Extent of *ipt* gene expression and resulting amount of cytokinins affect activities of carboxylation enzymes in transgenic plants

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Abstract

Three types of transgenic plants of *Solanum tuberosum* cvs. Kamýk and Oreb, and *Nicotiana tabacum* cvs. Maryland Mammoth and Trapezond were selected according to intensity of introduced *ipt* gene expression and resulting amount of synthesised cytokinins (CKs). In comparison with controls, original transgenic regenerants grown *in vitro* showed a massive increase of CK contents, in tobacco by 379 % and in potato by 159 % (MAS). Potato grown in soil from tubers of transgenic plants demonstrated a moderate increase (44 %) of CK contents (MOD). Transgenic tobacco grown from seeds *in vitro* did not show any significant change in CK contents (NOT). Initial ($RuBPC_i$ and $RuBPO_i$) and total ($RuBPC_t$) activities of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), and the activity of phospho*enol*pyruvate carboxylase (PEPC) were not significantly affected by the transformation in the NOT plants. In the MOD plants, the RuBPCO activities were stimulated by up to 34 % whereas the PEPC activity was decreased by 17 %. On the other hand, all the measured enzyme activities were 32 - 91 % lower in the MAS. Leaf area, fresh and dry masses, and chlorophyll and soluble protein contents also went down with increasing CK amounts in the transformants. Dependence of $RuBPC_i/RuBPO_i$ and $RuBPC_t$/PEPC ratios on the relative CK amounts in transgenic plants revealed that the individual enzyme activities were not affected uniformly. Endogenous CK contents in the MAS thus apparently exceeded an optimum needed for positive effects on many physiological traits and became a stress factor for such plants.

Additional key words: chlorophyll, *Agrobacterium tumefaciens*, *Nicotiana tabacum*, phospho*enol*pyruvate carboxylase, potato, ribulose-1,5-bisphosphate carboxylase/oxygenase, tobacco, *Solanum tuberosum.*

Introduction

Cytokinins (CKs) represent an important class of phytohormones that influence plants during their whole life. Biological activity and function of CKs were described by Mok and Mok (1994) and an impact of exogenously applied CKs on various aspects of plant physiology was extensively studied. There are numbers of papers dealing with the action of CKs on formation and function of photosynthetic apparatus in plants and tissue or cell cultures. Generally, CKs usually intensify biosynthesis and amounts of cell DNA and RNA through the heightened expression of the complete set of plastid genes together with nuclear genes coding for plastid functions (Lerbs *et al.* 1984, Parthier *et al.* 1985, Chen *et al.* 1993). That implies that CKs stimulate biosynthesis of proteins and photosynthetic pigments, formation of pigment-protein complexes and electron transport chain components, activity and photochemical efficiency of photosystem 2, photophosporylation and ATP content, activity and amount of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO), growth of chloro-

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Abbreviations: C_3, C_4 - reaction intermediates are three- or four-carbon acids, respectively; CAM - Crassulacean acid metabolism; Chl - chlorophyll; CK - cytokinin; DTT - dithiothreitol; f.m. - fresh mass; iP - isopentenyl adenine; iPR - isopentenyl adenine riboside; MAS - transgenic plants with massive rise in CK contents; MOD - transgenic plants with moderate rise in CK contents; NOT - transgenic plants without rise in CK contents; PEPC - phospho*enol*pyruvate carboxylase; $RuBPC_{i,t}$, $RuBPO_i$ - carboxylating, resp. oxygenating operation of ribulose-1,5-bisphosphate carboxylase/oxygenase, subscripts: i - initial, t - total; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; SD - standard deviation; SE - standard error of the mean; Z - zeatin; ZR - zeatin riboside.

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plasts and their quantity per cell, which ultimately leads to an increase in carbon dioxide assimilation, leaf area, biomass production, overall plant productivity, and better recovery after water stress or amelioration of its negative effect in some cases (*e.g.* Adedipe *et al*. 1971, Buschmann and Lichtenthaler 1977, Feierabend and De Boer 1978, White and Schmidt 1988, Wilhelmová and Kutík 1995, Rulcová and Pospíšilová 2001, Kulaeva *et al.* 2002, Vomáčka and Pospíšilová 2003, Pospíšilová and Baťková 2004). The above mentioned experiments were performed with various cell or tissue cultures, isolated chloroplasts, detached and attached green or greening cotyledons, primary leaves as well as normal leaves of various age. Extent of CK effects depends on the leaf or plant age and on phytohormone concentration. Results tend to be more potent with young leaves or plants. High CK concentrations may act as inhibitors.

CKs mimic salt treatment and elicit specific isoforms of phospho*enol*pyruvate carboxylase (PEPC) in *Mesembryanthemum* prior to switching from C_3 to CAM metabolism (Thomas *et al.* 1992). In certain cases, CKs substitute the effect of irradiation upon formation of plastids in plant tissues kept in darkness (Kusnetsov *et al.* 1994). Much attention has also been paid to exogenous CKs and leaf and plant ageing with similar findings. CKs slow down the decay of pigments and proteins of photosynthetic membranes and delay the degradation of all photosynthetic structures which results in the preservation of photosynthetic activity of assimilation organs (Čatský *et al.* 1996; for reviews see Šesták 1985, Synková *et al.* 1997b). Parallelly appliced CKs and abscisic acid, considered as antagonists in many cases, can co-operate in regulation of photosynthesis and transpiration by fine-tuned stomata opening (Pospíšilová 2003a).

Research on interaction between endogenous CKs and photosynthetic traits falls into the scope of views formed on the basis of action of exogenously applied ones. For example, a rise of biological activity of CKs was parallel with the development of photosynthetic apparatus during *Solanum* leaf ontogenesis (Borzenkova and Nefedova 1981). Lowering photosynthetic characteristics of *Helianthus* grown long in the environment enriched with CO_2 was accompanied by lowering the CK content (Gulyaev *et al.* 1989). Ageing of outer leaves of *Brassica* was also accompanied by a reduction of the CK contents (Thomas 1977). Individual CKs varied differently during ageing of *Phaseolus* cotyledons with their life span modified by various interventions (Wilhelmová *et al.* 2004). Ammonia salts increased the concentrations of chlorophylls and CKs in protocorms of *Epidendrum* (Mercier and Kerbauy 1991). Endogenous CKs together with other phytohormones participate in plant responses to stresses (*e.g.* Thomas *et al.* 1992; for review see Naqvi 1999). Roles of CKs under water stress were reviewed in general by Pospíšilová *et al.* (2000) and with respect to regulation of the stomatal function during and after water stress by Pospíšilová (2003b).

Content of endogenous phytohormones can be manipulated by methods of molecular biology and molecular genetics. Progress in gene engineering has enabled growth of the research on transgenic plants bearing genes for phytohormone biosyntheses such as the oncogene of bacterial origin called *ipt* gene (*tmr* or gene *4*). This gene originates from *Agrobacterium tumefaciens* T-DNA and codes for isopentenyl transferase, an enzyme catalyzing the limiting step in synthesis of CKs, all of which are adenine derivatives. Transgenic plants overproducing CKs often exhibit phenotypic signs of the hormone surplus (for details see Synková *et al.* 1997b), or sometimes display alterations in otherwise normally developing plants. Diverse patterns of general or localized CK overproductions can be found in plants by coupling the *ipt* gene with various promoter types. Among transformant types, differing by a degree of expression of the introduced gene, it is possible to select clones showing its long-lasting expression as opposed to normal plants where CK administration may induce increased degradation of endogenous CKs (Chatfield and Armstrong 1986) so that the plants have then less CKs than they had before the hormone application (Pospíšilová *et al.* 1993). Therefore the transgenic plants can be used as more convenient models for studying the interaction between CKs and photosynthetic traits under various conditions, *e.g.* stress ones or virus infection, than plants with externally applied artificial CKs.

Effect of elevated CK contents in plants expressing the *ipt* gene on diverse facets of growth and photosynthesis was investigated by Smart *et al.* (1991), Čatský *et al.* (1993a,b), Synková (1997), Synková *et al.* (1997a,b, 1999), Synková and Valcke (2001), Pospíšilová *et al.* (1997/98), *etc*. There is, however, almost no information regarding an impact of CK overproductions on the carboxylation enzymes RuBPCO and PEPC. One of a few reports on this topic is that of Jordi *et al.* (2000) which refers to interactions between CKs and RuBPCO contents in transgenic tobacco plants that express the *ipt* gene in senescing leaves. The aim of the present work was to reveal the effects of elevated internal CKs on the activities of RuBPCO and PEPC in *ipt* transformed C_3 plants.

Materials and methods

Plants: Original transgenic regenerants grown *in vitro* on solid agar medium and plants raised from seeds (tobacco) or tubers (potato) of the transformants were used. Transgenic potato (*Solanum tuberosum* L. cvs. Kamýk and Oreb) and tobacco (*Nicotiana tabacum* L. cvs. Maryland Mammoth and Trapezond) were derived by Ondřej *et al.* (1989, 1990, 1991). They used soil bacterium *A. tumefaciens* with transformation plasmid

systems (pAL4404) (pCB1339) for potato cv. Kamýk and both tobacco cultivars, or (pAL4404) (pCB1334) for potato cv. Oreb. The plasmid constructs pCB1334 and pCB1339 carried an *ipt* gene for CK synthesis under the control of its own promoter and a kanamycin resistance gene serving as a selection marker for transformed plant cells.

Transformed tissues were left to regenerate and later to establish clones on the solid Murashige and Skoog medium *in vitro* (100 cm^3 vessels) in several cycles first with and finally without antibiotics, growth regulators, and sucrose. By omitting sucrose fully autotrophic plants were obtained. These plants were termed original transgenic regenerants *in vitro*. Kanamycin resistant (*i.e.* transgenic) progeny of the tobacco cv. Maryland Mammoth was grown *in vitro* from seeds yielded by the original transgenic plants transferred to soil. Control *in vitro* plants were raised from seeds (tobacco) or internode segments (potato) and propagated by the same way. Vessels with transgenic and control plants were placed in an air-conditioned room under a photoperiod of 12 h, irradiance (400 - 700 nm) of 80 μmol(photon) m^{-2} s^{-1} (standard fluorescent tubes), temperature of 20 ºC, and humidity outside the vessels of 60 - 70 %. The plants were taken for experiments at the age of about 43 - 51 d (potato cv. Oreb and tobacco cv. Trapezond) or 62 - 66 d (tobacco cv. Maryland Mammoth) counting from the last propagation.

Tubers of the potato cv. Kamýk were generated from rooting transgenic plants transferred to soil with difficulty because the transgenic potato poorly rooted (Kostřica, personal communication). These tubers of the second clone generation were planted into pots (upper diameter 16.0 cm, height 14.5 cm) with soil, garden substrate, and sand (3:1:1) around 3 cm deep. The pots were placed in a greenhouse. Growing plants were regularly irrigated with tap water and side-dressed with a commercial fertiliser. In the time of experiments, the plants were 34 - 41 d old.

Enzyme assays: The plants *in vitro* cultivated in the controlled environment were sampled at least 2 h after beginning of the photoperiod. Leaves without petioles (tobacco) or the whole shoots (potato) of controls and green parts of transgenic teratomas were fast (< 2 s) removed from the vessels and frozen in liquid nitrogen. Three to five vessels were used per one sample. The pots with potato grown in the greenhouse were first moved into the cultivation room where the plants were acclimated for at least 2 h at 20 ºC and irradiance of ≈ 40 μmol m^{-2} s^{-1} at the upper leaves. Pieces from mature and fully developed young leaves (*i.e.* the fifth to ninth leaves counted from the first emerging leaf) were then immediately frozen in liquid nitrogen. Mixed samples from 10 - 15 both transgenic and control plants were made. Quick sampling of tissues into liquid nitrogen is a critical step. Measuring in crude extracts then enables to evaluate the *in vivo* RuBPCO activities ($RuBPC_i$ and $RuBPO_i$). The frozen samples were next powdered in liquid nitrogen by a mortar and pestle, weighed, and homogenised in 1 - 3 cm^3 of medium by another chilled mortar and pestle for about 1 min. In dependence on plant type, samples of 0.5 - 2.0 g(f.m.) cm^{-3} were used. The homogenisation medium was prepared using CO_2-free redistilled H_2O (boiled and bubbled with N_2) and prior to use it was further bubbled with N_2 for 10 min. It consisted of 100 mM Bicine, 10 mM $MgCl_2$, 10 mg cm^{-3} polyvinylpyrrolidone, 1 mM EDTA Na_2, at pH 8.0 (KOH rid of K_2CO_3), 5 mM dithiothreitol (DTT), 5 mM 2-mercaptoethanol, and 10 mg cm^{-3} polyvinylpolypyrrolidone (insoluble). DTT, 2-mercaptoethanol, and polyvinylpolypyrrolidone were added just before the homogenisation. The crude homogenate was centrifuged at 24 000 *g* and 4 ºC for 3 min in a closed Eppendorf microtube. The supernatant (enzyme extract) was kept ice-cold in the plugged microtube until assayed.

RuBPC and PEPC activities were determined at 25 ºC by the conventional coupled enzyme assays. In the series of enzymatic reactions, NADH oxidation by absorbance change at 340 nm was continuously monitored in optical cuvettes of 0.5 cm path length using a double beam spectrophotometer (*PU 8800*, *Philips-Pye Unicam*, Cambridge, UK). The reactions were routinely started by adding the enzyme extract. RuBPC activity was assayed according to Lilley and Walker (1974). Reaction medium of 1 cm^3 volume in a sample cuvette contained: 50 mM Bicine and 20 mM $MgCl_2$ at pH 8.2 (KOH), and freshly prepared 3.5 mM ATP, 3.5 mM phosphocreatine, 350 μM NADH, 80 nkat cm^{-3} each of glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate phosphokinase, and creatine phosphokinase, 400 μM RuBP Na_4, 10 mM $NaHCO_3$, and 50 mm^3 of the enzyme extract (non-activated or activated). Solution in a reference cuvette was without RuBP and $NaHCO_3$. RuBPCO in the extract was activated by 20 mM $MgCl_2$ and 10 mM $NaHCO_3$ at room temperature for 10 min. Assay with a non-activated extract reflects the initial ($RuBPC_i$) activity whereas assay using the activated one reflects the total ($RuBPC_t$) activity. PEPC activity measurement was based on the method by Lane *et al.* (1969). Reaction medium of 1 cm^3 volume in a sample cuvette contained: 50 mM Hepes (KOH) and 5 mM $MgCl_2$ at pH 7.2, and freshly prepared 160 μM NADH, 100 nkat cm^{-3} malate dehydrogenase, 42 nkat cm^{-3} lactate dehydrogenase, 4 mM PEP Na, 5 mM $NaHCO_3$, and 50 mm^3 of the enzyme extract. The reference cuvette did not contain PEP and $NaHCO_3$.

$RuBPO_i$ activity was determined from oxygen consumption in solution by the method of Kung *et al.* (1980). The oxygen consumption was monitored amperometrically by a Clark-type electrode at 25 ºC in a stoppered chamber filled with 5.2 cm^3 of reaction medium saturated with air deprived of CO_2 by passing through a column of soda lime (*Natrocalcid*, *Chemopetrol Spolana*, Neratovice, Czech Republic). The reaction medium consisted of 50 mM Bicine and 20 mM $MgCl_2$ at pH 8.2 (KOH), prepared CO_2-free like the homogenisation medium - *viz.* above, 400 μM RuBP (freshly prepared), 236.254 μM O_2, and 100 - 120 mm^3 of the enzyme extract (non-activated). The reaction was

started by adding the enzyme extract through a small hole in the stopper. The electrode was calibrated at 25 ºC using distilled H_2O saturated with nitrogen (oxygen concentration of 0 μM) or air (oxygen concentration of 236.25 μM).

Chlorophyll *a* and *b* contents were determined spectrophotometrically (*PU 8800*) in 80 % (v/v) acetone extract by a two-wavelength method (645 and 663 nm) against 80 % acetone in a reference cuvette (1 cm thick). To quantify the chlorophyll (Chl) amounts, Arnon-MacKinney equations were used (Šesták 1971). Samples were prepared by mixing an appropriate part of the crude homogenate for the enzyme assays with the necessary volume of 100 % acetone to make the 80 % acetone extract. After centrifuging (16 000 *g*, 5 min) and washing the pellet with 80 % acetone, the sample was obtained by combining the supernatants.

Soluble protein content was estimated by the biuret method which is based on a reaction of two peptide bonds with Cu^{2+} in an alkaline medium. A sample of the enzyme extract (supernatant) containing at least 1 mg of protein was precipitated with the same volume of ice-cold trichloroacetic acid (240 mg cm^{-3}). The precipitate was after centrifugation (800 *g*, several minutes) redissolved in such a volume of 100 mM NaOH that the protein concentration was in the range of 1 - 10 mg cm^{-3}. One volume of this solution was mixed with four volumes of the biuret reagent [1.5 g dm^{-3} $CuSO_4$, 6 g dm^{-3} $NaKC_4H_4O_6$ (tartrate), 30 g dm^{-3} NaOH]. A complex violet compound developed within 30 min. Absorbance at 545 nm was then read on the *PU 8800* spectrophotometer in a 0.5 cm thick cuvette. The reference cuvette contained the biuret reagent and 100 mM NaOH in the ratio of 4:1.

Cytokinin contents were determined according to Strnad *et al.* (1989) and Macháčková *et al.* (1993). Briefly, methanolic extracts (80 %) were first purified on P- and DEAE-cellulose columns and then on a *SepPak* cartridge where CKs caught. CKs were then separated by high performance liquid chromatography. Amount of individual CKs was finally evaluated by an ELISA test with polyclonal antibodies conjugated with alkaline phosphatase and p-nitrophenyl phosphate as a substrate.

Other measurements: Fresh leaf mass of the potted plants was estimated directly by weighing 0.5 cm^2 discs as fast as possible after their cutting. Small leaves from the *in vitro* plantlets were first put on water surface, then thoroughly dried and weighed. Dry mass of the samples was estimated after about 12 h drying at 105 ºC. Leaf area was determined on leaf copies by a scanner *Apple Graphics Tablet*.

Statistical treatment: Results are presented as mean ± standard error of the mean (SE), only leaf area as mean ± standard deviation (SD). Extreme values were excluded by non-parametric Dixon's test of extreme deviations. Significance of differences between controls and transformants was statistically appraised using non-parametric Mann-Whitney's test or parametric Student's *t*-test. For the statistical estimations, *SOLO* software, release *3.1* (*BMDP Statistical Software*, Los Angeles, USA) was applied. The differences were regarded as significant at a probability level $\alpha = 0.05$.

Results

CK contents: Isopentenyl transferase encoded by the *ipt* gene catalyses condensation of dimethylallyldiphosphate with AMP forming thus isopentenyl adenosine monophosphate. However, this ribotide is not the main CK of higher plants. Therefore, expression of the gene was probed as a change of the amount of derived CKs that were zeatin (Z), zeatin riboside (ZR), isopentenyl adenine (iP), and ispentenyl adenine riboside (iPR). The greatest total content of CKs was found in the original transgenic regenerants *in vitro*. The tobacco plantlets contained 141.4 μg(CKs) kg^{-1}(f.m.) in controls and 677.1 μg(CKs) kg^{-1}(f.m.) in transgenic regenerants; the potato ones then 140.0 μg(CKs) kg^{-1}(f.m.) in controls and 362.6 μg kg^{-1}(f.m.) in transgenic regenerants. It represents 379 % increase for transgenic tobacco and 159 % for transgenic potato in comparison with control plantlets. Transgenic potato originated from tubers showed moderately but still significantly enhanced (by 44 %) CK contents; they had 158.5 μg(CKs) kg^{-1}(f.m.) compared with 110.0 μg(CKs) kg^{-1}(f.m.) in the controls. Transgenic tobacco raised from seeds did not significantly differ from the control plants [106.0 and 115.2 μg(CKs) kg^{-1}(f.m.) in transgenic and control plants, respectively]. Thus according to expression of the *ipt* gene in the transgenic plants, three classes of the transformants were distinguished: the ones with massive (MAS), moderate (MOD), and no (NOT) increase of CK contents.

Transgenic plants with massively enhanced CK contents (MAS): Two independent transgenic lines of two plant species, potato cv. Oreb and tobacco cv. Trapezond, all showing markedly enhanced CK contents, were investigated. After several cycles of subcultivation on the agar medium without growth regulators, the transgenic regenerants showed marked signs of transformation and formed rootless teratomas characteristic by clusters of small, thick, and deformed leaves (Fig. 1*A*). Tobacco had few very short shoots and white regions at leaf bases. Potato showed either short, stout, and greatly branched stalks under a high level of *ipt* gene expression, or long, little branched stalks with irregularly small leaves in clones with lower (but still very high) expression of the gene. The teratomas

generally very little resembled control plantlets.

The transformation affected most of the studied plant characteristics (Table 1). Individual transgenic clones of both potato (4 and 13) and tobacco (5 and 7) were influenced similarly so results are presented as respective averages. The transgenic plants contained significantly less Chl *a*+*b* and soluble proteins than the controls. Chl *a*/*b* ratio was reduced only in potato cv. Oreb. Altered pigment composition may suggest diverse proportion of light-harvesting complexes and reaction centres in the transformants. All the measured enzyme activities were significantly lower in the transgenic clones. Thus, these regenerants had lower contents of PEPC as well as of active (deduced from initial activities) and activable (deduced from total activities) RuBPCO. In potato, RuBPCO activation state was lower in the controls (Table 2). This could indicate that transgenic plantlets mobilised their assimilation potential and the cells offset a drop of the total amount of RuBPCO with a relative increase of its active portion in order to best utilise the existing conditions. In tobacco, on the other hand, RuBPCO activation state was significantly lower in the transgenic plants although they contained markedly lower amount of active RuBPCO compared with the controls. It means that regulation mechanisms in the transgenic tobacco not only did not mitigate the consequences of the transformation, which is an opposite fact to that found in potato, but also did not manage to use the conditions like the controls did.

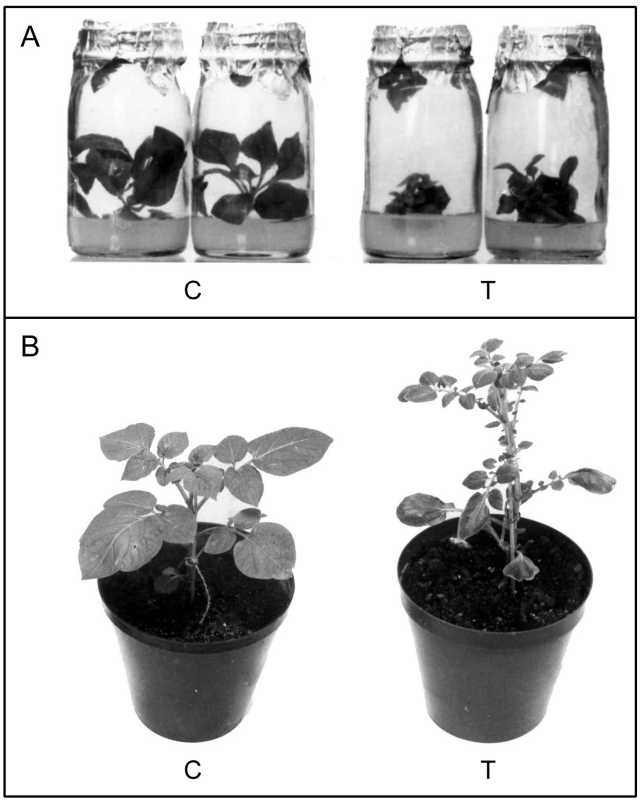

Fig. 1. Morphology of control (C) and *ipt* transgenic (T) plants: *Nicotiana tabacum* cv. Trapezond *in vitro* (*A*); *Solanum tuberosum* cv. Kamýk grown from tubers in soil (*B*).

Transgenic plants with moderately enhanced CK contents (MOD): This plant type was obtained from tubers of transgenic clones 43 and 178 of potato cv. Kamýk. Both the clones provided similar results and therefore their averages are presented. CK amounts were 1.44 times greater in the transformants which was manifested in plant habitus. The transformants were slightly higher and had more branched stems than the controls. The transgenic plants had also a greater amount of smaller, orbiculate leaflets and many stipules (Fig. 1*B*). Leaf area and leaf dry mass of the transformants were significantly reduced while their leaf fresh mass, soluble protein and Chl *a*+*b* contents, and Chl *a*/*b* ratio were not significantly different from the control plants (Table 1). The transformation caused a significant increase of $RuBPC_i$, $RuBPC_t$, and $RuBPO_i$ activities. This implies that the transgenic potato must have had a greater amount of both active and activable RuBPCO in the leaf tissue compared with the controls. RuBPCO activation state yet remained unchanged (Table 2). Surprisingly, PEPC activity was significantly lower in the transformants than in the controls (Table 1).

Table 1. Characteristics of *ipt* transgenic potato and tobacco with differently enhanced CK contents. Values (means ± SE or SD for leaf area, n = 5 - 10) represent percentages [%] of the respective controls. Original data were expressed on fresh mass basis. Leaf area, and leaf fresh and dry masses of the *in vitro* plantlets with massive CK accumulations were not measureable. *Asterisks* denote significantly different transgenic and control plants at the probability level $P < 0.05$.

	Solanum tuberosum Oreb (MAS)	Kamýk (MOD)	*Nicotiana tabacum* Trapezond (MAS)	Maryland Mammoth (NOT)
Leaf area	-	13.8 ± 4.9*	-	30.9 ± 13.1*
Leaf fresh mass	-	104.7 ± 9.4	-	95.5 ± 7.3
Leaf dry mass	-	81.8 ± 5.3*	-	97.5 ± 3.4
Chlorophyll *a*+*b*	17.9 ± 2.3*	99.4 ± 9.3	16.2 ± 2.4*	52.8 ± 12.0*
Chlorophyll *a*/*b*	13.8 ± 4.9*	98.1 ± 2.4	97.3 ± 1.0	116.5 ± 3.2*
Soluble protein	104.7 ± 9.4	100.8 ± 11.2	45.4 ± 3.2*	79.1 ± 17.6
$RuBPC_i$	81.8 ± 5.3*	133.8 ± 11.1*	8.9 ± 1.8*	91.0 ± 11.8
$RuBPC_t$	9.6 ± 1.1*	131.2 ± 7.6*	21.4 ± 2.6*	81.0 ± 7.4
$RuBPO_i$	23.0 ± 3.6*	108.5 ± 12.8*	55.1 ± 7.4*	131.9 ± 22.5
PEPC	51.2 ± 3.1*	82.9 ± 7.2*	67.6 ± 3.8*	130.7 ± 16.8

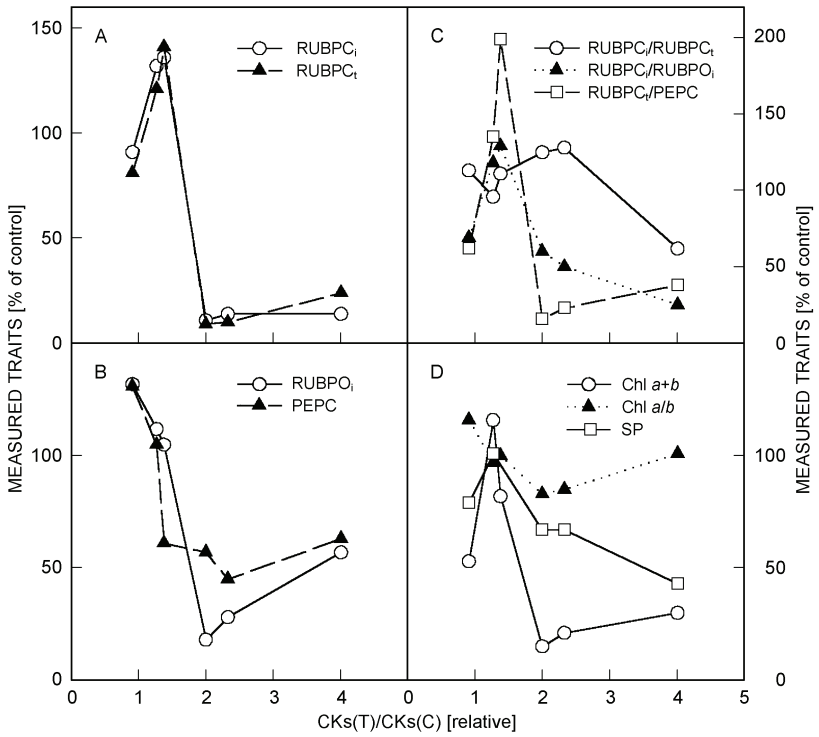

Fig. 2. Ratio of the averaged traits measured in transgenic (T) and control (C) plants in dependence on relative CK contents in the transgenic ones. Absolute values of CK contents (Z + ZR + iP + iPR) in individual plant types are: *N. tabacum* cv. Maryland Mammoth (NOT) - controls 115.2, transformants 106.0 μg kg^{-1}(f.m.); *S. tuberosum* cv. Kamýk (MOD) - controls 110.0, transformants 129.8 and 187.0 μg kg^{-1}(f.m.) (clones 178 and 43, respectively); *S. tuberosum* cv. Oreb (MAS) - controls 140.0, transformants 334.6 and 390.6 μg kg^{-1}(f.m.) (clones 4 and 13, respectively); *N. tabacum* cv. Trapezond (MAS) - controls 141.4, transformants 677.1 μg kg^{-1}(f.m.).

Transgenic plants without change in CK content (NOT): In plants of the clone 3 of transgenic tobacco cv. Maryland Mammoth I found CK amounts similar to those of control plants. These transgenic plantlets were raised from seeds of the original transformants. Their morphology and growth characteristics were not dramatically different from those observed in the controls. They were a little lower, had a reduced leaf area per plant and Chl *a*+*b* contents (Table 1). Increase of Chl *a*/*b* ratio indicated an alteration of the proportion between

reaction centres and light-harvesting complexes in the transformants. On the contrary, differences in soluble protein content, and leaf fresh and dry masses were not significant. All the studied enzyme activities were not significantly influenced by the transformation but initial and total RuBPC activities tended to be slightly lower whereas $RuBPO_i$ and PEPC activities, and RuBPCO activation state were somewhat higher.

Table 2. Ratios of enzyme activities in control and *ipt* transgenic plants showing differently enhanced CK contents. Means ± SE from 6 - 10 independent measurements are presented. In the columns, distinct *superscript letters* mark significant differences ($P < 0.05$) between controls and transformants.

Rise in CKs	Cultivar		$RuBPC_i/RuBPC_t$ [%]	$RuBPC_i/RuBPO_i$ [%]	$RuBPC_t$/PEPC [%]
MAS	Oreb	control	48.0 ± 4.2^a	0.54 ± 0.07^a	0.48 ± 0.04^a
		transformant	60.7 ± 3.2^b	0.29 ± 0.07^b	0.09 ± 0.02^b
	Trapezond	control	48.4 ± 1.6^c	5.60 ± 0.70^c	2.72 ± 0.15^c
		transformant	36.4 ± 1.1^d	1.50 ± 0.30^d	0.84 ± 0.11^d
MOD	Kamýk	control	59.0 ± 2.9^e	6.70 ± 0.60^e	5.00 ± 0.40^e
		transformant	60.6 ± 1.6^e	8.20 ± 1.10^e	8.50 ± 0.70^f
NOT	Maryland Mammoth	control	50.0 ± 3.2^f	3.30 ± 0.60^f	6.90 ± 0.80^g
		transformant	56.7 ± 1.3^f	2.30 ± 0.40^f	4.30 ± 0.50^h

Discussion

After the introduction of the *ipt* gene into plant genome, a rise in CK contents in cells followed by changes of plant morphology and physiology was expected. However, this assumption was not demonstrated unequivocally and transgenic plants showed an increase of CK contents in a very broad range. CK amounts of the original transgenic regenerants were up to 3 orders enhanced in comparison with the controls. CKs/auxin ratio is substantially higher in such plants (Ondřej *et al.* 1991). On the other hand, transgenic plants raised from seeds or tubers had just little elevated or unelevated CK contents. Similar results were obtained by Ondřej *et al.* (1989, 1990, 1991). Rate of gene expression depends among others on a site in the plant genome where the gene is incorporated and it can be time-dependent. This explains why the gene product may be in a lesser amount in the progeny. Moreoever, Schubert *et al.* (2004) described transgene silencing if the transcript level of an introduced gene exceeded a certain, gene-specific threshold. Transcript level-mediated silencing can then also account for the pronounced transgene expression variability among various transformant types. Čatský *et al.* (1993a) found CK contents decreasing with time in the same *in vitro* regenerant clones of potato cv. Oreb as in my work. They recorded the decrease not only in the transformants but also in the controls. Not all CKs were identified by the methods used, *e.g.* the glycosylated CKs. Nevertheless, amounts of those forms, which were assayed, provide an adequate picture about the extent of the *ipt* gene expression and its impact on physiology of the plant.

Transgenic plants bearing the CK synthesis gene can display normal phenotype and look like control plants or their phenotype can be substantially different (Thomas *et al.* 1995, Pospíšilová *et al.* 1997/98, Jordi *et al.* 2000). Such morphological abnormalities are inhibition of root formation, teratoma-like structures of multiple shoot meristems with small leaves, and short internodes in *in vitro* grown transformants (Thomas *et al.* 1995, Lexa *et al.* 2002), and reduced growth of stems, reduced apical dominance, reduced size of leaves, and increased number of short internodes in grafted or rooted plants (Synková *et al.* 1999). Degree of the morphological changes depends on promoters used for induction of gene expression and on the cultivation method (Synková and Valcke 2001). Specific conditions *in vitro* can contribute to the maintenance of great expression of the introduced gene and thus to a development of the CK overproduction syndrome. Here, in agreement with those facts, the most distinct morphologic differences were noticed between the control plants and the original transgenic regenerants *in vitro*. The transgenic plants of the next generation, *i.e.* tobacco from the seeds or potato from the tubers differed less (Fig. 1).

Leaf area could not be determined in the original transgenic plants *in vitro* because of miniature leaves. The other transformant types had three (tobacco) to seven (potato) times smaller leaf area even if no increase of CK contents was detected. Leaf area can thus serve as a simple marker of *ipt* gene expression in plants. Leaf fresh masses of the NOT and MOD were very similar compared to the respective controls. Leaf dry mass did not alter in the NOT but went down in the MOD (Table 1) suggesting that the latter transformants contained more water in its leaf tissue than the controls. Chl *a+b* as well as soluble protein contents diminished in the transformants with increasing amount of CKs and so their greatest fall (up to 85 %) was registered in the MAS (Fig. 2*D*). Other studies on transgenic plants bearing the *ipt* gene in various expression constructs generally confirmed the basic traits of such plants (Ondřej *et al.*

1990, Šiffel *et al.* 1992, Čatský *et al.* 1993a,b, Pospíšilová *et al.* 1997/98, Synková *et al.* 1999, Jordi *et al.* 2000, Synková and Valcke 2001, Lexa *et al.* 2002). These authors detected unaffected or slightly better photosynthetic characteristics (plant dry and fresh masses, relative water, soluble protein, and Chl *a+b* contents, Chl *a* fluorescence parameters, and electron transport and gas exchange capacities) in plants with just moderately increased CK contents. When CKs exceeded a certain amount in plant tissues, negative effects started predominating. Then mostly a decline of the photosynthetic parameters was observed. This is in agreement with outcomes of the studies dealing with impacts of externally added CKs. Various physiological processes in plants were inhibited when exogenous CKs were applied above some limit. For example, rates of net photosynthesis and transpiration, and stomatal conductance were negatively influenced in *Phaseolus* (Pospíšilová *et al.* 2001, Rulcová and Pospíšilová 2001). However, it is not easy to find any limit because CK effects strongly depend on plant species, CK type and its content, and less on the way of application.

Very steep drop of $RuBPC_i$, $RuBPC_t$, and $RuBPO_i$ activities was recorded with increasing CK contents in the transgenic plants (Fig. 2*A,B*). In the MAS, all the RuBPCO activities falled by up to 91 %. Conversely, a 9 - 34 % stimulation of the RuBPCO activities was found in the MOD (Table 1). This stimulation is similar to that induced by using an optimal amount of external CKs (Lerbs *et al.* 1984, Nath and Mishra 1990, Chernyad´ev 1994). $RuBPC_i$ and $RuBPC_t$ activities changed in parallel, that is, if $RuBPC_i$ activity was significantly different between the transgenic and control plants, so was $RuBPC_t$. But there was no correlation between the CK amounts and RuBPCO activation state. The activation state only sharply decreased at the highest CK ratio in the transformants and controls (Fig. 2*C*).

Amperometric measurement of the RuBPO activity is susceptible to an incidence of larger experimental errors than the measurement of the RuBPC one. CO_2 activates both these reactions, but it is also a substrate for carboxylation and thus acts as a competitive inhibitor of the oxygenase reaction. Measured total RuBPO activity (after the *in vitro* RuBPCO activation with 10 mM HCO_3^- and 20 mM Mg^{2+}) was always lower than the respective initial RuBPO activity (data not shown). The inhibition of oxygenase activity by CO_2 transferred into the reaction medium together with the activated enzyme could be the reason. Therefore only the initial RuBPO activities are given in this work. Another source of result variabilities may be dilution of the enzyme extract after its addition into the bulk of reaction mixture. Activating CO_2 can then much easily dissociate from a part of RuBPCO molecules causing their inactivation.

PEPC is an enzyme of cytoplasmic origin and is much less influenced by CKs than the chloroplast enzymes (Herrmann *et al.* 1992, Chernyad´ev 1994). However, CK effects on PEPC are scarce in literature. Here, the highest PEPC activity was determined in the transgenic tobacco with unaffected amount of CKs. The activity did not show so strong decrease with increasing CK contents compared to RuBPCO and it fluctuated around 50 % of control over the broad range of the relative CK contents (Fig. 2*B*). Apparently, there must have existed at least another factor, in addition to enhanced CK syntheses, influencing PEPC activity in the transformed plants.

$RuBPC_i/RuBPO_i$ ratio was higher than one, as was logically expected, with a surprising exception for the *in vitro* potato cv. Oreb where the ratio was inverse (Table 2). In this case, the RuBPO activity was greater than the RuBPC one. It suggests that a sort of an inhibitor/ activator had to act on the RuBPCO carboxylase/ oxygenase activity, respectively, although during almost the whole photoperiod CO_2 concentration inside the cultivation vessels was near the compensation value (Pospíšilová *et al.* 1989) and all CO_2 consuming and evolving reactions were in equilibrium. Concurrently, the activity of PEPC was also greater than that of $RuBPC_t$ in those plants which is clear from the $RuBPC_t/PEPC < 1$ (Table 2). In comparison with the controls, $RuBPC_i/RuBPO_i$ did not significantly change in the NOT and MOD. On the other hand, this ratio was significantly smaller in the MAS (Fig. 2*C*). However, an increase of the $RuBPO_i$ relative to RuBPCi was noticed in both the transformants and controls grown *in vitro*. Reasons for such a selective change are unclear. An extra consumption of oxygen in addition to the RuBPO reaction might be a cause. RuBP is a fairly labile compound susceptible to decomposition. McCurry *et al.* (1978) predicted in plant tissues the existence of a nonspecific metalloenzyme (containing Cu) able to oxidise RuBP owing to its instability.

Dependence of the $RuBPC_t/PEPC$ ratio on the relative CK contents reveals that the increased synthesis of CKs in the transgenic plants influenced the RuBPC activity more than the PEPC one (Fig. 2*C*). Excluding the MOD where the $RuBPC_t/PEPC$ ratio was significantly greater in the transformants, this ratio was significantly lower in the remaining transgenic plant types compared with the controls. It was even lesser than one in some cases indicating that the PEPC activity exceeded the $RuBPC_t$ one (Table 2). For comparison, *e.g.*, RuBPC/PEPC ratios ranged from 1.87 to 6.23 in polymorphous leaves of *Populus euphratica* (Wang *et al.* 1997).

There are not many articles dealing with an impact of endogenously manipulated amount of CKs in transgenic plants on enzyme activities. Lexa *et al.* (2002) showed that *ipt*-transgenic *Nicotiana* (with several dozen times increase in content of total CKs) displayed lowered nitrate reductase activity compared with control wild-type plants. Similarly, the controls grown in medium containing benzyladenine looked and behaved like the transgenic plants. Moreover, the addition of benzyladenine into the growth medium eliminated any differences between the control and transgenic plants during induction of nitrate reductase activity with nitrate (Lexa *et al.* 2002). Catalase activity was antagonistically modulated in shoot (inhibition) and cell (up to double

increase) cultures of *Nicotiana* in comparison with controls (Petit-Paly *et al.* 1999) or it remained unchanged in various types of transgenic *Nicotiana* (Synková and Valcke 2001). The latter authors studied also activities of other antioxidant enzymes in *ipt*-transformed *Nicotiana*. They found that the activities of glutathione reductase, various classes of peroxidases (ascorbate, guaiacol, and syringaldazine), and superoxide dismutase reflected not only the consequences of the transformation itself (elevated CK contents) but the plant age, growth conditions, and water stress as well (Synková and Valcke 2001). Hence the outcomes are often ambiguous and depend on many internal and external factors and their interactions during plant life.

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