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Chlorophyll synthesis retardation and ultrastructural alterations to *Solanum tuberosum* chloroplasts in *Solanum nigrum* cells

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Abstract

Photosynthetic pigment contents of the second sexual generation of a cybrid plant (C-18-1) resulting from *Solanum nigrum* genome and *Solanum tuberosum* plastome were compared to those of the original (*S. nigrum*). Chloroplast ultrastructure alterations among *S. tuberosum*, cybrid, and *S. nigrum* were also studied. Leaf segments of both the cybrid and *S. nigrum* plants were cultured on shoot induction medium [B5 supplemented with 0.56 g m^{-3} benzylaminopurine (BAP)] for one week in light, to induce adventitious bud formation. These leaf segments were then placed in darkness for 5 weeks to form a white shoot. The respective cybrid plant had the same phenotype of the fusion recipient plant (*S. nigrum*) and was fertile. The rate of photosynthetic pigment biosynthesis in the white cybrid shoots was lower than that of the original plant shoots after subjecting the two plants to the same conditions of different irradiation periods (0, 2, 4, 6, 8, and 10 d). At the 10-d irradiation period of two white shoot plants, the total pigment content of *S. nigrum* shoot increased approximately 3-fold over that of the cybrid shoot. Numbers of grana and thylakoids as well as chloroplast size were decreased in cybrid cells in comparison to those in *S. tuberosum* cells. Under atrazine stress, while the chloroplast ultrastructure of the cybrid cells (atrazine sensitive) was strongly influenced, the chloroplasts of *S. nigrum* (atrazine resistant) were not affected.

Additional key words: atrazine; carotenoids; cybrid; electron microscopy; granum; thylakoid.

Introduction

Transfer of cytoplasmic genes after protoplast fusion has been obtained as a result of independent segregation of nuclei and cytoplasmic organelles in the primary protoplast fusion product (Nehls *et al.* 1986, Morgan and Maliga 1987). The ability to get functional cybrids containing nuclear genome of one species with plastome or chondriome of other species *via* protoplast fusion is a good tool for studying the nuclear-organelle interaction in plants (Nehls *et al.* 1986, Kushnir *et al.* 1987, Morgan and Maliga 1987, Perl *et al.* 1990, 1991, Hassanein *et al.* 1993, Grosser *et al.* 1996).

The genetic background of the respective cybrid plant (C-18-1) was proved using RFLP and isoenzyme analyses (Hassanein *et al.* 1993). The atrazine (2-ethylamino-4-chloro-isopropylamino-1.3.5-triazine) resistant character is presumed to be located on the plastidal DNA. The main action of atrazine on plants is the inhibition of photosynthesis in the chloroplast (Chua and Gillham 1977, Callahan *et al.* 1989, Mattoo *et al.* 1989). Triazine-resistant chloroplast membranes exhibit changes in lipid composition (Pillai and St.John 1981, Lehoczki *et al.* 1985). In addition, triazine-resistant chloroplasts are characterized by an increase of grana stacks and thylakoid grana stacking, lower Chl *a/b* ratio, and a decrease of starch content (Burke *et al.* 1982, Vaughn and Duke 1984).

Substitution of *S. nigrum* chloroplasts by those of *S. tuberosum* created an atrazine-sensitive cybrid plant. Physiological and isoenzymatic studies on nuclear-organelle interaction of the respective cybrid were previously reported (Hassanein 1998, Hassanein *et al.* 1998). The most obvious conclusion obtained in these studies was the reduction of photosynthetic pigment contents of the cybrid in comparison to that of the original plant which may be due to the incompatibility between the *S. nigrum* genome and *S. tuberosum* plastome. Consequently, the following question has been risen: Has the ultrastructural organization of the

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Abbreviations: Car - carotenoids; Chl - chlorophyll; TEM - transmission electron microscopy.

S. tuberosum chloroplast components been changed due to their existence in *S. nigrum* cells? Therefore, the rates of pigment biosynthesis (Chl *a*, Chl *b*, Car) and ultrastructure of chloroplast (organization, size, and number) of both cybrid and original plants were investigated in the present study.

Materials and methods

Shoot culture: The cybrid plant (C-18-1) containing *S. nigrum* genome and *S. tuberosum* plastome was originally obtained (Hassanein *et al.* 1993) by fusion of an albino plastid mutant of *S. nigrum* (Sn-F-W2) and a diploid clone (St-H2 258) of *S. tuberosum* L. (Binding *et al.* 1978, 1987). The obtained seeds of the respective cybrid and original plants were disinfected by dipping in 5 % chlorox solution for 5 min followed by 5 min dipping in 75 % ethanol. Seeds were germinated on hormone-free B5 medium (Gamborg *et al.* 1968). Seedlings were then cut at epicotyl or hypocotyl and transferred to B5 or MS (Murashige and Skoog 1962) media without phytohormones to establish shoot culture. Shoots were cultured for 2 weeks to induce high multiplication. All shoot cultures were maintained under 16 h daily irradiation with 400 μmol m^{-2} s^{-1} at 25±1 °C without humidity control.

Atrazine treatment: About 30 shoots of each of cybrid and original plant shoots were grown on B5 medium with sucrose concentration reduced to 1 % (m/v) and supplemented with 0.1 mM atrazine (Binding *et al.* 1982).

Formation of white shoots: White shoots of the cybrid and *S. nigrum* were obtained using two experimental procedures: (*1*) Adventitious bud formation on fully expanded leaf segments of both *S. nigrum* and cybrid plants was induced by placing the leaf explants on B5 basal medium supplemented with 0.56 g m^{-3} BAP at 16 h daily irradiation with 100 μmol m^{-2} s^{-1} for one week. (*2*) The initiation and formation of white shoots was accomplished by transferring the Petri dishes containing the leaf segments into complete darkness at 25±1 °C for 5 weeks. Then shoots were cut (2-3 cm long) and transferred to new medium containing low cytokinin concentration (0.1 g m^{-3}). A group of sixty such shoots was cultured in three Petri dishes (9 cm diameter) and considered as a replicate for each period (0, 2, 4, 6, 8, 10 d) of irradiation with 400 μmol m^{-2} s^{-1} at 25±1 °C daily for 16 h. The control plants were grown under the same irradiaton for 6 weeks.

Photosynthetic pigment determination: After the desired irradiation periods (0, 2, 4, 6, 8, 10 d) the upper parts of white shoots (containing three or four leaves) of the cybrid and *S. nigrum* plants were ground in 80 % acetone to extract the pigments. Contents of Chl *a*, Chl *b*, and Car were spectrophotometrically determined (Metzner *et al.* 1965). Pigment contents of plant shoots subjected to the above irradiation periods were then compared to those of control shoots grown for 6 weeks at 16 h daily irradiation (400 μmol m^{-2} s^{-1}).

Ultrastructural observations and quantitation of chloroplasts: Samples (about 1 mm^2) for transmission electron microscopy (TEM) were fixed by 2.5 % glutaraldehyde in 0.05 M phosphate buffer (pH 7.00) for 3 h at room temperature. Samples were rinsed several times with 0.05 M phosphate buffer and then were post-fixed in OsO_4 for 2 h at room temperature. Samples were rinsed several times with the 0.05 M phosphate buffer, dehydrated in a graded acetone series, and embedded in the medium of Spurr (1969). Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate. Specimens were viewed 30 times with a *Jeol-1010* transmission electron microscope at 100 kV and the numbers of chloroplasts per cell profile were counted. Sizes of chloroplasts were measured as long axes.

Results and discussion

The functional cybrid interaction between the nuclear genes of plant species and organelles' genes of other species is of considerable relevance in plant biology. The genus *Solanum* is suitable for the study of this interaction (Binding *et al.* 1986, Nehls *et al.* 1986, Perl *et al.* 1991). The second sexual generation of the cybrid (C-18-1) was fertile (Hassanein *et al.* 1993). The cybrid shoot was light-green and bleached under atrazine stress while the original plant (*S. nigrum*) was dark green. The respective cybrid had a similar phenotype as the fusion recipient plant (*S. nigrum*) under both tissue culture and greenhouse conditions (leaf shape, leaf and stem hairs, flower shape, fruit shape). Kushnir *et al.* (1987) report that the fertile cybrids possessing genome and plastome

of plant species belonging to different genera are genetically functional, however, the cooperation between genome and plastome is not perfect and shows some abnormalities.

Under normal cultivation (16-h daily irradiation with 400 μmol m^{-2} s^{-1} at 25±1 °C), the photosynthetic pigment contents of the cybrid were lower than those of the original plant shoots (Table 1). In agreement with the results of Hassanein *et al.* (1998), the light-green colour of the cybrid was due to the significant decrease in contents of photosynthetic pigments. This may reflect a limited compatibility between the *S. nigrum* genome and the *S. tuberosum* plastome. Similar results were obtained by Perl *et al.* (1990) when the plastome of the donor was similar to that of the recipient species.

When the white shoots of both the cybrid and original were subjected to graded periods of irradiation (0, 2, 4, 6, 8, and 10 d), a progressive increase in the pigment contents was found (Table 1). However, the pigment contents of the cybrid plant shoots were always lower than those of the original shoots. Prior to irradiation, the contents of Chl *a*, Chl *b*, and Car of white shoots of both cybrid and original plants (Table 1) were approximately the same. The quantity of total pigments (Chl *a* + Chl *b* + Car) of the cybrid (Table 1) increased approximately 27-fold from 0 to 10 d of irradiation, which was *ca.* 46 % of that formed in shoots grown under normal condition during 48 d. In original plant shoots, the quantity of total pigments (Table 1) increased 76-fold from 0 to 10 d irradiation, and it was *ca.* 87 % of that formed in shoots under normal condition. Content of Chl *a* of the cybrid increased about 41-fold from 0 to 10-d while the Chl *b* content increased only about 18-fold. In original plant shoots (*S. nigrum*), the Chl *a* content increased about 109-fold from 0 to 10 d, whereas Chl *b* content increased only about 49-fold. The Chl *a/b* ratio of the white cybrid shoot was 1.15 at 0-d and increased to 2.60 at 10-d. In the 0-d, the original shoots (*S. nigrum*) had Chl *a/b* of 1.03 and at 10 d it was 2.32. In general, the Chl *a/b* ratio of cybrid shoots at all periods was lower than that of original (*S. nigrum*). The Chl *a* content of both the cybrid and original increased more rapidly than the Chl *b* or Car contents at all irradiation periods. This delay in Chl synthesis of the cybrid plant shoots can be explained by incompatibility between the *S. tuberosum* plastome and *S. nigrum* genome.

Table 1. Photosynthetic pigment contents [mg kg^{-1}(FM)] of white cybrid and white original (*S. nigrum*) shoots after irradiation for 0, 2, 4, 6, 8, 10, and 48 d (16 h daily, 400 μmol m^{-2} s^{-1}, at 25±1 °C). Percentage of total pigment content after different irradiation periods was related to that of control plants grown for 48 d under the same conditions). Each value represents the mean ± SD of three replicates. Differences from 0-d irradiation values were highly significant at $p < 0.05$.

	Irradiation [d]	Chl *a*	Chl *b*	Car	total	[%]	Chl *a/b*
cybrid	0	8.3±0.3	7.2±0.2	6.3±0.3	21.8±0.8	1.71	1.15
	2	29.7±1.6	25.3±1.8	20.0±0.8	75.0±3.1	5.89	1.17
	4	157.0±10.5	74.0±6.3	60.0±3.0	291.0±15.0	22.84	2.12
	6	254.0±24.9	116.0±10.5	106.0±12.1	476.0±46.6	37.36	2.19
	8	289.0±18.1	122.0±8.6	115.0±8.5	526.0±34.9	41.29	2.37
	10	338.0±29.6	130.0±12.3	117.0±19.8	585.0±52.4	45.92	2.60
	48	736.0±58.5	283.0±18.5	255.0±14.9	1274.0±91.7	100.00	2.60
	LSD 0.05	52.3	19.4	16.9	87.0		
original	0	7.9±0.6	7.5±0.5	4.6±0.4	19.8±1.2	1.1	1.03
	2	177.0±19.0	140.0±23.1	91.0±8.1	408.0±49.3	23.4	1.26
	4	405.0±42.0	225.0±18.3	140.0±29.6	770.0±84.0	44.3	1.80
	6	598.0±30.2	269.0±15.4	204.0±21.0	1071.0±64.9	61.7	2.22
	8	810.0±65.2	349.0±24.2	269.0±15.1	1438.0±103.6	82.2	2.32
	10	856.0±60.0	369.0±22.6	281.0±20.0	1506.0±101.3	86.7	2.32
	48	989.0±75.0	420.0±29.7	328.0±24.4	1737.0±127.3	100.0	2.35
	LSD 0.05	78.6	39.0	34.1	143.7		

The mean numbers and sizes of chloroplasts, grana, and thylakoids of examined plants per profile of 30 cells, 100 chloroplasts, and 300 grana, respectively (Table 2), showed important differences. Chloroplasts of *S. tuberosum* were *ca.* 4.2 μm long (Fig. 1*A*). Grana stacks occupied a large portion of the chloroplast volume.

Table 2. Number (mean ± SD) of chloroplasts, grana, and thylakoids per profile of photosynthetic cell, chloroplast, and grana of examined plants, respectively.

	S. tuberosum	Cybrid untreated	treated	*S. nigrum* untreated	treated
Chloroplast	6.42±1.16	8.00±2.00	8.75±2.01	9.50±2.02	9.00±2.41
Grana	18.29±2.49	11.43±1.39	4.14±0.69	14.85±2.19	14.52±1.27
Thylakoids	11.80±1.92	8.40±1.14	9.10±1.31	14.80±1.30	11.83±1.79

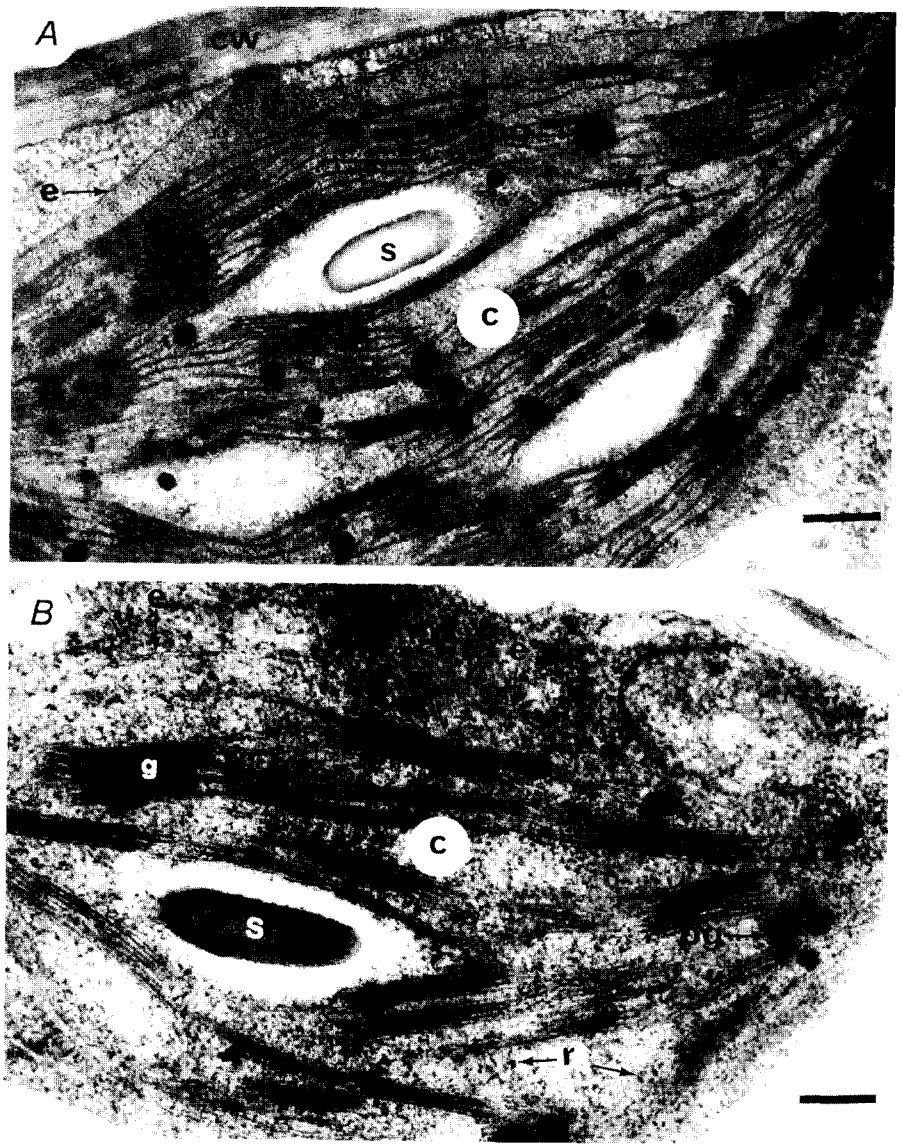

Fig. 1. Electron micrographs show the fine structure of chloroplast of the mesophyll cell from *S. tuberosum* (*A*) or from cybrid containing *S. tuberosum* plastome and *S. nigrum* genome (*B*). Grana stacks are interconnected by long (*A*) or short (*B*) stroma lamellae. Stroma matrix contains abundant plastoglobuli and starch grains. Numbers of grana stacks, grana thylakoids, and plastoglobuli in *A* are less compared to *B*. Scale bars: 265 nm (A). 185 nm (B). *c*, Chloroplast; *g*, granum; *e*, envelope; *er*. endoplasmic reticulum; *n*, nucleus; *nu*: nucleolus; *pc*, prochloroplast; *pg*, plastoglobuli, *s*: starch grains; *v*: vacuoles.

They were as electron opaque as the envelope, but relatively more electron opaque than the stroma. The *S. tuberosum* chloroplast in *S. nigrum* cells (cybrid) was *ca.* 2.3 μm long (Fig. 1*B*). Grana stacks occupied a small portion of the chloroplast volume. Grana thylakoids were much more electron opaque than the envelope and stroma. The chloroplast stroma was rich in ribosomes and its matrix was similar to that surrounding the chloroplasts. Chloroplasts of both the *S. tuberosum* and cybrid contained starch grains and plastoglobuli. However, the number of plastoglobuli of *S. tuberosum* chloroplasts in *S. tuberosum* cells was higher than that of the cybrid cells. Fayez (1998) found that chloroplasts of soybean (sensitive to photosynthetic herbicides) contained many plastoglobuli. The cybrid and *S. nigrum* mesophyll cells contained about the same number of chloroplasts, higher than cells of *S. tuberosum*. The increase in the number of *S. tuberosum* chloroplasts upon their transfer into *S. nigrum* cells was accompanied with a drastic decrease in the number of grana and thylakoids as well as the size of the chloroplasts. Therefore, the reduction of photosynthetic pigment contents may be, among others, due to the detected disruption of the chloroplast organization. Disruption of some chloroplasts was observed even in intergeneric heterokaryons (Hodgson and Rose 1984). Of course, chloroplast development depends not only on a functional cell metabolism but also on the activity of nucleus genes (Lichtenthaler 1984). Hence chloroplast disruption is a reflection of nuclear-plastid incompatibility (Rose *et al.* 1990).

Genes controlling the phenotypes of eukaryotes are present in the nucleus. However, some important characters of eukaryotes are controlled by genes of cytoplasmic organelles such as atrazine resistance (plastidal gene). The substitution of *S. nigrum* chloroplasts (atrazine-resistant) by those of *S. tuberosum* (atrazine-sensitive) resulted in atrazine sensitive cybrid.

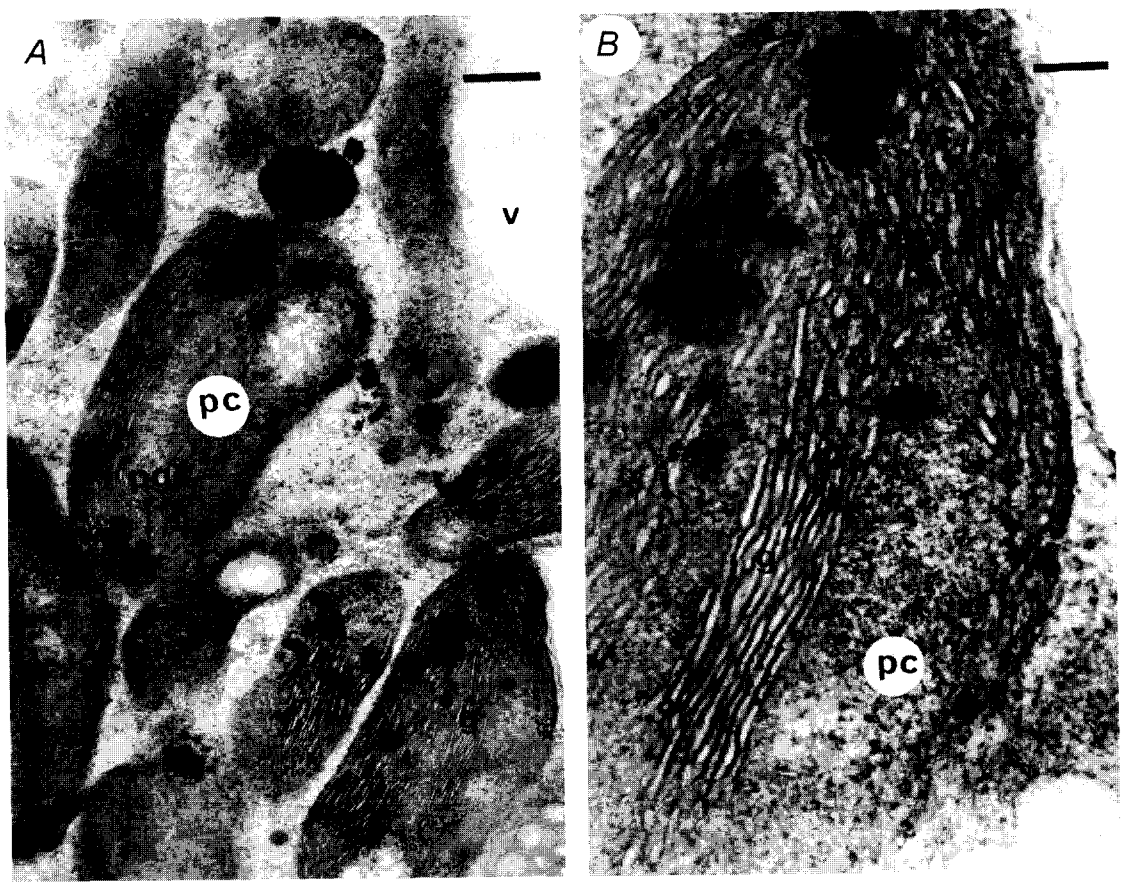

Fig. 2. Electron micrographs show the fine structure of chloroplasts of the mesophyll cell from cybrid treated with 0.1 mM atrazine for two weeks. Atrazine herbicide modified the chloroplasts due to destruction the pigments, and they appear to be in the phase of prochloroplasts. Grana thylakoids are dilated and they are much less electron opaque than in the untreated cybrid (Fig. 1*B*). Grana stacks decreased in number while thylakoids were increased in length. Plastoglobuli are aggregated on groups within chloroplasts. Starch grains are disappeared in the plastids. Scale bars: 500 (*A*) or 155 (*B*) nm.

Ultrastructural alterations were observed in atrazine-treated cybrid (Fig. 2*A*,*B*) compared to that of the untreated cybrid (Fig. 1*B*). Their plastids are randomly distributed through the cytoplasm lacking evident grana (Fig. 2*A*). They were *ca.* 2.25 μm long. Grana thylakoids (Fig. 2*B*) were dilated and much less electron opaque than those in the untreated cybrid (Fig. 1*B*). Grana stacks decreased in number while the thylakoid length increased. They appeared to be run in one direction and appressed virtually all along their entire length. Plastoglobuli were aggregated within the chloroplast. Starch grains had disappeared from the plastids (Fig. 2). Generally, photosynthetic herbicides induce destruction of pigments and modification of chloroplasts ultrastructure (Lichtenthaler 1984, Barry *et al.* 1990, Fayez 1998).

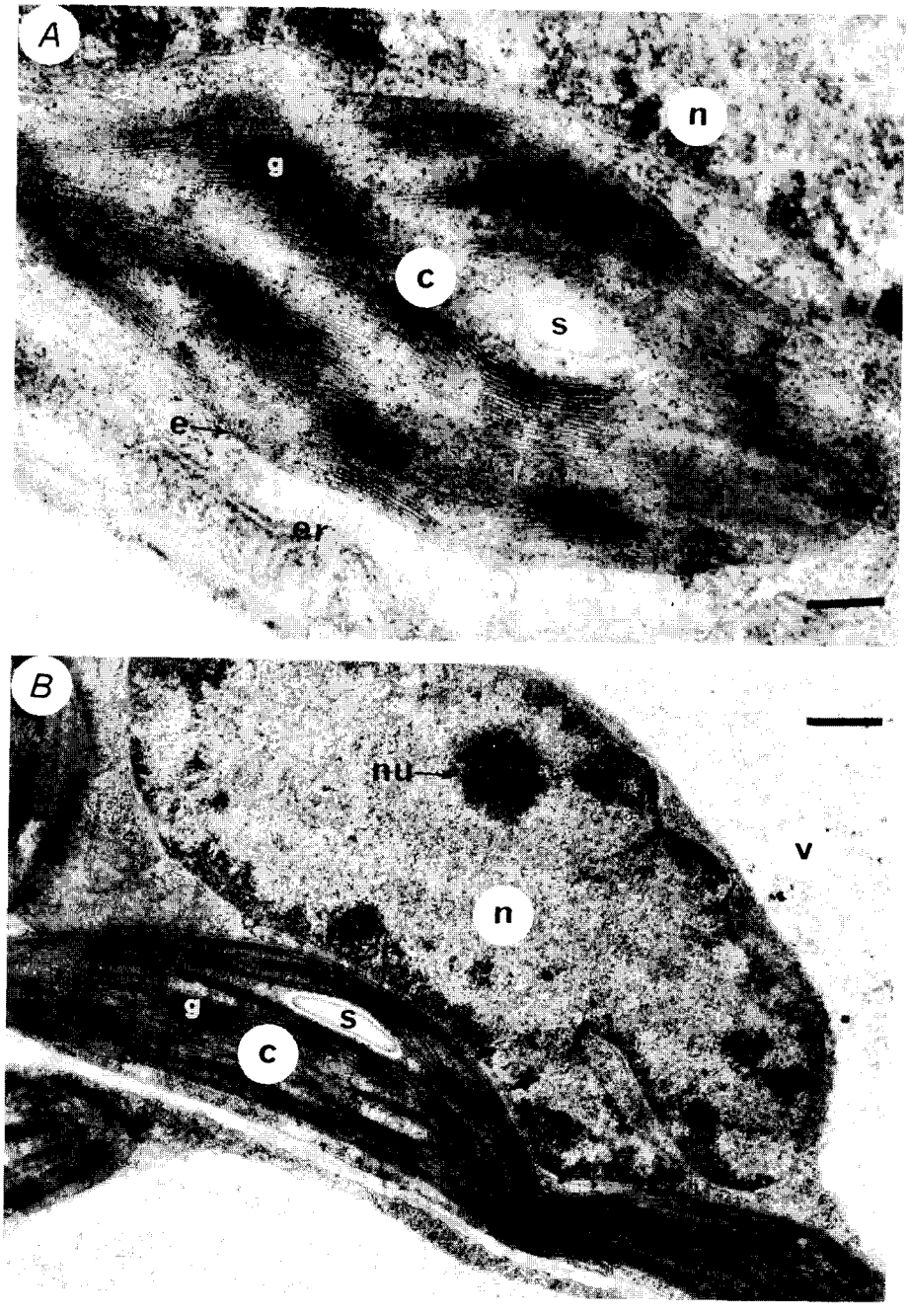

Fig. 3. Electron micrographs show the fine structure of chloroplast, nucleus, and endoplasmic reticulum of the mesophyll cell of *S. nigrum* untreated (*A*) or treated with 0.1 mM atrazine (*B*) for 20 d. (*A*): Grana stacks are interconnected by short stroma lamellae and occupy a greater portion of chloroplast volume. Stroma is poor in plastoglobuli but rich in ribosomes. (*B*): Chloroplasts are not affected by atrazine and exhibit a well organized grana. Scale bars: 240 (*A*) or 500 (*B*) nm.

The chloroplasts of *S. nigrum* (atrazine-resistant) were *ca.* 3.52 μm long (Fig. 3*A*) and had many ribosomes. Grana thylakoids were relatively more electron opaque than the stroma. The chloroplasts of *S. nigrum* (Fig. 3*A*) showed an increase in the number of grana thylakoids compared to chloroplasts of *S. tuberosum* within the cybrid (Fig. 1*B*) or of *S. tuberosum* cells (Fig. 1*A*). In addition, they were very poor in plastoglobuli in comparison with those of *S. tuberosum*. The cell cytoplasm of *S. nigrum* was rich in ribosomes and rough endoplasmic reticulum (Fig. 3*A*). The ultrastructure differences between atrazine resistant and sensitive plastids reported herein are similar to those reported by Vaughn and Duke (1984) or Lichtenthaler *et al.* (1982). Chloroplasts of *S. nigrum* were not affected by 0.1 mM atrazine (Fig. 3*B*). Under atrazine stress the chloroplast length of *S. nigrum* was similar (*ca.* 3.69 μm) to that in untreated plants.

Tracing and analyzing the incompatibilities and incongruities of species from different taxa can be attempted by cell grafting (Moore and Walker 1983, Binding *et al.* 1987) and production of somatic hybrid or cybrid (Nehls *et al.* 1986, Rose and Thomasand 1990, Grosser *et al.* 1996, Sigareva and Earle 1997). Chimeric *S. nigrum* with *S. tuberosum* plants examined microscopically exhibited nearly perfect cooperation (Binding *et al.* 1987). In addition, while the respective cybrid has been getting normal fertility through different sexual generations, somatic hybrid plants were never fertile and got vigour growth with dark-green colour (Hassanein *et al.* 1993). On the other side, the cybrid generations usually contained less photosynthetic pigments than the original plant. This indicates that incompatibility is different in the creative somatic products between these two species and needs further studies.

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