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Maize F_1 hybrid differs from its maternal parent in the development of chloroplasts in bundle sheath, but not in mesophyll cells: Quantitative analysis of chloroplast ultrastructure and dimensions in different parts of leaf blade at the beginning of its senescence

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Abstract

The quantitative changes of chloroplast ultrastructure and dimensions in mesophyll (MC) and bundle sheath (BSC) cells, associated with the onset of leaf senescence, were followed along the developmental leaf blade gradient of the third leaf of maize (*Zea mays* L.). To ascertain whether the rapidity of structural changes associated with the transition of chloroplasts from mature to senescent state is a heritable trait, the parental and the first filial generations of plants were used. The heterogeneity of leaf blade, associated with the development of maize leaf (with the oldest regions at the apex and the youngest ones at the base) was clearly discernible in the ultrastructure and dimensions of chloroplasts; however, there were differences in the actual pattern of chloroplast development between both genotypes as well as between both cell types examined. While the course of MC chloroplasts' development at the onset of leaf senescence in maize hybrid followed that of its parent rather well, this did not apply for the BSC chloroplasts. In this case, each genotype was characterized by its own distinguishable developmental pattern, particularly as regards the accumulation of starch inclusions and the associated changes of the size and shape of BSC chloroplasts.

Additional key words: chloroplast development; electron microscopy; genetic variability; granal thylakoids; leaf blade heterogeneity; peripheral reticulum; plastoglobuli; starch inclusions; *Zea mays*.

Introduction

A characteristic feature of plants with NADP-ME type of C_4 photosynthesis is a spatial compartmentation of various processes linked to the photosynthetic carbon metabolism: the initial fixation of atmospheric CO_2 by phospho*enol*pyruvate (PEP) carboxylase (PEPC), its conversion into C_4 organic acids, its subsequent release from these acids to provide higher CO_2 concentrations for RuBPCO (in order to increase the carboxylation efficiency of this enzyme at the expense of its oxygenation activity), and the regeneration of PEP as the primary acceptor of CO_2. These processes take place in two different photosynthetic cell types, *i.e.* the bundle sheath cells (BSCs) surrounding the vascular bundles, and the mesophyll cells (MCs), which in turn surround the BSCs. The arrangement of these cells in leaves of NADP-ME plants follows the pattern referred to as the Kranz anatomy (Kranz is the German word for "wreath"), where the two cell layers give the appearance of wreath surrounding each vein (Furbank and Foyer 1988, Nelson and Langdale 1989, Nelson and Dengler 1992, Edwards *et al.* 2001, Leegood 2002, Brown *et al.* 2005). The BSCs and MCs differ in their structural properties; the most conspicuous feature of BSCs being their larger size compared to MCs, their thick cell walls, and the centrifugal or centripetal

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Abbreviations: BSC – bundle sheath cell; F_1 – the first filial generation; L/W – ratio of chloroplast cross-section length to width; MC – mesophyll cell; ME – malic enzyme; PEP – phospho*enol*pyruvate; PS2 – photosystem 2; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase.
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(NAD-malate type) arrangement of chloroplasts (Nelson and Langdale 1989, Dengler *et al.* 1994, Leegood 2002, Brown *et al.* 2005).

The BSCs and the MCs show various differences in the ultrastructure of chloroplasts, related to their different role in photosynthetic carbon fixation processes. The high demand for NADPH and ATP in MCs (where it is needed for the conversion of oxaloacetate to malate and of pyruvate to PEP, as well as for other non-photosynthetic processes demanding high supply of reducing equivalents and energy, *e.g.* nitrogen assimilation, biosynthesis of lipids) necessitates the preference for a linear photosynthetic electron transport in thylakoid membranes of these cells, and thus for a high amount and photochemical activity of PS2, associated with high granality of MC chloroplasts' thylakoids (Chow *et al.* 2005, Majeran *et al.* 2005). On the other hand, the biosynthesis and the accumulation of starch take place preferentially (but not exclusively) in the BSCs, which are often filled up with starch inclusions (Spilatro and Preiss 1987, Nelson and Langdale 1989, Lunn and Hatch 1995, Majeran *et al.* 2005).

The distinctive role of BSC and MC chloroplasts in photosynthetic processes of NADP-ME type C_4 plants is further reflected in the cell-specific expression of genes coding for various chloroplast proteins (Sheen and Bogorad 1987, 1988, Kubicki *et al.* 1994, Furumoto *et al.* 2000, Edwards *et al.* 2001, Hahnen *et al.* 2003, Majeran *et al.* 2005). The regulation of this cell-specific expression occurs at both transcriptional and post-transcriptional levels and is closely linked to the existence of Kranz anatomy. It seems that the default pattern of MC photosynthesis even in C_4 plants is a C_3 mechanism and that the switch of MCs to C_4 photosynthesis depends on their close proximity to BSCs and on the availability of photon energy (Nelson and Langdale 1989, Nelson and Dengler 1992, Cousins *et al.* 2003, Hahnen *et al.* 2003, Majeran *et al.* 2005). The C_4 specialization of BSCs is similarly light-dependent, requires their adjacent position to veins, and is influenced also by their procambial/meristematic origin (Nelson and Dengler 1992, Jankovski *et al.* 2001, Majeran *et al.* 2005).

The whole process of the differentiation of BSC and MC chloroplasts during the development of photosynthetic tissue in C_4 plants has been rather extensively studied using various methods of plant anatomy, biochemistry, and molecular biology (Nelson and Langdale 1989, Nelson and Dengler 1992, Langdale and Kidner 1994, Edwards *et al.* 2001, Majeran *et al.* 2005). Many of these studies were made on maize (*Zea mays* L.) and took the advantage of the unique characteristics of this grass species. Leaves of plants belonging to the grass family have been for a long time ideal model objects for the study of chloroplast development, as they show a distinctive, well-defined developmental gradient from base to tip during their growth. This gradient can be observed for the content of photosynthetic pigments, the activity of photosynthetic and other metabolic processes, the amounts of mRNA, proteins, and lipids, the content and organisation of chloroplast DNA, *etc*. (*e.g.* Leech *et al.* 1973, Kirchanski 1975, Perchorowicz and Gibbs 1980, Miranda *et al.* 1981a,b, Martineau and Taylor 1985, Langdale *et al.* 1987, 1988, Nelson and Langdale 1989, Nelson and Dengler 1992, Nishioka *et al.* 1993, Williams *et al.* 1993, Šesták and Šiffel 1997, Oldenburg and Bendich 2004), as well as for chloroplast ultrastructure and dimensions (Kirchanski 1975, Rascio *et al.* 1980, Wellburn *et al.* 1982, Kutík 1985, 1992, 1998, Nishioka *et al.* 1993).

The above-mentioned developmental pattern of chloroplasts along the grass leaf blade is partly discernible in mature, fully developed leaves as well (Kutík *et al.* 2001, Kołodziejek *et al.* 2003) and becomes again more pronounced with the onset of leaf senescence (Martinoia *et al.* 1983, Biswal and Biswal 1988, Chonan *et al.* 1991, Kutík 1985, 1998, Kutík *et al.* 2001, Matile 1992, Sakai *et al.* 1999, Biswal *et al.* 2003, Kołodziejek *et al.* 2003). However, not only there can be differences in this respect among various grass species (Kołodziejek *et al.* 2003), but such variability can be observed also within individual species (different genotypes can display different rates of chloroplast development) (Kutík and Kočová 1996, Kutík *et al.* 1999, 2001, Vičánková and Kutík 2005). Whether the rapidity of structural changes associated with the transition of chloroplasts from mature to senescent state is a heritable trait, is not known. Thus, we had decided to make a quantitative study of the ultrastructural heterogeneity of chloroplasts in various parts of leaf at the beginning of its senescence in two generations of maize (parental and F_1). Our main purpose was to ascertain whether the developmental pattern showed by the filial generation follows that of its maternal parent, and whether there are any differences in this respect between MC and BSC chloroplasts.

Materials and methods

The ultrastructure and dimensions of chloroplasts were evaluated in MCs and BSCs of three different parts of the third leaf of maize. Plants of two maize genotypes (the inbred line 2023 and its F_1 hybrid 2023×CE810; 2023 being the maternal parent of this hybrid) were grown in planting dishes filled with garden soil and placed in a growth chamber (*Klimabox RK1-007, Kovodružstvo Slaný*, Czech Republic) under conditions of 16/8 h day/night period, 25/16 °C temperature, 70/80 % relative air humidity, and 230–470 μmol m^{-2} s^{-1} photon flux density. They were allowed to grow under these conditions till maturity of the third leaf was reached. Small pieces of leaf blade for the evaluation of chloroplast ultrastructural and dimensional parameters were taken 24 d

after the date of sowing from three parts of leaf blade: the basal one (at approx. one quarter of the entire leaf blade length, measured from the base), the middle one, and the apical one (at approx. three quarters of the entire leaf blade length). In relation to leaf width, the position of the samples was always midway between the central rib and the leaf margin. The collection of samples was repeated again four days later, *i.e.* on 28-d-old plants, when the tip of the third leaf begun to display senescence symptoms.

The leaf blade samples were first fixed with glutaraldehyde followed by osmic acid treatment, dehydrated through ethanol series, and embedded into Spurr's low viscosity resin (see Kutík *et al.* 1999). Transverse ultrathin sections were then prepared from the embedded objects that were first contrasted with a saturated uranyl acetate solution followed by the lead citrate solution (Reynolds 1963). The microphotographs were taken using the transmission electron microscope *Phillips EM 300* (the Netherlands) (primary magnifications 7 000×, final magnifications 30 000×), scanned, and imported into *Lucia* image analysis system for the quantification of selected ultrastructural and dimensional parameters of MC and BSC chloroplasts. The volume densities of granal thylakoids, plastoglobuli, peripheral reticulum, and starch inclusions were determined, together with the total area, the length (L) and the width (W) of chloroplast cross sections. The shape of the chloroplasts was inferred from the L/W ratio.

The ultrastructural and dimensional parameters of chloroplasts were evaluated in four plants of each genotype for each sampling date, each plant being represented by five randomly selected chloroplasts from the respective leaf part and cell type. Data from both sampling dates were pooled together for the purposes of statistical analysis. The differences between both genotypes, between individual leaf parts, and between MC and BSC chloroplasts were determined by the analysis of variance accompanied by Tukey-Kramer's tests; all 20 values of each parameter representing each respective sample were used as the source data for this analysis. The mutual relationships between the parameters examined were investigated by correlation analysis (Pearson's correlation coefficient). Each sample (representing the respective cell type/leaf part/plant age/genotype) was represented here by four values (averaged from five chloroplasts for each individual plant) of each ultrastructural or dimensional parameter. Similar analysis was used to ascertain whether there exists any parallelism in the development of MC or BSC chloroplast ultrastructure in the inbred line and its hybrid; the initial data for this analysis were the means of each parameter, calculated from all 20 values evaluated for the respective cell type/leaf part/plant age/genotype. All statistical evaluation was made with the *CoStat* (version *6.204*) programme (*CoHort Software*, Monterey, CA, USA).

Results

The representative samples of chloroplast cross sections from mesophyll and bundle sheath cells of the basal, middle, and apical parts of leaf blade are shown in Fig. 1. The average area of these cross sections in the youngest leaf part examined (*i.e.* the basal part of leaf of 24-d-old plants) was rather similar in both cell types, but the hybrid was at this stage characterized by larger BSC chloroplasts compared to the inbred line, and the area and the width of BSC chloroplasts' cross-sections in this genotype were significantly larger compared to the MC chloroplasts' ones. In almost every other case the size of BSC chloroplasts highly and significantly exceeded that of the MC ones (Table 1).

The average area of MC chloroplasts' cross-sections slightly increased in both genotypes with the increasing distance from the leaf base and the genotypes did not differ much in this parameter. This increase in the total area of chloroplast cross sections was related more to the increase of chloroplast width than chloroplast length. However, there was a significant drop in the size of MC chloroplasts' cross sections in the apical part of leaf of 28-d-old plants (this leaf part already displayed the visible symptoms of senescence, *i.e.* yellow colour, dry tissue at the extreme leaf tip), that was caused by the diminution of the length of chloroplasts' cross-sections. Thus, the shape of MC chloroplasts gradually changed from rather flat one observed in the youngest tissue (the basal part of leaf of 24-d-old plants) to the more rounded one in the oldest tissue (the apical part of leaf of 28-d-old plants) (Fig. 1). The relationship between the inbred line and the hybrid as regards the L/W ratio calculated for MC chloroplasts also changed with the increasing age of leaf blade tissue, with the hybrid genotype exceeding the inbred one in younger leaf parts, and the reverse situation in more aged leaf parts (Table 1).

The BSC chloroplasts of the inbred line were always less rounded than those of the hybrid and these differences between genotypes in the L/W ratio were usually statistically significant. The increasing trend, observed for the average area of chloroplast cross sections in mesophyll cells, was partly discernible in the bundle sheath cells as well, but there were two great deviations: the middle and the apical parts of leaf of 24-d-old hybrid plants were characterized by extremely large chloroplasts (this applied both for the length and the width of the chloroplast cross sections). No drop in the BSC chloroplasts' dimensions was observed for the already senescing leaf apex of 28-d-old plants (Table 1).

As expected, the thylakoid membranes constituted the largest proportion of MC chloroplasts' inner volume, and the volume density of thylakoid grana in this type of chloroplasts was approximately 40 %. This applied for all

Table 1. Dimensions of chloroplasts of mesophyll cells (MC) and bundle sheath cells (BSC) in basal, middle, and apical parts of the third leaf of two maize genotypes (I − inbred line 2023, H − its F_1 hybrid 2023×CE810). A − area [μm^2], L − length [μm], W − width [μm]. Means ± standard errors of mean (SEM) are shown, together with the statistical significance of the differences between genotypes (**significant with $p\leq0.01$, *significant with $p\leq0.05$, ns − non-significant), between individual leaf parts (the letters ABCD denoting the differences for the particular row of the table; only those parts marked with different letters significantly differ at $p\leq0.05$), and between MC and BSC chloroplasts (**significant with $p\leq0.01$, *significant with $p\leq0.05$, ns − non-significant).

		Base				Middle				Apex			
		24-d old plants		28-d old plants		24-d old plants		28-d old plants		24-d old plants		28-d old plants	
		MC chloroplasts											
A	I	9.04±0.55^{BCD}	ns	8.56±1.26^{CD}	ns	12.68±0.99^{AB}	ns	11.34±1.21^{ABC}	ns	14.40±0.78^{A}	*	7.33±0.74^{D}	
	H	8.27±0.77^{CD}		10.07±1.19^{BC}		13.05±1.05^{AB}		10.26±1.30^{BC}		16.96±1.13^{A}		5.11±0.41^{D}	
L	I	6.72±0.27^{AB}	ns	5.51±0.40^{BC}	ns	6.68±0.33^{AB}	ns	6.17±0.39^{B}	ns	8.08±0.43^{A}	*	4.09±0.24^{C}	
	H	7.13±0.40^{AB}		6.05±0.51^{AB}		7.28±0.30^{A}		5.52±0.52^{B}		7.53±0.51^{A}		3.17±0.25^{C}	
W	I	1.57±0.07^{B}	*	1.86±0.15^{AB}	ns	2.34±0.12^{A}	ns	2.07±0.21^{AB}	ns	2.24±0.12^{A}	ns	2.00±0.11^{AB}	
	H	1.35±0.07^{B}		1.91±0.18^{AB}		2.20±0.12^{A}		2.07±0.21^{A}		2.50±0.12^{A}		2.00±0.11^{A}	
L/W	I	4.35±0.18^{A}	**	3.23±0.30^{A}	ns	2.97±0.18^{A}	ns	4.98±1.86^{A}	ns	3.66±0.18^{A}	*	2.12±0.15^{A}	
	H	5.38±0.28^{A}		3.39±0.30^{B}		3.41±0.18^{B}		2.70±0.21^{B}		3.12±0.24^{B}		1.64±0.14^{C}	
		BSC chloroplasts											
A	I	8.32±0.73^{D}	**	18.60±2.12^{BC}	ns	16.90±0.70^{BCD}	**	25.79±3.07^{AB}	*	13.67±0.74^{CD}	**	32.62±4.27^{A}	ns
	H	14.28±1.44^{C}		19.40±1.74^{C}		87.89±4.59^{A}		17.88±1.02^{C}		61.71±9.01^{B}		24.24±6.41^{C}	
L	I	6.78±0.40^{B}	ns	8.54±0.66^{AB}	ns	8.63±0.28^{AB}	**	10.93±1.19^{A}	**	8.80±0.40^{AB}	*	11.29±1.43^{A}	ns
	H	7.79±0.47^{B}		8.15±0.33^{B}		14.13±0.48^{A}		7.29±0.29^{B}		12.05±0.75^{A}		7.24±1.18^{B}	
W	I	1.50±0.07^{D}	**	2.62±0.16^{ABC}	ns	2.57±0.14^{BC}	**	3.48±0.37^{AB}	ns	1.86±0.07^{CD}	**	3.56±0.38^{A}	ns
	H	2.44±0.17^{B}		2.97±0.16^{B}		6.72±0.28^{A}		3.24±0.20^{B}		6.02±0.72^{A}		3.08±0.49^{B}	
L/W	I	4.56±0.22^{A}	**	3.37±0.26^{A}	ns	3.57±0.24^{A}	**	3.81±0.77^{A}	ns	4.79±0.22^{A}	*	3.38±0.39^{A}	*
	H	3.41±0.23^{A}		2.83±0.14^{AB}		2.14±0.08^{B}		2.40±0.17^{AB}		2.84±0.86^{AB}		2.42±0.16^{AB}	
		Statistical significance of the differences between MC and BSC chloroplasts											
A	I	ns		**		**		**		ns		**	
	H	**		**		**		**		**		**	
L	I	ns		**		**		**		ns		**	
	H	ns		**		**		**		**		**	
W	I	ns		**		ns		**		**		**	
	H	**		**		**		**		**		*	
L/W	I	ns		ns		ns		ns		**		**	
	H	**		ns		**		ns		ns		**	

three leaf parts and both plant ages, as well as for both genotypes examined, the only exception being the apical part of leaf of the 28-d-old plants. The relative partial volume of granal thylakoids in this part of leaf significantly decreased both in the inbred line and in the hybrid; however, the decrease observed in the hybrid was much more pronounced compared to the inbred line. The BSC chloroplasts also contained some grana, but their amount was very low compared to the MC chloroplasts. The basal part of leaf of 24-d-old plants, as well as the leaf apex of 28-d-old plants, was characterized by comparatively high relative partial volume of granal thylakoids in both genotypes examined. Otherwise, no specific trend was observed for this chloroplast compartment in the BSC chloroplasts. The inbred line generally showed higher grana volume density compared to the hybrid; in some cases, the differences between genotypes were statistically significant (Table 2).

The BSC chloroplasts of the hybrid were rather filled up with starch and the relative partial volume of starch inclusions varied between 25 and 57 %. The values of the volume density of starch inclusions in the inbred line were similar, but this applied only for 28-d-old plants; all leaf parts examined in the younger plants showed significantly lower amounts of starch (between 5 and 10 %) and the inbred thus significantly differed from the hybrid in these cases. The amount of starch in MC chloroplasts was extremely small; however, some starch inclusions were usually found in all leaf parts examined (more suspiciously in the hybrid) and there were no statistically significant differences among various leaf parts regardless of plant age (Fig. 1, Table 2).

The number and size of plastoglobuli increased with the developmental stage of chloroplasts, both in MCs and BSCs. Although this increase was rather gradual in MC chloroplasts of both genotypes, the individual leaf parts did not significantly differ in this parameter, with the exception of the leaf apex of 28-d-old plants that was characterized by the highest relative partial volume of plastoglobuli. The same applied for the BSC chloroplasts of the hybrid. As regards the inbred line, we found several statistically significant differences between

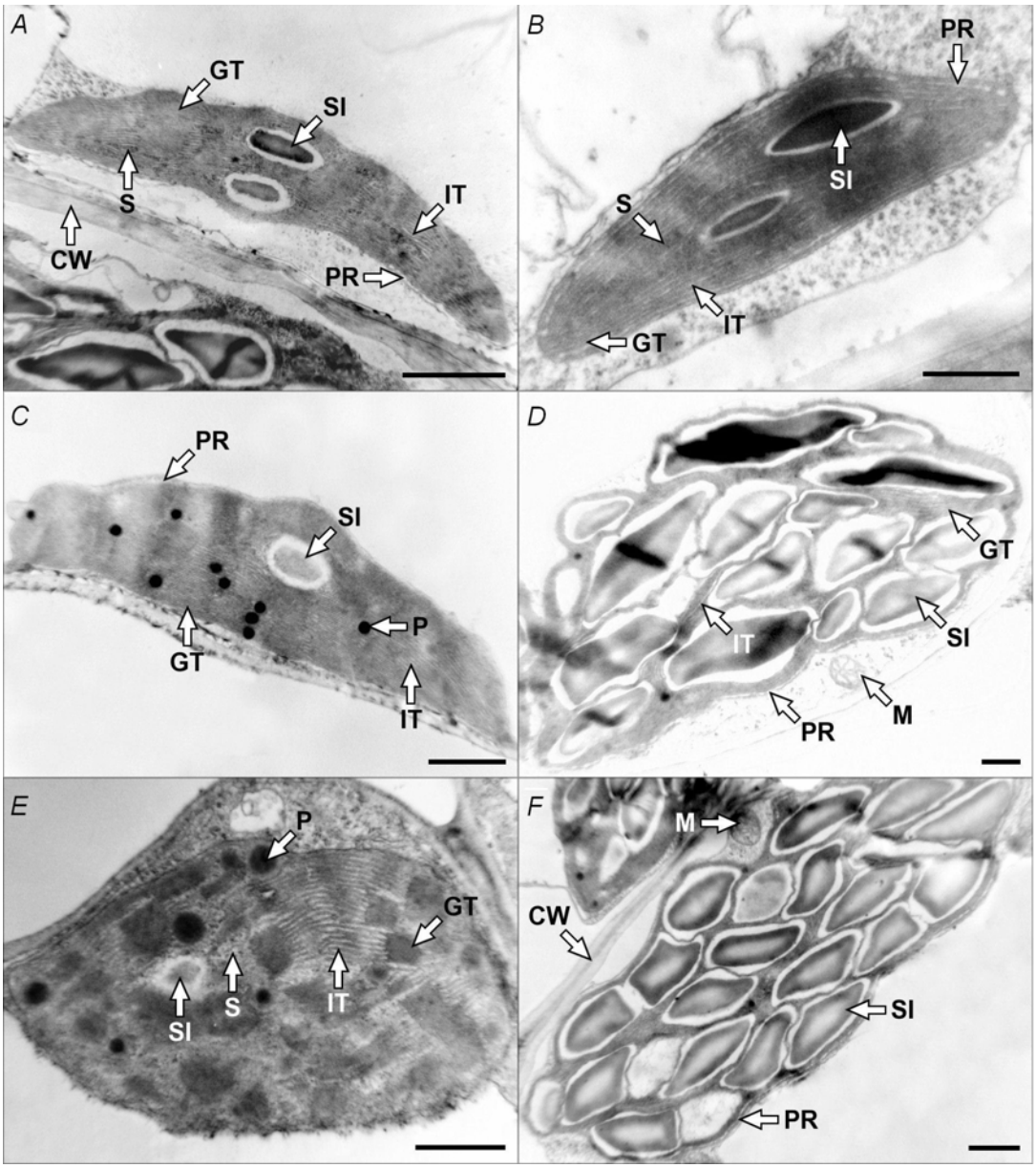

Fig. 1. Transmission electron micrographs of chloroplast cross sections taken from mesophyll (MC) and bundle sheath (BSC) cells in three parts of the third leaf of maize F_1 hybrid 2023×CE810. The cross-sections of MC (*A, C, E*) and BSC (*B, D, F*) chloroplasts in the basal (*A, B*), middle (*C, D*), and apical (*E, F*) parts of leaf are shown. CW – cell wall, GT – granal thylakoids, IT – intergranal thylakoids, M – mitochondrion, P – plastoglobulus, PR – peripheral reticulum, S – stroma, SI – starch inclusion. Bar − 1 μm.

various parts of leaf blade in the volume density of plastoglobuli, and the inbred line also showed significantly higher values of this parameter compared to the hybrid. In all cases, the MC chloroplasts contained significantly more plastoglobuli than the BSC ones (Table 2).

The percentage of MC chloroplast cross sections constituted by the peripheral reticulum varied between 1.0 and 2.0 in the hybrid, and between 1.8 and 3.5 in the inbred line. Similar values of this parameter were observed in the BSC chloroplasts, but the variability between individual leaf parts was somewhat higher. When there were some statistically significant differences between both types of chloroplasts, the volume density of peripheral reticulum in the MC ones invariably exceeded that of the BSC ones. However, with the exception of BSC chloroplasts of the inbred line, we did not find any statistically significant differences among leaf parts and there was also no specific trend as regards the differences between genotypes (Table 2).

The relationship between various ultrastructural and dimensional parameters of chloroplasts in MC or BSC, as well as between both cell types, was examined using the correlation analysis. The relative partial volume of granal thylakoids in MC chloroplasts significantly and positively

Table 2. Ultrastructural parameters of chloroplasts of the mesophyll cells (MC) and bundle sheath cells (BSC) in basal, middle, and apical parts of the third leaf blade of two maize genotypes (I − inbred line 2023, H − its F_1 hybrid 2023×CE810). All values in %. GT − granal thylakoids, PG − plastoglobuli, PR − peripheral reticulum, SI − starch inclusions. Means ± standard errors of mean (SEM) of volume densities of the respective chloroplast compartments are shown, together with the statistical significance of the differences between genotypes (**significant with $p\leq0.01$, *significant with $p\leq0.05$, ns − non-significant, nd − non-determined), between individual leaf parts (the letters ABCD denoting the differences for the particular row of the table; only those parts marked with different letters significantly differ at $p\leq0.05$), and between MC and BSC chloroplasts (**significant with $p\leq0.01$, *significant with $p\leq0.05$, ns − non-significant).

		Base				Middle				Apex			
		24-d old plants		28-d old plants		24-d old plants		28-d old plants		24-d old plants		28-d old plants	
		MC chloroplasts											
GT	I		41.98±2.56^{A}		34.91±3.01^{AB}		39.57±2.44^{A}		41.44±2.03^{A}		37.63±1.76^{A}		27.60±2.52^{B}
	H	ns	38.37±2.65^{A}	ns	41.83±2.84^{A}	ns	42.45±2.12^{A}	ns	41.71±1.92^{A}	ns	40.63±1.63^{A}	**	16.96±2.27^{B}
PG	I		0.15±0.05^{B}		2.30±0.48^{B}		0.45±0.08^{B}		3.00±0.42^{B}		4.56±1.28^{B}		11.28±2.28^{A}
	H	ns	0.21±0.06^{B}	ns	1.34±0.31^{B}	ns	0.64±0.15^{B}	ns	2.75±0.45^{B}	*	1.72±0.23^{B}	**	19.52±1.75^{A}
PR	I		3.23±0.32^{A}		2.04±0.64^{A}		1.77±0.21^{A}		3.47±0.73^{A}		2.41±0.68^{A}		2.95±0.46^{A}
	H	*	2.05±0.30^{A}	ns	1.66±0.35^{A}	ns	1.81±0.20^{A}	**	1.31±0.24^{A}	ns	1.45±0.14^{A}	*	1.75±0.24^{A}
SI	I		0±0.00^{A}		0.24±0.23^{A}		0.04±0.03^{A}		1.86±1.02^{A}		0.83±0.23^{A}		1.28±0.50^{A}
	H	*	0.64±0.29^{A}	ns	0.87±0.31^{A}	*	1.65±0.76^{A}	ns	3.57±1.58^{A}	ns	0.99±0.29^{A}	ns	0.99±0.35^{A}
		BSC chloroplasts											
GT	I	**	9.66±1.10^{A}		2.74±0.58^{D}	**	8.38±0.92^{AB}		3.18±0.42^{CD}	**	4.84±0.53^{BCD}		7.77±2.31^{ABC}
	H		4.84±0.40^{AB}	ns	1.84±0.19^{B}		0.89±0.19^{B}	ns	3.13±0.41^{AB}		1.59±0.27^{B}	ns	6.99±2.81^{A}
PG	I		0±0.00^{D}		0.50±0.24^{BCD}	**	0.18±0.06^{CD}	*	1.00±0.23^{B}	**	0.74±0.14^{BC}		1.78±0.23^{A}
	H	nd	0±0.00^{B}	*	0.01±0.01^{B}		0±0.00^{B}		0.32±0.18^{B}		0.07±0.02^{B}	ns	1.57±0.32^{A}
PR	I	**	3.51±0.23^{A}		1.42±0.26^{BC}		1.02±0.24^{C}		1.02±0.25^{C}	**	2.30±0.18^{B}		1.60±0.35^{BC}
	H		2.00±0.21^{A}	ns	2.46±1.41^{A}	ns	0.65±0.13^{A}	ns	1.11±0.23^{A}		0.78±0.14^{A}	ns	1.81±0.49^{A}
SI	I	**	5.85±1.59^{B}		31.08±3.07^{A}	**	8.82±1.69^{B}		34.20±2.97^{A}	**	5.98±0.77^{B}		32.96±3.52^{A}
	H		26.97±2.71^{BC}	ns	33.56±2.45^{BC}		57.40±1.96^{A}	ns	32.35±1.73^{BC}		38.67±1.95^{B}	ns	24.80±4.66^{C}
		Statistical significance of the differences between MC and BSC chloroplasts											
GT	I	**		**		**		**		**		**	
	H	**		**		**		**		**		**	
PG	I	**		**		*		**		**		**	
	H	**		**		**		**		**		**	
PR	I	ns		ns		*		**		ns		*	
	H	ns		ns		**		ns		ns		*	
SI	I	**		**		**		**		**		**	
	H	**		**		**		**		**		**	

correlated with the area of chloroplast cross sections both in the inbred and in the hybrid. On the other hand, the volume density of plastoglobuli significantly decreased with increasing length of MC chloroplasts' cross-sections, and the correlation between the volume densities of granal thylakoids and plastoglobuli was negative as well (again in both genotypes). The hybrid also showed significant negative relationship between the volume density of plastoglobuli and the area of chloroplasts' cross-sections or the L/W ratio; this type of correlation was not found for the inbred line. All other possible relationships between MC chloroplasts' parameters were statistically non-significant (as regards the Pearson's correlation coefficient) (Table 3).

Other types of ultrastructural relationships were found for the BSC chloroplasts. The relative partial volume of the starch inclusions significantly and negatively correlated with the volume densities of granal thylakoids. The amount of starch was positively related to the chloroplast size, while the reverse was true for the volume density of peripheral reticulum or granal thylakoids and the BSC chloroplasts' width. The width of the BSC chloroplasts' cross-sections significantly increased with their length. All these relationships were found in both genotypes, but there were also several other statistically significant correlations observed either only in the inbred line or in its hybrid. The positive correlation between the volume density of plastoglobuli and the dimensional parameters of BSC chloroplasts was found in the inbred line only, whereas in the hybrid this relationship was either negative or statistically non-significant. The inbred L/W ratio was related to the relative partial volume of starch inclusions (negatively) and peripheral reticulum (positively). On the other hand, the statistically significant correlations observed only in the hybrid included the inverse relationship between the length (or area) of BSC chloroplasts' cross-sections and the volume density of peripheral reticulum or granal thylakoids, and between volume densities of plastoglobuli and starch inclusions (Table 3).

Table 3. Relationships between individual ultrastructural and/or dimensional parameters of chloroplasts of mesophyll cells (MC) and bundle sheath cells (BSC) evaluated as the correlations between the respective parameters in maize inbred 2023 and its F_1 hybrid 2023×CE810. Only those correlations that were statistically significant for at least one genotype are shown as the values of Pearson's correlation coefficient (r) ± standard errors (SE), together with the level of statistical significance (** − significant with $p \leq 0.01$, * − significant with $p \leq 0.05$, ns − non-significant). TG − the volume density of granal thylakoids, PG − the volume density of plastoglobuli, PR − the volume density of peripheral reticulum, SI − the volume density of starch inclusions, A − the total area of chloroplasts' cross-sections, L − the length of chloroplasts' cross-sections, W − the width of chloroplasts' cross-sections.

Chloroplast	Relationship	r ± SE(r) Inbred		r ± SE(r) Hybrid	
MC	TG – PG	–0.503±0.184	*	–0.772±0.136	**
	TG – A	0.422±0.193	*	0.510±0.183	*
	PG – A		ns	–0.446±0.191	*
	PG – L	–0.519±0.182	**	–0.701±0.152	**
	PG – L/W		ns	–0.606±0.170	**
BSC	TG – SI	–0.483±0.187	*	–0.455±0.190	*
	TG – L		ns	–0.459±0.189	*
	TG – W	–0.406±0.195	*	–0.434±0.192	*
	PG – SI		ns	–0.465±0.189	*
	PG – A	0.529±0.181	**		ns
	PG – L	0.449±0.191	*	–0.438±0.192	*
	PG – W	0.434±0.192	*		ns
	PR – A		ns	–0.478±0.187	*
	PR – L		ns	–0.473±0.188	*
	PR – W	–0.580±0.138	**	–0.501±0.185	*
	PR – L/W	0.421±0.193	*		ns
	SI – A	0.631±0.166	**	0.748±0.142	**
	SI – L	0.425±0.193		0.838±0.117	**
	SI – W	0.747±0.142	**	0.826±0.120	**
	SI – L/W	–0.448±0.191	*		ns
	L – W	0.712±0.150	**	0.907±0.090	**
MC – BSC	TG (MC) – TG (BSC)		ns	–0.565±0.176	**
	TG (MC) – PG (BSC)	–0.422±0.193	*	–0.615±0.168	**
	TG (BSC) – PG (MC)		ns	0.623±0.167	**
	TG (BSC) – A (MC)		ns	–0.431±0.192	*
	TG (BSC) – L (MC)		ns	–0.418±0.194	*
	PG (MC) – PG (BSC)	0.786±0.132	**	0.802±0.128	**
	PG (MC) – A (BSC)	0.551±0.178	**		ns
	PG (MC) – L (BSC)	0.418±0.194	*		ns
	PG (MC) – W (BSC)	0.408±0.195	*		ns
	PG (BSC) – SI (MC)	0.493±0.186	*		ns
	PG (BSC) – L (MC)	–0.562±0.176	**	–0.530±0.181	**
	PG (BSC) – L/W (MC)		ns	–0.464±0.189	*
	SI (MC) – A (BSC)	0.546±0.179	**		ns
	SI (MC) – L (BSC)	0.642±0.163	**		ns
	SI (MC) – W (BSC)	0.450±0.190	*		ns
	SI (BSC) – A (MC)		ns	0.413±0.194	*
	SI (BSC) – L (MC)	–0.558±0.177	**		ns
	A (MC) – A (BSC)		ns	0.506±0.184	*
	A (MC) – L (BSC)		ns	0.554±0.178	**
	A (MC) – W (BSC)		ns	0.584±0.173	**
	A (BSC) – L (MC)	–0.511±0.183	*		ns
	L (MC) – L (BSC)		ns	0.426±0.193	*
	L (MC) – W (BSC)	–0.466±0.189	*		ns
	W (MC) – W (BSC)		ns	0.465±0.189	*

We found also some mutual interdependence of the ultrastructural (or dimensional) parameters of chloroplasts in MCs and BSCs. In this case, the differences between both genotypes were even more marked. The relative partial volume of granal thylakoids in MC chloroplasts was negatively correlated to the relative partial volume of plastoglobuli in BSC chloroplasts in both the inbred line and its hybrid, the interrelationship

between the plastoglobuli volume densities in both types of chloroplasts was a positive one, and the relative partial volume of plastoglobuli in BSC chloroplasts was negatively related to the length of MC chloroplasts' cross-sections. Other statistically significant values of Pearson's correlation coefficient were unique either for one or the other genotype. The inbred line was characterized by several positive relationships between the volume density of plastoglobuli or starch inclusions in MC chloroplasts and some dimensional parameters of BSC chloroplasts. The length of MC chloroplasts' cross-sections was also negatively related to the area, the width, or the starch amount of BSC chloroplasts in this genotype. The hybrid showed negative correlations between the relative partial volumes of granal thylakoids of BSC chloroplasts and the MC chloroplast dimensions, the same applied for the relationship between BSC and MC chloroplasts' granal thylakoids, while the volume density of plastoglobuli in MC chloroplasts was inversely related to the volume density of BSC chloroplasts' grana. There were also several positive interrelationships between dimensions of MC and BSC chloroplasts in this genotype, as well as the positive correlation between the area of MC chloroplasts' cross-sections and the amount of starch in BSC chloroplasts (Table 3).

The examination of the relationship between the inbred line and its hybrid, as regards various ultrastructural and dimensional parameters of MC or BSC chloroplasts, revealed that the development of MC chloroplasts (as inferred from the examination of chloroplasts in three different parts of leaf blade) in leaves of the hybrid followed the behaviour of its parent rather well (the only non-significant correlations were found for the volume density of peripheral reticulum and the L/W ratio). On the other hand, no such similarity was found for the BSC chloroplasts, with the exception of significant correlation in the volume density of plastoglobuli (Table 4).

Table 4. Relationship between maize inbred line 2023 and its F_1 hybrid 2023×CE810 evaluated as the correlations between the average values of selected ultrastructural or dimensional parameters of chloroplasts of the mesophyll (MC) or bundle sheath (BSC) cells, determined for three different parts of the third leaf blade in 24-d-old and 28-d-old plants. The values of Pearson's correlation coefficient (r) ± standard errors (SE) are shown, together with the level of statistical significance (**significant with $p \leq 0.01$, *significant with $p \leq 0.05$, ns − non-significant).

Parameter	MC chloroplasts r ± SE(r)	BSC chloroplasts r ± SE(r)
Granal thylakoids	0.835±0.275 *	0.409±0.456 ns
Plastoglobuli	0.946±0.162 **	0.900±0.219 *
Peripheral reticulum	–0.138±0.495 ns	0.300±0.478 ns
Starch inclusions	0.922±0.193 **	–0.193±0.491 ns
Cross-section area	0.941±0.169 **	–0.224±0.487 ns
Cross-section length	0.914±0.202 *	–0.294±0.478 ns
Cross-section width	0.922±0.193 **	–0.193±0.491 ns
Cross-section length/width	0.492±0.435 ns	0.634±0.387 ns

Discussion

For our study of the changes in chloroplast ultrastructure and dimensions during the beginning of leaf senescence, we chose two genotypes of maize: the inbred line 2023 and its direct descendant, F_1 hybrid 2023×CE810, which inherited its chloroplasts maternally from the 2023 inbred. As this hybrid showed somewhat faster development compared to its parent, during the first sampling date, when the third leaves of the inbred line have just completed their transition into the maturity stage, the corresponding leaves of the hybrid were already fully mature. Four days later, at the second sampling date, the apical parts of leaves of hybrid plants showed full symptoms of senescence (yellow colour, dry tip), the middle part of leaf was light-green and the basal one was fully green, whereas the entire length of 2023 leaves was green and only the extreme tip of leaves has begun to get yellow. Thus, we could compare the course of chloroplast development in relation to the different onset of leaf senescence in both genotypes.

From the beginning of leaf maturity, MC chloroplasts in our samples of leaf blade were characterized by well-developed thylakoid grana that usually contained between 15 and 30 thylakoids. Their volume density stayed the same in almost all leaf parts examined (so that, presumably, they have already reached their full extent at the first sampling date) and, despite the developmental lead of hybrid genotype over its parent (and despite its different activity of PS2 compared to the inbred line, see Kutík *et al.* 2001), there were no differences between both genotypes in this parameter. Only at the senescing apical part of leaf of 28-d-old plants we found a significant drop in the volume densities of granal thylakoids, and the difference between both genotypes manifested itself, as this drop was more pronounced in the hybrid. The decay of thylakoid membranes, and particularly the diminishing of the grana, is a typical phenomenon associated with the senescence of chloroplasts (Naito *et al.* 1981, Biswal and Biswal 1988, Matile 1992, Kutík *et al.* 1999, 2001, Zavaleta-Mancera *et al.* 1999, Prakash *et al.* 2001, Biswal *et al.* 2003, Kołodziejek *et al.* 2003). Similar sign of chloroplast senescence is the increase in the number and size of plastoglobuli, small particles accumulating the products of thylakoid lipids and chlorophyll breakdown, carotenoids and carotenoid esters (Naito *et al.* 1981, Tevini and Steinmuller 1985, Biswal and Biswal 1988, Matile 1992, Kutík *et al.* 1993, Biswal 1995, Kutík 1998, Matile *et al.* 1989, Biswal *et al.* 2003). Such increase was clearly

discernible in the senescing apical part of leaves at the second sampling date, and, again, the hybrid exceeded its parent in this parameter. The association between the degradation of thylakoid membranes and the accumulation of the products of their breakdown in plastoglobuli was thus obvious and was confirmed also by the results of the correlation analysis.

However, the inverse relationship between thylakoids and volume densities of plastoglobuli applied only for the MC chloroplasts. Besides the fact that the BSC chloroplasts had much less thylakoids (which is one of their well-known characteristics, see *e.g.* Kirchanski 1975, Nishioka *et al.* 1993, Vičánková and Kutík 2005) compared to the MC ones, and the relative volume density of their plastoglobuli was also lower than that of the plastoglobuli in MC chloroplasts, no direct correlation between these two chloroplast compartments was found in this case. The reason for this lies probably in the different pattern of leaf blade heterogeneity observed for the volume densities of plastoglobuli and grana in BSC chloroplasts. Whereas an increasing trend from the youngest leaf base to the oldest leaf apex, similar to the one observed in MC chloroplasts and concordant with the gradual advancement of chloroplast senescence, was discernible for the plastoglobuli volume density, the BSC grana behaved differently. The high volume density of thylakoid grana found in the basal part of leaves was in good agreement with findings of other authors, who observed that the amount of granal thylakoids of the BSC chloroplasts decreases during their development and that this decrease is light-dependent (Brangeon 1973, Kirchanski 1975, Nishioka *et al.* 1993). As the leaf base of maize leaves is more shielded from light by leaves of higher insertions, larger grana are only to be expected here. However, surprisingly high volume density of BSC chloroplasts' grana was found also in the senescing leaf apex. We observed similar results when comparing BSC chloroplasts in the middle third of leaf blade of young, mature, and senescing maize leaves (Vičánková and Kutík 2005). The stacking of thylakoid membranes could be in this case rather secondary, due to the fact that BSC chloroplasts at this developmental stage were fully packed with starch granules and there was thus less possibility of thylakoid spacing. The superiority of the inbred line over its progeny, shown for the granal thylakoids' volume density in BSC chloroplasts, could be possibly associated with its slightly lagging development (more discernible in younger plants), but similar superiority was observed also for plastoglobuli (*i.e.* symptom of senescence), and this discrepancy must be therefore caused by some unknown factors related to genetic differences between both genotypes.

Peripheral reticulum is a rather elusive chloroplast compartment: its role in the chloroplast function is still far from being clear. It is typical for C_4 plants and probably participates in the transport of primary assimilates (malate, aspartate) from MC to BSC (Laetsch and Kortschak 1972, Chapman *et al.* 1975, Kirchanski 1975, Hudák 1997, Kratsch and Wise 2000). This agrees well with our findings that the volume density of this compartment in MC chloroplasts of mature leaf blade was mostly higher than in the BSC ones. The inbred line usually showed greater size of peripheral reticulum in MC chloroplasts compared to the hybrid (in younger plants this applied for BSC chloroplasts as well); this was perhaps also related to the delay in chloroplast development displayed by the parental line (*i.e.* the transport processes between MCs and BSCs were fully active even in the oldest part of 28-d-old inbred plants).

The size and shape of chloroplasts also changes with their development (Naito *et al.* 1981, Kutík 1985, Kura-Hotta *et al.* 1990, Kutík and Kočová 1996, Kutík *et al.* 1999, 2001, Zavaleta-Mancera *et al.* 1999, Biswal *et al.* 2003) and we observed slight gradual enlargement of MC chloroplasts until the stage of senescence was reached by leaf tissue. This increase in the size was caused by the rise of chloroplast height (*i.e.* width of their cross-sections), so that the chloroplasts gradually acquired more rounded shape. In the senescing part of leaf apex, this rounding of MC chloroplasts was very suspicious, and at the same time there was a sharp drop in their size as well—another evidence of their transition to senescing state (Kura-Hotta *et al.* 1990, Kutík *et al.* 1999, Sakai *et al.* 1999). Again, the slight delay in the development of the inbred genotype after its hybrid could be seen here. The BSC chloroplasts, which are generally larger than those of MCs (Kirchanski 1975, Yoshimura *et al.* 2004), also displayed this increasing trend but their shape stayed more-or-less the same in all parts of leaf tissue examined and no decrease in the BSC chloroplasts' size was observed in the senescing leaf apex. The MC and BSC chloroplasts thus differ in the course of their development. The inbred line was characterized by more flat BSC chloroplasts compared to its progeny; this difference could be partly caused by the larger amount of starch inclusions that filled up the BSC chloroplasts of hybrid and caused their "swelling".

As regards the accumulation of starch in the BSC chloroplasts, there were interesting differences between both genotypes. The leaves of 24-d-old plants of the inbred line, which were at the beginning of their maturity, did not yet contain much starch inclusions, probably due to the fact that the production of 3-phosphoglycerate by the Calvin-Benson cycle (followed by its transport to MC chloroplasts, reduction to triosephosphates, and return to BSC chloroplasts, where it is used for starch synthesis) had not yet reached its full efficiency. On the other hand, the hybrid, which was ahead of its parent in the development of leaves, had already sufficient photosynthetic metabolism and its synthesis and accumulation of starch in BSC chloroplasts was well advanced. Four days later, both genotypes showed similar amounts of starch, and with the advancement of leaf tissue senescence, the parental genotype even slightly gained over its

progeny. The assessment of the changes in volume density of starch inclusions in BSC chloroplasts (and, consequently, of BSC chloroplasts' shape and dimensions), brought actually the most interesting piece of information about the genotypic differences in chloroplast ultrastructure, and about the possible heritability of the changes associated with chloroplast development. Whereas the development of MC chloroplasts during the onset of senescence of maize leaves occurred with about the same rapidity (judging from the changes of almost all ultrastructural and dimensional parameters examined) in the inbred line as in its hybrid, this did not apply for the BSC chloroplasts. Here we observed significant correlations between parent and its progeny only for the volume density of plastoglobuli, but the synthesis of starch and its accumulation in the starch inclusions of BSC chloroplasts followed different course in each genotype. Thus, the different nuclear genetic background of hybrid asserted itself in this case.

Other differences between both genotypes examined involved mostly the correlations among various ultrastructural and/or dimensional characters of chloroplasts. Thus, for example, the increase of plastoglobuli with the increasing BSC chloroplast size (*i.e.* the symptoms of advancing development of chloroplasts) observed in the inbred line was not followed by similar trend in the hybrid; on the contrary, the reverse was true. The enlargement of MC chloroplasts was accompanied by similar enlargement of BSC chloroplasts in the hybrid but not in its parent (again, probably due to the differences in the advancement of starch accumulation and the related "swelling" of chloroplasts). We can thus conclude that although several parameters related to the arrangement of chloroplast inner structure indeed seem to be directly inherited from parent to its progeny, this does not apply generally. Moreover, there are differences in the inheritance of chloroplast ultrastructural or dimensional parameters between two basic chloroplast types found in the photosynthetic tissue of NADP-ME type of C_4 plants. Whereas the course of MC chloroplast development at the onset of leaf senescence in maize hybrid simply follows that of its parent, this is not true for the BSC chloroplasts, where each genotype has its own distinguishable pattern of chloroplast development.

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