Cytokinin-induced activity of antioxidant enzymes in transgenic P*ssu-ipt* tobacco during plant ontogeny

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Abstract

Cytokinin (CK) content and activities of several antioxidant enzymes were examined during plant ontogeny with the aim to elucidate their role in delayed senescence of transgenic P*ssu-ipt* tobacco. Control *Nicotiana tabacum* L. (cv. Petit Havana SR1) and transgenic tobacco with the *ipt* gene under the control of the promoter of small subunit of Rubisco (P*ssu-ipt*) were both grown either as grafts on control rootstocks or as rooted plants. Both control plant types showed a decline in total content of CKs with proceeding plant senescence. Contrary to this both transgenic plant types exhibited at least ten times higher content of CKs than controls and a significant increase of CK contents throughout the ontogeny with maximal values in the later stages of plant development. Significantly higher portion of *O*-glucosides was found in both transgenic plant types compared to control ones. In transgenic plants, zeatin and zeatin riboside were predominant type of CKs. Generally, P*ssu-ipt* tobacco exhibited elevated activities of antioxidant enzymes compared to control tobacco particularly in the later stages of plant development. While in control tobacco activity of glutathione reductase (GR) and superoxide dismutase (SOD) showed increasing activity up to the onset of flowering and then gradually decreased, in both transgenic types GR increased and SOD activity showed only small change throughout the plant ontogeny. Ascorbate peroxidase (APOD) was stimulated in both transgenic types. The manifold enhancement of syringaldazine and guaiacol peroxidase activities was observed in transgenic grafts throughout plant ontogeny in contrast to control and transgenic rooted plants, where the increase was found only in the late stages. Electron microscopic examination showed higher number of crystallic cores in peroxisomes and abnormal interactions among organelles in transgenic tobacco in comparison with control plant. The overproduction of cytokinins resulted in the stimulation of activities of AOE throughout the plant ontogeny of transgenic P*ssu-ipt* tobacco.

Additional key words: ascorbate peroxidase, catalase, glutathione reductase, guaiacol peroxidases, superoxide dismutase, syringaldazine peroxidase.

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Abbreviations: AOE - antioxidant enzymes; APOD - ascorbate peroxidase; BSA - bovine serum albumine; CAT - catalase; CK - cytokinins; C - control type rooted tobacco; C/C - control grafted plants; DAB - 3,3´-diaminobenzidine; DTT - dithiotreitol; GPOD - guaiacol peroxidase; GR - glutathione reductase; *ipt* - the gene for isopentenyl transferase; PAGE - polyacrylamide gel electrophoresis; PC - peroxisomal cores; P*ssu* - promoter sequence of the gene coding for small subunit of Rubisco; SPOD - syringaldazine peroxidase; SOD - superoxide dismutase; T - transgenic rooted plants; T/C - transgenic grafts; TEM - transmission electron microscopy.
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Introduction

Cytokinins (CK) are plant hormones involved in regulations of plant growth and development, as well as the stress response (Haberer and Kieber 2002). In spite of the recent progress in CK signal transduction studies (for review see Aoyama and Oka 2003), mechanisms of their action still remain to be fully elucidated. CK functions have often been studied with advantage in systems with elevated CK contents, either by the application of exogenous CKs or using plants overexpressing CK biosynthetic gene. Problems with uptake and transport limit an application of exogenous CKs, while on the other hand, a constitutive expression of the *ipt* gene constantly disturbs CK homeostasis (Kamínek *et al*. 1997) and this induces stress responses. This was manifested by, *e.g.*, salt stress symptoms, such as an accumulation of proline and osmotin (Thomas *et al*. 1995). In spite of the fact that CK overproducing systems do not fully reflect the situation in normal plants, their use has allowed to obtain numerous relevant data on CK mode of action (*e.g*. Kamínek *et al*. 1997).

CKs are important retardants of senescence. Correlation between CK content in leaves or xylem sap of normal plants and the onset of senescence was reported by Badenoch-Jones *et al*. (1996). The positive effect of increased CK contents on delay of senescence was also monitored in tobacco plants expressing the *ipt* gene (Gan and Amasino 1997). CKs may retard senescence directly by scavenging or interferring with free radicals, which are proposed to be involved in this process (Leshem *et al*. 1981, Miller 1992). CKs were also reported to modulate activities of various antioxidative enzymes, *e.g*. peroxidase (Chaloupková and Smart 1994), superoxide dismutase (SOD) (Kurepa *et al*. 1997), and catalase (CAT) (Petit-Paly *et al*. 1999). Hormone treatment that induced resistance to viral infection also enhanced the activity of enzymatic systems for detoxification of active oxygen species (Clarke *et al*. 2000). Activities of antioxidant enzymes (AOE) and their response to stress depend on plant developmental stage. While the increase in activities of SOD, CAT, glutathione reductase (GR) etc. was observed in young tissues as a response to stress, no change or even decline was found in later stages of plant or tissue development (Asada *et al*. 1977, Casano *et al*. 1994).

Our previous studies showed that regardless of permanent mild water stress, plant and leaf senescence was considerably delayed in transgenic tobacco with elevated CK content (Synková *et al*. 1999). Activities of several AOE were differently modulated by imposed water stress and growth conditions compared to control wild type (Synková and Valcke 2001).

Because the expression and enzyme activities are affected by processes of plant development and senescence and also by CKs, we focused our study on oxidative metabolism in P*ssu-ipt* transgenic tobacco throughout the plant ontogeny. Detailed CK analysis was done at several distinct phenological stages to elucidate if overproduction of CKs is stable throughout the plant ontogeny in transgenic tobacco. *In situ* localisation of peroxidase, electron microscopic observations of a close association of organelles, and a more detailed analysis of the structure of peroxisomal cores was done to distinguish the importance of those aspects in the adaptation to stress caused by overproduction of cytokinins.

Materials and methods

Plants and growth conditions: Control tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) was referred as C. Control grafts (C/C) were made from control C shoots grown *in vitro*, grafted onto C rootstock. Transgenic tobacco containing *ipt*-gene under the control of the promoter for the small subunit of Rubisco (P*ssu-ipt*) was generated by means of the *Agrobacterium tumefaciens* transformation system and grown *in vitro* as shoots unable to form roots. The transgenic shoots were grafted on C rootstock (T/C) as described by Beinsberger *et al*. (1992). P*ssu-ipt* transgenic plants (T), *i.e*. the autogamic progeny of the transgenic grafts, which are able to form a small root system, were selected on agar medium with kanamycin (*in vitro*) and then transferred into soil. In order to reduce plant heterogeneity, P*ssu-ipt* plants of moderate growth rate were selected.

Generally, two types of plants were used in our experiments: *a*) plantlets grown from seeds (C, T), which were sown on agar, precultivated for about three weeks *in vitro*, and then transferred to soil; *b*) *in vitro* precultivated shoots (T/C, C/C), which were used after three weeks of cultivation as scions and grafted on rootstocks of C grown in soil before and after grafting. Although the absolute age of control type and both transgenic types was different, the samples were taken from the plants in the comparable stages of plant development: *iv* = during *in vitro* precultivation, VY = very young, a stage of a vegetative growth with total number of 6 leaves, V = vegetative growth (about 12 leaves), FB = at the onset of flowering, plants forming the first flower buds, F = flowering plants, FS = plants flowering and forming seeds. Leaf samples were taken from the central part of the first young fully developed leaf, frozen in liquid nitrogen and kept at -70 °C until use.

All plants were grown after *in vitro* precultivation in pots with soil substrate in a greenhouse from January till September under day/night temperature of 25/18 °C, and relative humidity (RH) 60 %. Natural irradiance [overall integrated mid-values, *ca*. 500 μmol(photon) m^{-2} s^{-1}] was prolonged to 16 h by additional illumination (*AgroSon T* and *HT9* lamps, *ca*. 200 μmol m^{-2} s^{-1}).

Cytokinin analysis: Homogenized leaf samples were extracted overnight in Bieleski´s solvent (Bieleski 1964). Before centrifugation at 24 000 *g* for 15 min at 4 °C, deuterated standards for CKs (*Apex International*) were added. Extracts were purified using *DEAE-Sephadex* (to separate nucleotides), C18 cartridges, and immunoaffinity columns. In this way CKs were separated into three fractions: *a*) free bases, ribosides, and *N9*-glucosides; *b*) ribotides, determined as ribosides after cleavage by alkaline phosphatase; and *c*) *N7*- and *O*-glucosides. Quantitative analysis of CKs was performed as described in Witters *et al*. (1999) by micro liquid chromatography with column switch configuration coupled to positive ion electrospray tandem mass spectrometry (*Finnigan LCQ*).

Enzyme activities: Leaf tissue (1 g fresh leaf matter) was homogenized in 5 cm^3 of ice cold buffer (0.1 M Tris-HCl, pH 7.8) containing 1 % of polyvinylpyrrolidone and 1 mM dithiotreitol (DTT) and centrifuged at 20 000 *g* for 10 min. The supernatant obtained was immediately used for enzyme activity measurements. Frozen equivalents were later used for soluble protein determination according to Bradford (1976). Activities of enzymes were determined spectrophotometrically (Helios α, *Thermo-Spectronics*, USA) and the a specific activity in μmol of respective product formed or disappeared per mg of total soluble proteins per min was given.

Glutathione reductase (GR, EC 1.8.1.7.) activity was assayed by the oxidation increase of NADPH at 340 nm according to Goldberg and Spooner (1984). Catalase (CAT, EC 1.11.1.6) was detected at 240 nm as the rate of decomposition of H_2O_2 as described by Aebi (1984). Total ascorbate peroxidase (APOD, EC 1.11.1.11) activity was determined as the decrease in absorbance of ascorbate at 298 nm by the method of Gerbling *et al*. (1984). Peroxidase (EC 1.11.1.7) activity (GPOD) was assayed as an increase of absorbance at 436 nm with quaiacol (18 mM) by the method of Amako *et al.* (1994). Syringaldazine peroxidase (SPOD) was determined with syringaldazine as substrate and absorbance at 530 nm was measured according to Imberty *et al*. (1984).

Superoxide dismutase (SOD, EC 1.15.1.1.) isozymes patterns and activities were obtained after separation by gradient 7 - 14 % nondenaturing polyacrylamide gel electrophoresis (PAGE). Aliquots of supernatants corresponding to 55 μg of protein per lane were used. SOD isozymes were detected *in situ* in the gel by photochemical nitroblue tetrazolium (NBT) staining method according to Beauchamp and Fridovich (1971). Stained gels were scanned and the relative activity of SOD was estimated as the sum of intensities of individual bands.

Purification of peroxisomal cores: Peroxisomal cores (PC) were purified by Percoll density gradient centrifugation of crude particle fractions from tobacco leaves according to the method of Tenberge *et al*. (1997). To prepare a crude particle fraction, 200 g of tobacco leaves were homogenized in the extraction medium (0.3 M mannitol, 20 mM pyrophosphate buffer (pH 7.5), 1 mM EDTA, 0.1 % BSA), and after filtration centrifuged at 1 000 *g* for 15 min. The pellet was twice washed, resuspended and recentrifuged at 10 000 *g* for 10 min. The resulting pellet was resuspended in 2 cm^3 of washing medium (without BSA) and layered on top of a discontinuous Percoll step gradient (20, 38, 80 %). After centrifugation at 40 000 *g* for 30 min, four main bands were distinguished. The fraction obtained from the 80 % and 38 % Percoll interface was enriched in PC. The fraction was diluted with double volume of washing medium and centrifuged at 4 000 *g* for 15 min. The precipitated cores were resuspended in 10 mM phosphate buffer (pH 7.2). The suspension was used for measurements of enzyme activity and for electron microscopic evaluation.

***In situ* localization of peroxidases:** Histological staining for peroxidase activity was carried out using 3,3´-diaminobenzidine (DAB) on cryo-sections made from leaf tissue (Feltlová 2000). Freshly harvested leaves were cut into small pieces (*ca*. 3 × 3 mm), vacuum infiltrated by fixation medium containing 0.25 % (v/v) glutaraldehyde and 3 % (m/v) p-formaldehyde in PBS buffer [NaCl (135 mM), KCl (2.7 mM), KH_2PO_4 (1.5 mM), and K_2HPO_4 (8 mM), pH 7.2]. After 2 h, the samples were washed in PBS buffer and transferred through series of buffers with increasing sucrose concentration (from 0.1 M to 1.8 M) into 1.8 M sucrose solution and then frozen at -70 °C. Cryo-sections were cut on cryomicrotome (*Cryotome*, *Cryostat Shandon*, Pittsburg, USA) and then transferred through the serie of buffers with decreasing sucrose concentration into TBS [Tris (50 mM), NaCl (150 mM), pH 7.6] at room temperature. Staining was done by incubation of the sections with DAB (50 mg per 100 cm^3) in the presence of H_2O_2 (5 mM) for 15 min. After thorough washing by deionized water and dehydration through a serie of solutions with increasing ethanol concentration permanent preparations were made. The samples were examined by light microscopy (*Nicon Eclipse E600*, Japan) equipped with a CCD camera.

Transmission electron microscopy (TEM): Leaf samples were taken from the central part of the young fully developed leaf. Small pieces of tissue or

suspensions of peroxisomal cores were stained by OsO_4 and aquaeous uranyl acetate after the overnight fixation in 3 % glutaraldehyde in 50 mM PIPES buffer (pH 7.5) at 4 °C. After several washes and dehydration through alcohol series the samples were embedded in Spurr´s resin. For TEM, ultrathin sections were cut and stained by uranyl acetate and Reynold´s lead citrate and examined in *JEM 1010* (*Jeol*, Japan) equipped with a CCD camera.

Images taken from serial sections were used for three-dimensional (3D) reconstructions. This was done by the program *IMOD 2.42*. Lattice parameters of crystal basic unit cell of peroxisomal cores were obtained from the Fourier analysis of images using *MRC Cambridge Image Processing System* (1994) software.

The suspensions of PC were also examined by the negative staining using uranyl acetate on copper grids covered by carbon layer. For the core size estimation, images were processed and evaluated using *LUCIA* software. Besides of 3D reconstructed PC, sections with maximal area of PC were exclusively taken into account.

Statistical analysis: Four independent series of plants were used in this experiment. Each value represents the mean of at least 20 independent measurements, except CKs determination, where triplicates were taken. Data were statistically analyzed by a one way analysis of variance, combined with Fisher's LSD test (α = 0.05, two tailed).

Results

Cytokinin contents: The results of CK analysis were presented in five groups with the aim to distinguish the different groups: *O*-glucosides (zeatin-9-riboside-*O*-glucoside, zeatin-*O*-glucoside, dihydrozeatin-9-riboside-*O*-glucoside, dihydrozeatin-*O*-glucoside), *N*-glucosides (*i.e*. zeatin-7-glucoside, zeatin-9-glucoside, dihydrozeatin-7-glucoside, dihydrozeatin-9-glucoside, isopentenyladenine-7-glucoside, isopentenyladenine-9-glucoside), isoprenoid free bases and ribosides (*i.e*. zeatin, zeatin-9-riboside, isopentenyladenine, isopentenyladenine-9-riboside), isoprenoid derived bases and ribosides (dihydrozeatin, dihydrozeatin-9-riboside), and ribotides.

Total CK contents varied significantly throughout the plant ontogeny in all plant types. Maximal values in both control types were reached in vegetative stages and then they declined. In C/C, CK contents were even slightly lower than in C (Fig. 1*A*,*B*). Constitutive expression of *ipt* gene caused in both transgenic plants (T/C and T) a significant increase of CK contents in comparison to controls in all stages of plant development with higher contents in the later stages (Fig. 1*C*,*D*). The striking differences between control type and transgenic tobacco were observed also in abundance of individual CK types. Free bases and ribosides represented 15 - 38 % of

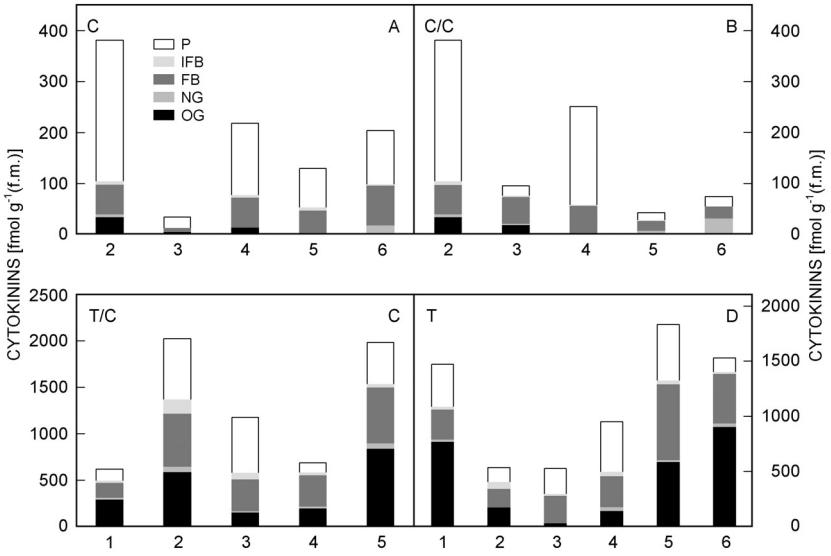

Fig. 1. Content of cytokinins in several stages of plant development (1 - during *in vitro* precultivation ; 2 - very young, in the stage of vegetative growth with total number of 6 leaves; 3 - vegetative growth (about 12 leaves); 4 - at the onset of flowering, plants forming the first flower buds; 5 - flowering plants; 6 - plants flowering and forming seeds) in control rooted tobacco C (*A*), in control grafts C/C (*B*), transgenic grafts T/C (*C*), and transgenic rooted tobacco T (*D*). CKs are presented in five groups: OG - *O*-glucosides, NG - *N*-glucosides, FB - free bases + ribosides, IFB - isoprenoid derived bases + ribotides, P - all ribotides. The part corresponding to each group represents the mean value of sum of respective CKs.

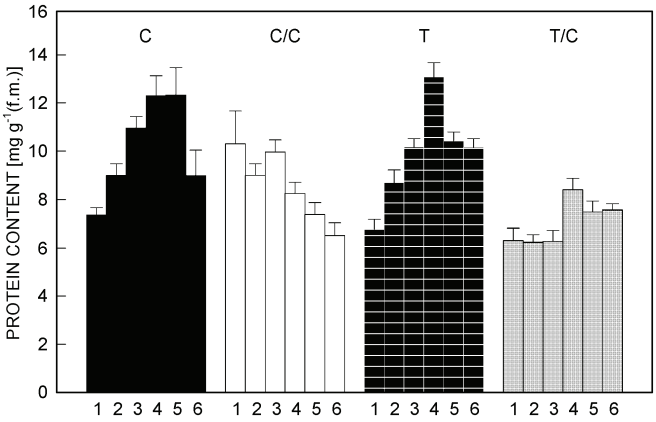

Fig. 2. Total soluble protein contents in several stages of plant development (see Fig. 1 for abbbreviations). Means ± SE, n = 19.

total CKs in C, while the content of ribotides decreased from 73 to 52 % during plant ontogeny. *O*-glucosides were detected in the early stages of development and represented up to 13 % of the total CK content in C. However, no *O*-glucosides were detected after the onset of flowering in C. C/C showed rather a different composition of CKs, *i.e*. free bases and ribosides represented about 55 to 20 %, and ribotides from 23 to 77 % with the maximum values in FB for the latter ones. Content of *N*-glucosides increased from 2 to 42 % in C/C during ontogeny in contrast to C, where only very low contents were found. In T/C, free bases and ribosides represented from 15 to 49 % of the total CKs, with the maximal value in FB. Ribotides represented about 50 % of total CKs in the earlier stages and about 15 % in the later stages of development. Low content of *N*-glucosides was observed during plant ontogeny (up to 3 %). However, the content of *O*-glucosides increased in the later stages of development up to 71 %, while in the earlier stages it represented about 20 % of the total CKs. In T a similar trend was found as in T/C, *i.e*. the content of ribotides in the later stages decreased from 48 % in FB to 8 % in FS, the content of *O*-glucosides increased up to 59 % of the total CKs, and the content of free bases and ribosides (30 - 40 %) was more or less steady.

Antioxidant enzymes: Soluble protein content calculated per g(f.m.) was dependent on plant age and plant type (Fig. 2). In C, protein content gradually increased during plant ontogeny, a decline was found in FS stage. In C/C the highest protein contents were found in the early stages of plant development and then a gradual decline was observed. Transgenic rooted plants (T) showed a similar course as C, while T/C exhibited very small changes and the lowest protein contents during the plant ontogeny. Both transgenic types showed the maximum of proteins in FB stage.

CAT activity was significantly dependent on plant type and also on the developmental stage of plants (Fig. 3). In both control types, CAT activity was the lowest during *in vitro* cultivation (Fig. 3*A*,*C*) and then doubled during VY stage. In C, CAT activity increased further moderately in the flowering stages. Contrary to this, a significant decline in CAT activity was observed in C/C in the later stages of plant development starting FB stage. During *in vitro* cultivation CAT activity was significantly higher in both transgenic types than in C. In transgenic T/C a moderate increase of CAT activity was found throughout plant ontogeny. In T, CAT activity showed maximum in VY and V stage. Lower CAT activity was found after the onset of flowering, another increase was observed in FS.

GR activity was significantly elevated in both transgenic types compared to C and a gradual increase was found in the later stages of plant development (Fig. 3*B*,*D*). Control tobacco showed maximal GR activity in V stage and the gradual decline in the later stages. In C/C the steady state level of GR activity was mainated throughout the plant ontogeny.

Activities of SOD were dependent on plant type and also on the developmental stage (Fig. 3). In both control types the maximal SOD activity was reached in V and FB stages (Fig. 3*A*,*C*). Contrary to this, both transgenic types showed lower but steady activities throughout the plant ontogeny (Fig. 3*C*,*D*). In T/C a significant increase was found only in VY stage.

Compared to controls, transgenic T and T/C showed also differences in an isozyme composition of SOD (Fig. 4). Six isozymes was present in both control plants in V and FB stages. Their number decreased to 2 - 3 during *in vitro* cultivation and also in the later stages of plant ontogeny. In T and T/C lower number of isozymes was present throughout the plant ontogeny. The maximal number of four was reached in VY and V stage in T/C, and in FB and F stage in T.

Activity of APOD did not change significantly during plant ontogeny in C and C/C (Fig. 5*A*,*C*). Both transgenic types exhibited higher APOD activity compared to

controls (Fig. 5*B*,*D*). T showed the increased APOD activity particularly after the onset of flowering. In T/C enhanced APOD activity was observed during *in vitro* cultivation. After the drop in APOD activity in VY stage, a gradual moderate increase was observed in the following stages (Fig. 5*D*).

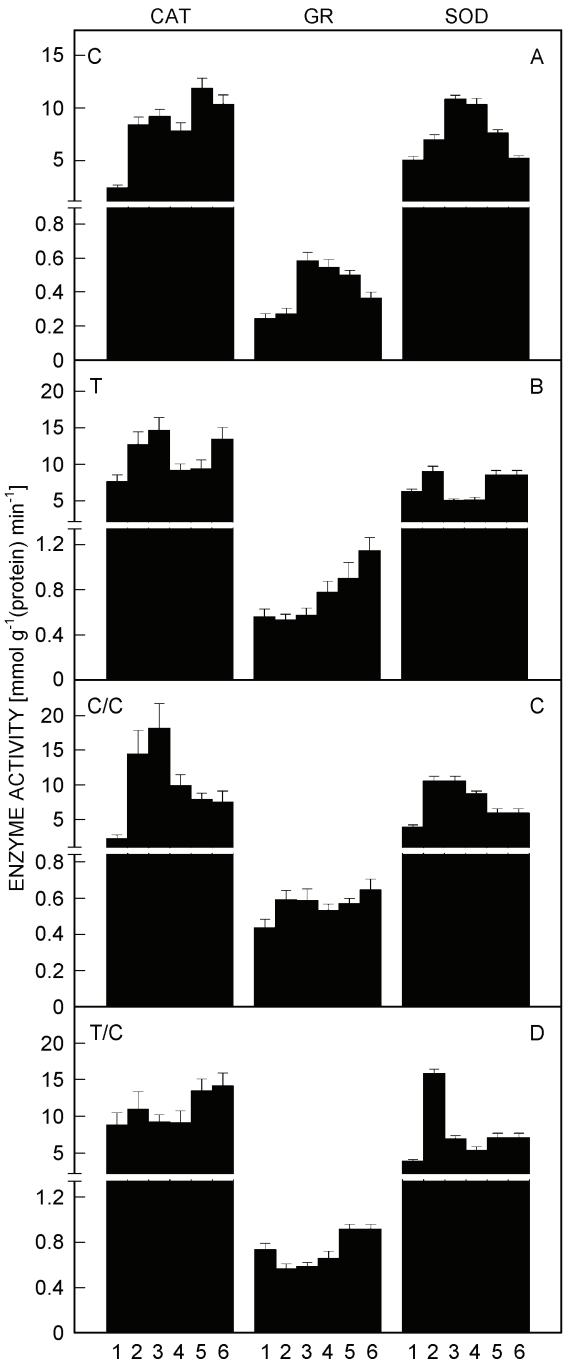

Fig. 3. Activities of catalase (CAT), glutathione reductase (GR), and superoxide dismutase (SOD) in several stages of plant development (see Fig. 1 for abbbreviations). Enzyme activities were calculated in μmol of corresponding substrate consumed per mg of soluble protein per min. Means ± SE, n = 19.

***In situ* peroxidase localization:** Leaf cryo-sections stained by DAB for peroxidase activity showed that peroxidases were predominantly localized in cell walls, particularly of epidermal cells (Fig. 6). Significantly stronger stain intensity in T/C compared to C showed no difference between samples taken from the leaf in V (Fig. 6*B*,*D*) and FS stage (Fig.6 *A*,*C*). This correlated with enhanced activities of GPOD and SPOD in T/C determined spectrophotometrically in leaf extracts (Fig. 5).

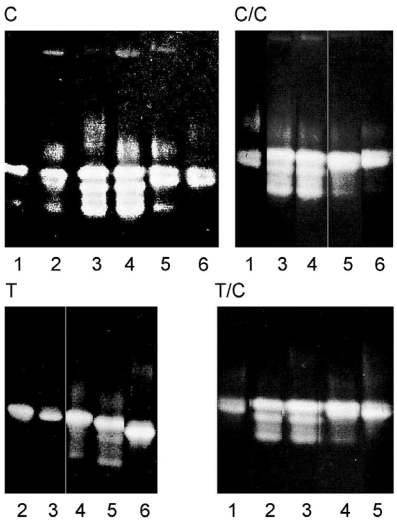

Fig. 4. Isozyme patterns of SOD in several stages of plant development (see Fig. 1 for abbbreviations).

GPOD and SPOD activities showed a similar course in all plants (Fig. 5). They were high in all plants during *in vitro* cultivation, then decreased in VY stage, and gradually increased in the later stages starting at the onset of flowering. T/C exhibited manifold enhancement of peroxidase activities compared to others throughout the plant ontogeny (Fig. 5*D*).

Ultrastructure of organelles: TEM showed a very close association among organelles in transgenic tobacco, particularly in T/C (Fig. 7*B*). Mitochondrion and/or peroxisome was often captured inside „cups“ formed by chloroplast envelope. The incidence of this phenomenon increased with the plant age. This was not observed in control plants (Fig. 7*A*).

Higher number of peroxisomal cores (PC) in peroxisomes was detected in transgenic T/C and T compared to C particularly in the later stages of plant development (Fig. 8*A*). Both Spurr embedded leaf samples and negative stained suspensions of isolated PC showed that the size of cores changed moderately (Fig. 8*C*). In T/C, the maximal size of PC was reached in F stage. In T, the larger PC were observed in V stage and the smallest in F stage (Fig. 9).

The lattice parameters of basic crystal unit cells of PC were calculated from images of Spurr embedded samples

as: a = 8 nm, b = 9.3 nm, γ = 92° (Fig. 8*B*). 3D reconstruction of cores proved a regular shape of quadrangular block or a cube of variable size (Fig. 8*D*), which took up to 60 % of total volume of peroxisomes.

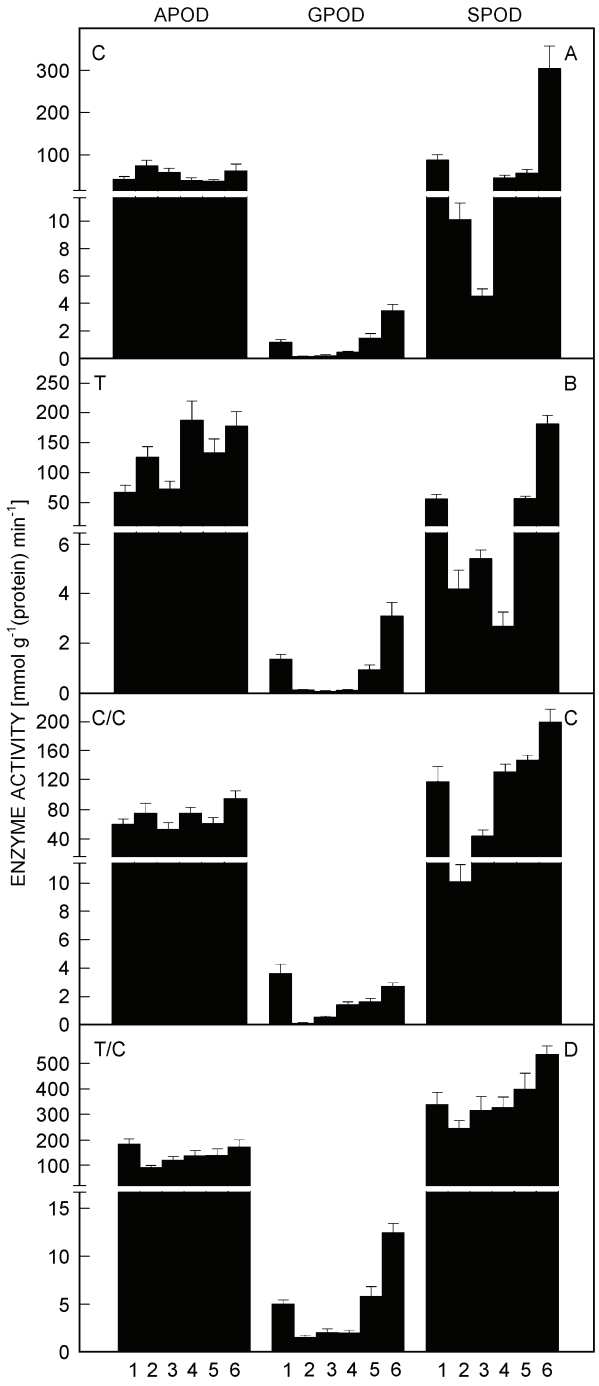

Fig. 5. Activities of ascorbate peroxidase (APOD), quaiacol peroxidase (GPOD), and syringaldazine peroxidase (SPOD) in several stages of plant development (see Fig. 1 for abbbreviations). Enzyme activities were calculated in μmol of corresponding substrate consumed per mg of soluble protein per min. Means ± SE, *n* = 19.

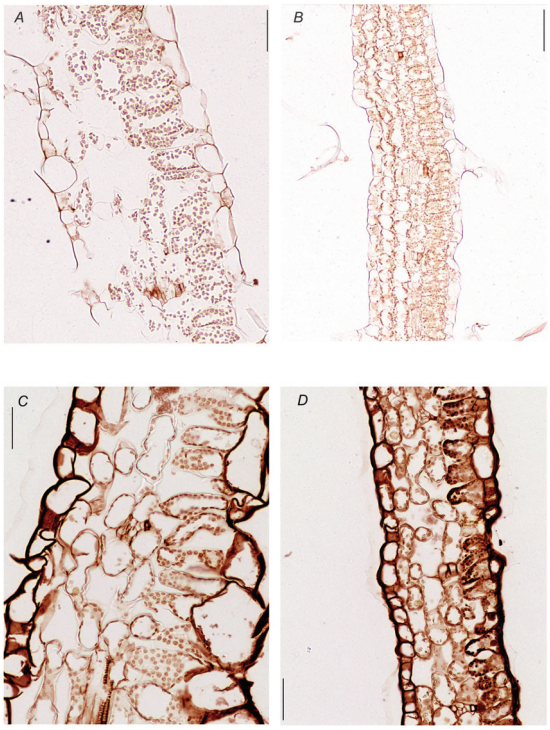

Fig. 6. Cryosections of the young mature leaf stained by DAB in control rooted tobacco in the stage of flowering plants (*A*), and in the vegetative stage (*B*). P*ssu-ipt* grafts in the stage of flowering plants (*C*), and in the vegetative stage (*D*). *Scale bar* = 50 μm.

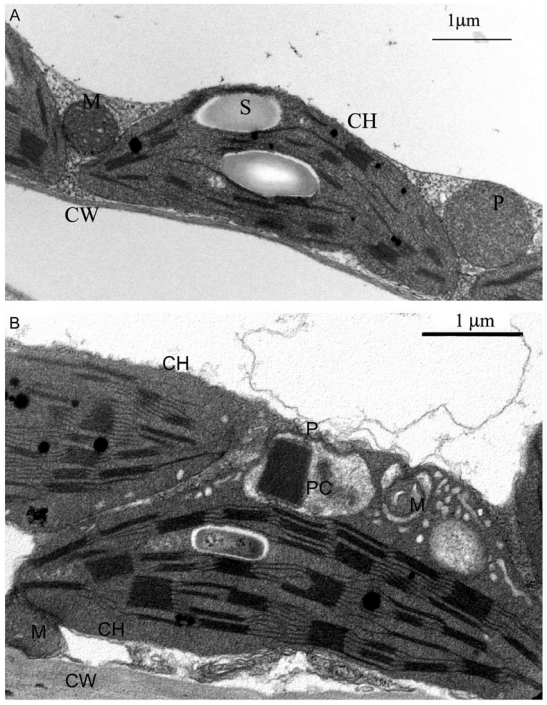

Fig. 7. Ultrastructure of chloroplast in leaf of control tobacco (*A*) and in transgenic P*ssu-ipt* tobacco (*B*). CH - chloroplast, CW - cell wall, M - mitochondrion, P - peroxisome, PC - peroxisomal core, S - starch.

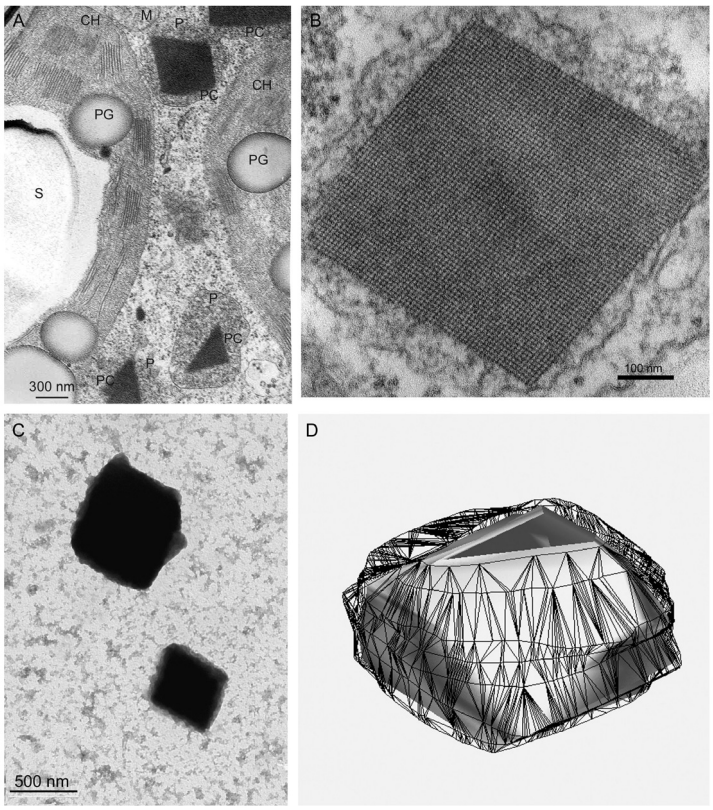

Fig. 8. Peroxisomes (P) and peroxisomal cores (PC) in P*ssu-ipt* tobacco: *A* - peroxisomes in parenchyma cell, *B* - detail view of peroxisomal core, *C* - isolated suspension of catalase crystals after negative staining, *D* - 3-D reconstruction of peroxisome (*black lines*), the peroxisomal core is inside (*grey cube*). PG - plastoglobuli, the other abbreviations are the same as in Fig. 7.

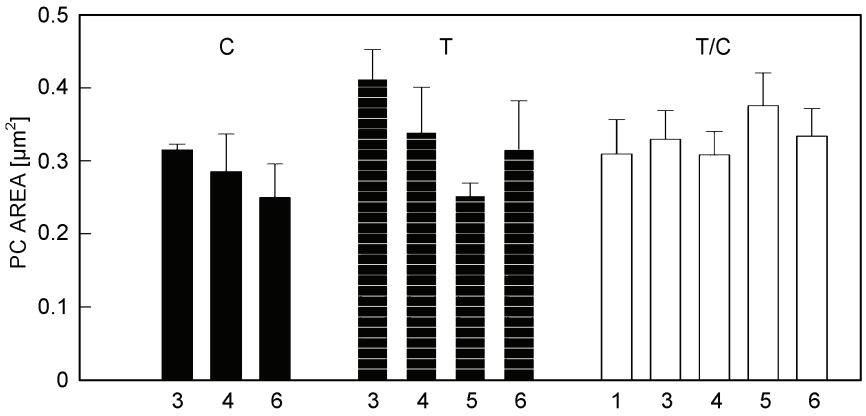

Fig. 9. Size of peroxisomal cores in control rooted tobacco (C), P*ssu-ipt* grafts (T/C), and P*ssu-ipt* rooted plants (T). (see Fig. 1 for other abbbreviations). Means ± SE, $n = 9$.

Discussion

The introduction of *ipt* gene resulted in the manifold increase of endogenous cytokinins in P*ssu-ipt* tobacco which persisted throughout the plant ontogeny. Compared to control tobacco the most pronounced accumulation of CKs was found in the later stages of the plant development. This correlates with results of Geuns *et al*. (1997) who found higher CKs contents in older leaves of P*ssu-ipt* tobacco within the plant insertion gradient. Although we compared the same leaf insertion level throughout plant ontogeny, the results show the

increasing accumulation of CKs in mature leaves.

Only little is known from the literature about changes in CKs levels during plant ontogeny except for early stages of development and/or senescence. In our experiments, control tobacco (C and C/C) showed higher CKs contents in VY and FB stages. The endogenous CKs content declined in the later stages of plant development, starting at the onset of flowering. This was also observed by Mercier and Endres (1999) in *Tillandsia recurvata*. An enhancement of CKs content was found in the juvenile and flowering stages, *i.e*. phases characterized by intense organ formation. Contrary to this, no such a dependence was observed in both P*ssu-ipt* tobacco types. They showed elevated contents of total CKs and also the difference in an abundance of CKs types with significantly increasing portion of *O*-glucosides in the later stages of plant ontogenesis. Those conjugates represent CKs storage forms, because of reversibility of *O*-glycoconjugation. They are resistant to cytokinin oxidase, enzyme which degrades CKs by a cleavage of their N_6-unsaturated isoprene side chain. Their biological activity reported in various bioassays may be connected with their hydrolysis and release of active CKs due to action of β-glucosidases (Vaňková 1999). We found that from individual CKs, Z and Z9R predominated in transgenic tobacco and *N*-glycosylated CKs represented only marginal portion of total CKs. Increased content of Z in transgenic plants with *ipt* gene reported elsewhere (Medford *et al*. 1989) was accompanied with increased contents of *N*-glycosylated forms, which was proposed as an important pathway involved in the homeostatic control of the pool size of active CKs. Glucosylation of the purine ring, which occurs at positions N_7 and N_9, results in irreversible inactivation of CKs (Vaňková 1999). Eklof *et al*. (1996) observed that transformation with *ipt* gene resulted in increased contents of Z, Z7G, and Z9R+ZMP, the content of Z9R+ZMP being greater in young tissues than in old ones. In agreement with our results, the content of *O*-glycoconjugates and ribotides increased in older transgenic plants (Eklof *et al*. 1996).

Our results shows that the content of CKs forms, which are considered active ones, was at least ten times higher in transgenic leaf tissue than in non-transgenic plant type throughout the plant ontogeny. There is enough evidence that high activity of AOE (Lacan and Baccou 1998) and CKs (Gan and Amasino 1997) correlate with delayed senescence. Natural regulations of plant development and senescence include also down regulation of genes of AOE during senescence, which opens the way for the final cell destruction (*e.g*. Price *et al*. 1994). However, CKs are believed to counteract partly this trend. This was proved by, *e.g.*, Dhindsa *et al*. (1982) who found that kinetin inhibited a decline in CAT activity. Recently, Dertinger *et al*. (2003) proved higher AOE activities and higher contents of ascorbic acid and glutathione in old leaves of transgenic P_{SAG12}-*ipt* tobacco, where CK production was stimulated by the onset of plant senescence.

Our experiments showed that activity of AOE were modulated differently in control and P*ssu-ipt* transformed tobacco during plant ontogeny. Besides peroxidases, control plants exhibited high AOE activities in the early stages of plant development, *i.e.* usually until the onset of flowering (FB), and a decline or no change in the later stages. Casano *et al*. (1994) suggested that higher AOE activities in young tissues under non-stress conditions correspond to higher photosynthetic and metabolic activity. In our experiments, transgenic plants (T) and grafts (T/C) showed an increase of CAT, GR, and APOD activity in the later stages of plant development contrary to controls. All those enzymes are involved in H_2O_2 scavenging. CAT is mainly associated with removing the bulk of H_2O_2 in microbodies, APOD (with its higher affinity for H_2O_2) and GR are both involved in the ascorbate - glutathione cycle. Although their properties and requirements are different, they function effectively in parallel. Enhanced activity of AOE is generally associated with an acclimation to elevated amounts of active oxygen species and the increase in stress tolerance (Dat *et al*. 2000). It seems that H_2O_2 accumulation plays an important role in transgenic P*ssu-ipt* tobacco and could be one of the effects of CK overproduction. Moreover, H_2O_2 was proved clearly to function as a signalling molecule during abiotic stress and activate the expression of defense proteins (Dat *et al*. 2000). In our previous study, we have proved that an expression of pathogenesis related proteins such as PR-1b protein and proteins with chitinase activity was induced already during *in vitro* cultivation of P*ssu-ipt* tobacco contrary to control type (Synková *et al*. 2004). In the present paper, activity of AOE involved in H_2O_2 metabolism was increased in all transgenic plants grown *in vitro* compared to controls.

Transgenic grafts also exhibited a significant enhancement of syringaldazine and guaiacol peroxidase activities during the plant ontogeny compared to other plant types. Peroxidases were predominantly localized in cell walls of palisade parenchyma and epidermis. Carpin *et al*. (1999) suggested that the epidermis contains a strong peroxidase activity which could be involved in the polymerization of phenolics. Our preliminary experiments showed that transgenic grafts contain high amount of various phenolic substances that are natural substrates for peroxidases (Cvikrová, unpublished results). This could be a consequence of stress caused not only by CK overproduction but also the effects of grafting that brings about a tissue damage during the procedure. Wounding was proved to stimulate H_2O_2 generation systemically in tomato leaves (Orozco-Cardenas and Ryan 1999). Moreover, peroxidases are known to be involved in many important metabolic processes including wound healing (Smith *et al*. 1994), lignin formation in the cell walls (Gross 1980), and in the catabolism of indoleacetic acid (Gazaryan *et al*. 1996).

High contents of phenolics, low auxin content (Synková *et al*. 1999), and high peroxidase activities could be of a great importance in shoots of P*ssu-ipt* tobacco. This probably causes that P*ssu-ipt* shoots are not able to form its own roots. This was also reported in non-rooting *rac* tobacco mutant, which is characterized by a higher lignin content, a higher soluble peroxidase activity, more free and conjugated putrescine and higher contents of benzyladenine and isopentenyladenosine in comparison with control plants (Faivre-Rampant *et al*. 2002).

In our study, the increase of CAT activity in the older transgenic plants correlates also with larger and more frequent peroxisomal cores in peroxisomes of those plants (Fig. 9). We have observed smaller crystal unit cells in CAT crystalls in transgenic tobacco than, *e.g.*, Tenberge *et al*. (1997) in sunflower cotyledons or Sato *et al*. (1993) in beef liver. However, formation of different types of cores suggests that differences in the molecular structure of CAT might be also responsible for the capability of one CAT type to build different crystals (Harris and Holzenburg 1995). Tenberge *et al*. (1997) proved that PC have CAT activity and that the enzymatic activity of CAT was barely affected by its integration into the cores. Kleff *et al*. (1997) proved that 59 and 55 kD CAT isozymes in peroxisomes of sunflower cotyledons were rather specifically divided into the core and matrix, respectively. They concluded that cores *in vivo* represent a subcompartment active in H_2O_2 decomposition. The higher need to decompose more effectively H_2O_2 could be the reason for a close association of organelles that we observed in transgenic plants (see Fig. 7).

Our results show that transgenic P*ssu-ipt* tobacco accumulated biologically active cytokinins throughout plant ontogeny contrary to control tobacco, where the decline in cytokinin content was found. Overproduction of cytokinins seems to affect directly activities of antioxidant enzymes, particularly those involved in H_2O_2 metabolism, because the enhancement of activities with increasing plant age was observed in both types of transgenic plants contrary to control types. The close association of chloroplasts, mitochondria and peroxisomes observed in transgenic grafts, might be the adaptation to long-lasting stressful environment within the cell.

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