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Low-temperature induced changes in the ultrastructure of maize mesophyll chloroplasts strongly depend on the chilling pattern/intensity and considerably differ among inbred and hybrid genotypes

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Abstract

The ultrastructure and dimensions of chloroplasts in leaf mesophyll cells were quantitatively examined in three parental inbred lines of maize (*Zea mays* L.) and their four hybrids subjected to two types of four-week low-temperature (LT) treatment: the abrupt onset of chilling temperatures ("severe chilling", SC) and the gradual, more moderate one ("moderate chilling", MC). The relationship between the response of individual genotypes to one or the other type of chilling was analyzed as well as the possibility to predict the behaviour of chloroplasts in hybrids from that of their parents. Although selected parameters of chloroplast ultrastructure (*e.g.* volume densities of granal and intergranal thylakoids, plastoglobuli, and peripheral reticulum) and dimensions changed due to the exposure of maize plants to LT, no general pattern of such changes was found for this species due to the observed intraspecific variability. The response of some genotype to SC could not be predicted from its behaviour under MC (and *vice versa*) and no clear rules could be applied for the inheritance of chloroplast response to chilling in the general sense. Thus, great caution should be always taken when interpreting the results of studies aimed at the dissection of chloroplast ultrastructure as affected by LT, particularly in case such studies are made with one genotype or under one type of chilling only.

Additional key words: inheritance; peripheral reticulum; photosynthesis; plastoglobuli; stress; thylakoid; volume density.

Introduction

A majority of higher plants is negatively affected by environmental conditions that do not meet the requirements for optimum plant growth and development. Such conditions can induce diverse changes in plant morphology, anatomy, and physiology that manifest itself from the whole plant level to the cellular or sub-cellular ones. Changes in the structure and function of photosynthetic apparatus are usually classified among the early symptoms of plant stress (even in its mild or moderate form) and photosynthetic organelles—chloroplasts—act as the major cellular sensors of adverse environment. Virtually every abiotic stress factor has strong influence on chloroplasts and changes in chloroplast ultrastructure and development are commonly used as the evidence of plant stress. Various studies describe the ultrastructure of chloroplasts in plants stressed by high/low temperature (*e.g.* Jagels 1970, Taylor and Craig 1971, Kimball and Salisbury 1973, Smilie *et al.* 1978, Ristic and Cass 1992, Sharkova and Bubolo 1996, Hudák and Salaj 1999, Kratsch and Wise 2000, Vani *et al.* 2001, Semenova 2004, Sowinski *et al.* 2005, Xu *et al.* 2006, Holzinger *et al.* 2007), high/low irradiance (Jagels 1970, Wheeler and Fagerberg 2000), drought (Ristic and Cass 1992, McCain 1995, Yamane *et al.* 2003, Zellnig *et al.* 2004, Haisel *et al.* 2006), salinity (Khavari-Nejad and Mostofi 1998, Fidalgo *et al.* 2004, Yamane *et al.* 2004), nutrient or oxygen deficiency (Ladygin 2003, 2005), or soil pollution (Djebali *et al.* 2005, Li *et al.* 2006, Papadakis *et al.* 2007).

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Abbreviations: ANOVA – analysis of variance; F_1 – the first filial generation; LT – low temperature; MC – moderate chilling; MeC – mesophyll cell; SC – severe chilling; VD – volume density.

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Chilling temperatures (their range is usually given as 0–15 °C for plants of temperate origin and 10–25 °C for plants of tropical or subtropical origin—Hudák and Salaj 1999) cause various structural alterations of chloroplasts. The most common one is the swelling of thylakoid membranes, the diminishment or loss of grana, the diminishion of the total thylakoid volume, and (after the prolonged or strong chilling) even the disintegration of thylakoids (Taylor and Craig 1971, Smilie *et al.* 1978, Murphy and Wilson 1981, Wise *et al.* 1983, Gemel *et al.* 1986, Čiamporová and Trgiňová 1996, 1999, Hudák and Salaj 1999, Pinhero *et al.* 1999, Kratsch and Wise 2000, Kutík *et al.* 2004). Another change commonly associated with the exposure of plants to low temperature is the increase in the number and size or relative partial volume of plastoglobuli (Čiamporová and Trgiňová 1996, 1999, Hudák and Salaj 1999, Kutík *et al.* 2004). Reduction in the size and number of starch inclusions or their complete disappearance from chloroplasts has been also depicted as a symptom of chilling stress (Taylor and Craig 1971, Kimball and Salisbury 1973, Murphy and Wilson 1981, Musser *et al.* 1984, Čiamporová and Trgiňová 1996, Hudák and Salaj 1999, Kratsch and Wise 2000) but in some cases of long-term hardening of plants at chilling temperatures, the accumulation of starch has occurred (Hudák and Salaj 1999). Several studies of chloroplast ultrastructure in plants subjected to chilling describe the increase in the volume of peripheral reticulum (*i.e.* the structure arising from the inner membrane of chloroplast envelope, probably of transport significance—Taylor and Craig 1971, Wise *et al.* 1983, Kutík *et al.* 2004). The shape and size of photosynthetic organelles often change as well: chloroplasts become more rounded and sometimes swell up (Kimball and Salisbury 1973, Kratsch and Wise 2000, Kutík *et al.* 2004). Chloroplast stromuli (or protrusions) are absent or rare in cold conditions (Holzinger *et al.* 2007).

Although the above-described changes of chloroplast structure can be considered as the general symptoms of chilling stress, the specific way of their manifestation depends on several other factors as well. For example, the combination of low temperature (LT) and water deficiency increases the chloroplast injury, and the same is true for chilling in the light, as compared to the chilling in the dark (Jagels 1970, Kratsch and Wise 2000). Short-term or long-term exposures of plants to LT result in different changes of chloroplast ultrastructure, which means that the duration of cold and the possibility of chloroplast recovery under optimum temperatures is also important (Taylor and Craig 1971, Wise *et al.* 1983, Wu *et al.* 1997, Kratsch and Wise 2000). Some species are chilling-resistant and their plastids remain intact and more-or-less unaffected by LT, others show cold-hardening (acclimation) and gradually develop various mechanisms that enable their photosynthetic organelles to cope with such unfavourable conditions; thus, a wide interspecific variability exists in this respect (Taylor and Craig 1971, Kimball and Salisbury 1973, Wise *et al.* 1983, Gemel *et al.* 1986, Kratsch and Wise 2000). Intraspecific variability has been observed as well (though not so often) and several authors described the different response of chloroplast ultrastructure to chilling for individual genotypes or cultivars of one species (Čiamporová and Trgiňová 1996, 1999, Pinhero *et al.* 1999, Kutík *et al.* 2004).

For some time, our laboratory has been engaged in the monitoring of chloroplast structure and function in stressed plants, focusing on the intraspecific variability in these characteristics, particularly the differences among inbred and hybrid genotypes (Körnerová and Holá 1999, Holá *et al.* 2003, 2004, 2007, Pechová *et al.* 2003, Kutík *et al.* 2004). Working with maize (*Zea mays* L.), we have observed not only the superiority of F_1 hybrids over their inbred parental lines regarding their photosynthetic efficiency under chilling conditions, but—perhaps more interestingly—the dependence of such genotypic differences on the pattern of chilling, *i.e.* whether the LT-stress is induced rapidly or gradually (Holá *et al.* 2003). This paper presents the results of the parallel study made with the purpose to ascertain: (*1*) whether such dependence (previously observed for some functional photosynthetic parameters) is shown also for the structural parameters of chloroplasts, (*2*) whether the changes in chloroplast ultrastructure resulting from the abrupt exposure of plants to LT and characteristic for individual genotypes are related to those observed in plants of the same genotypes subjected to more moderate type of chilling, and (*3*) whether the response of F_1 hybrids to such chilling patterns could be predicted from the response of their parental inbred lines.

Materials and methods

The ultrastructure of chloroplasts was studied in mesophyll cells of mature leaves of three maize (*Zea mays* L.) inbred lines (2013, CE704, and CE810) and their F_1 hybrids (2013×CE810, CE704×CE810, CE810×2013, and CE810×CE704; the maternal parent being the first genotype of the respective cross). The inbred lines differed in photosynthetic and yield performance and all hybrids were characterized by a positive heterotic effect when grown in the field (Holá *et al.* 1999). The kernels of all genotypes were obtained from Maize Breeding Station CEZEA in Čejč, Czech Republic. Plants were cultivated in planting dishes with garden soil, placed firstly in the glasshouse with optimum temperature (24–27/16–20 °C, day/night) till the appearance of the first leaf (*i.e.* 9–10 d after the sowing date). After that, the seedlings were divided into two groups of equal size representing two temperature treatments: one remained in the optimum conditions (control), the other was transferred to chilling conditions. Their cultivation then proceeded for 4 weeks. Two patterns of chilling were used: (*1*) "severe chilling" (SC), *i.e.* the abrupt onset of low temperature (15–23/1–8 °C, day/night) followed by the slow increase in the day temperatures (till 22–30/1–8 °C, day/night) during the 4-week period of plant cultivation; and (*2*) "moderate chilling" (MC), *i.e.* the gradually decreasing temperatures (from 22–30/11–13 °C, day/night at the beginning to 15–23/4–9 °C, day/night at the end of 4-week period). During the whole time, plants were well-watered with tap water, the relative air humidity was kept between 60–80/90–100 % day/night, and no additional irradiation was applied.

Four experimental series were made and each experimental variant (genotype/temperature treatment/chilling pattern) was originally represented by 60–80 plants (the majority of these plants was used for the biochemical studies of photosynthetic parameters, that took place at the same time; for results of these studies see Holá *et al.* 2003) and 4 plants representative of each variant were randomly chosen from this number for the analysis of chloroplast ultrastructure. The youngest mature (*i.e.* fully developed) leaf from each plant was used for the collection of samples, *i.e.* the third or the fourth one from the control plants and the second or the third one from the chilling-stressed plants. According to Bongard-Pierce *et al.* (1996), the initial maize leaves up to the fourth one display juvenile features and are all formed during embryo development (Vega *et al.* 2002).

Several small pieces (approximately 4 mm^2) were cut from the middle third of each leaf blade as the samples for the transmission electron microscopy. They were prepared according to the standard procedure (Kutík *et al.* 1999): the double fixation with glutaraldehyde followed by osmic acid treatment, the dehydration through ethanol/propylene oxide series, and the embedding into Spurr´s low viscosity resin. For the orientation in the embedded objects, semithin sections (thickness about 1 µm) were stained by toluidine blue solution. Chloroplast ultrastructure was evaluated on transverse ultrathin sections (thickness approximately 60–90 nm) of the objects contrasted with uranyl acetate followed by lead citrate solution (Kutík *et al.* 1999). The transmission electron microscopes *Philips EM 300* and (later) *Philips EM 268 Morgagni* (*Philips*, The Netherlands) were used at the primary magnification of about 7 000×. The length and the width of nearly median cross-sections of mesophyll cell (MeC) chloroplasts were determined on electron microphotographs at the final magnification of about 30 000× and the approximate volume of chloroplast was then calculated from these parameters as the volume of circular ellipsoid. On the same microphotographs, the volume densities VD (relative partial volumes) of main chloroplast compartments—granal and intergranal thylakoids, peripheral reticulum, starch inclusions (those were usually absent and, subsequently, were not included in the analysis), plastoglobuli, and stroma (including also the small periplastidial space between the outer and the inner chloroplast envelope membrane)—were evaluated using stereological grids with regularly distributed points. This evaluation was made after simplified Weibel (1979) formula [$Est.\ V_v = P_\alpha/P_\beta$, where $Est.\ V_v$ is the estimated mean VD of a particular component (*e.g.* system of thylakoids), P_α is the number of test points from a square lattice grid falling on the structural profile of this component, and P_β is the total number of test points falling within the boundary of higher order structural profile (*e.g.* chloroplast cross section)]. For each sample, five chloroplast sections were evaluated. Consequently, 20 MeC chloroplasts (chosen at random from several mesophyll cells) were analysed for each experimental variant.

The possible sources of the intraspecific variability in MeC chloroplast ultrastructure and dimensions were initially assessed by 3-way ANOVA with 5 % probability level as the upper limit of statistical significance. The response of these parameters to chilling treatment was then expressed for each genotype as the ratio of the mean value of the respective parameter recorded under chilling to the appropriate mean value in the control (separately for both chilling patterns), and these ratios were used for the calculation of Pearson's correlation coefficients in order to examine the possibility of the relationship between the response of individual genotypes to SC or MC or the existence of some correlations between the examined genotypes. All statistical evaluations were made with the *CoStat* (version *6.204*) statistical software (*CoHort Software*, Monterey, CA, USA).

Results

The exposure of young maize plants to LT had a profound effect on the ultrastructure of chloroplasts in the MeCs of their leaves. Several changes usually associated with the unfavourable environment were observed: the decrease in the VD of thylakoids (mostly granal, but often intergranal ones as well), the increase in the VD of plastoglobuli and peripheral reticulum (Table 1, Fig. 1*A–C*). However, the variability in these parameters was due not only to the temperature treatment in itself, but also to the differences between SC and MC conditions (with the exception of the VD of intergranal thylakoids and the ratio of the length and the width of chloroplast cross-sections), and to the differences between genotypes examined (with the exception of the parameters concerning the thylakoid membranes) (Table 2). Moreover, the first- and the second-degree interactions between these three sources of variation were usually also statistically significant (Table 2), implying that MeC chloroplasts show distinct response to low temperature depending on the type of chilling treatment and that individual genotypes of maize respond differently to chilling both in the general sense and in relation to the chilling pattern.

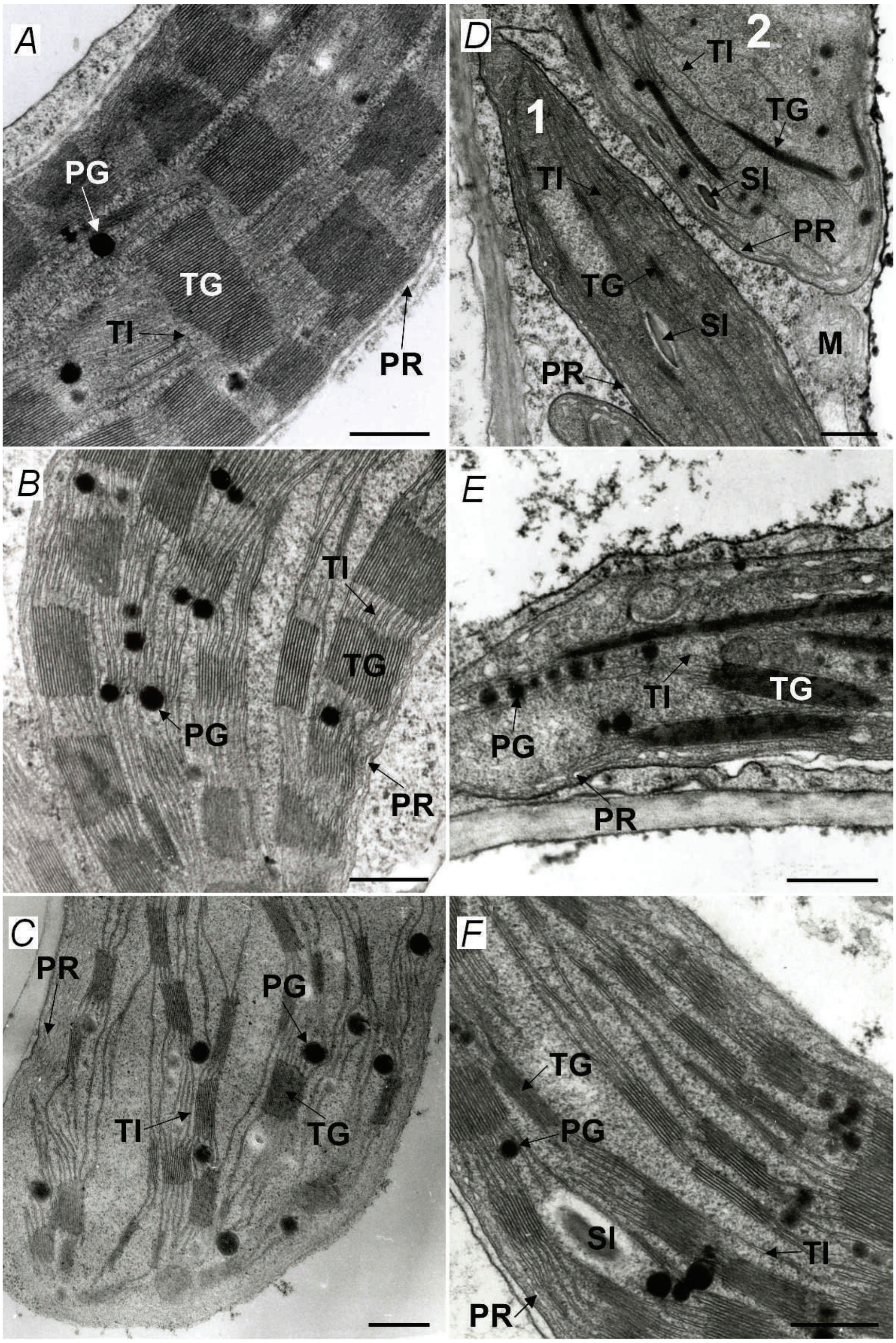

Fig. 1. Transmission electron micrographs of nearly-median chloroplast cross sections taken from leaf mesophyll cells (with the exception of *D*) of maize plants of F1 hybrid CE810×CE704 (*A*–*C*) or parental inbred line CE810 (*D*–*F*). Plants were grown either under optimum conditions (*A*) or subjected to the "moderate chilling" treatment (*B*) or the "severe chilling" treatment (*C*–*F*). Disruptions of chloroplast ultrastructure (particularly largely altered grana) were very rarely seen both in bundle-sheath cell chloroplasts (*D*) and mesophyll cell chloroplasts (*E*). Starch inclusions in the mesophyll cell chloroplasts were infrequent (*F*). 1 – nearly normal bundle sheath cell chloroplast, 2 – bundle sheath cell chloroplast with much altered ultrastructure. M – mitochondrion, PG – plastoglobulus, PR – peripheral reticulum, SI – starch inclusion, TG – granal thylakoids, TI – intergranal thylakoids. Bar = 0.5 µm.

Table 1. The response of selected parameters of ultrastructure and dimensions of chloroplasts in leaf mesophyll cells of maize to low temperature conditions. The percentages of the mean value of the respective parameter recorded under chilling to the appropriate mean value under control conditions are shown for three inbred (2013, CE704, CE810) and four hybrid (2013×CE704, CE704×CE810, CE810×2013, CE810×CE704) genotypes of maize grown under two chilling patterns ("severe chilling" – SC, and "moderate chilling" – MC). The statistical significance of the differences between the mean values recorded under chilling *vs*. control conditions is indicated by * and ** (0.05 and 0.01 levels of statistical significance, respectively). TG – granal thylakoids, TI – intergranal thylakoids, TT – total thylakoids, PG – plastoglobuli, PR – peripheral reticulum (or, more precisely, the volume densities of the respective chloroplast compartments), Gr – granality (*i.e.* the ratio of granal to total thylakoids' volume densities), L/W – the ratio of the length and the width of nearly median chloroplast cross-sections, V – the approximate chloroplast volume.

	Genotype	TG	TI	TT	PG	PR	Gr	L/W	V
SC	2013	80.18*	84.34	81.91*	73.87*	128.83*	97.40	89.43	54.43*
	CE704	77.89*	81.44*	79.27*	134.44	202.45*	99.02	80.87*	106.69
	CE810	72.49**	82.38**	76.11**	95.79	146.70**	95.13	88.23*	121.32*
	2013×CE810	78.40*	91.47	83.52*	132.29	122.22*	93.78	109.11	108.55
	CE704×CE810	74.34*	97.07	82.56*	155.11*	131.50*	90.84*	106.26	146.11*
	CE810×2013	89.91	96.39	92.42*	133.70*	129.82*	97.17	104.03	109.67
	CE810×CE704	67.79*	77.13*	71.29*	166.67*	155.37*	95.93	71.75*	178.90
MC	2013	84.88*	73.10*	80.50*	160.73*	135.32*	104.32	91.28	157.49*
	CE704	72.33*	86.07*	77.63*	110.13	123.66	92.78*	85.69*	142.17*
	CE810	80.83**	91.74*	84.82**	151.28**	125.93*	94.52*	90.27	80.37
	2013×CE810	85.66*	85.34	85.54*	133.33*	117.19	100.40	95.27	110.44
	CE704×CE810	98.39	84.19*	92.73*	105.16	119.42	106.26	95.83	136.12*
	CE810×2013	89.46*	99.88	93.00*	152.81*	132.27*	96.30	107.72	149.03*
	CE810×CE704	95.90	95.08	95.59	79.46	67.17	100.06	97.46	135.26*

Table 2. The results of three-way ANOVA applied to the selected parameters of chloroplast ultrastructure and dimensions in mesophyll cells (for abbreviations see legend to Table 1). Plants of three inbred and four hybrid genotypes of maize were grown under two chilling patterns ("severe chilling", SC and "moderate chilling", MC), each comprising two temperature treatments (control, *i.e.* optimum, or low temperature). The probability levels for the individual sources of variation are shown.

Source of variation	TG	TI	TT	PG	PR	Gr	L/W	V
Chilling pattern (P)	0	0.234	0	0	0	0	0.054	0
Temperature treatment (T)	0	0	0	0	0	0.012	0	0
Genotype (G)	0.260	0.286	0.112	0	0	0.464	0	0
P×T	0.004	0.943	0.007	0.940	0	0.085	0.477	0.038
P×G	0.064	0	0	0	0	0.015	0	0
T×G	0.034	0.103	0	0.434	0.043	0.709	0.001	0.005
P×T×G	0.009	0.193	0.002	0	0	0.140	0.089	0

The inbred line 2013 showed significant decrease in the VD of thylakoid membranes to about 80 % of the control values, both under SC and MC. Its granality, *i.e.* the ratio of granal to total thylakoid VDs, did not suffer from LT (Table 1). The change of MeC chloroplast shape towards more rounded one (reflected by the decrease in the ratio of the length and the width of chloroplast cross-sections) was discernible but statistically non-significant in this inbred line and there were no differences between both patterns of chilling treatment regarding this parameter (Table 1). The VD of peripheral reticulum significantly increased (to approx. 130 % of the values observed for the control plants) due to the chilling; again, there were no differences between SC and MC (Table 1). However, the response of the approximate volume of MeC chloroplasts to LT strongly depended on the severity of chilling stress in this genotype: the values of this parameter significantly decreased after the SC (to about 60 % of the control) but increased (to about 160 % of the control) after the MC, and similar response was observed also for the VD of plastoglobuli (Table 1).

The response of the second inbred line examined (CE704) to LT differed in several aspects from that shown by the 2013 genotype (Table 1). The decrease in the thylakoid VD (independent of the chilling pattern) and the decrease in the ratio of the length and the width of MeC chloroplast cross-sections (in this case statistically significant) were also observed, as well as the increase in the VD of peripheral reticulum. However, this increase was markedly higher after the SC treatment (about 200 % of the control values) compared to the MC treatment (about 120 % of the control). The VD of plastoglobuli increased after either type of LT treatment (more after the SC) but these changes were not statistically significant.

Regarding the changes in the volume of MeC chloroplasts, the values of this parameter significantly increased only after the MC treatment but not after the SC one.

The inbred line CE810 showed still another type of response (Table 1), particularly regarding the approximate volume of MeC chloroplasts (that increased to about 120 % of the control values after the SC treatment and decreased to about 80 %—albeit non-significantly—after the MC) and the VD of plastoglobuli (increase to about 150 % of the control after the MC, no difference from the control in the SC series). The increase in the VD of peripheral reticulum was more pronounced after the SC compared to the MC, the same applied for the decrease in the thylakoid VD.

As regards F_1 hybrids, no general trend in the changes of the ultrastructure, size, or shape of their MeC chloroplasts due to LT was observed and each one responded differently to chilling in general and to SC or MC as well (Table 1). The VD of granal thylakoids decreased after the SC treatment in all four hybrids examined, but in the CE810×2013 hybrid this change was statistically insignificant and amounted only to about 90 % of the control values (contrary to the other hybrids, where the decrease was more pronounced). The values of this ultrastructural parameter recorded for the hybrid plants subjected to the MC were either similar to the control ones (in the CE704×CE810 and CE810×CE704 hybrids) or slightly but significantly lower (in the other two hybrids). For the VD of intergranal thylakoids, the situation was different: no change of this parameter due to either type of the chilling treatment was observed in the 2013×CE810 and CE810×2013 hybrids, the genotype CE810×CE704 showed significant decrease of the VD of intergranal thylakoids (to about 70 % of the control) after SC but no change after the MC, whereas the reverse was true for the CE704×CE810 hybrid. The granality was usually not affected by LT in either of the F_1 hybrids or chilling patterns examined.

The differences between hybrids and the differences between their responses to both patterns of chilling were even more marked for the other two parameters of MeC chloroplast ultrastructure (Table 1). The 2013×CE810 hybrid showed a similar increase in the plastoglobuli VD (to about 135 % of the control) or the VD of peripheral reticulum (to about 120 % of the control) under both types of chilling. Its reciprocal hybrid CE810×2013 displayed similar (if slightly more pronounced) response as regards the VD of peripheral reticulum, but the VD of its plastoglobuli increased more after MC compared to SC. The response of both F_1 hybrids that had the CE704 inbred line as one of their parents was opposite: high increase (usually to about 160 % of the control values) of both peripheral reticulum and plastoglobuli VDs due to the SC, no significant increase (or, in the CE810×CE704 hybrid, even statistically significant decrease) of these parameters due to the MC.

No statistically significant changes were usually observed in the ratio of the length and the width of chloroplast cross-sections (with the exception of MeC chloroplasts in leaves of the CE810×CE704 hybrid, that were significantly more rounded after SC treatment compared to the control plants), but there were again significant differences in the approximate chloroplast volume (Table 1). This parameter did not respond to either type of chilling in the F_1 hybrid 2013×CE810, but it significantly increased after the MC but not after the SC in the CE810×2013 genotype, significantly, but rather similarly increased under both chilling patterns in the CE704×CE810, and increased to a higher degree after SC compared to the MC in its reciprocal hybrid.

Although MeC chloroplasts in leaves of plants subjected to LT showed various changes of their ultrastructure or dimensions, these changes usually were merely quantitative and chloroplasts with greatly disrupted system of thylakoid membranes were seen only very rarely (and only under SC) (Fig. 1*E*). In some cases, such damaged chloroplasts were observed also in bundle sheath cells (Fig. 1*D*). Starch was seen only exceptionally in MeC chloroplasts under chilling (Fig. 1*F*) and the majority of MeC chloroplasts studied did not contain any starch inclusions.

To ascertain whether there is a relationship between the response of MeC chloroplast ultrastructure to SC and MC, the values of Pearson‘s correlation coefficient were calculated for all parameters examined, separately for the inbreds and F_1 hybrids. This analysis (Table 3) indicated that almost no such correlation exists, with the possible exception of the VD of plastoglobuli or peripheral reticulum where it seems that the response of these parameters to SC could be inversely related to the response

Table 3. The relationship between the response of inbred or hybrid maize genotypes to “severe chilling”, SC and “moderate chilling”, MC as determined by the analysis of the selected parameters of mesophyll cell chloroplast ultrastructure and dimensions (for abbreviations see legend to Table 1). The ratios of the mean value of the respective parameter recorded under SC or MC to the appropriate mean value under control conditions were used as the initial values for the correlation analysis. The values of Pearson’s correlation coefficient ± its standard error [r±SE(r)] and the respective probability levels (*p*) are shown.

Parameter	Inbreds r±SE(r)	*p*	Hybrids r±SE(r)	*p*
TG	0.092±0.996	0.941	–0.587±0.573	0.413
TI	–0.811±0.585	0.398	–0.248±0.685	0.752
TT	–0.638±0.771	0.560	–0.329±0.668	0.671
PG	–0.982±0.188	0.120	–0.958±0.202	0.042
PI	–0.809±0.587	0.400	–0.903±0.304	0.097
Gr	–0.044±0.999	0.972	–0.958±0.203	0.042
L/W	0.999±0.041	0.026	0.083±0.705	0.917
V	–0.797±0.604	0.413	0.200±0.693	0.800

Table 4. The relationship between three inbred (2013, CE704, CE810) and four hybrid (2013×CE704, CE704×CE810, CE810×2013, CE810×CE704) maize genotypes, as determined by the analysis of the response of the selected ultrastructural parameters of leaf mesophyll cell chloroplasts (the volume densities of granal and intergranal thylakoids, plastoglobuli and peripheral reticulum) to two types of chilling treatment. The ratios of the mean value of the respective parameter recorded under "severe chilling", SC or "moderate chilling", MC to the appropriate mean value under control conditions were used as the initial data for the correlation analysis. The values of Pearson's correlation coefficient ± its standard error [r±SE(r)] are shown in the upper half of the table (above diagonal), the respective probability levels (p) are shown in the lower half of the table (below diagonal).

	2013	CE704	CE810	2013×CE810	CE704×CE810	CE810×2013	CE810×CE704
2013		0.468±0.361	0.914±0.166	0.645±0.312	0.209±0.399	0.767±0.262	–0.149±0.404
CE704	0.243		0.726±0.281	0.703±0.291	0.729±0.280	0.639±0.314	0.698±0.292
CE810	0.002	0.041		0.794±0.248	0.446±0.365	0.872±0.200	0.211±0.616
2013×CE810	0.084	0.052	0.019		0.823±0.232	0.966±0.106	0.504±0.353
CE704×CE810	0.619	0.040	0.268	0.012		0.664±0.305	0.785±0.253
CE810×2013	0.026	0.088	0.005	0	0.072		0.322±0.387
CE810×CE704	0.724	0.054	0.616	0.203	0.021	0.437	

shown to MC (however, the correlation coefficients, though of rather high values, were not statistically significant).

Another correlation analysis was made for the four main parameters of MeC chloroplast ultrastructure (*i.e.* the VDs of granal and intergranal thylakoids, plastoglobuli, and peripheral reticulum) with the purpose to find out whether the response of F_1 hybrids to SC or MC follows that of their parents. In about half of the cases, there was a significant correlation between one of the hybrids and its maternal (usually) or paternal parent, but considering that several such correlations were found also for the unrelated genotypes (*e.g.* between inbred lines 2013 and CE810 or CE704 and CE810), it appears that no particular parent-hybrid relationship regarding the response of young maize plants to chilling probably exists (Table 4).

Discussion

Changes in chloroplast structure and function are usually regarded as a good indicator of plant stress and various features of chloroplasts have been described as distinctive signs of the exposure of a plant to some unfavourable environmental factor. A general consensus exists in the scientific literature dealing with this topic, indicating that chloroplasts in a plant stressed by a prolonged chilling should display disturbances of the thylakoid membranes, the increased number and/or size of plastoglobuli, the greater volume of peripheral reticulum, that they should swell up, and that their shape should change from flattish to a more rounded one (Hudák and Salaj 1999, Kratsch and Wise 2000, Kutík *et al.* 2004). Thus far, the picture seems to be rather clear and unambiguous. However, several studies have pointed out that the combination of chilling with another stress factor, the interruption or the following of chilling period by a period/s of more normal temperatures, or the inclusion of a greater collection of genotypes into the analyzed material can—often substantially—change this picture and present a more intricate view of the response of chloroplast ultrastructure to low temperature (Kratsch and Wise 2000, Kutík *et al.* 2004).

In our previous studies of chloroplast ultrastructure made with various maize genotypes we have observed significant variability in the development of chloroplasts and the response of individual genotypes to unfavourable environment (Pechová *et al.* 2003, Kutík *et al.* 2004). Such variability was often accompanied by differences in chloroplast function as well, and could change depending on the growing conditions the plants were subjected to (Pechová *et al.* 2003, Holá *et al.* 2004, 2007). During the study of the response of photosynthetic apparatus to LT we found that the relationship between various genotypes regarding the function of main photosynthetic complexes of their thylakoid membranes (*i.e.* photosystems 1 and 2) under abruptly induced chilling stress is different from the relationship observed under more moderate, gradual chilling (Holá *et al.* 2003). To get more information about this phenomenon, we made further analysis in order to ascertain whether this applies also for parameters of chloroplast structure and dimensions. Our results confirm this expectation and show that the response of chloroplast structure to LT needs not necessarily follow the customary course, the whole situation being more interesting as the plants were subjected to rather long period of chilling that should indeed induce the ultrastructural changes usually described in the literature. Moreover, the genotypes we examined were not originally regarded as being particularly different in their sensitivity/tolerance to chilling.

The only type of chilling-caused ultrastructural change of mesophyll chloroplasts that could be described as a more generally applicable (observed in all genotypes and both chilling patterns) and that agreed with the data presented in other studies was the decrease in the amount of thylakoid membranes. However, even there were some differences between both chilling patterns as well as among genotypes, as the response of some F_1 hybrids to abrupt exposure to LT was less pronounced compared to more slow MC. This could be perhaps caused by the ability of these plants to gradually acclimate to the unfavourable environment, so that their photosynthetic apparatus should have time to "prepare" for the approaching sub-optimum temperatures. As the inbred lines often show worse response of photosynthetic apparatus to chilling compared to their hybrids (probably due to their homozygosity) (Du *et al.* 1999, Fracheboud *et al.* 1999, Körnerová and Holá 1999, Holá *et al.* 2003, 2007), this would not apply so much for the parental genotypes we also examined. The granal thylakoids were usually affected more than the intergranal ones, which agrees well with our previous findings (as well as those of other authors) that the activity of photosystem 2 (located predominantly in the grana) decreases more strongly than the activity of photosystem 1 under SC stress (Allen and Ort 2001, Holá *et al.* 2003).

As regards other ultrastructural changes usually described as characteristic for the LT-induced stress, we cannot justly confirm their general applicability. Consider *e.g.* the expected increase in the VD of plastoglobuli: though we certainly found this to be true for some of our genotypes, the other ones displayed no changes or even significant decrease of this parameter (even in the relatively small genotypic set we have examined). Moreover, several genotypes showed one type of response under SC and the opposite response under MC. The same applied for the VD of peripheral reticulum and for the changes in chloroplast size or shape. Consequently, due to this great genotypic variability we could draw no general inferences regarding the behaviour of MeC chloroplasts in leaves of plants subjected to different chilling patterns and we suggest an extreme caution in interpreting the results of similar studies made with only one or even two or three genotypes.

As we have found no significant correlations between the changes of MeC chloroplast ultrastructure or dimensions, caused by SC and MC, no predictions could be really made for the response of some genotype to *e.g.* SC based on its response under the other type of chilling. What could be perhaps more important from the practical point of view is our finding that no general rules apply for the inheritance of the chloroplast response to chilling, either. There was no particular correlation between parents and their progeny regarding this (or, more precisely, similar correlation as that found between related genotypes was also found between the unrelated ones). The expression of the genetic effects participating in the determination of MeC chloroplast ultrastructure in parents and their progeny strongly depended on the type of chilling we have applied, and we have observed similar phenomenon for various functional photosynthetic characteristics including the parameters often considered as suitable secondary selection traits (unpublished). Thus, even if some genotype should be identified as a good genetic donor in a breeding programme aimed at the improvement of chilling tolerance based on the results of a study of photosynthetic parameters made in one type of LT-conditions, our prediction would be that it will probably behave differently under another chilling pattern and that the passing of such tolerance to its progeny (and its expression in the descendant genotypes) is by no means guaranteed.

Our results and conclusions can be perhaps summarized as follows: though both inner structure and dimensions of MeC chloroplasts in maize leaves certainly change due to the exposure of plants to LT, no pattern of such changes can be found that would be generally applicable for this species (due to great intraspecific variability), no prediction of the response of some genotype to one type of chilling can be made from its behaviour under different chilling pattern, and no unambiguous rules apply for the inheritance of chloroplast response (at least as regards their structure and dimensions) to chilling in the general sense. The results of studies aimed at the dissection of chloroplast ultrastructure as affected by LT should be therefore always interpreted very cautiously.

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