Changes in the Content of Chlorophyll and Redox Components of the Thylakoid Membrane during Development and Senescence of Beech *(Fagus sylvatica)* Leaves

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Leaves from 145-year-old and 44-year-old beech trees were harvested during 1991–1993. Chlorophyll (Chl) and redox components of the thylakoid membrane, including P-700, cytochrome *f* (Cyt *f*) and D_1 protein, were determined with the following results. Chl *a*+*b*, P-700 and Cyt *f* per unit of fresh weight (FW), dry weight (DW) and leaf area (LA) increase significantly during leaf development. This can be attributed to a massive membrane synthesis and new thylakoid formation in the cells. The Chl *a*/Chl *b* ratio decreases with the synthesis of Chl during the development of beech leaves and is reduced further with Chl breakdown in the stage of senescence. When expressed on the basis of Chl, a reduction in the amount of P-700 and Cyt *f* is observed during the growth stage, indicating a marked enlargement of the functional photosynthetic unit. During senescence of beech leaves, Chl breaks down, and P-700 and Cyt *f* per unit of FW, DW and LA decrease dramatically with the decline in Chl levels. However, when expressed on the basis of Chl, P-700 remains relatively constant, while Cyt *f* shows a pronounced increase. The uncommon pattern of Cyt *f* change in senescent beech leaves and the relationship between the amount of Chl and the content of Cyt *f* throughout an entire growth period of beech leaves are discussed.

Introduction

The highly dynamic and flexible nature of the thylakoid membrane is well known. The relative stoichiometries of PS II : PS I : Cyt b_6/f complexes and the Chl-antenna sizes for the two photosystems are neither identical nor constant over the time, as they can vary in different plant species and in response to different light regimes (Melis, 1991; Anderson, 1992). P-700 per Chl is about 1.6–2.0 mmol (mol Chl)$^{-}$ in chloroplasts of some higher plants (Hope, 1993), and this concentration is relatively constant in a wide range of growth illuminances, whereas PS II and Cyt *f*, on a basis of Chl, increase in sun (or high irradiance-acclimated) plants when compared to shade (or low irradiance-acclimated) plants. Furthermore, the plants growing under sunlight (or high irradiance) have less LHC II and a relatively high ratio of Chl *a*/Chl *b* (Wild *et al.,* 1986; Anderson *et al.,* 1988; Hope, 1993).

The photosynthetic pigments and electron-transport elements also vary during the stages of leaf development and senescence. During the greening of barley and jackbean seedlings, the Chl *a*/Chl *b* ratio rapidly falls and the photosynthetic units are small compared to those of mature chloroplasts (Alberte *et al.,* 1972; Boardman, 1977). It has also been reported that a marked reduction of Cyt b_6/f complex on the basis of Chl is seen in mature barley leaves (Holloway *et al.,* 1983), as well as in spruce needles during the summer months (Flammersfeld and Wild, 1992). In addition, plants grown under intermittent light conditions could also serve as a model for the photosynthetic apparatus during an early stage of

Abbreviations: Chl, chlorophyll; Cyt, cytochrome; DW, dry weight; DTE, dithioerythritol; EDTA, ethylenedinitrilo tetraacetic acid, disodium salt dihydrate (Titriplex III); FW, fresh weight; HEPES, N-(2-hydroxyethyl)-piperazine-N′-(2-ethanesulfonic acid); LA, leaf area; LHC, light-harvesting chlorophyll *a/b* protein; PS, photosystem; Polyclar AT, water-insoluble polyvinylpyrrolidone for binding phenols.

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chloroplast development (Argyroudi-Akoyunoglou and Akoyunoglou, 1970; Melis, 1984; Marquardt and Bassi, 1993).

During leaf senescence Chl is degraded. This change is frequently accompanied by a reduction in the ratio of Chl *a*/Chl *b*, while photosynthetic activity also decreases rapidly (Šesták, 1977; Jenkins *et al.*, 1981; Kura-Hotta *et al.*, 1987; Hidema *et al.*, 1991). No significant change in the amount of PS I-complex protein on the basis of Chl was found with advancing senescence in oat and bean leaves (Ben-David *et al.*, 1983). However, according to the more detailed study of Robert *et al.* (1987), the PS I protein on the basis of thylakoid protein remains constant only in the early stage of senescence in bean leaves and begins to decrease in later stages. A decrease in the amount of PS I protein on the basis of leaf area in senescent *Lolium* plants was also reported, although the process is delayed in shaded leaves (Mae *et al.*, 1993).

In contrast to the patterns of the complex change in P-700, uniform decrease of Cyt b_6/f with progressing senescence has been repeatedly reported in some higher plants expressed in term of different bases; for example, in barley, oat and bean on the basis of Chl and fresh weight (Ben-David *et al.*, 1983; Holloway *et al.*, 1983), in bean on the basis of thylakoid protein (Robert *et al.*, 1987), and in rice and *Lolium* on the basis of leaf area (Hidema *et al.*, 1991, Mae *et al.*, 1993).

Beech is the most common broadleaf tree in Germany. Although a change of lipid composition during the senescence of beech leaves has been reported (Tevini *et al.*, 1985), little research has been done on the photosynthetic membrane of beech leaves. In this communication we report on our study in changes of Chl $a+b$ and of redox components of beech leaves through the entire growth period, especially during the phase of development and senescence.

Materials and Methods

Materials

Beech leaves used in these experiments were from either the Zierenberg site or the botanical garden of the Johannes Gutenberg University of Mainz.

The Zierenberg site is located in the central part of Germany, *ca.* 450 m above sea level near the city of Kassel. It belongs to the forestry office of Wolfhagen in Hessia. In the forest where our investigations took place, the main plant species are beech and stinging nettle *(Urtica dioica)*, indicating a surplus of nitrogen supply in the soil. The beech trees from which our samples were taken grow at the north-east side of a mountain. They were all about 145 years old and had an average height of about 35 m.

At the Zierenberg site 4 harvests took place every year during 1991 and 1992. At each harvest, 4 trees which showed similar development and senescence rhythm were selected for the experiment. The twigs from the top of the trees were shot down and the leaves were immediately broken into pieces in liquid nitrogen and then kept in storage at −80 °C. The samples from each tree were harvested separately. The harvests were made on the following dates in 1991: 28/5, 1/7, 19/8, 10/9; and in 1992: 20/6, 8/7, 27/8, 14/10.

In the botanical garden a 44-year-old beech tree was used in this experiment. A few small twigs exposed to the light were cut with a pair of shears and the leaves were treated by the above-mentioned method. In 1993, the beech tree began to develop new leaves from buds in the middle of April; abscission was in the middle of October. After bud break leaves of the tree were harvested once a week in the first and second week, and then once every two weeks. The samples were taken on the following dates in 1993: 23/4, 1/5, 14/5, 1/6, 15/6, 1/7, 14/7, 2/8, 17/8, 1/9, 16/9, 3/10.

Thylakoid isolation

The isolation of thylakoids was performed according to the method used for spruce needles (Wild *et al.*, 1993) with modified media. The isolation medium consisted of 50 mM Na-pyrophosphate/HCl (pH 7.0), 5 mM $MgCl_2$, 0.4 mM $KHSO_3$, 5 mM DTE, 500 mM sorbitol, 10% (w/v) polyethylene glycol 6000; the resuspension medium of 50 mM HEPES/KOH (pH 7.0), 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, 5 mM NaCl, 500 mM sorbitol; and the shock medium of 10 mM HEPES, 10 mM $MgCl_2$, 10 mM NaCl. The storage medium was the same as the resuspension medium, but with only 250 mM sorbitol.

Determination of P-700 and Cyt f

The tests were carried out according to the methods used for spruce needles (Wild *et al.*, 1993), but with reduced concentrations of detergent and polyclar AT. The medium for the measurement of P-700 was made up of 50 mM K-phosphate buffer (pH 7.0), 0.2% Triton X-100, 0.4 M saccharose, 0.5% (w/v) polyclar AT. The medium for Cyt *f* determination was 50 mM K-phosphate buffer (pH 6.5), 0.75% Triton X-100, 0.3 M sorbitol, 0.5% (w/v) polyclar AT.

Determination of D_1 protein (Q_B protein)

D_1 protein was determined by [^{14}C]atrazine titration performed according to the method of Tischer and Strotmann (1977) modified by Wild *et al.* (1988).

Determination of Chl a+b and Chl a/Chl b

0.1–0.15 g pieces of beech leaf were ground in a Micro-Dismembrator U (Braun Co., Melsungen) with liquid nitrogen and then extracted with 10 ml 80% acetone. The total Chl *a+b* and the ratio Chl *a*/Chl *b* were determined according to Ziegler and Egle (1966). This method is well suited for handling relatively hard leaf material and enables a large number of such samples to be treated quickly.

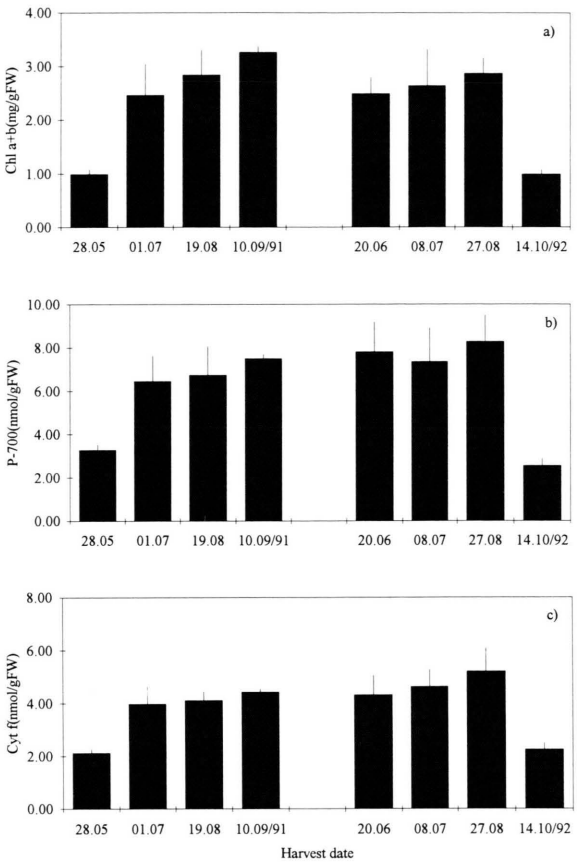

Fig. 1. Contents of Chl *a+b* (mg/g FW) (a), P-700 (nmol/g FW) (b) and Cyt *f* (nmol/g FW) (c) in beech leaves harvested at the Zierenberg site in 1991–1992. The bars indicate the standard deviation of the values obtained from 4 trees.

Results

Chl a+b and Chl a/b

The amount of Chl *a+b* and the ratio Chl *a*/Chl *b* in beech leaves taken from the Zierenberg site during 1991–1992 are shown in Fig. 1 a and Fig. 2 a. Compared to the leaves from the first 1991 harvest (May 28), the leaves from the second harvest (July 1) showed a pronounced increase in the amount of Chl *a+b* (Fig. 1 a) and a decrease in the Chl *a*/Chl *b* ratio (Fig. 2 a); this corresponds to a typical developmental process of beech leaves from spring to early summer. In the senescent leaves obtained at the fourth harvest in 1992 (October 14) the Chl content was much lower than the value at the third harvest in 1992 (August 27) (Fig. 1 a), while the Chl *a*/Chl *b* ratio showed a clear decrease over the same interval (Fig. 2 a). For each year the Chl *a*/Chl *b* ratio is highest in spring, then decreases in summer and autumn until abscission (Fig. 2 a).

P-700, Cyt f, D_1 protein

P-700 and Cyt *f* on the basis of FW increased substantially during development of beech leaves from the first to the second 1991 harvest (Fig. 1 b, 1 c); a similar pattern of change could also be seen when expressed on the basis of DW and LA (Table I). However, when it was calculated on the basis of Chl both P-700 and Cyt *f* decreased (Fig. 3 a, 3 b). The ratio P-700/Cyt *f* remained almost unchanged during the development (Fig. 2 b).

Table I. P-700 and Cyt *f* contents (expressed as nmol/g DW and mmol/m^2 LA) in the beech leaves harvested at the Zierenberg site in 1991 and 1992.

	1991				1992			
Dates of harvest	28/5	1/7	19/8	10/9	20/6	8/7	27/8	14/10
P-700 (nmol/g DW)	11.73 ± 1.26	15.78 ± 2.54	13.75 ± 3.36	16.42 ± 0.11	16.76 ± 3.22	16.83 ± 4.10	18.28 ± 2.83	5.43 ± 0.80
P-700 (μmol/m^2 LA)	0.48 ± 0.06	0.88 ± 0.09	1.16 ± 0.19	1.03 ± 0.23	1.05 ± 0.17	0.94 ± 0.11	1.00 ± 0.21	0.29 ± 0.03
Cyt *f* (nmol/g DW)	7.55 ± 0.67	9.70 ± 1.37	8.35 ± 1.09	9.69 ± 0.44	9.22 ± 1.60	10.54 ± 1.84	11.44 ± 1.93	4.74 ± 0.42
Cyt *f* (μmol/m^2 LA)	0.31 ± 0.03	0.55 ± 0.06	0.72 ± 0.06	0.60 ± 0.10	0.58 ± 0.10	0.59 ± 0.02	0.63 ± 0.15	0.26 ± 0.03

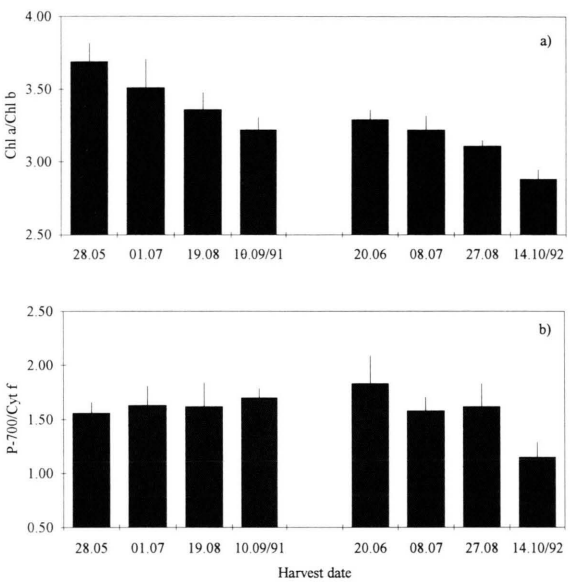

Fig. 2. The ratio Chl *a*/Chl *b* (a) and P-700/Cyt *f* (b) in beech leaves harvested at the Zierenberg site in 1991–1992. The bars indicate the standard deviation of the values obtained from 4 trees.

In the senescent leaves taken at the fourth 1992 harvest both P-700 and Cyt *f* on the basis of FW, DW and LA declined dramatically (Fig. 1b, 1c, Table I). However, based on Chl, P-700 was nearly constant, while Cyt *f* showed a significant increase (Fig. 3a, 3b). The quotient P-700/Cyt *f* decreased and showed a ratio approximately equal to 1 (Fig. 2b), indicating that P-700 breaks down more rapidly than Cyt *f* in thylakoid membranes.

Table II shows the contents of D_1 protein on the basis of Chl, FW, DW and LA in the beech leaves from 6 harvests at the Zierenberg site during 1991–1992. Since the leaves taken at the first harvest in 1991 and at the fourth harvest in 1992 did contain only small amounts of Chl *a*+*b*, the [^{14}C]-atrazine titration method for D_1 protein determination could not be applied and therefore the samples could not be measured at this time. A significant decrease in D_1 protein on the basis of Chl was observed from the second to the third 1991 harvest (in August); when expressed on the basis of FW, DW and LA (Table II) the reduction could be also detected. In 1992 no difference in the amount of D_1 protein was found in the leaves obtained from 3 harvests during summer.

Correlation of Chl a+b and Cyt f

In order to investigate in more detail the changes in the thylakoid membrane in a complete year's cycle, especially the relationship between Chl *a*+*b* and Cyt *f*, a beech tree growing in the botanical garden of the Johannes Gutenberg University of Mainz was used in our experiments as well. As can be seen from Fig. 4, the Chl *a*+*b* content per unit of FW in beech leaves increased very

Table II. D1 protein content (expressed as mmol/mol Chl, nmol/g FW, nmol/g DW and mmol/m^2 LA) in the beech leaves harvested at the Zierenberg site in 1991 and 1992 (n.d. = not determined).

	1991				1992			
Dates of harvest	28/5	1/7	19/8	10/9	20/6	8/7	27/8	14/10
(mmol/mol Chl)	n.d.	2.76 ± 0.23	1.80 ± 0.27	1.83 ± 0.14	2.11 ± 0.41	1.88 ± 0.25	1.97 ± 0.22	n.d.
(nmol/g FW)	n.d.	7.47 ± 1.67	5.79 ± 1.75	6.60 ± 0.34	5.75 ± 0.91	5.35 ± 0.58	6.32 ± 1.25	n.d.
(nmol/g DW)	n.d.	18.26 ± 3.65	11.88 ± 4.15	14.65 ± 0.73	12.26 ± 1.56	12.23 ± 1.84	13.95 ± 2.75	n.d.
(mmol/m^2 LA)	n.d.	1.02 ± 0.15	0.98 ± 0.14	0.90 ± 0.17	0.78 ± 0.15	0.62 ± 0.02	0.76 ± 0.19	n.d.

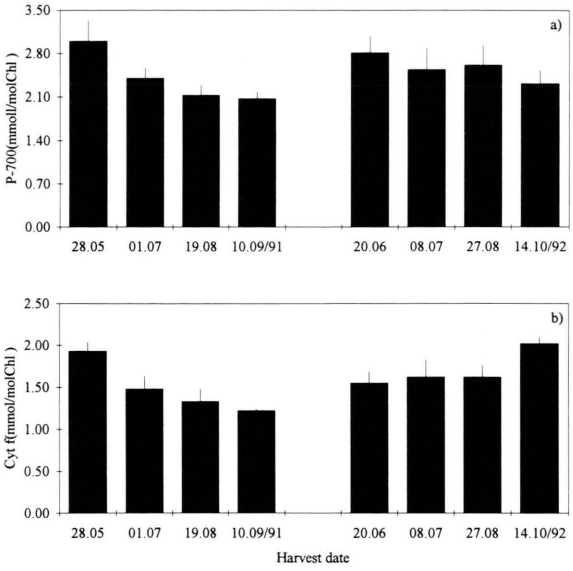

Fig. 3. Cyt *f* (a) and P-700 (b) expressed as mmol/mol Chl in beech leaves harvested at the Zierenberg site in 1991–1992. The bars indicate the standard deviation of the values obtained from 4 trees.

rapidly in the first 4 weeks of the developmental period (in spring) and reached its maximal level in the tenth week (in summer), then decreased gradually until abscission. In comparison to Chl *a*+*b*, the change of Cyt *f* content in the thylakoid membranes, expressed per unit of Chl, showed a reverse pattern during the entire beech leaf growth period. Fig. 5 shows the relationship between Chl *a*+*b* and Cyt *f* obtained from beech leaves from the both sites. The common function can be expressed as formula (1):

$$(\text{Cyt } f) = 2.237 - 0.279\ (\text{Chl}) \qquad (1)$$
$$R = 0.8805\ (n = 44,\ p < 0.001)$$

Discussion

The stoichiometry of light-harvesting complexes associated with the two photosystems, as measured by the ratio Chl *a*/Chl *b*, has been demonstrated by several workers (Alberte *et al.*, 1972; Kura-Hotta *et al.*, 1977; Melis, 1991; Marquardt and Bassi, 1993). Our data show that the Chl *a*/Chl *b* ratio decreases significantly with the increase in Chl content during the growth stage of beech leaves, and is reduced drastically with Chl decline during senescence. This is consistent with what has been reported for chloroplast development and senescence in other common higher plants (Argyroudi-Akoyunoglou and Akoyunoglou, 1970; Alberte *et al.*, 1972; Boardman, 1977; Melis, 1984; Kura-Hotta *et al.*, 1987; Marquardt and Bassi, 1993; Tevini *et al.*, 1985).

The present study also indicates that Chl, P-700 and Cyt *f* per unit of FW, DW and LA increase significantly with beech leaf development. This can be attributed to massive membrane synthesis and new thylakoid formation during the greening stage. However, when expressed on the basis of Chl the levels of P-700 and Cyt *f* decrease progressively during the development of beech leaves, which implies that the functional photosynthetic units (indicated by the ratio Chl/Cyt *f*) have

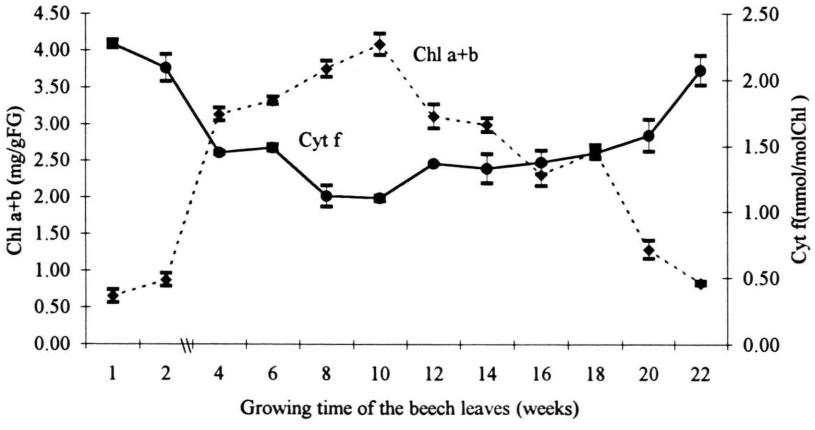

Fig. 4. Changes in the content of Chl *a*+*b* (mg/g FW) (·····) and of Cyt *f* (mmol/mol Chl) (——) in leaves harvested from a beech tree in the botanical garden throughout an entire beech leaf growth period in 1993. The standard deviation is from 3 replicates.

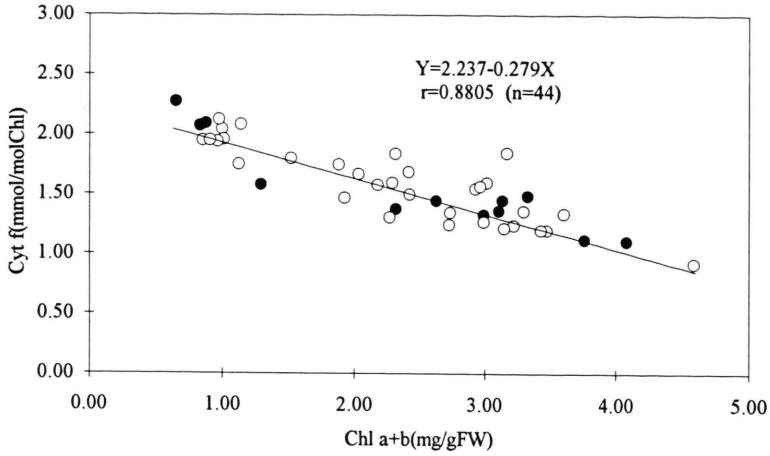

Fig. 5. The correlation between Chl $a+b$ and Cyt f obtained from two sites. The open symbols indicate the data from the Zierenberg site in 1991 and 1992 and the solid symbols the data from the botanical garden in 1993.

been markedly enlarged through the increase in the levels of Chl-protein complexes, especially LHC II. Thus beech leaves seem to share the common development pattern of leaves shown in other higher plants (Armond and Arntzen, 1976; Boardman, 1977; Marquardt and Bassi, 1993).

The rapid rate of turnover in D_1 protein is well known. It has also been reported that the synthesis of D_1 protein continues throughout the senescence of bean leaves (Robert *et al.*, 1987). In the present study we found that D_1 protein levels decreased significantly between the second and third harvest in 1991. An interpretation of the phenomenon might be the enlargement of the photosynthetic unit size of photosystem II at the relatively late stage of development. On the other hand, the decrease of the D_1 protein could also be an indicator of photoinhibition caused by strong-light intensity.

No significant change in P-700 on the basis of Chl for the senescent beech leaves was found in this study. This is consistent with what has been detected in bean and oat leaves (Ben-David *et al.*, 1983). However, as previous investigations have indicated, the relative content of PS I protein in the thylakoid membrane is variable at various stages of senescence (Robert *et al.*, 1987), and is influenced by environmental factors, such as light conditions (Mae *et al.*, 1993). It is not clear whether the changes in PS I are also different in the various phases of the senescence processes of beech leaves, or whether a rapid PS I decline takes place only after abscission.

It has been repeatedly reported that the content of Cyt f in the thylakoid membranes decreases in senescent leaves in some higher plants (Holloway *et al.*, 1983; Ben-David *et al.*, 1983; Robert *et al.*, 1987), and declines more rapidly than Chl in the senescence period of rice and *Lolium* leaves (Hidema *et al.*, 1991; Mae *et al.*, 1993). Furthermore, the authors suggested that the decline of Cyt b_6/f could be the main cause for the decrease in photosynthetic activity in the senescent leaves. We found of particular interest that the Cyt f content on the basis of Chl increases markedly in the senescent beech leaves. The relatively high Cyt f concentration observed in our present study suggests that the pattern of change in Cyt f during leaf senescence could differ between plant species.

A highly significant correlation between Chl levels in beech leaves and the Cyt f content in the thylakoid membranes throughout the complete growth period of the beech leaves has been demonstrated in the present study. In the early stage of development the chloroplasts of higher plants have no or only poorly developed grana, and the morphological change of chloroplasts during ontogenesis are primarily characterized by the development of grana (Armond and Arntzen, 1976; Šesták, 1977). On the other hand, according to the studies of Terashima (1985a, 1985b), during the development of beech leaves the chloroplasts should change gradually with depth from sun to shade type having highly stacked grana. Thus, after development and differentiation the chloroplasts in a mature leaf have more grana thylakoids than those in an immature leaf. Taking into account the relationship between the thylakoid

structure and Cyt *f* concentration in sun and shade leaves, it is not difficult to understand that the mature beech leaves contain relative less Cyt *f* in the photosynthetic membranes. Holloway *et al.* (1983) have reported that Cyt *f* on the basis of Chl decreases continuously during the development of barley leaves; this is in accordance with the results of our present study of beech leaves. Furthermore, the previous studies have also shown that the ultrastructural changes during the chloroplast development in some higher plants are very similar (Boardman, 1977; Šesták, 1977). We believe that the relationship observed between the level of Chl and the Cyt *f* content in thylakoid membranes is a common feature of leaf development in higher plants.

Cyt b_6/f is the functional link between the co-regulation of electron transfer and light-harvesting processes (Anderson, 1992), because Cyt b_6/f complex is involved not only in linear and cyclic electron transfer and proton translocation, but also in the regulation of state transitions. The molecular mechanism of state transitions, where the redox state of Cyt b_6/f controls the activity of LHC II kinase, resulting in changes in the organization of the components in the thylakoid membrane, is well documented (Gal *et al.*, 1990; Vallon *et al.*, 1991). There are additional indications that Cyt b_6/f is one of the molecular targets for change of the composition in the chloroplasts and optimization of photosynthetic function under varying light conditions (Wild, 1979; Wilhelm and Wild, 1984; Anderson, 1992). Whether the Cyt b_6/f is also a key regulatory complex for the co-ordinated modulation of composition of thylakoid membranes during the mainly genetically controlled development of leaves requires further investigation.

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