ratio of the wild type was also observed. If the LL → HL transition occurred in the 10th day of growth, then the Chl *b* deficiency in the y_9y_9 mutant failed to appear. Instead, primary leaves from mutant soybean plants behaved in a manner identical to that of wild-type plants.

The results of Fig. 2 strongly suggest that low-light intensity allows for the efficient acquisition of Chl *b* by the y_9y_9 chloroplast and promotes the fast development of the light-harvesting antenna of PS II. Once the Chl antenna of PS II is fully developed, however, it is not possible any more to revert the plants to the Chl-*b*-deficient phenotype upon transfer to a high-light environment. Such results appear to be incompatible with alternative (a) above.

Measurements similar to those in Fig. 2 were extended to include the leaf chlorophyll content, the chlorophyll-to-carotenoid ratio, and the leaf surface area as a function of the age of the plants. Fig. 3A shows the chlorophyll per leaf area in wild-type (solid circles) and y_9y_9 soybean plants (open circles) grown under high light (HL), under low light (LL) and upon transition of low-light-grown plants to high-light conditions (LL → HL). The transition occurred on either the 7th or the 10th day of growth. The results support the notion that y_9y_9 plants display lower chlorophyll content per leaf area under high-light conditions. Such a difference is not detected beyond the 10th day of growth under low-light conditions. As with the Chl *a*/Chl *b* ratio, a transition of low-light-grown

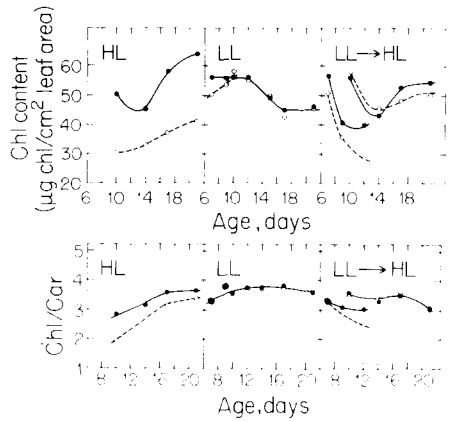

Fig. 3. (A) The chlorophyll content per leaf area from wild-type (solid circles) and Clark y_9y_9 mutant soybean plants (open circles) as a function of plant age. Leaves were harvested from plants grown in the greenhouse (HL), or in the growth chamber under a 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity (LL). Low-light grown plants were transferred from the growth chamber to the greenhouse (LL → HL) either on the 7th or on the 10th day after germination. (B) Chlorophyll/carotenoid (w/w) ratio in leaf discs from wild-type (solid circles) and Clark y_9y_9 mutant soybean plants (open circles) as a function of plant age.

plants to high light will generate a differential response between wild-type and y_9y_9 only if it occurs on the 7th day of growth, or earlier. If the transition occurs on the 10th day, or later, no differential response is observed (Fig. 3A right-hand panel LL → HL).

Fig. 3B show the chlorophyll/carotenoid (Car) ratio in wild-type (solid circles) and y_9y_9 plants (open circles) grown under high-light (HL) and low-light (LL) conditions, and upon a transition of low-light-grown plants to high-light conditions (LL → HL) on the 7th or 10th day of growth. The results show that under HL conditions y_9y_9 leaves display a lower Chl/Car ratio than the wild-type, suggesting that the effect of the mutation cannot be attributed to a lack of carotenoids in the y_9y_9 mutants (see also Refs. 11, 12). Moreover, the Chl/Car differences in wild-type and mutant disappear under low-light conditions (LL). The lower Chl/Car ratio in the y_9y_9 can reappear only if low light-grown plants are transferred to high-light conditions on the 7th day, or earlier, following germination of the seedlings. A transition of low-light-grown plants to high-light intensity produces

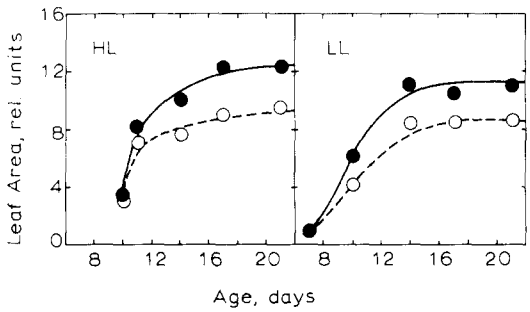

Fig. 4. The leaf surface area as a function of plant age. Soybean wild-type (solid circles) and y_9y_9 mutant plants (open circles) were grown in the greenhouse under direct sunlight (HL) or in a growth chamber under reduced light-intensity (LL).

no effect if it occurs on the 10th day of growth, or later.

Fig. 4 presents a measurement of the leaf surface area as a function of the age of the plant. The leaf growth pattern is the same in wild-type (solid circles) and y_9y_9 mutant (open circles), except that wild-type leaves are larger by about 30% when fully grown. The rate of leaf growth appears somewhat greater under high-light (HL) when compared to low-light (LL) conditions. In terms of our understanding of the phenomena presented in Fig. 2 and Fig. 3, it is important to observe that leaves 10 days old or older, probably expand through cell elongation only, whereas 7-day-old leaves probably expand through active cell division. This may be a useful observation, although the relation between cell growth and chloroplast development is not understood.

The results presented above clearly suggest that chloroplasts in the primary leaves of the y_9y_9 soybean mutant become fully developed by the 10th day of growth under low-light conditions and, thereupon, lose the developmental characteristics of the Chl *b* deficiency. Under high-light conditions, however, they remain in a prolonged state of 'development', clearly unable to acquire the full complement of chlorophyll in their photosynthetic units. Such results suggest that the adverse effect of the mutation manifests itself during, or even before, the assembly of the LHC II in the chloroplast. If the lesion prevents the stable formation of LHC II complexes, it is implied that unstable LHC II components would be degraded in the chloroplast, as stated in alternative (b) above. This led us to investigate the relative polypeptide composition of thylakoid membranes from wild-type and y_9y_9 mutant chloroplasts at various stages of 'development'.

Fig. 5 compares densitometric scans of Coomassie-stained SDS-polyacrylamide gel electrophoresis in analysis of thylakoid membranes from wild-type (WT, Chl *a*/Chl *b* = 3.7) and y_9y_9 soybean mutant (y_9y_9, Chl *a*/Chl *b* = 5.0). The most pronounced polypeptides in the wild type are those associated with the LHC II, migrating in the 27–29 kDa region. In thylakoid membranes from y_9y_9 mutant chloroplasts, the most abundant proteins appear migrating in the 12–15 kDa region, representing at least two polypeptides with apparent molecular masses of 15.1 and 12.4 kDa. These polypeptides, whose function is totally unknown, are also present in wild-type chloroplasts in the early stages of development (Fig. 5, upper trace), but they are absent from the mature chloroplast. Fig. 6 presents a quantitation of the relative amount of protein in the 12–15 and 27–29 kDa regions as a function of the Chl *a*/Chl *b*,

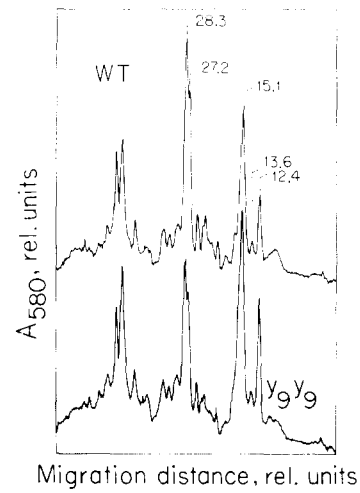

Fig. 5. Densitometric scans of Coomassie stained SDS-polyacrylamide gel electrophoresis of thylakoid membrane proteins from 10-day-old wild type (upper) and Clark y_9y_9 soybean mutant plants (lower). Plants were grown in the greenhouse. Lanes were loaded with 10 μg chlorophyll. The protein bands in the 27–29 kDa region correspond to polypeptides of the LHC II. Those in the 12–15 kDa region are novel thylakoid bound proteins of unknown function, found in developing chloroplasts. The Chl *a*/Chl *b* ratio of the WT sample was 3.7 and that of the mutant was 5.0.

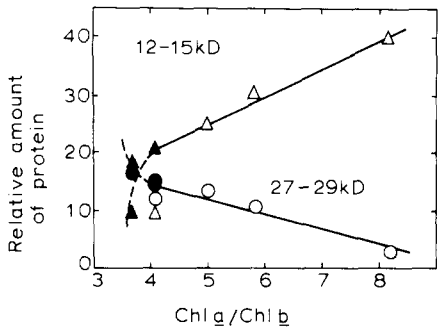

Fig. 6. Relative Coomassie stain incorporation in the 27–29 kDa and 12–15 kDa regions plotted as a function of the Chl *a*/Chl *b* ratio of thylakoid membranes isolated from wild-type (solid symbols) or Clark y_9y_9 soybean mutant plants (open symbols). The relative amount of protein was calculated from the integrated area of the respective band in densitometric scans similar to those of Fig. 5.

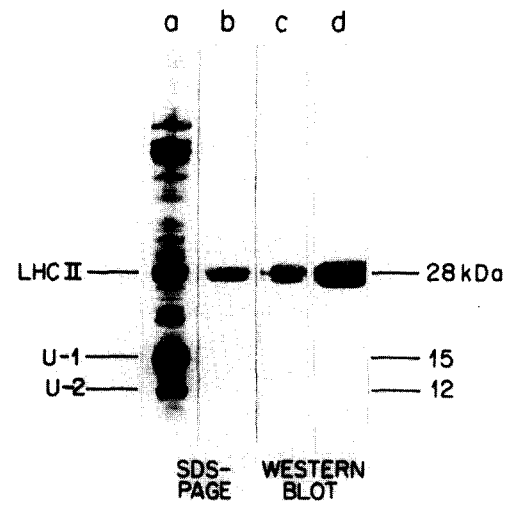

Fig. 7. Composition and immunological reaction of thylakoid membrane polypeptides from y_9y_9 soybean mutant chloroplasts. Coomassie blue stained SDS-polyacrylamide gel electrophoresis of thylakoids (lane a) and of purified LHC II (lane b). Western blot of thylakoid membranes (lane c) and of purified LHC II (lane d) probed with anti-LHC II polyclonal antibody. Note the major proteins U-1 (15.1 kDa) and U-2 (12.4 kDa) in lane a. The Chl *a*/Chl *b* ratio of the chloroplasts was 8.2.

which is an indirect measure of the chloroplast development stage. It is observed that at low Chl *a*/Chl *b* values (fully developed chloroplasts) the relative amount of protein in the 12–15 kDa region is low and that in the 27–29 kDa region is clearly more pronounced. At higher Chl *a*/Chl *b* ratios, the relative amount of 27–29 kDa protein is reduced, whereas that of the 12–15 kDa region increases. Thus, it appears that the relative content of the polypeptides in the 12–15 kDa region is inversely proportional to that of LHC II present in the membrane. This observation raised the question of whether one or both of the pronounced bands in the 12–15 kDa region might originate from the partial proteolysis of membrane-bound LHC II polypeptides that, for some reason, failed to assemble and/or incorporate into the functional photosynthetic unit of PS II. To test for this hypothesis, polypeptides from SDS gel electrophoresis of y_9y_9 thylakoid membranes (Chl *a*/Chl *b* = 8.2, Fig. 7, lane a) were transferred to nitrocellulose and, subsequently, probed with a polyclonal antibody raised against purified LHC II (Fig. 7, lane c). As evidenced in this Western blot analysis, the antibody gave a positive reaction with polypeptides of the LHC II in the 28 kDa region, but no cross-reactivity with the 15.1 kDa protein (U-1) or the 12.4 kDa protein (U-2) occurred. For comparison purposes, Fig. 7 shows the migration of purified LHC II (lane b) and its cross-reaction with the same antibody (lane d). Further experiments were conducted to eliminate the possibility of contribution in the 12–15 kDa region from subunits of the ATP synthetase and/or from the small subunit of the ribulosebisphosphate carboxylase/oxygenase. Thus, repeated washings of the isolated lamellae with pyrophosphate [13] and/or with NaBr and EDTA [14,15] failed to remove U-1 and U-2 from the thylakoid membrane. In support of this, a Western blot analysis with antibody against the ribulosebisphosphate carboxylase/oxygenase failed to show any cross reactivity with either U-1 or U-2 (results not shown). It is suggested that polypeptides migrating in the 12–15 kDa region (Fig. 7, lane a) are novel proteins whose role may be linked with the mechanism of chloroplast development and whose precise function remains to be elucidated.

Discussion

Virescent mutations in higher plants and algae affect the normal greening process of chloroplasts.

A number of these mutations are known to produce plants in which the chlorophyll content per leaf area is a function of leaf age: young leaves have reduced chlorophyll content, while older leaves accumulate chlorophyll to nearly maximal levels. In some cases, the chlorophyll deficiency selectively applies to Chl *b*, resulting in plant with higher Chl *a*/Chl *b* ratios [11,12,16–19]. Virescent phenotypes may have quite different origins. Some are the result of chloroplast mutations [20], while others originate from nuclear mutations. To the latter category belongs the y_9y_9 soybean mutant.

The expression of the y_9y_9 phenotype is manifested by a low chlorophyll content in the leaves, higher Chl *a*/Chl *b* ratio, and lower amounts of LHC II and LHC I in the photosynthetic unit of PS II and PS I, respectively. Equally important is the observation of relatively large amounts of polypeptides in the 12–15 kDa region, whose function is currently unknown. The expression of the mutation in the y_9y_9 is strongly affected by the light intensity during plant growth; however, it could not be correlated with a carotenoid deficiency in the leaves.

Results presented in this work appear to favor the hypothesis of a lesion affecting the assembly and/or incorporation of LHC II into the functional photosynthetic unit of PS II. The precise step affected by the mutation is not clear, neither is the control of the y_9y_9 phenotype by light intensity. It must be observed that the development of the photosynthetic unit of photosynthesis is a complex process involving the coordinated interaction of a number of biosynthetic pathways and the expression of both nuclear and organelle genomes. Such complexity suggests the existence of several regulatory processes, many of which have not yet been identified. Noteworthy in this respect is the appearance of polypeptides in the 12–15 kDa region (U-1 and U-2 in Fig. 7) which are dominant under conditions of maximum phenotypic expression in the y_9y_9 Chl-*b*-deficient mutant. Preliminary Western blotting experiments failed to identify these polypeptides as proteolytic fragments of specific subunits from the LHC II. However, the question of whether U-1 and U-2 represent proteolytic fragments of LHC II polypeptides may not be successfully addressed with antibodies against LHC II because U-1 and U-2 may originate from the inner, hydrophobic portions of the molecule which may not elicit a strong antigenic reaction. Hence, the lack of cross-reactivity between U-1, U-2 and anti-LHC II (Fig. 7, lane c), may not be of unequivocal significance in terms of their identification, and further testing may be needed.

The possibility that polypeptides in the 12–15 kDa region play an as yet unknown regulatory role during chloroplast development is supported by the observation that they are not unique to mutant chloroplasts but are also detected in wild-type plants during the early stages of leaf development (Fig. 5, upper). Additional preliminary results showed the presence of these polypeptides in a variety of intermittent light plastids (pea, barley, bean) as well as in etioplasts (unpublished results). It may be of interest to speculate that U-1 and U-2 play a role in chloroplast development, either as 'assembly proteins' that facilitate the incorporation of newly synthesized subunits of the LHC II into a functional photosynthetic unit, or as membrane-bound proteinases active in the degradation of unstable components of the LHC II. Either alternative is consistent with the observation that proteins in the 12–15 kDa region are present in the thylakoid membrane during the process of chloroplast development only and are virtually absent from the fully mature chloroplast. Work in other laboratories has indicated the presence of early light-induced proteins (ELIP) that appeared migrating in the 15–17 kDa region. Working with etioplasts, Apel [21] and Meyer/Kloppstech [22] reported the early appearance of polypeptide(s) of unknown function, migrating in the 15–17 kDa region. The relation of U-1 and U-2 with the early light-induced proteins is not known; nevertheless, it must be noted that the latter peaked within 6 h of illumination of etioplasts and then decayed within 20 h in the light [23]. In contrast, U-1 and U-2 appeared stable in the mutant chloroplasts for several days under continuous illumination.

Clearly, we are only now beginning to address questions on the control of chloroplasts development and on the regulation of the LHC II assembly and organization in higher plant chloroplasts. Elucidation of the origin and physiological role of

proteins in the 12–15 kDa region might prove useful in this endeavor.

Acknowledgements

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