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The ultrastructure of chloroplasts, content of photosynthetic pigments, and photochemical activity of maize (*Zea mays* L.) as influenced by different concentrations of the herbicide amitrole

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Abstract

The effect of three different concentrations of amitrole (AM), a bleaching herbicide affecting carotenogenesis, on chloroplast ultrastructure, photosynthetic pigment contents, and photochemical activity was studied in two maize genotypes differing in photosynthetic characteristics. The content of photosynthetic pigments in leaves of plants treated with low (20 μM) AM concentration was similar to control plants and no damaging effect of the herbicide on the ultrastructure of either mesophyll (MC) or bundle-sheath (BSC) cell chloroplasts was observed. Higher (60 and 120 μM) concentrations of AM caused a significant decrease in the content of carotenoids (especially xanthophylls), which was followed by photooxidative destruction of chlorophylls and some alterations of chloroplast ultrastructure. MC chloroplasts appeared more sensitive to the damaging effect of AM compared to BSC chloroplasts. A significant decrease in the amount of both granal and intergranal thylakoids in MC chloroplasts was observed with the increasing concentration of AM. As regards BSC chloroplasts, rapid decrease in the volume density of starch inclusions was found in plants treated with higher concentrations of AM. When 120 μM AM was used, both MC and BSC chloroplasts contained just a few thylakoid membranes that were strongly altered. The changes in the ultrastructure of MC chloroplasts were accompanied by the changes in their photochemical activity. The formation of chloroplast protrusions after treatment of plants with AM as well as in control plants was also observed.

Additional key words: 3-amino-1,2,4-triazole; carotenoids; chlorophylls; Hill reaction; photosystems 1 and 2.

Introduction

The photosynthetic apparatus localised in chloroplasts is one of the most sensitive systems to various stress factors, both environmental and anthropogenic. Carotenoids (Cars) belong to the most important protective components of photosynthetic pigment-protein complexes. Their function in chloroplast thylakoid membranes is complex (see, *e.g.*, Demmig-Adams *et al.* 1996). Their ability to absorb photons (especially in the blue region of spectrum) enables them to act as additional light-harvesting pigments besides chlorophylls (Chls). They are also necessary for the assembly and stabilisation of various complexes of thylakoid membranes (namely photosystem 2

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Abbreviations: AM, amitrole (3-amino-1,2,4-triazole); BSA, bovine serum albumin; BSC, vascular bundle sheath cell; Car, carotenoid; Chl, chlorophyll; DCMU, 3-(3`,4`-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; HPLC, high performance liquid chromatography; HRA, Hill reaction activity; LHC, light-harvesting complex; MC, mesophyll cell; PAR, photosynthetically active radiation; PBS, buffer used in immunocytochemistry; PS, photosystem.
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and LHC), where they fulfil an important structural role (Hoober and Eggink 1999, Phillip *et al.* 2002). However, their most important function in the chloroplast is probably the protection of photosynthetic apparatus from photodestruction caused by strong irradiance or other stress factors (Siefermann-Harms 1987). Their importance for successful development and function of the photosynthetic apparatus is clearly obvious when working with Car-deficient plants. Such plants can be obtained either by treatment with various so-called bleaching herbicides (*e.g.* amitrole, norflurazon, fluridone, flurtamone, and others; see, *e.g.* Sandmann and Böger 1982) or as Car-free mutants, such as *vp9* and *vp2* of maize (*Zea mays* L. – La Rocca *et al.* 2000a), *im* of *Arabidopsis thaliana* L. – Wetzel *et al.* 1994), or *albino* mutant of sunflower (*Helianthus annuus* L. – Fambrini *et al.* 1993).

Amitrole (AM, 3-amino-1,2,4-triazole) can be successfully used to obtain plants with damaged chloroplasts and non-functional photosynthetic apparatus. The effect of this herbicide on plant protective systems seems to be manifold. For example, the inhibition of catalase followed by increased concentration of H_2O_2 in peroxisomes was observed in potato (*Solanum tuberosum* L.) plants treated with AM (Muraja-Ljubičić *et al.* 1999). However, the most conspicuous changes due to the application of this herbicide were found in the content of Cars. AM inhibits cyclisation of lycopene, which leads to the accumulation of its precursors (phytoene and phytofluene; La Rocca *et al.* 2001). The formation of β-ionone or ε-ionone rings is a necessary step in the conversion of lycopene to either β-carotene (and subsequently to zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin) or α-carotene (which is a precursor of lutein; Hirschberg 2001). Therefore the inhibition of this reaction can have dangerous consequences for the structure and function of the whole photosynthetic apparatus. This interruption of the Car synthetic pathway affects negatively the other photosynthetic pigments as well; in the absence of β-carotene the degradation of Chls occurs (Wrischer *et al.* 1992).

The changes in synthesis and/or degradation of Cars and Chls described above are usually followed by changes in the structure of thylakoid pigment-protein complexes, which in turn are associated with alterations in the ultrastructure of chloroplasts. These alterations depend on the concentration of herbicide used, irradiance (Anderson and Robertson 1960), and temperature (Rascio *et al.* 1996, La Rocca *et al.* 1998, 2001). However, most studies concerned with the changes in chloroplast ultrastructure due to AM deal only with the effects of high AM concentrations (125 μM AM: Zito *et al.* 1995, Agnolucci *et al.* 1996, Rascio *et al.* 1996; 200 μM AM: La Rocca *et al.* 2000a,b, Dalla Vecchia *et al.* 2001). Such concentrations lead to the nearly complete destruction of chloroplasts. The use of lower concentrations of AM should facilitate the production of plants with different amounts of Cars, and the correlation between the contents of photosynthetic pigments and the ultrastructure of chloroplasts could therefore be analysed by more subtle methods.

Maize, as a typical C_4 plant belonging to NADP-ME group, exhibits so-called Kranz-type anatomy of leaves with characteristic presence of two distinct cell types (mesophyll and bundle sheaths) containing chloroplasts which differ in many parameters. The possible differences in response of these two types of chloroplasts to various degrees of Car deficiency and its relationship to photosynthetic activity have not—as far as we know—yet been described. The main purpose of this work was therefore to prepare (by the use of AM) maize plants differing in the amount of photosynthetic pigments (especially Cars), to analyse the changes in ultrastructure of MC and BSC chloroplasts in leaves of these plants, and to correlate the level of chloroplast destruction to photosynthetic processes which take place there.

Materials and methods

Plants: Seeds of two genotypes (CE704 and CE810) of maize (*Zea mays* L.) were obtained from the Maize Breeding Station CEZEA in Čejč (Czech Republic). They were germinated for 24 h in water and then placed in nutrient solution (Hoagland 3) with different concentrations of amitrole (20, 60, and 120 μM) or without AM (control plants). Plants were grown in a growth chamber (*Klimabox RK1-007, Kovodružstvo Slaný*, Czech Republic) with the duration of light period 16 h, temperature 21/16 °C, relative humidity 70/80 %, and irradiance 400 μmol m^{-2} s^{-1} PAR. 18 d after germination of seeds, leaf tissue samples were taken from the middle third of leaf blade of the mature third leaves. These samples were used for the transmission electron microscopy and stereological analysis of chloroplast ultrastructure, polarographic measurement of photochemical activity of isolated chloroplasts, and the determination of the contents of photosynthetic pigments.

Analysis of chloroplast ultrastructure: Leaf blade samples were double fixed (glutaraldehyde/osmic acid), dehydrated in ethanol/propylene oxide series, and embedded into Spurr's low viscosity resin. Chloroplast ultrastructure was evaluated on transverse ultra-thin sections of embedded objects contrasted with uranyl acetate followed by lead citrate treatment (Kutík *et al.* 1999) using a transmission electron microscope *Philips EM 300*. The volume densities (relative partial volumes, Gundersen and Jensen 1987) of five chloroplasts for each sample were counted stereologically on microphotographs using morphometric grids with regularly distributed points as described by Kutík *et al.* (1999). Four plants were

evaluated for each variant. In MC chloroplasts, the volume densities of granal and inter-granal thylakoids, peripheral reticulum, starch inclusions, and plastoglobuli were measured and the remaining volume of stroma was counted into 100 %, whereas in BSC chloroplasts only the volume densities of starch inclusions and plastoglobuli were determined. The differences between variants were tested by the Mann-Whitney test, using the 5 % level of statistical significance.

Immunocytochemical analysis: The leaf blade samples were fixed for 2 h in 4 % paraformaldehyde and 0.25 % glutaraldehyde fixative in 0.1 M cacodylate buffer, dehydrated in ethanol, and embedded into *London Resin White* medium. The ultra-thin sections picked up on nickel grids were incubated for 10 min on 1 % bovine serum albumin (BSA) in PBS and treated with the mouse primary antibody against the apoprotein of LHC2 (which labelled both LHC1 and LHC2). After washing with 1 % BSA in PBS, the sections were incubated with colloid gold conjugated with goat-anti-mouse antibodies. The sections were then washed and stained with uranyl acetate for 10 min and examined with the electron microscope (*CM-10 Philips*). Control experiments were performed similarly, but the incubation of the ultra-thin sections with the primary antibody was excluded.

Photochemical activity of chloroplasts: The analysis of photochemical activity of isolated MC chloroplasts was performed as described in Körnerová and Holá (1999). Hill reaction activity (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 added as an artificial electron-acceptor, and a constant temperature of 25 °C was maintained in the measurement chamber (Bartoš *et al.* 1975). Activity of PS1 was measured similarly as the consumption of oxygen by the suspensions of isolated MC chloroplasts. 0.15 mM reduced DCPIP was used as an artificial donor of electrons and 0.1 mM methyl viologen as an electron acceptor. The inhibitor of PS2 activity in this case was 0.1 mM DCMU. Neither HRA nor PS1 activity was analysed in plants treated with 120 μM AM due to the apparent absence of photosynthetic pigments in MC chloroplasts in those plants.

Contents of photosynthetic pigments were determined in acetone extracts of leaf discs by HPLC (*Spectra-Physics,* San Jose, USA) using a reverse phase column (*Sepharon* SGX C18, 5 μm particle size, 150×3 mm, *Tessek,* Prague, Czech Republic). The solvent system was acetonitrile : methanol : water (80 : 12 : 6) followed by 100 % methanol, and the gradient was run from 8 to 12 min. The flow rate was 16.7 mm^3 s^{-1}, the detection wavelength was 445 nm.

Results and discussion

The negative effect of AM was observed almost from the beginning of plant development. As expected, the damage was most pronounced in plants treated with the highest (120 μM) concentration of AM, whereas application of lower concentrations of herbicide (20 or 60 μM) resulted in less perceptible changes. 120 μM AM-treated plants were generally much smaller and could not be cultivated longer than about 18 d, which was obviously due to the fact that the organic nutrients stored in grains were completely consumed at this time. Under normal conditions, the beginning of photosynthetic activity and synthesis of new, energetically rich metabolites would soon compensate loss of these nutrients (used in the first phases of plant development). However, as the application of AM strongly diminishes the amount of photosynthetic pigments in leaves, the consequent damage to photosynthetic apparatus can be severe and no functional chloroplasts might be present in plants treated with high concentration of herbicide. This, of course, would ultimately result in a premature death of plants, as observed in our case.

Differences in the amount of photosynthetic pigments in leaves due to the application of different concentrations of AM were already seen in the outward appearance of plants. Both control and 20 μM AM-treated plants had fully green leaves, whereas the leaves of plants treated with 60 μM AM were pale yellow-green (*i.e.* with diminished amount of Chls and Cars). The leaves of plants treated with the highest concentration of AM were completely white (*i.e.* without photosynthetic pigments). These observations were further supported by HPLC analysis of photosynthetic pigments in tissue of both genotypes. The content of Cars in leaves of AM-treated plants decreased in a concentration-dependent manner (Table 1). A slight decrease in the amount of total Cars—to about 93 % of that found in control plants—was observed in the plants treated with 20 μM AM. A much lower amount of total Cars was found after treatment of plants with 60 μM AM (about 33 % of control). The plants treated with the highest concentration of AM (120 μM AM) contained very limited amounts of protective Cars (0.7 % of control in CE810 to 1.7 % of control in CE704). The loss of xanthophyll cycle pigments (*i.e.* violaxanthin, zeaxanthin, and antheraxanthin) together with the loss of neoxanthin was mainly responsible for this decrease in content of total Cars; this agrees well with some earlier observations (Agnolucci *et al.* 1996, Dalla Vecchia *et al.* 2001, Hirschberg 2001, La Rocca *et al.* 1998). Lutein (synthesised from α-carotene; Young *et al.* 1997) and β-carotene seemed to be less susceptible to AM. However, analysis of the changes in the ratio of lutein and neoxanthin (as representative pigments of LHC) to β-carotene (as a representative Car of reaction

centres) revealed a greater relative decrease in the amount of β-carotene compared to these two xanthophylls. This, together with the significant loss of the pigments of xanthophyll cycle, suggests that the Cars synthesised through β-carotene pathway are more susceptible to AM. AM probably inhibits the cyclisation of lycopene (Dalla Vecchia *et al.* 2001) and the formation of β-ionone ring could be more sensitive than the formation of ε-ionone ring (characteristic for α-carotene and lutein).

The protective role of Cars in plants is widely accepted (Demmig-Adams *et al.* 1996, Young *et al.* 1997). Their absence in chloroplasts leads to severe damage of pigment-protein complexes of thylakoid membranes, which is accompanied by loss of Chls (Anderson and Robertson 1960, Young *et al.* 1997). Moreover, if the Car/xanthophylls binding sites in the complexes can not be occupied, the whole complex becomes instable and Chls will be degraded. The analysis of the content of Chls in leaves of plants treated with various concentrations of AM revealed that even the lowest concentration used (20 μM) had negative influence on these pigments (their amount decreased to about 90 % of control; Table 1). Plants treated with 60 μM AM contained about 49 % of the amount of Chls found in the control plants, and plants treated with 120 μM AM were nearly devoid of Chls (0.7 to 1.1 % of control). The decrease in total Chl concentration was probably due mainly to the decrease in Chl *a* content. This pigment appeared to be more sensitive to AM compared to Chl *b*. Similar results were reported by Kushwaha and Bhowmik (1999) for cucumber (*Cucumis sativus* L.) treated with isoxaflutole, another herbicide inhibiting Car biosynthesis. The greater susceptibility of Chl *a* to AM-induced photooxidative damage was reflected also in the values of Chl *a/b* ratio (Table 1), which decreased with increasing concentration of herbicide. These results, together with the changes observed in the content of individual Cars, suggest that reaction centres of photosystems might be more sensitive to photooxidative damage caused by AM treatment compared to LHC.

Table 1. Contents of photosynthetic pigments [mg m^{-2}] together with Hill reaction activity (HRA) and activity of photosystem 1 (PS1) [μmol(O_2) m^{-2} s^{-1}] in mesophyll chloroplasts isolated from maize plants of two genotypes (CE704, CE810) untreated (C) or treated with different concentrations of amitrole (20, 60, and 120 μM). Plants treated with the highest concentration of AM contained very small amounts of pigments in plant tissue and therefore some values are not given. Car, total carotenoids; V, violaxanthin; A, antheraxanthin; Z, zeaxanthin; Chl, chlorophyll; n.d., not determined. Means ± standard errors of mean (SEM).

	Characteristic	C	20 μM	60 μM	120 μM
CE704	Car	46.6±0.0	43.7±0.1	15.0±0.7	0.8±0.0
	neoxanthin	8.3±0.0	7.0±0.1	1.5±0.1	0.0
	lutein	17.9±0.1	17.7±0.1	8.2±0.5	0.5±0.0
	V+A+Z	6.0±0.1	4.8±0.1	1.1±0.1	0.0
	β-carotene	14.5±0.1	14.2±0.2	4.3±0.1	0.3±0.0
	Chl *a+b*	311.0±0.7	281.5±0.7	154.8±6.5	3.4±0.1
	Chl *a/b*	3.33±0.02	3.10±0.00	3.01±0.02	n.d.
	HRA	7.41±0.00	6.07±0.70	3.80±0.00	n.d.
	PS1	12.60±0.21	14.29±0.22	12.81±0.27	n.d.
CE810	Car	43.1±0.0	26.9±10.9	14.1±0.3	0.0
	neoxanthin	9.1±0.1	7.3±0.2	1.7±0.1	0.0
	lutein	14.9±0.1	14.9±0.1	7.4±0.2	0.2±0.1
	V+A+Z	5.4±0.0	4.5±0.1	1.0±0.0	0.0
	β-carotene	13.7±0.1	13.5±0.1	4.1±0.1	0.1±0.0
	Chl *a+b*	268.5±0.2	249.1±1.8	132.3±2.0	0.0
	Chl *a/b*	3.36±0.02	3.01±0.02	2.85±0.03	n.d.
	HRA	11.23±0.00	9.70±0.69	4.75±0.00	n.d.
	PS1	19.88±0.48	15.66±0.12	11.38±0.18	n.d.

Analysis of the photochemical activities of PS1 and PS2 in isolated MC chloroplasts revealed a great sensitivity of PS2 (Table 1). Hill reaction activity (which is a measure of the activity of PS2) significantly decreased with an increasing concentration of herbicide in both genotypes studied. The highest difference was observed between control and 60 μM AM-treated plants (photochemical activity of isolated chloroplasts in 120 μM AM-treated plants could not be measured due to the nearly complete absence of photosynthetic pigments in MC chloroplasts). On the other hand, the activity of PS1 was affected less, which suggests that this pigment-protein complex is more resistant to the photooxidative damage. Similar results were found by Bolychevtseva *et al.* (1995).

Treatment of plants with various concentrations of AM affected not only the content of photosynthetic pigments or photochemical activity of thylakoid pigment-protein complexes, but also the chloroplast ultrastructure (Figs. 1 and 2). MC and BSC chloroplasts in leaves of

control plants were characterised by well-organised thylakoid membranes (see also Kutík *et al.* 2001). MC chloroplasts contained both granal and intergranal thylakoids, whereas thylakoid membranes of BSC chloroplasts were largely non-appressed; these chloroplasts also contained great amounts of starch inclusions. Mesophyll chloroplasts of plants treated with 20 μM AM were ultrastructurally similar to chloroplasts of control plants. However, stereological methods (Table 2) revealed some differences in chloroplast ultrastructure. Statistically significant differences between MC chloroplasts of control and 20 μM AM-treated plants of genotype CE704 were found only for the volume density of starch inclusions and of genotype CE810 for the volume density of starch inclusions and plastoglobuli. On the other hand, the ultrastructure of BSC chloroplasts was more influenced by this low concentration of AM. The volume density of starch inclusions in BSC chloroplasts of 20 μM AM-treated plants of CE704 genotype decreased into about 53.9 % of the values found in control plants and into about 48.1 % in the case of genotype CE810. The decrease in the amount of starch inclusions in chloroplasts is probably connected with either various stress factors or advancing senescence of plants (Mostowska 1997, Kutík 1998). In our case, the decrease observed in BSC chloroplasts of 20 μM AM-treated plants was accompanied by slight tendency of MC chloroplasts of CE810 genotype to accumulate starch under these conditions.

Table 2. Ultrastructural characteristics of mesophyll (MC) and bundle sheath cell (BSC) chloroplasts in two genotypes of maize (CE704 and CE810) untreated (C) or treated with 20 μM amitrole: volume densities [%] of appressed (A) and non-apressed (NA) thylakoids, starch inclusions (ST), plastoglobuli (PL), peripheral reticulum (PR), stroma (S), and proportion [%] of appressed thylakoids to all thylakoids (G). Means ± standard error of mean (SEM). Statistical significance of the differences between AM-treated and untreated plants (D1) or between genotypes (D2) as proven by Mann-Whitney test is also given ($^{**}p \leq 1$ %, $^{*}p \leq 5$ %, $^{-}p > 5$ %).

		CE704			CE810			D2	
		C	20 μM	D1	C	20 μM	D1	C	20 μM
MC	A	28.10±1.76	28.29±1.74	-	26.05±1.58	23.37±1.43	-	-	-
	NA	23.34±1.19	21.00±1.24	-	21.17±1.54	18.77±1.34	-	-	-
	G	54.37±1.97	57.08±2.43	-	55.19±2.80	55.24±2.61	-	-	-
	ST	0.10±0.06	0.00	*	1.11±0.32	1.24±0.43	*	**	**
	PL	1.37±0.17	2.10±0.19	-	2.01±0.18	1.68±0.18	*	*	-
	PR	3.84±0.34	3.68±0.20	-	4.78±0.39	3.85±0.34	-	-	-
	S	43.25±2.28	44.92±1.91	-	44.89±1.84	51.09±1.89	-	-	-
BSC	ST	26.00±1.81	14.07±1.77	**	19.14±1.27	9.20±1.36	**	**	*
	PL	0.23±0.05	0.42±0.07	**	0.44±0.08	0.43±0.09	-	*	-

The decrease of Cars to about 33 % of control in plants treated with 60 μM AM was accompanied by dramatic damage of MC chloroplasts especially in genotype CE810 (Fig. 2). MC chloroplasts in leaves of this genotype showed great changes, similar to those observed in plants treated with the highest AM concentration. They were amoeboid in shape and contained only a few thylakoids. MC chloroplasts of genotype CE704 (Fig. 1) subjected to the same experimental conditions were less damaged: they had normal shape and very reduced amount of thylakoids, occasionally being swollen a bit. On the other hand, BSC chloroplasts in leaves of plants treated with 60 μM AM of both genotypes were relatively unaffected. They, however, contained almost no starch inclusions but their thylakoid membranes were still preserved. As it is unlikely to expect that bundle sheath cells contain different AM amount compared to mesophyll, it is probable that BSC chloroplasts are less sensitive to oxidative damage than MC chloroplasts. These two types of chloroplasts differ in the amount of antioxidant compounds (Doulis *et al.* 1997) and this difference seems to be even more pronounced under stress conditions (Pastori *et al.* 2000). Moreover, BSC chloroplasts are relatively physically isolated from oxygen evolved by MC chloroplasts during primary photosynthetic reactions and thus also from reactive oxygen species produced there.

Mesophyll cells in leaves of 120 μM AM-treated plants contained greatly damaged chloroplasts with only a few anomalous thylakoid membranes, sometimes grouped to form very electron-dense masses. Thylakoids were usually either localised only in a part of chloroplast or surrounded the inner chloroplastic membrane. Connections of thylakoids to the inner chloroplastic membrane were also occasionally observed (not shown). Similar phenomenon was found in early stages of chloroplast development (Hudák 1997). BSC chloroplasts in leaves of 120 μM AM-treated plants had very limited amount of thylakoids and never contained starch inclusions. Immunocytochemical analysis of ultra-thin sections of leaf fragments from 120 μM AM-treated plants revealed a great decrease in the amount of LHC particles both in MC and BSC chloroplasts as compared to control plants. However, the decrease in BSC chloroplasts (not shown) was not so prominent as in MC chloroplasts (Fig. 3),

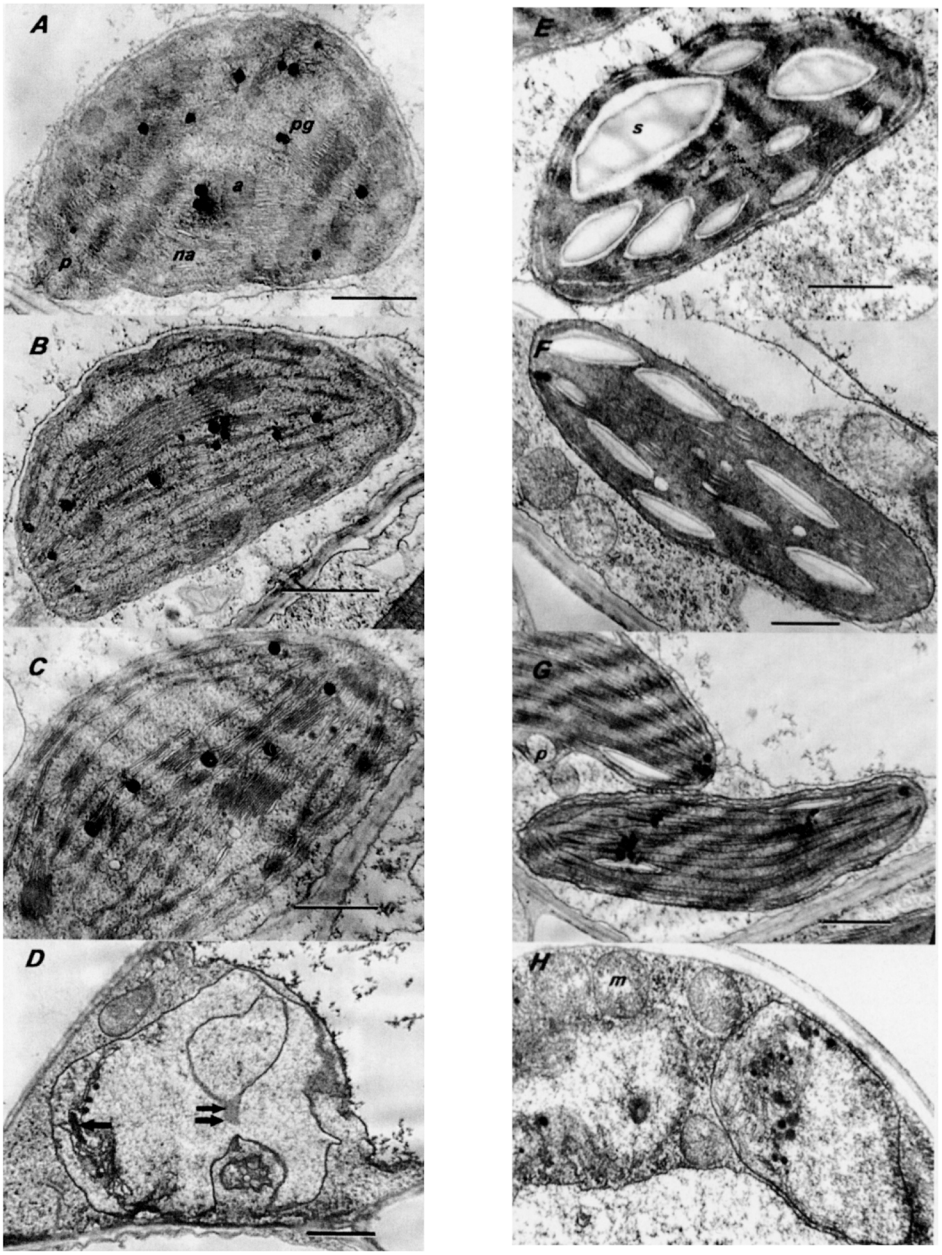

Fig. 1. Chloroplasts of mesophyll cells (MC; *A–D*) or bundle sheath cells (BSC; *E–H*) in the middle part of mature third leaf of maize genotype CE704. MC chloroplast of a leaf of control plant exhibits a well organised thylakoid membranes – appressed (*a*) and non-appressed (*na*) thylakoids, plastoglobuli (*pg*), and a small protrussion (*p*). (*B*) MC chloroplast of a plant treated with low concentration of AM (20 μM), similar to A. (*C*) MC chloroplast of a plant treated with 60 μM AM characterised by a decrease of thylakoid volume density. (*D*) A greatly damaged MC chloroplast of a plant treated with 120 μM AM. Chloroplast contains a few, irregularly distributed thylakoids which are sometimes appressed (*arrows*) and form electron dense material. Angle wise cut thylakoid membranes (*double arrows*) are also seen. An undamaged mitochondrium is also shown. (*E*) BSC chloroplast of control plant with large starch inclusions (*s*). (*F*) Decrease in volume density of starch inclusions in BSC chloroplast of a plant treated with 20 μM AM. (*G*) Increasing concentration of AM (60 μM) causes a great decrease of starch volume density. A small protrusion is also seen (*p*). (*H*) BSC chloroplast of a plant influenced by 120 μM AM shows absence of starch inclusions and very damaged thylakoids. Undamaged mitochondria (*m*) are also seen. Bars = 1 μm.

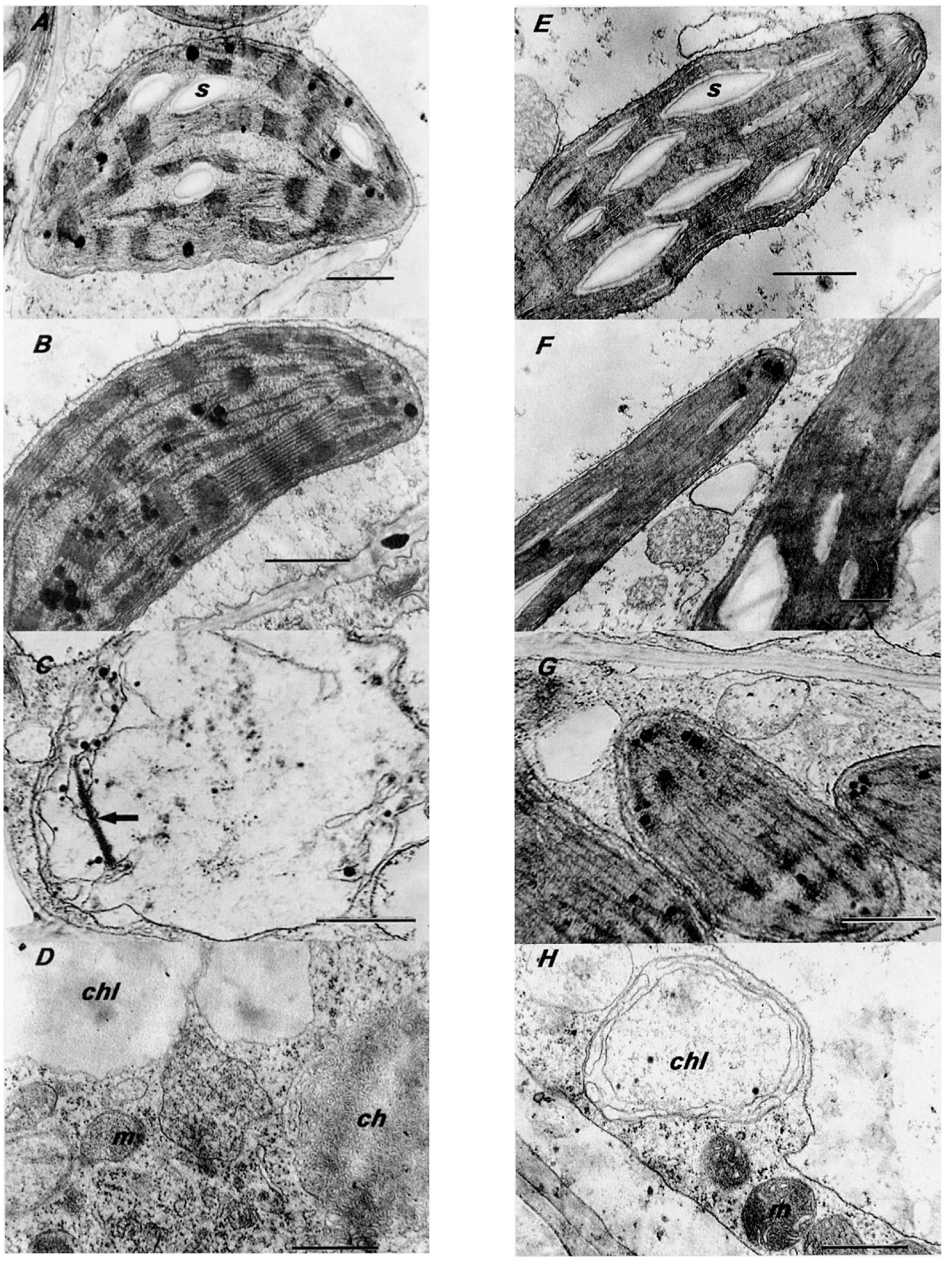

Fig. 2. Chloroplasts of mesophyll cells (MC; *A–D*) or bundle sheath cells (BSC; *E–H*) in the middle part of mature third leaf of maize genotype CE810. (*A*) MC chloroplast of a control plant exhibits a well-organised thylakoid membranes and starch inclusions (*s*). (*B*) MC chloroplast of a plant treated with low concentration of AM (20 μM) that contains no starch inclusions. (*C*) MC chloroplast of a plant treated with 60 μM AM is greatly damaged. Chloroplast contains only a few irregularly distributed thylakoids which are appressed (*arrow*) and form electron dense material. (*D*) Greatly damaged mesophyll cell of a plant treated with 120 μM AM with mitochondria (*m*), chloroplasts devoid of any thylakoids (*chl*) or chloroplasts containing crystalline material (*ch*) resembling prolamelar bodies in etioplasts. (*E*) BSC chloroplast of control plant with large starch inclusions (s). (*F*) Decreased volume density of starch inclusions in BSC chloroplast of a plant treated with 20 μM AM. (*G*) Increasing concentration of AM (60 μM) causes a great decrease of starch volume density. (*H*) BSC chloroplast (*chl*) of a plant influenced by 120 μM AM shows absence of starch inclusions and very damaged thylakoids. Undamaged mitochondria (*m*) are also seen. Bars = 1 μm.

suggesting again a lesser sensitivity of BSC chloroplasts to AM. Loss of LHC apoproteins due to the application of high AM concentration was reported also by Dalla Vecchia *et al.* (2001) together with a loss of other Chl *a*-binding polypeptides (*e.g.* D1, D2, CP43, CP47, and 22 kDa proteins of PS2).

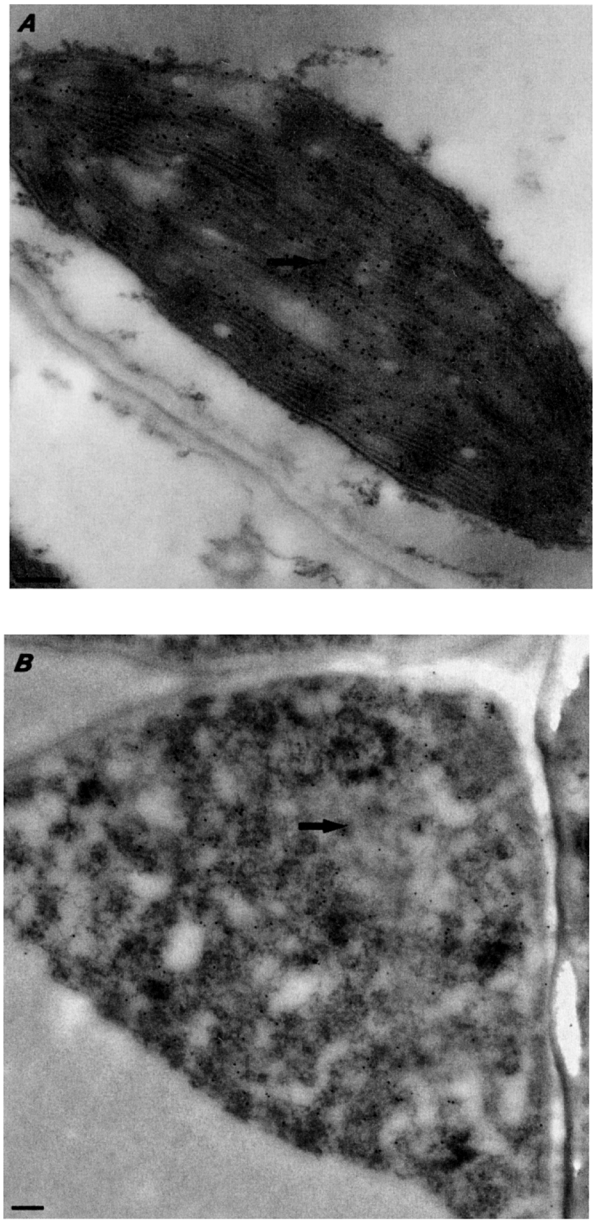

Fig. 3. Immunocytochemical localisation of LHC labelled by 10 nm gold particles (*black dots - arrow*) in MC chloroplast of genotype CE810 of a control plant (*A*) and of a plant treated with 120 μM AM (*B*). Bars = 0.2 μm.

Ageing chloroplasts or chloroplasts exposed to various stress conditions accumulate plastoglobuli in their stroma. Plastoglobuli contain lipids from degenerating photosynthetic membranes and some investigations performed on Car-free plants indicate that they can also contain precursors of α- and β-carotene, *i.e.* phytoene and phytofluene (Dahlin and Ryberg 1986). The results of our study of chloroplast ultrastructure in leaves of AM-treated plants suggested that the amount of plastoglobuli practically did not change with the application of different concentrations of AM. This suggests that the degree of degeneration of photosynthetic membranes is limited by the amount of photosynthetic membranes formed during chloroplast development. Lower contents of Cars and Chls, which act as stabilisers during LHC assembly and are necessary for the correct development of thylakoid membranes (Wettstein *et al.* 1995, Hoober and Eggink 1999), allowed here the formation of only a few thylakoids. The plastoglobuli in chloroplasts of AM-treated plants could therefore contain only low amount of thylakoid lipids and no increase in their volume density was needed.

Our observation that some chloroplasts formed tubular protrusions into cytoplasm, filled with peripheral reticulum and stroma and lacking thylakoids, was fairly interesting (Fig. 4). These stroma-filled tubules, recently named stromules (Gray *et al.* 2001), are usually more abundant in cells containing relatively small plastid volume. Therefore the formation of such protrusions may enable plastids to increase their surface area in order to optimise metabolic or signal-transduction processes which require movement of various molecules across the plastid envelope. Stromules also interconnect plastids (Gray *et al.* 2001). Plastids with protrusions, so-called amoeboid plastids, have been described also by other authors; they occur mainly during senescence or as a result of some stress factor (Hudák 1997). We suppose that the presence of chloroplast protrusions observed in our study could be connected with cultivation conditions (growth in nutrient solution is not optimal for maize, but was the only possible method to ensure that plants with different amount of Cars will be obtained) rather than with the AM treatment (these protrusions were found both in control and AM-treated plants).

Although the reaction of both genotypes to AM treatment was in many aspects similar, some interesting differences were also observed. The genotypes did not much differ in the content of photosynthetic pigments. CE704 contained slightly more Chls and Cars (except neoxanthin) compared to CE810, but the differences were not statistically significant. This applied both for control and AM-treated plants regardless of the concentration of AM used. The decrease in the content of photosynthetic pigments associated with the increasing concentration of AM was also similar in both genotypes. Some differences were found only for the content of β-carotene or lutein when plants were treated with 120 μM AM. In this case, CE810 was slightly more affected than the second genotype. On the other hand, the differences between both genotypes in photochemical activity of isolated MC chloroplasts were distinct. CE810 displayed higher activites of both PS1 and PS2, which applied especially for plants untreated with AM. This trend was conserved in AM treated plants as well, but the differences between both genotypes tended to diminish with increasing concentration of AM. This suggests that the PS1 complex (and to some extent the PS2 complex, too) in MC chloroplasts

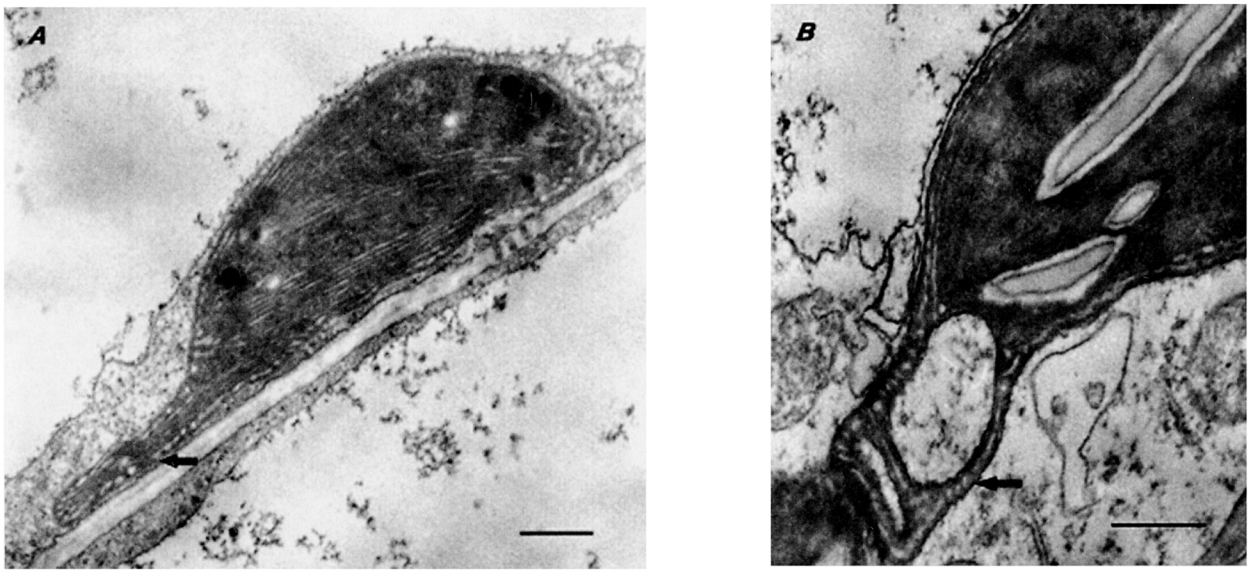

Fig. 4. (*A*) MC chloroplast with a protrussion (*arrow*) of a control plant of genotype CE704. (*B*) BSC chloroplast with a protrusion (*arrow*) of a plant of CE810 genotype treated with 20 μM AM. Bars = 1 μm.

of genotype CE810 is probably more susceptible to photooxidative damage than in CE704 genotype.

The lesser susceptibility of CE704 to such damage was further supported by the analysis of chloroplast ultrastructure. The different reaction of both genotypes to AM was clearly evident especially in MC chloroplasts of plants treated with 60 μM AM (see above). Statistical analysis of differences between both genotypes was performed only in control and 20 μM AM-treated plants. In control plants, both MC and BSC chloroplasts of genotype CE810 were characterised by significantly higher volume density of plastoglobuli compared to CE704; this difference disappeared when 20 μM AM-treated plants were analysed. As regards starch inclusions, their volume density in MC chloroplasts of both control and 20 μM AM-treated plants was significantly higher in CE810 compared to CE704, whereas in BSC chloroplasts the situation was reverse. Taken together, these results confirm our earlier observations that also suggested that CE704 is able to withstand well various stress conditions (*e.g.* low temperature, water deficit; Holá, personal communication). Finally, not only the intra-species variability in the photosynthetic apparatus can be responsible for differences between these two genotypes after the AM treatment but also differences in the rates of uptake and mobility of the herbicide or different metabolic activities relating to the herbicide effects. These features were unfortunately not studied.

Thus our study of chloroplast ultrastructure, photosynthetic pigment contents, and photochemical activity of chloroplasts in maize plants showed that various levels of Car deficiency induced by AM are reflected in various changes of chloroplast ultrastructure. The mesophyll chloroplasts were more affected compared with their counterparts in bundle sheath cells; the negative influence of the partial or total loss of Cars was obvious especially in the alterations in the amount and organisation of thylakoid membranes. These structural changes were connected also to the changes in the function of thylakoid pigment-protein complexes; of these, PS2 was more susceptible to AM-induced damage than PS1. Even the lowest concentration of the herbicide unfavourably affected successful development of chloroplasts. However, some differences between two genotypes examined in the reaction to AM-treatment were also observed, suggesting the existence of intra-specific variability in the response of the photosynthetic apparatus to development in photodamaging conditions.

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