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The development of chloroplast ultrastructure and Hill reaction activity during leaf ontogeny in different maize (*Zea mays* L.) genotypes

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Abstract

Changes in Hill reaction activity (HRA) and ultrastructure of mesophyll cell (MC) chloroplasts were studied during the ontogeny of third leaf of maize plants using polarographic oxygen evolution measurement, transmission electron microscopy, and stereology. The chloroplast ultrastructure was compared in young (actively growing), mature, and senescing leaves of two different inbreds and their reciprocal F1 hybrids. Statistically significant differences in both HRA and MC chloroplast ultrastructure were observed between different stages of leaf ontogeny. Growth of plastoglobuli was the most striking characteristic of chloroplast maturation and senescence. The chloroplasts in mature and senescing leaves had a more developed system of thylakoids compared to the young leaves. Higher HRA was usually connected with higher thylakoid volume density of MC chloroplasts.

Additional key words: chloroplast dimensions; electron microscopy; peripheral reticulum; photosynthesis; plastoglobuli; starch; stereology; stroma; thylakoids.

Introduction

During ontogeny of photosynthetically active leaves (from their unfolding to yellowing), the ultrastructure of chloroplasts in the mesophyll cells substantially changes (for reviews see Kutík 1985, 1998, Hudák 1997). The main features of this development are increase of chloroplast size in maturing leaves and their diminishing during leaf senescence, accumulation of starch in the chloroplasts of just mature leaves, accumulation of plastoglobuli material during leaf senescence, and changes in quantity of the thylakoid system and in thylakoid stacking degree during whole leaf

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ontogeny. The thylakoid membrane system grows, acquires shade character (large grana are formed), and the thylakoids finally dilate and are destructed.

Maize is an economically important, frequently studied crop with NADP-malate type of C4 photosynthesis and chloroplast dimorphism (*e.g.*, Rascio *et al.* 1984, Rüffer-Turner *et al.* 1984, Wang and Hu 1988, Wrischer 1989, Evert *et al.* 1996, Roth *et al.* 1996). Differentiation of dimorphic chloroplasts in the cells of mesophyll (MC) and bundle sheaths (BSC) of maize leaves was followed from the stage of (more or less identical) proplastids by Brangeon (1973a,b), Nishioka *et al.* (1993), Wang *et al.* (1993), *etc.* The cultivar differences in photosynthetic characteristics within this species have also been often studied (*e.g.*, Fousová and Avratovščuková 1967, Heichel and Musgrave 1969, Crosbie *et al.* 1978, Gaskel and Pearce 1981, Avratovščuková 1983, Mehta *et al.* 1989, 1992, Rocher *et al.* 1989, Kočová 1992, Krebs *et al.* 1996).

We have studied mesophyll cell (MC) chloroplast development in two maize inbreds differing in HRA and their reciprocal F1 hybrids. Preliminary results of this study were presented in Kutík and Kočová (1996). As far as we know, this is the first attempt to evaluate genotypic differences in photochemical activity of chloroplasts together with the changes of chloroplast ultrastructure during leaf ontogeny in maize (for a similar attempt in pea see Vaishlya *et al.* 1998). Such study can contribute to the understanding of relationships between chloroplast structure and function.

Materials and methods

Maize (*Zea mays* L.) plants were grown from seeds obtained from Maize Breeding Station CEZEA in Čejč (Czech Republic). Two inbred lines (CE813 and CE829) displaying high and low HRA, respectively, and their F1 hybrids (CE813×CE829, CE829×CE813) were used. Plants were cultivated in soil and watered with modified Knop's nutrient solution and tap water in a growth chamber (*Klimabox RK1-007, Kovodružstvo Slaný*, Czech Republic) at day/night regime 16/8 h, irradiance 500/0 μmol m^{-2} s^{-1} (PAR), temperature 25/16 °C, and relative air humidity 70/80 %. The length and appearance of leaf blade were recorded during the third leaf (numbering from coleoptile as leaf zero) ontogeny: from the young leaves having about one quarter of final length (2-week-old plants) up to the senescing (yellowing) ones (6-7 week-old plants). Leaf tissue samples were taken approximately three hours after the beginning of light period, at 09:00 h. Each genotype was represented by at least four plants during the different stages of ontogeny. The measurements were taken in two separate sets of experiments (in 1994 and 1995).

The middle third of leaf blade was used both for the isolation of photochemically active chloroplasts and for electron microscopic and stereological evaluation of chloroplast ultrastructure. For HRA analysis, 2 g of leaf tissue were homogenised in 40 cm^3 of sucrose medium (0.4 M sucrose, 0.05 M $MgCl_2$, 0.05 M Tris-HCl, pH 7.0) for 18 s (*Thurmix 302*, maximal revolutions). The homogenate was filtered through 8 layers of gauze and centrifuged at 1000×*g* for 10 min. The pellet was resuspended in

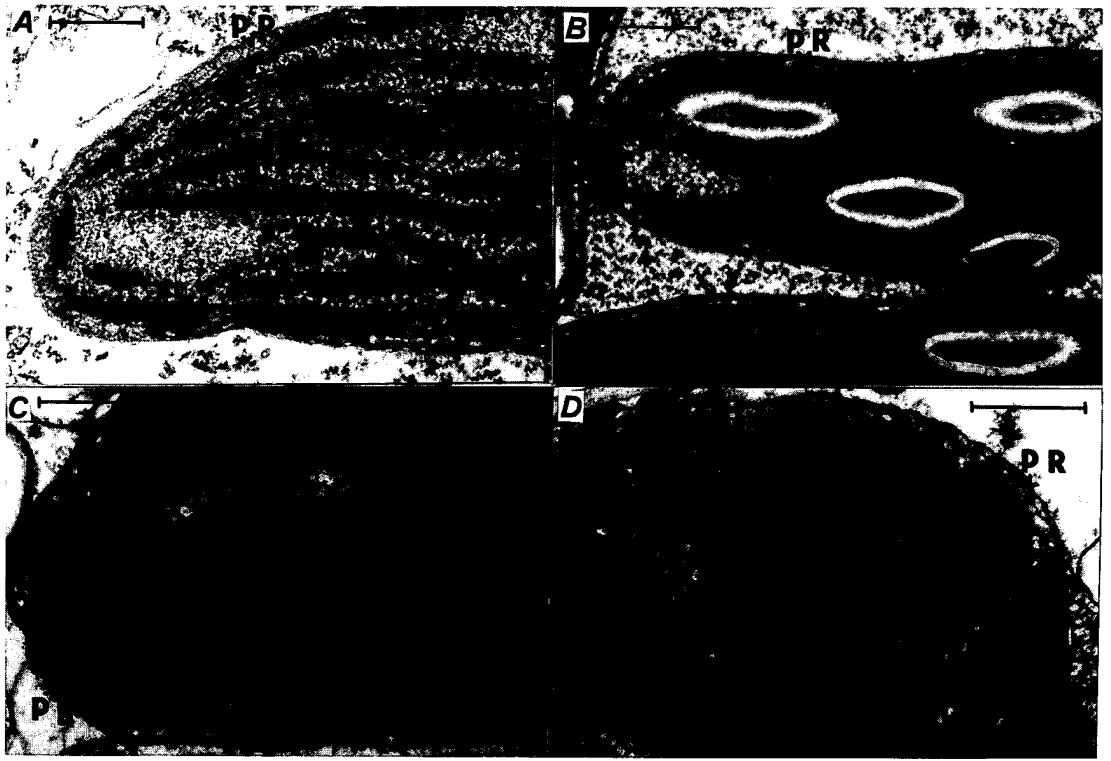

Fig. 1. Transmission electron micrographs of chloroplast cross sections taken from the third leaf mesophyll (MC) and bundle sheath (BSC) cells of maize inbred line CE829: (*A*) young leaf, MC chloroplast; (*B*) young leaf, BSC chloroplast; (*C*) mature leaf, MC chloroplast; (*D*) senescing leaf, MC chloroplast. The system of chloroplast thylakoids contains many grana from stacked thylakoids in MC but not in BSC chloroplasts where large starch inclusions (S) are visible. In senescing leaves, parallel orientation of thylakoids is partially lost and large plastoglobuli (P) are seen in chloroplasts. Vesicles of peripheral reticulum (PR) are visible in all chloroplast sections. Bar = 0.5 μm.

0.8 cm^3 of resuspension medium (0.4 M sucrose, 0.006 M $MgCl_2$, 40 % glycerol, 0.05 M Tris-HCl, pH 7.0), and stored in dark and cool place. Each step of isolation procedure was performed at 0 to 4 °C. HRA was measured polarographically as the amount of oxygen formed by the chloroplast suspensions in the light (750 μmol m^{-2} s^{-1} PAR); 0.007 M $K_3[Fe(CN)_6]$ was present as an artificial electron-acceptor and constant temperature of 25 °C was maintained in the measurement chamber (made according to Bartoš *et al.* 1975). Each sample was measured 4 to 6 times. The content of chlorophyll (*a*+*b*) in chloroplast suspensions was determined spectrophotometrically in 80 % aqueous acetone (Porra *et al.* 1989).

Small samples of the leaf blades used for electron microscopic and stereological evaluation were fixed with 5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer containing sucrose, pH 7.3, followed by treatment with 2 % (m/v) osmic acid in the same buffer, 2 h both. They were then embedded into Spurr (1969) low viscosity resin *via* propylene oxide after dehydration in a graded ethanol series. Chloroplast ultrastructure was evaluated on transverse ultrathin sections of embedded objects contrasted with saturated solution of uranyl acetate in 70 % aqueous ethanol, followed by lead citrate solution treatment according to Reynolds (1963), 20 min both, using transmission electron microscope *Philips EM 300* at primary magnifications of about 8 000×. Anatomical structure of leaf blades was checked on semithin sections from the same objects after their staining with 1 % solution of toluidine blue in 1 % sodium tetraborate.

Ultrastructure of chloroplasts in MC was stereologically evaluated in young, mature (fully developed, non-growing), and senescing (yellowing) leaves of inbred lines, and in mature leaves of F1 hybrids. The length, width, and area of (approximately median) chloroplast cross sections were determined at final magnifications of about 40 000× on electron microphotographs. Volume densities (relative partial volumes, see, *e.g.*, Gundersen and Jensen 1987) of granal and intergranal thylakoids, peripheral reticulum, starch inclusions, plastoglobuli, and remaining stroma were counted stereologically on the same microphotographs using morphometric grids with regularly distributed points. Five chloroplasts were evaluated for each leaf sample. The differences between genotypes as well as between different stages of ontogeny were tested by Student's *t*-test, using the 5 % level of statistical significance as the critical one.

Results

The chloroplasts in MC and bundle sheath cells (BSC) of the leaves studied showed dimorphism typical for maize as a plant of the NADP-ME subtype of C4 photosynthesis. This dimorphism was seen as differences in ultrastructure, shape, and dimensions of chloroplasts in young, mature, and senescing leaves. The differences between chloroplasts from leaves of different age (MC as well as BSC) were also apparent, concerning shape of chloroplast cross sections, arrangement of the thylakoid system, size of starch inclusions and plastoglobuli (Fig. 1).

The evaluation of HRA and size and ultrastructure of MC chloroplasts is summarized in Tables 1 to 4. Generally, HRA values continually increased from young to mature leaves and decreased during leaf senescence in all genotypes examined. The highest HRA values in the inbred line CE813 were found for chloroplasts from mature leaves in both experimental series, and in CE829 for young leaves in the first experimental series but for mature leaves in the second series. MC chloroplasts of both F1 hybrids showed the highest photochemical activity in mature leaves (other values not shown).

Thylakoid volume density increased from young to mature leaves in both inbred lines and both series, whereas that of stroma decreased inversely. The volume density of granal thylakoids increased during whole leaf ontogeny. The density of intergranal ones increased in CE813 genotype in both series from young to mature leaves and decreased from mature to senescing leaves whereas the changes in CE829 had no specific trend. The highest volume density of peripheral reticulum was found in mature leaves of both inbreds in both series. However, the ontogenic differences in this characteristic were usually not significant. On the other hand, volume density of plastoglobuli greatly increased in both inbreds during whole leaf ontogeny in both experimental series. MC chloroplasts frequently contained small starch inclusions. Their volume density was negligible in both inbreds in the first experimental series. In the second series, the amount of starch was higher and the volume density of starch inclusions was the highest in mature leaves.

The shape and dimensions of MC chloroplasts as judged by the area, length, and width of chloroplast cross sections have also changed during leaf ontogeny. The most evident was slight change of chloroplast shape from rather flat in the young leaves to more round in the senescing ones. Changes in the area of chloroplast cross sections (*i.e.*, in chloroplast size) were less unambiguous (see Tables 1 and 2). However, in the CE829 genotype, the chloroplasts in mature leaves were larger than in young ones for both experimental series.

Analysis of genotypic differences in photochemical activity of chloroplasts isolated from equally aged leaves showed statistically significant differences for mature and senescing leaves. CE813 displayed higher HRA values compared to the other genotypes (including hybrids) and CE829 had generally a low photochemical activity. Volume density of thylakoids in MC chloroplasts of mature leaves was higher and stroma volume density was lower in CE813 compared to CE829 in both experimental series. Other genotypic differences in thylakoid characteristics as well as in volume densities of starch inclusions and plastoglobuli were not significant or differed between both series (see Table 4). There were no significant differences between genotypes in volume density of peripheral reticulum.

As to the chloroplast shape and dimensions, chloroplast cross section length in mature and senescing leaves was higher in CE829 compared to CE813 for both experimental series. The same was true for chloroplast cross section area in mature leaves. Chloroplast cross section length/width ratio in the senescing leaves was higher in CE829 compared to CE813 genotype in both experimental series.

Table 1. Structural and functional characteristics of mesophyll chloroplasts in young, mature, and senescing third leaves of four maize genotypes: Hill reaction activity, HRA [mmol(O_2) kg^{-1}(chlorophyll) s^{-1}]; volume densities [% of chloroplast volume] of granal thylakoids (GT), intergranal thylakoids (IT), all thylakoids (T), peripheral reticulum (PR), starch inclusions (SI), plastoglobuli (P), and stroma (S); chloroplast cross section area, SA [μm^2], length, SL [μm], or width, SW [μm]. The first experimental series. The mean values ± standard error of mean (SEM) are presented.

	CE813			CE829			CE813×CE829	CE829×CE813
	Young	Mature	Senescing	Young	Mature	Senescing	Mature	Mature
HRA	32.68±0.73	37.06±0.50	27.53±0.38	32.44±0.75	26.34±0.24	16.00±0.10	35.32±0.31	32.18±0.33
GT	36.91±1.56	42.66±1.63	48.45±1.07	31.97±1.37	36.96±2.06	38.40±1.81	38.77±1.88	40.88±1.57
IT	12.48±0.96	20.07±1.14	16.28±0.78	13.48±1.25	20.19±1.14	22.94±1.15	22.29±1.11	17.55±0.89
T	49.38±2.01	62.73±1.55	64.77±1.11	45.42±1.96	57.13±2.11	61.34±1.90	61.06±1.55	58.43±1.69
PR	4.56±0.33	5.82±0.51	4.98±0.24	5.24±0.72	5.78±0.40	5.04±0.50	6.09±0.60	4.82±0.35
SI	0.06±0.03	0.11±0.07	0.08±0.04	0±0	0.12±0.04	0±0	0.46±0.16	0.25±0.13
P	0.15±0.03	1.04±0.13	3.17±0.23	0.08±0.04	1.40±0.14	3.50±0.49	2.03±0.32	1.11±0.15
S	45.86±2.07	30.32±1.41	27.01±1.05	49.27±2.17	35.57±1.91	30.13±1.88	30.38±1.46	35.40±1.65
SA	7.48±0.55	6.89±0.32	6.91±0.24	7.17±0.44	9.32±0.46	6.84±0.46	7.53±0.52	8.89±0.57
SL	4.61±0.18	4.13±0.09	3.92±0.08	4.88±0.19	5.30±0.20	4.25±0.16	4.49±0.18	4.99±0.17
SW	2.11±0.10	2.20±0.12	2.22±0.06	1.90±0.07	2.32±0.10	2.06±0.12	2.16±0.10	2.26±0.10
SL/SW	2.28±0.14	1.97±0.10	1.81±0.06	2.61±0.10	2.38±0.14	2.19±0.14	2.17±0.14	2.22±0.08

Table 2. Structural and functional characteristics of mesophyll chloroplasts in young, mature, and senescing third leaves of four maize genotypes. The second experimental series. Abbreviations are the same as in Table 1. The mean values ± SEM are presented.

	CE813			CE829			CE813×CE829	CE829×CE813
	Young	Mature	Senescing	Young	Mature	Senescing	Mature	Mature
HRA	25.79±1.70	42.88±0.42	35.83±1.09	26.72±1.27	30.65±0.81	24.90±0.61	30.91±0.27	30.68±0.46
GT	20.50±1.55	33.78±1.61	36.86±2.48	28.83±2.00	33.41±1.43	37.93±0.90	40.60±2.13	39.56±1.88
IT	15.01±0.86	22.92±1.00	19.32±0.85	14.50±1.48	13.75±0.70	19.13±0.94	22.73±1.35	27.03±1.37
T	35.52±1.66	56.80±2.00	56.17±2.73	43.31±1.92	47.16±1.61	57.04±1.20	63.31±2.16	66.59±2.10
PR	4.39±0.35	5.55±0.32	4.60±0.39	5.41±0.64	6.13±0.46	4.68±0.53	5.59±0.40	5.10±0.28
SI	0.04±0.04	0.79±0.19	0.04±0.03	0.10±0.06	0.43±0.11	0.04±0.04	0.92±0.28	0.96±0.20
P	0.21±0.05	1.36±0.15	4.45±0.78	0.15±0.04	0.96±0.11	3.50±0.40	1.11±0.10	1.19±0.16
S	59.89±1.55	35.50±2.10	34.75±2.19	51.04±2.16	45.34±1.46	34.75±1.22	29.08±2.26	26.17±2.12
SA	6.45±0.44	7.48±0.52	8.47±0.62	4.86±0.39	8.87±0.58	9.99±0.75	6.67±0.69	5.68±0.33
SL	4.31±0.17	4.58±0.15	4.25±0.17	3.91±0.18	5.10±0.15	4.95±0.23	4.70±0.21	4.30±0.17
SW	1.98±0.08	2.18±0.11	2.59±0.11	1.58±0.08	2.30±0.11	2.52±0.13	1.77±0.12	1.71±0.05
SL/SW	2.21±0.09	2.17±0.08	1.67±0.06	2.56±0.13	2.29±0.10	2.02±0.10	2.78±0.14	2.54±0.10

Table 3. The ontogenic differences in the same characteristics as in Table 1. The statistical significances for the respective *t*-tests are presented.

	Young/mature				Mature/senescing			
	CE813		CE829		CE813		CE829	
	Series 1	Series 2	Series 1	Series 2	Series 1	Series 2	Series 1	Series 2
HRA	0	0	0	0.04	0	0	0	0
GT	0.02	0	0.05	0.07	0	0.31	0.60	0.01
IT	0	0	0	0.65	0.01	0.01	0.10	0
T	0	0	0	0.13	0.29	0.85	0.15	0
PR	0.04	0.02	0.50	0.37	0.10	0.07	0.25	0.05
SI	0.50	0	0.01	0.01	0.68	0	0	0
P	0	0	0	0	0	0	0	0
S	0	0	0	0.04	0.07	0.81	0.05	0
SA	0.36	0.05	0	0	0.96	0.44	0	0.24
SL	0.02	0.24	0.11	0	0.09	0.15	0	0.60
SW	0.57	0.16	0	0	0.84	0.01	0.11	0.20
SL/SW	0.08	0.72	0.20	0.11	0.15	0	0.34	0.07

Discussion

The development of chloroplast ultrastructure during leaf ontogeny is strongly associated with changes in chloroplast photochemical activity (see reviews in Šesták 1985). However, both inter- and intraspecific variability in structural and functional characteristics of chloroplasts exists during this development. To examine this phenomenon, we studied changes in HRA together with several structural characteristics of MC chloroplasts during the third leaf ontogeny of two different maize inbreds and their reciprocal F1 hybrids. In maize, HRA was connected mostly with MC chloroplasts having high proportion of stacked thylakoids.

As regards ontogenic development of the chloroplasts in both inbreds, some common features were evident. The increase of plastoglobuli compartment found during leaf development (number or size of plastoglobuli, or both) is one of the most characteristic phenomena accompanying chloroplast senescence (see Kutík 1985, 1998, Hudák 1997 for reviews). The products of breakdown of thylakoid lipids are probably accumulated in this compartment. Similarly, the size of thylakoid membrane system increases and stroma volume decreases from young to mature leaves. The increase of thylakoid compartment is apparently positively correlated with increasing HRA in developing leaves. Stacked thylakoid membranes are the main site of photosystem 2 and thus they are connected with HRA. However, we found some differences between the examined genotypes during leaf senescence. The increase in volume density of granal thylakoids in CE813 was compensated by the decrease in intergranal ones, which means that lesser number of larger grana was formed (probably by grana merging). Similar phenomenon was observed in the chloroplasts under shade conditions (see, *e.g.*, Lichtenthaler *et al.* 1984). On the contrary, volume density of granal and intergranal thylakoids increased simultaneously in senescing chloroplasts

of the CE829 genotype, which displayed lower photochemical activity. The chloroplasts in senescing leaves had large volume density of stacked thylakoids but relatively low HRA. Hence the quality of thylakoid membranes probably also changes during maize leaf development. MC chloroplasts in the senescing maize leaves studied were apparently not the gerontoplasts (senescent chloroplasts designated to destruction, see Sitte 1977). Chloroplast "vacuolation", thylakoid breakdown or envelope membranes rupture (see also description of senescent plastids in Hudák 1997) were observed only rarely and the HRA of respective leaves was not low.

Table 4. Genotypic differences between CE813 (13), CE829 (29), or their reciprocal F1 hybrids in the same characteristics as in Table 1. The statistical significances for the respective *t*-tests are presented.

	Young 13/29	Mature 13/29	13/13×29	13/29×13	29/13×29	29/29×13	13×29/29×13	Senescing 13/29
Exp. ser. 1								
HRA	0.82	0	0.02	0	0	0	0	0
GT	0.02	0.04	0.13	0.44	0.52	0.14	0.40	0
IT	0.53	0.94	0.17	0.09	0.20	0.08	0	0
T	0.17	0.04	0.45	0.07	0.14	0.63	0.26	0.10
PR	0.39	0.96	0.73	0.11	0.68	0.08	0.08	0.91
SI	0.03	0.90	0.05	0.33	0.05	0.33	0.31	0.22
P	0.14	0.06	0.01	0.70	0.08	0.16	0.01	0.48
S	0.26	0.03	0.98	0.03	0.04	0.95	0.03	0.12
SA	0.66	0	0.30	0	0.01	0.55	0.09	0.87
SL	0.31	0	0.08	0	0	0.17	0.07	0.04
SW	0.10	0.43	0.83	0.68	0.28	0.69	0.48	0.19
SL/SW	0.06	0.03	0.25	0.06	0.30	0.36	0.73	0.01
Exp. ser. 2								
HRA	0.68	0	0	0	0.77	0.97	0.69	0
GT	0	0.87	0.02	0.03	0.01	0.01	0.72	0.60
IT	0.77	0	0.91	0.02	0	0	0.03	0.88
T	0	0	0.03	0	0	0	0.28	0.77
PR	0.17	0.31	0.95	0.29	0.38	0.06	0.32	0.91
SI	0.39	0.12	0.71	0.54	0.12	0.03	0.91	1.00
P	0.34	0.03	0.17	0.42	0.30	0.24	0.69	0.29
S	0	0	0.04	0	0	0	0.35	1.00
SA	0.01	0.20	0.18	0	0.02	0	0.21	0.12
SL	0.11	0.02	0.65	0.23	0.14	0	0.15	0.02
SW	0	0.43	0.02	0	0	0	0.67	0.68
SL/SW	0.03	0.36	0	0.01	0.01	0.10	0.17	0

The peripheral reticulum is a chloroplast membrane system not involved in primary reactions of photosynthesis. It is therefore not surprising that we did not find any specific association between ontogenetic changes in HRA and the volume density of peripheral reticulum. As for starch inclusions, MC chloroplasts of the maize genotypes examined contained very small amount of starch (inclusions were identified only

morphologically) which agrees with the chloroplast dimorphism syndrome of NADP-ME C4 plants.

We also found several differences in shape and dimensions of chloroplasts from young to senescing leaves. The observed changes are in accordance with previous studies on chloroplast development during leaf ontogeny reviewed in Kutík (1985, 1998) and Hudák (1997). The larger area and length of chloroplast cross sections in the mature leaves of the CE829 genotype compared to the CE813 may somewhat compensate for the smaller thylakoid volume density in the first genotype.

The explanation of some differences observed between both experimental series (see Tables 1 and 2) is difficult. The maize plants were grown in the growth chamber under the same conditions, the experimental setup was the same. One reason for these differences could be relatively small number of plants studied. Four plants were examined for each ontogenic stage in both experiments concerning chloroplast ultrastructure and dimensions. Such number is common in time-consuming stereological evaluations, but cannot fully cover all the possible variability between individual plants. The quality of seed material used (one year difference between experimental series) could also play a role.

The results of this study show the existence of both ontogenic and, in a smaller extent, genotypic differences in Hill reaction activity as well as in chloroplast ultrastructure of maize MC chloroplasts during leaf development. They can contribute to the better understanding of the complex structure-function relationships in the photosynthetic apparatus.

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