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Changes in chloroplast ultrastructure in P*ssu-ipt* tobacco during plant ontogeny

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Abstract

Changes in chloroplast ultrastructure and total content of endogenous cytokinins (CK) were studied during different phases of plant development in transgenic P*ssu-ipt* tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1). Permanent overproduction of CK was found in both rooted (SE) and grafted (G) P*ssu-ipt* plants in all phases of plant development with the peak in vegetative and flowering phase in the latter ones. No such a correlation was observed in SE on the contrary to control non-transgenic plants (SR1) and grafts (SRG), which showed also CK increase at juvenile and flowering phases. No significant differences in parameters of chloroplast ultrastructure, such as length of chloroplast, starch content, granum width, and number of thylakoids per granum, were proved between chloroplasts from young mature leaves of control and transgenic tobacco during plant ontogeny. Nevertheless, several anomalies in the ultrastructure of cell organelles were found in P*ssu-ipt* tobacco. Amoeboid shape of chloroplasts was often observed in connection with "tubular clusters" resembling peripheral reticulum. The distinct crystalline structures located in chloroplasts might be formed by LHC protein aggregates. Smaller crystals of unknown composition were found also in mitochondria. Numerous crystalline cores were present in peroxisomes. The alterations might be the result of imbalance of phytohormone content, degradation effect of CK overproduction, or the example of acclimation to permanent stress.

Additional key words: chloroplast ultrastructure; cytokinins; grafting; granum; leaf age; *Nicotiana*; peroxisome; phytohormones; thylakoid; transgenic plants.

Introduction

The role of cytokinins (CK) in plants is fairly complex. They affect a variety of processes involved in the regulation of development, maintenance, and senescence of photosynthetic apparatus. CK together with light promote de-etiolation and play the most important role in plastid development (Parthier 1979). At advanced senescence, degeneration of plastid membrane systems and stroma may be reversed by CK action (Gan and Amasino 1997, Zavaleta-Mancera *et al.* 1999). The effect of CK was examined mainly in plants with hormones applied exogenously, usually during early phases of chloroplast development from etioplasts (Naito *et al.* 1981, Colijn *et al.* 1982, Bracale *et al.* 1988, Branca *et al.* 1994). Benzyladenine (BA) or kinetin both promote grana formation and stacking, nevertheless, the extent of the effect depends on the phase of plant development. Caers and Vendrig (1986) showed that BA affected early chloroplast differentiation, whereas in the later phases BA stimulated plastid multiplication. Wilhelmová and Kutík (1995) observed induction of grana stacking in tobacco grown *in vitro* caused by increasing BAP contents.

Endogenous CK content and effect of irradiance on plastid ultrastructure and differentiation were studied in an *Arabidopsis* mutant by Chory *et al.* (1994) and in

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Abbreviations: BA, benzyladenine; CK, cytokinins; G, transgenic graft; LHC, light-harvesting complex proteins; PFD, photon flux density; P*ssu-ipt*, gene for isopentenyl transferase under a control of the promoter for the small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase; SE, transgenic rooted tobacco; SR1, control non-transgenic tobacco; SRG, control non-transgenic graft.

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a moss mutant by Reski and Abel (1992). Transgenic plants with *ipt*-gene under the control of various promoters overproducing endogenous CK were mainly used in studies of CK metabolism and relations with other growth hormones (*e.g.* Eklöf *et al.* 1996). Less attention was devoted to ultrastructural aspects of their action. Our previous research was focused on the effects of elevated CK content on photosynthesis and water regime (Synková *et al.* 1997a, 1999). Nevertheless, electron microscopic observations revealed an occurrence of anomalous vesicles and large amount of swollen thylakoids in calli of P*ssu-ipt* transformed tobacco (Beinsberger *et al.* 1991). This was later confirmed also in P*ssu-ipt* plants (Synková *et al.* 1997a). Changes or anomalies in plastid ultrastructure have been observed in various mutant plants (*e.g.* Knoth 1975, Susalla and Mahlberg 1975, Dinkins *et al.* 1997). Also chloroplasts from plants responding to environmental stresses undergo more or less extensive ultrastructural alterations (for review see Mostowska 1997).

In this paper we focused on the changes of chloroplast ultrastructure in P*ssu-ipt* transgenic tobacco during plant ontogeny. We tested if CK overproduction is responsible for anomalous structures found in P*ssu-ipt* tobacco.

Materials and methods

Plants: Control, non-transformed tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was in this work defined as a control wild type (SR1). Control non-transformed grafts were made from SR1 shoots grafted onto SR1 rootstock (SRG). Transgenic tobacco containing a supplementary *ipt*-gene under a control of the promoter for the small subunit of RuBPCO (P*ssu-ipt*) was generated by means of the *Agrobacterium tumefaciens* transformation and grown as grafts (G) on wild type rootstock as described by Beinsberger *et al.* (1992). The kanamycin resistant progeny of the transgenic grafts developed slowly to mature plants with a small root system (SE). In order to reduce plant heterogeneity, P*ssu-ipt* plants of moderate growth rate were selected.

Because the life span of plant is different in control wild type and both transgenic types, the samples were taken from the plants at the discrete phases of plant development: *iv* = plant during *in vitro* pre-cultivation, VY = juvenile plant in vegetative phase (plants with about 6 leaves), V = vegetative growth (plants with about 12 leaves), FB = plants at the onset of flowering forming the first flower buds, F = flowering plants, FS = plants forming seeds, VIV = viviparous leaves formed by transgenic plants.

All plants were grown after *in vitro* pre-cultivation in pots with soil in a greenhouse from January till September under 25/18 °C day/night and 60 % relative humidity. Day irradiance [overall integrated mid-values were *ca.* 500 μmol (quantum) m^{-2} s^{-1}] was prolonged by the additional irradiation [PFD *ca.* 200 μmol(quantum) m^{-2} s^{-1}] to 16 h.

Leaf samples were taken from the central part of the young fully developed leaf if not stated otherwise. Small pieces of tissue were stained by osmium tetraoxide and aquaeous uranyl acetate after overnight fixation in 3 % glutaraldehyde in 50 mM PIPES buffer (pH 7.5) at 4 °C. After several washes and de-hydration through alcohol series, samples were embedded in Spurr's resin. For light microscopy, semi-thin sections were stained with toluidine blue. For electron microscopy, ultra-thin sections were cut and stained by uranyl acetate and Reynold's lead citrate and examined in transmission electron microscope *Philips EM* equipped by CCD camera. Ultrastructural parameters such as chloroplast length, relative starch area, grana width, and number of thylakoids per granum were evaluated on sections throughout the largest axis of chloroplast by image analysis software *Lucia*.

Cytokinin extraction, purification, and analysis was done according to Benková *et al.* (1999) using HPLC linked to a mass spectrometer. Total CK contents were calculated as a sum of all three fractions (*1* – free bases, ribosides, and N9-glucosides; *2* – ribotides; *3* – N7- and O-glucosides).

Statistical analysis was done by *ANOVA* and Student's *t*-test. Three independent series of all plants were used for sample preparation. For each sample, an average of 10 sections and about 50 randomly chosen mesophyll cells were examined.

Results

Cytokinin content: P*ssu-ipt* transgenic rooted plants (SE) showed significantly higher CK content throughout the whole plant ontogeny and only moderate differences among the different phases compared to control (Table 1). Transgenic grafts exhibited even higher hormone content than SE, particularly in the phases of vegetative growth and flowering. In control SR1 the highest CK content was found in young plantlets during the early phase of vegetative growth (VY), while at the later phases some decrease was observed. In SRG even lower CK content was found after the onset of flowering.

Number of chloroplasts: During *in vitro* cultivation negligible differences in average number of chloroplasts per one mesophyll cell were found among SR1 and transgenic tobacco (Table 2). After plant transfer to *ex vitro* condition, SR1 exhibited increasing number of plastids with the peak at the onset of flowering (FB) and a decline during later development. Contrary to this, in both transgenic types the chloroplast number was stable throughout the ontogeny. Higher number was found in G compared to SE.

Chloroplast size was estimated as the length of chloroplast cross-sections. SR1 and G showed increasing size of plastids during plant ontogeny with the larger ones at later phases of plant development (Fig. 1*A*). No significant difference in the size of chloroplasts between young fully developed and senescent leaves was found at flowering phase in SR1 and SE, although in SE the dif-ference was significantly smaller (Fig. 2*A*). SE and SRG exhibited more uniform plastid size during the ontogeny with peak at the phase of seed forming phase (Fig. 1*A*). Moderate differences in chloroplast size between young and senescent leaves were observed in SRG and G (Fig. 2*A*).

Table 1. Total CK content at different phases of plant development (*iv* = *in vitro*, VY = early vegetative phase, V = vegetative phase, FB = flower buds, F = flowering, FS = seeds forming phase) in control non-transgenic tobacco (SR1 – rooted plants, SRG – grafted plants) and P*ssu-ipt* transgenic tobacco (SE – rooted plants, G – grafts). Means of triplicate determination. nd = not determined. Statistically significant differences at p=0.05 are marked by *different letters*.

Phase	SR1	SRG	G	SE
iv	nd	nd	627^{c}	1478^{c}
VY	384^{a}	nd	3228^{d}	539^{ac}
V	37^{b}	97^{ab}	1184^{c}	530^{ac}
FB	225^{a}	253^{a}	871^{c}	1019^{c}
F	131^{a}	44^{b}	4266^{d}	1835^{c}
FS	206^{a}	77^{b}	nd	1532^{c}

Table 2. Effect of plant type (SR1 = control non-transgenic tobacco, SE = rooted P*ssu-ipt*, G = grafted P*ssu-ipt* tobacco) and plant development (*iv* = *in vitro*,V = vegetative phase, FB = flower buds, F = flowering, FS = seeds forming phase) on the number of chloroplasts per section of palisade parenchyme cell. Means ± SE. Statistically significant differences at p = 0.05 are marked by *different letters*.

Phase	Plant SR1	SE	G
iv	11.7 ± 4^{a}	14.3 ± 4^{a}	14.9 ± 3^{a}
V	24.5 ± 7^{b}	15.5 ± 4^{a}	27.2 ± 4^{b}
FB	42.3 ± 3^{c}	17.8 ± 3^{a}	25.0 ± 4^{b}
F	16.0 ± 3^{a}	17.3 ± 4^{a}	22.1 ± 3^{ab}
FS	23.7 ± 4^{b}	19.1 ± 5^{ab}	17.6 ± 4^{a}

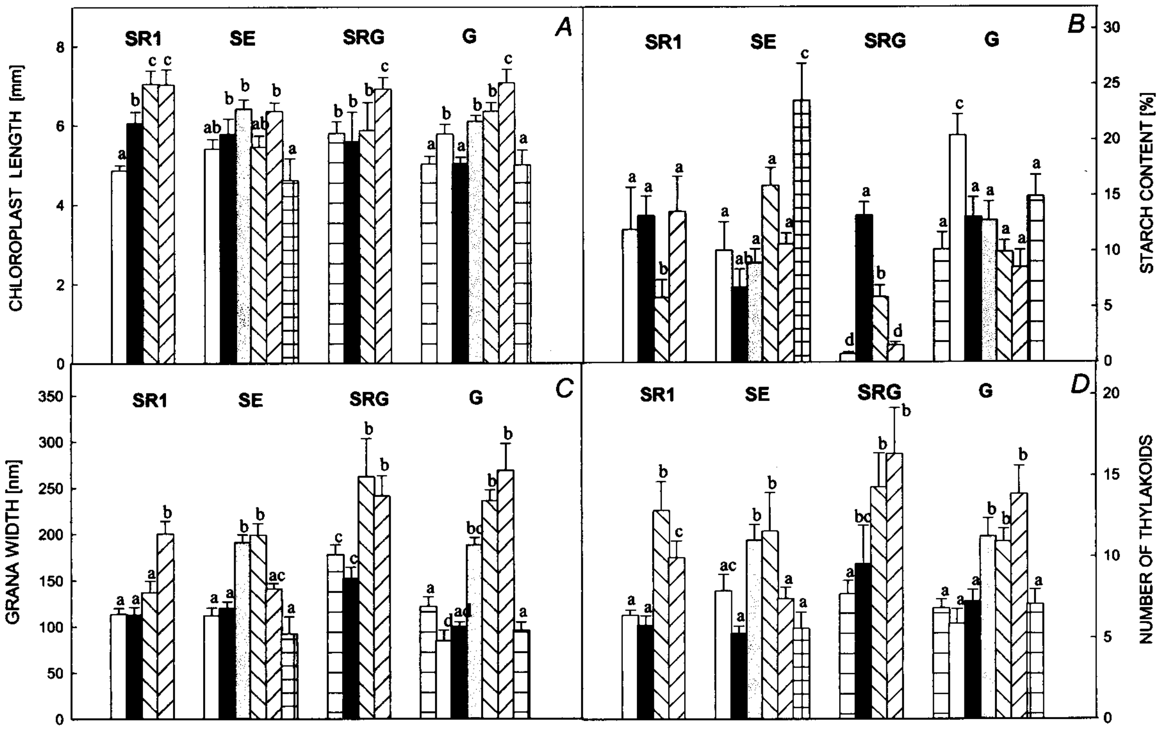

Fig. 1. Ultrastructural parameters of chloroplasts from young fully developed leaf of non-transgenic rooted plants (SR1), control grafts (SRG), transgenic rooted plants (SE), and transgenic grafts (G) at different phenological phases: *iv* = *in vitro* (▤);VY = early vegetative phase (☐); V = adult vegetative phase (■); FB = the first flower buds (▦); F = flowering phase (▧); FS = seed forming phase (▨); VIV = viviparous shoots (⊞). (*A*) The average length of chloroplast cross sections; (*B*) starch content calculated as percent total chloroplast area; (*C*) average width of grana, and (*D*) average number of thylakoids per granum. Means ± S.E. *Different letters* mark statistically significant differences at p = 0.05.

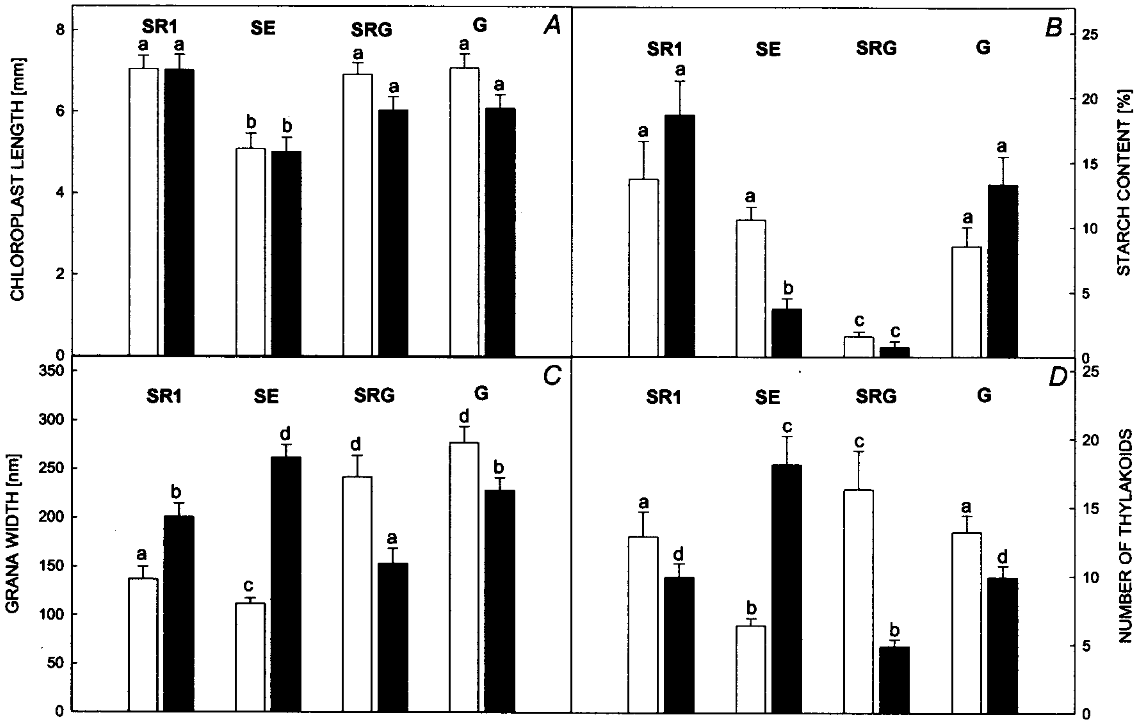

Fig. 2. Ultrastructural parameters of chloroplasts from young fully developed leaf (*empty columns*) and from senescent leaf (*full columns*) of non-transgenic rooted plants (SR1), control grafts (SRG), transgenic rooted plants (SE), and transgenic grafts (G) at flowering. (*A*) Average length of chloroplast cross sections; (*B*) starch content calculated as percentage of total chloroplast area; (*C*) average width of grana; and (*D*) average number of thylakoids per granum. Means ± S.E. *Different letters* mark statistically significant differences at $p = 0.05$.

Relative starch content was estimated as a percentage of the total area of chloroplast cross-section and the area occupied by starch grain (Fig. 1*B*). Very low starch content was found in SRG, particularly in *in vitro* shoots and at the phase of forming seeds (FS). SR1 and SE exhibited only small differences in starch content among developmental phases, except for the decline at flowering phase in the former one and a significant increase in viviparous shoots in the latter one. G showed also stable content of starch throughout plant ontogeny, although significant increase was observed in very young plants (VY). Differences in starch content between young fully developed and senescent leaves at flowering stage were significant only in SE, where lower content was found in senescent leaves (Fig. 2*B*) similarly as in SRG. SR1 and G showed opposite effect, starch content was lower in younger leaves.

Grana stacking: Size of grana was estimated from granum width and number of thylakoids stacked in one granum (Figs. 1*C,D* and 2*C,D*). SR1 showed increasing stacking of thylakoids during plant development with broader stacks at the phase of forming seeds. The apparent disproportion between number of thylakoids and grana width was found at F and FS phase. In SRG and G higher grana were found at F and FS. In SE a decrease in granum size was observed at FS. Both viviparous shoots (SE and G) showed lower grana stacks. Comparison of granum width and number of thylakoids per granum between young fully developed and senescent leaves at F (Fig. 2*C,D*) proved lower thylakoid stacking in older leaves in SRG and G, on the contrary to SR1 and SE.

Chloroplast ultrastructure: Chloroplasts from SR1 and SRG showed developed ultrastructure with distinct envelopes and grana, regular shape, small or larger starch grains, and plastoglobuli. While small amounts of starch and plastoglobuli were found in chloroplasts of the younger plants, the number and size increased with plant age. Detailed views of grana at the vegetative (VY) and flowering (F) phases are in Fig. 3 (SR1 in *A* and *B*, respectively). Less regular shape of plastids, sometimes swollen thylakoid membranes, and larger light plastoglobuli were observed at the later phases of plant ontogeny in transgenic SE (Fig. 3*C,D*) and during the whole plant development in G (Fig. 3*E,F*). Less regular or irregular shape of grana stacks was also observed. Although the majority of plastids showed more or less normal ultrastructure, a small but significant part of chloroplasts from transgenic G and SE exhibited anomalies in their ultrastructure. Irregular or amoeboid shape of plastids was often accompanied with distinctive peripheral

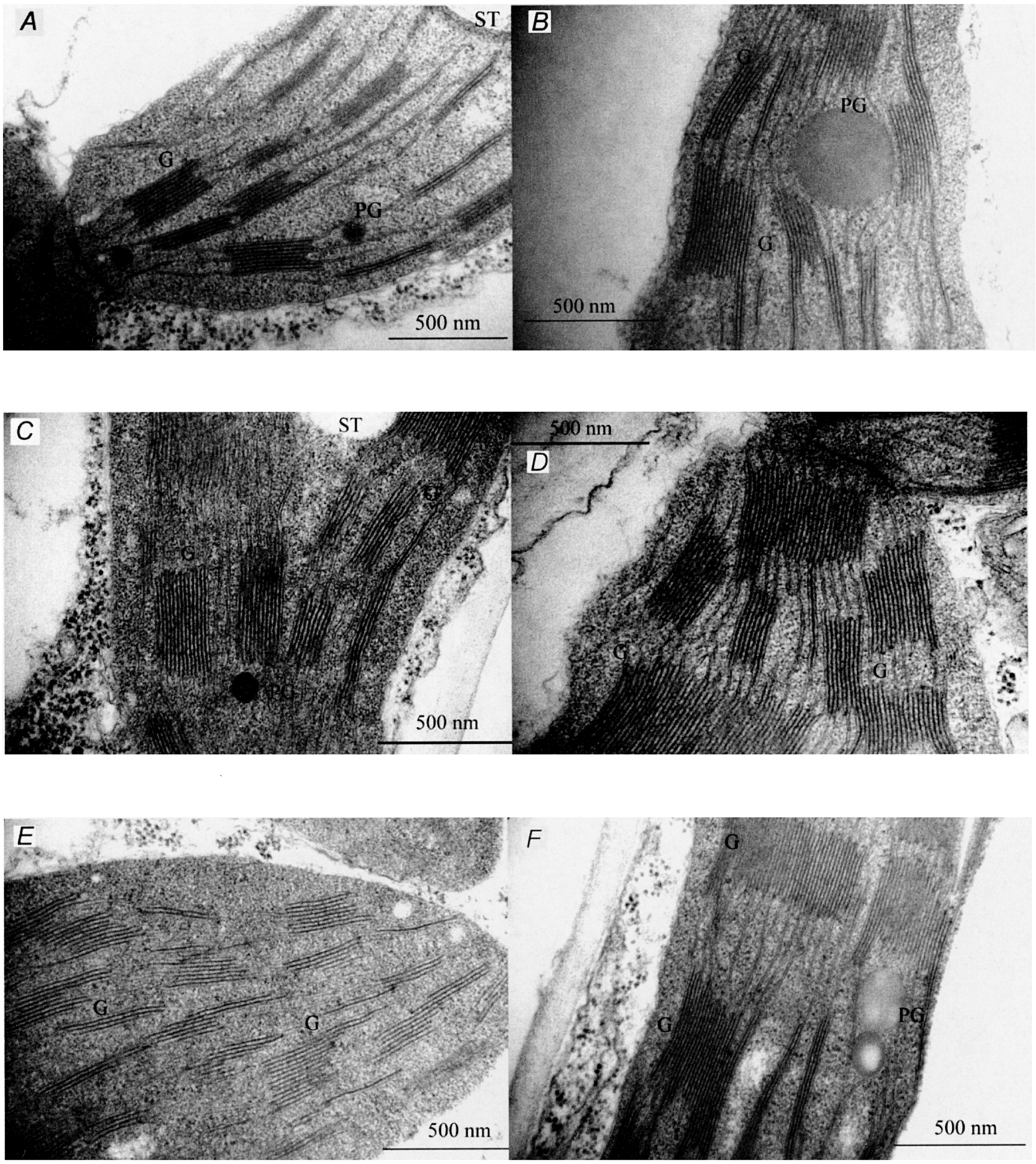

Fig. 3. Detailed view of chloroplasts of non-transgenic rooted plants (*A, B*), transgenic rooted plants (*C, D*), and transgenic grafts (*E, F*) at vegetative phase and at flowering, respectively. G = grana; PG = plastoglobuli; ST = starch.

reticulum. Inclusions or protrusions into cytoplasm formed by chloroplast envelope were frequently observed (Fig. 4*A*). Often other organelles such as mitochondrion or peroxisome were located inside the cups (Fig. 4*B*). The most prominent particles often found in chloroplasts were large protein crystals with a fine membrane like structure (Fig. 4*C,D,E*). They were observed particularly at the later phases of ontogeny, starting at the onset of flowering, although they could be found at all developmental phases in G.

The occurrence of large, probably protein bodies inside plastid stroma, which were more or less filled by electron dense material (Fig. 4*F*), was restricted to younger plants and viviparous shoots.

Crystallic cores in peroxisomes and paracrystalline and amorphous inclusions in mitochondria were found in transgenic tobacco more often than in SR1 (Fig. 5).

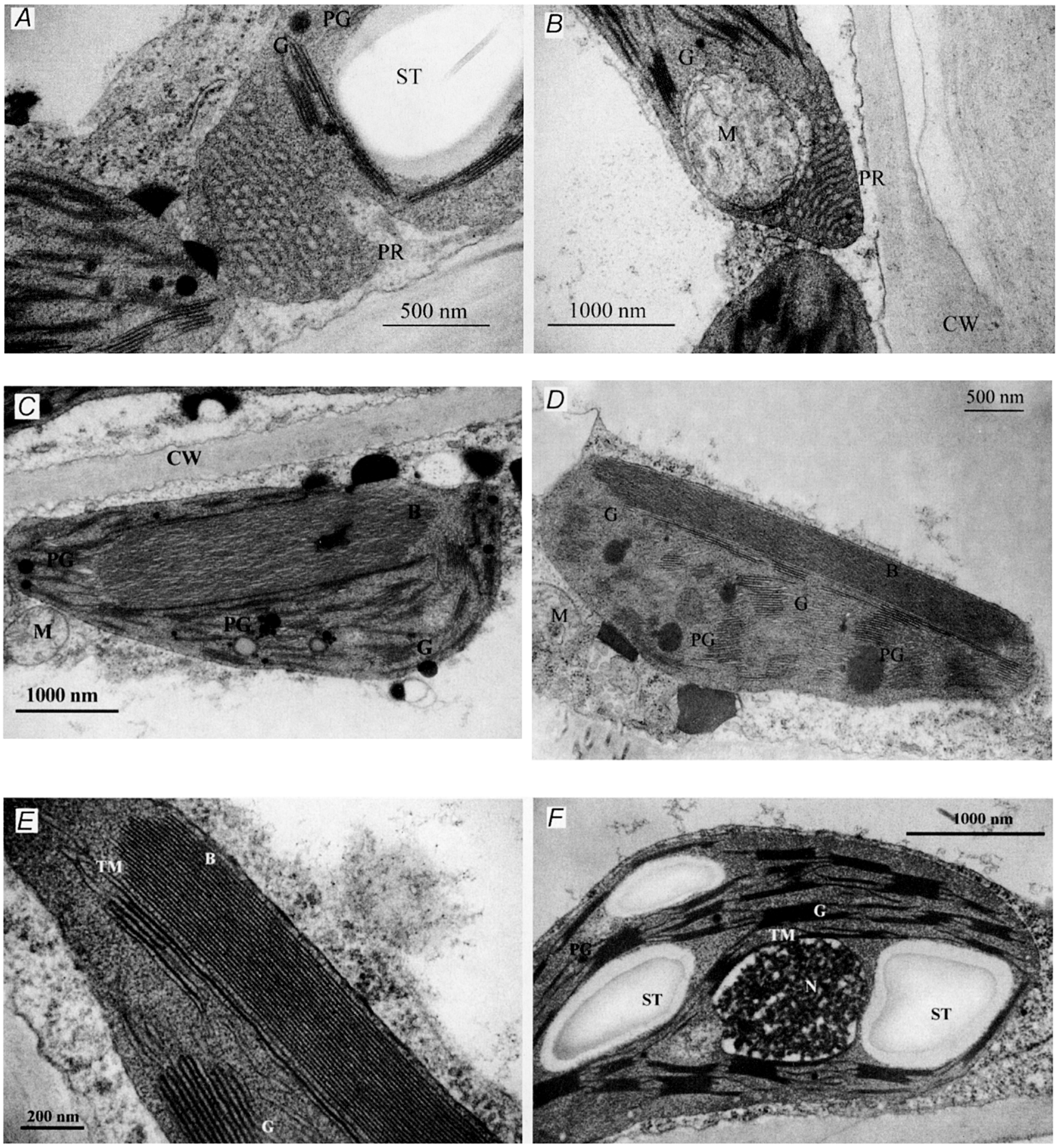

Fig. 4. Electron micrographs of anomalies in chloroplast ultrastructure found in P*ssu-ipt* tobacco. (*A*) Peripheral reticulum; (*B*) mitochondrion inside the cup formed by peripheral reticulum; (*C, D*) two different forms of large crystalloid inside chloroplast; (*E*) detailed view of lamellar structure of this crystalloid; (*F*) protein body inside chloroplast. B = crystalloid; CW = cell wall; G = granum; M = mitochondrion; N = protein body; PG = plastoglobuli; PR = peripheral reticulum; ST = starch; TM = thylakoid membranes.

Discussion

Although CK metabolism has been extensively studied recently (Mok and Mok 2001), only a few studies documented the changes in hormone contents throughout the plant life cycle (Slovin and Cohen 1988). Mercier and Endres (1999) concluded from their studies of *Tillandsia recurvata* that in developmental phases characterised by intense organ formation, such as juvenile and flowering phases, there was an enhancement of CK content. This is in agreement with our results. Introduction of the *ipt* gene under various promoters caused usually overproduction of endogenous CK, which also was the case of P*ssu-ipt* plants (Synková *et al.* 1997b). Control non-transgenic tobacco (SR1 and SRG) showed higher CK contents at VY and FB phases. Nevertheless, there was no such dependence in transgenic plants. CK contents were more than five times higher in SE compared to SR1 and even higher in transgenic grafts (G) during all phases of plant life cycle. This means that CK overproduction was not a temporary phenomenon and could affect plant metabolism during the whole plant life. Prolonged life span, one of the well-known CK effects (Gan and Amasino 1997), was observed in P*ssu-ipt* transgenic tobacco.

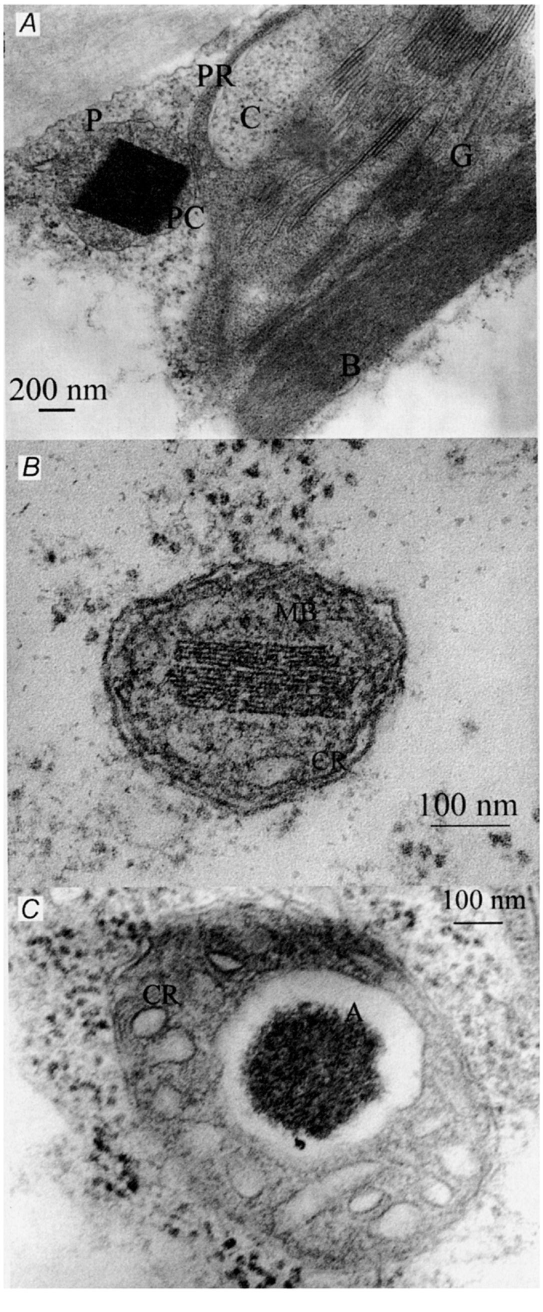

Fig. 5. Ultrastructure of peroxisome (*A*) and mitochondria (*B*, *C*) from P*ssu-ipt* tobacco. A = amorphous inclusion in mitochondrion; B = chloroplast crystalloid; C = cytoplasm; CR = crystae; G = granum; MB = mitochondrial crystalline structure; P = peroxisome; PC = peroxisomal cores; PR = peripheral reticulum; ST = starch; TM = thylakoid membranes.

The number of chloroplasts per palisade parenchyma cells increases and chloroplasts grow more in length than in breadth during leaf growth (Čatský and Šesták 1997). The number of chloroplasts per cell declines slowly until the last phase of leaf senescence, when rapid degradation of plastids takes place (Ono *et al.* 1995). In our experiment the ultrastructural parameters of mature chloroplasts were studied in the leaves of the same insertion level and the same phase of leaf development but at the different phases of plant life cycle. Nevertheless, some changes in the amount and size of chloroplasts were observed in control non-transgenic tobacco (SR1) in contrast to both transgenic SE and G. Less and smaller chloroplasts were found in SE independently on developmental phase.

Starch accumulation was not significantly affected by exogenously applied CK (Bracale *et al.* 1988, Branca *et al.* 1994). However, Markarova and Kolchugina (1998) reported presence of large starch grains in chloroplasts of *Ficus elastica* callus treated by BA. The amount of starch may increase or decline during later phases of leaf ontogeny (Čatský and Šesták 1997). High starch content is typical for chloroplasts of plants grown under high irradiance (Lichtenthaler and Meier 1984). P*ssu-ipt* transgenic tobacco accumulated starch significantly in viviparous shoots and in very young plants (G, Fig. 1*B*). Accumulation of starch may indicate depressed translocation of metabolites and may interfere with photosynthesis (Laza *et al.* 1993). Non-transgenic grafts (SRG) exhibited very low starch contents throughout the ontogeny except for vegetative phase in contrast to others. Limitations in nitrogen supply or more advanced senescence process observed in SRG could be caused probably by grafting itself.

Even though transgenic P*ssu-ipt* tobacco suffers permanently from a mild water stress because of an inadequate root system (Synková *et al.* 1999), permanent overproduction of endogenous CK might be responsible for the alterations in chloroplast ultrastructure. This is supported by more frequent occurrence of anomalies in transgenic grafts even at early phases of plant development than in rooted transgenic plants, while water stress was more pronounced in the latter ones (Synková *et al.* 1999).

Amoeboid plastids in meristematic tissues are considered a temporary phase of plastid development. Pleiomorphic forms conspicuously increase the plastid surface area and lead to more efficient exchange of metabolites (Hudák 1997). This might be also the case of P*ssu-ipt* tobacco, where surface area can be even increased by peripheral reticulum similar to that reported, *e.g.*, in *Nymphoides indica* (van Steveninck *et al.* 1972) and in a mutant of *Lemna* (Monselise *et al.* 1984). In P*ssu-ipt* tobacco, systems of 'channel' like vesicles often included other organelles, which was proved by serial sectioning. This might support the idea that organelles need to communicate more closely under certain conditions (Mckenzie and McIntosh 1999). Hedtke *et al.* (1999) suggest that such an inter-organellar cross-talk exists and that lack of chloroplast activity leads to an increase in mitochondrial gene copy number and an elevated level of mitochondrial transcripts.

Some lamellae-bound crystals present in the intrathylakoid space of chloroplasts have been identified as ribulose-1,5-bisphosphate carboxylase/oxygenase (Sprey 1976, Vapaavuori 1986). The plastid stroma may also contain deposits of protein phytoferritin (Hudák 1997). However, the most prominent crystalline structure found in P*ssu-ipt* chloroplasts (Fig. 4*C,D,E*) resembles partly structures called "macrograna". They were found in various mutant plants (*e.g.* Knoth 1975, Dinkins *et al.* 1997), during senescence (Hudák 1981), and after treatment by herbicides (Agnolucci *et al.* 1996, Ogawa *et al.* 2001) and heavy metals (Mostowska 1997). In contrast to macrograna, which seem to be giant grana stacks, the structures observed in P*ssu-ipt* chloroplasts form larger or smaller protein crystals with a finer lamellar structure. Although their composition and physiological function is still unknown, our hypothesis suggests that those particles are formed by LHC protein aggregating in a form of 2D crystals which constitute membrane stacks. Such aggregates of LHC may develop during degradation of the photosynthetic apparatus (Šiffel and Vácha 1998) or during senescence (Prakash *et al.* 2001). Rather scarce occurrence of similar structures in senescent leaves of control tobacco could support this idea. The presence of certain bands in fluorescence emission spectra indicates an accumulation of LHC aggregates in isolated P*ssu-ipt* chloroplasts (Šiffel, unpublished). Moreover, CK promote also the expression of genes encoding the protein of LHC (*e.g.* Teyssendier de la Serve *et al.* 1985).

The function of large protein bodies inside chloroplast stroma is questionable (Fig. 4*F*) and we did not find any reference to this phenomenon. Nevertheless, they occurred particularly in young grafts during the fast vegetative growth or in vivipary, where mostly undifferentiated cells were present. This could indicate a function as reservoir of proteins or material utilised later in plastid development.

Crystalline lamellar structures found in mitochondria (Fig. 5*B*) resemble those found in chloroplasts, but their structural components are even more speculative. Their occurrence has not been reported in the literature to our knowledge. Other more or less amorphous particles present in mitochondrion (Fig. 5*C*) could be products of mitochondrial metabolism. A similar phenomenon was observed in mitochondria of salt-resistant tobacco cells accumulating proline (Shevyakova *et al.* 1998).

Crystalline cores (Fig. 5*A*) sometimes present in peroxisomes had been considered storage components until recent studies proved that their predominant protein is catalase, which differs in its primary structure from the catalase in peroxisomal matrix (Kleff *et al.* 1997). As the number of peroxisomes with cores significantly increased in transgenic tissues, we suppose that cores could be made of enzymatically active catalase that can improve protection against reactive oxygen species.

Some of the above mentioned alterations were observed under stress conditions or during advanced senescence. Therefore they could be the result of degradation caused by long-term stress probably due to CK overproduction or genetic transformation itself. However, another possibility is that the changes might represent the plant acclimation to that stress. Closer co-operation among plant organelles demonstrated here is needed to withstand such unfavourable conditions. Nevertheless, more detailed study of the particular crystalline structures in chloroplast, mitochondrion, and peroxisome is necessary to elucidate their components, structural units, and function.

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