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High level of endogenous cytokinins in transgenic potato plantlets limits photosynthesis

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Abstract

Introduction of the gene for cytokinin synthesis into potato genome lead to a manifold increase in the level of cytokinins (zeatin, zeatin riboside, isopentenyladenine, isopentenyladenosine) in plantlets grown *in vitro*. The increasing cytokinin level was associated with increasing tendency to teratoma formation, to decreasing leaf net photosynthetic rate and to increasing dark and light respiration rates and CO_2 compensation concentration. During plantlet (or teratoma) ontogeny, net photosynthetic rate increased simultaneously with the decrease in cytokinin level. High level of endogenous cytokinins was associated also with lower photochemical activities of both photosystems in isolated chloroplasts.

Introduction

There is a rather extensive evidence that exogenously applied cytokinins (mostly kinetin, 6-benzylaminopurine, benzyladenine, benzimidazole, zeatin, *etc.*) affect various photosynthetic characteristics and distribution of newly formed biomass. The main effects are mainly stimulations of cell division and chloroplast development, of differentiation processes, of expression of photosynthetic genes, of syntheses of DNA, RNA, proteins, enzymes of carbon fixation, components of electron transport chain (plastoquinone, *P* 700), carbonic anhydrase, chloroplast pigments and enzymes of their synthetic pathways, of activities of photosystems, non-cyclic and cyclic photophosphorylation and ATPase, which result in a stimulation of net photosynthetic rate, modification of respiratory activities and an increase in leaf area and biomass formation (*e.g.* Legocka 1989, Vaňková *et al.* 1991, Dominov *et al.*

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Abbreviations: Chl - chlorophyll, iP - isopentenyladenine, iPA - isopentenyladenosine, P_N - net photosynthetic rate, PS - photosystem, R_D - dark respiration rate, R_L - photorespiration rate, Z - zeatin, ZR - zeatin riboside, ψ_w - water potential, ψ_s - osmotic potential, ψ_p - pressure potential.

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1992, Guéra and Sabater 1992, for further references see the annual bibliographies - Šesták and Čatský 1974-1991). The pattern of the response to cytokinin application varies with environmental factors (temperature, irradiance, water supply), and with the age of leaf or plant as cytokinins enhance juvenile characteristics and retard degradative processes, *e.g.* of chlorophyll. Cytokinins also modulate the expression of photosynthetic genes (*e.g.* Teyssender *et al.* 1985, Flores *et al.* 1986, Abdelghani *et al.* 1991, Chory *et al.* 1991, Schmitt and Piependock 1992).

Transgenic plants with endogenously increased cytokinin level have been produced by methods of gene engineering, mainly as parent material for breeding (Ooms 1987, Ondřej *et al.* 1989, 1990), but the information on their photosynthetic and production behaviour is very rare (Šiffel *et al.* 1992).

The introduction of the gene 4 for elevated cytokinin synthesis into plant genome may be a purposeful experimental tool, but high expression of the gene leads in some cases to malformed plants, not useful in plant breeding. These plants, however, provide a good model of contrasting plant types that differ in biomass distribution.

The aim of this study was to determine ontogenetic changes in net photosynthetic rate and their control by CO_2 concentration in morphologically different transgenic potato plants, cultivated *in vitro,* that possess different cytokinin levels and cytokinin to auxin ratios and hence different shoot/root ratio.

Material and methods

Transgenic plants of potato (*Solanum tuberosum* L. cv. Oreb) carrying gene for cytokinin synthesis were obtained by Ondřej *et al.* (1989, 1990a) using the *Agrobacterium tumefaciens* (pAL4404) (pCB1334) strain. The experimental transgenic and control plants were cultivated from internode segments on the Murashige and Skoog (1962) medium containing 2 % saccharose and 0.7 % agar, in an air-conditioned box at temperature of 25 °C and irradiance of 200 μmol(photon) m^{-2} s^{-1} (high-pressure mercury lamps).

Chloroplast isolation: Chloroplasts were isolated from plants about 3 weeks old by the modified method of Robinson (1984). Shoots (stems with leaves) (5 g) were ground in a *Turrax* homogeniser (*Janke & Kunkel*, Staufen, Germany) with 50 cm^3 of the isolation medium (0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes-KOH, 2 mM isoascorbate, 0.5 % bovine serum albumin and 1 % polyvinylpyrrolidone, pH 7.5). The brei was squeezed through a gauze and the filtrate was centrifuged at 3000 × *g* for 3 min. The pellet was resuspended in 6 cm^3 of 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes-KOH (7.5 pH), underlayered with 4 cm^3 of the same medium plus 40 % *Percoll* in the centrifuge cell, and then centrifuged at 4500 × *g* for 10 min. The pellet of chloroplasts was resuspended in the above medium and used for the determination of photochemical activities.

Photochemical activities: The activities of Photosystem 1 (PS1) (tetramethyl-*p*-

phenylenediamine red. → methylviologen) and Hill reaction (PS2) (H_2O → ferricyanide or H_2O → *p*-phenylenediamine) were determined amperometrically at 350 μmol(photon) m^{-2} s^{-1} and 25 °C as oxygen uptake or evolution.

Cytokinins were determined by the ELISA test after HPLC resolution of individual cytokinins in methanolic extracts purified by P-cellulose and DEAE-cellulose chromatography and *Sep-pak* cartridge (Strnad *et al.* 1990, Macháčková *et al.* 1993).

Chlorophyll contents were determined in 80 % acetone extracts of whole shoots with spectrophotometer *PU-8800* (*Philips Scientific*, Cambridge, U.K.) by the method of Arnon (1949).

CO_2 gas exchange: Rates of net photosynthesis (P_N) and respiration (R_D) were determined as CO_2 influx in a closed gas exchange system with an infra-red gas analyser (*Junkalor*, Dessau, Germany) in a CO_2 concentration range from 20 to 1200 mg m^{-3}, leaf temperature 20 °C and saturating irradiance (400-700 nm) of 860 μmol m^{-2} s^{-1} (*cf.* Čatský and Tichá 1975, Kaše and Čatský 1983). Photorespiration rate (R_L) and CO_2 compensation concentration (Γ) were calculated from CO_2 dependence of P_N.

Shoot water and osmotic potentials were measured by a droplet thermocouple psychrometer joined with a *Keithley Microvolt Ammeter 150 B* at a temperature of 25 ± 0.002 °C. Water potential was determined on a living tissue and osmotic potential was determined after the same sample had been frozen (-18 °C, 16 h) and thawed. Pressure potential was calculated as the difference between water and osmotic potentials.

Dry mass was determined after drying samples at 90 °C to constant mass.

Results and discussion

The transformed tissues showed extreme teratoma appearance in the first subcultivation, but later the teratoma tissues had a tendency to normalization in different degree, in dependence on the clone used. After 6 months of cultivation, three independently transformed clones (Nos. 1334-1, 1334-4, 1334-13) with different teratoma differentiation were used for further study (Ondřej *et al.* 1990).

Transformed tissues of all three clones showed a considerable increase in Z, ZP, iP and iPA levels (Table 1). The clone 1334-1 (not shown) contained the highest level of cytokinins, followed by clones 4 and 13. The clone 1334-1, however, was not suitable for determining photosynthesis due to its morphology. The indol-3-yl-acetic acid levels showed an increase of 60-80 % in comparison with control (Macháčková, unpublished).

Control plantlet or teratoma P_N increased with ontogeny of the culture (19, 40 and 68 d). In the clones 4 and 13, P_N was very low or respiration prevailed over photosynthesis (Fig. 1).

Table 1. Contents of endogenous cytokinins in control and transgenic potato planlets of different age. (Clone 1334-1 not shown.)

Clone	Plant age [d]	Cytokinin content [μg kg^{-1}(f.m.)] Z	ZR	iP	iPA	Sum
Control	19	57.9	63.1	47.6	62.9	231.5
	40	27.7	58.2	29.1	35.2	149.2
	68	traces	35.3	34.3	47.8	117.4
1334-4	19	125.3	239.7	91.8	139.2	596.0
	40	138.5	289.3	80.7	154.3	662.8
	68	44.0	114.5	47.1	122.4	328.0
1334-13	19	216.0	302.4	105.6	139.4	763.4
	40	1233.3	1308.8	545.1	842.6	3929.8
	68	347.8	525.4	191.2	267.7	1332.1

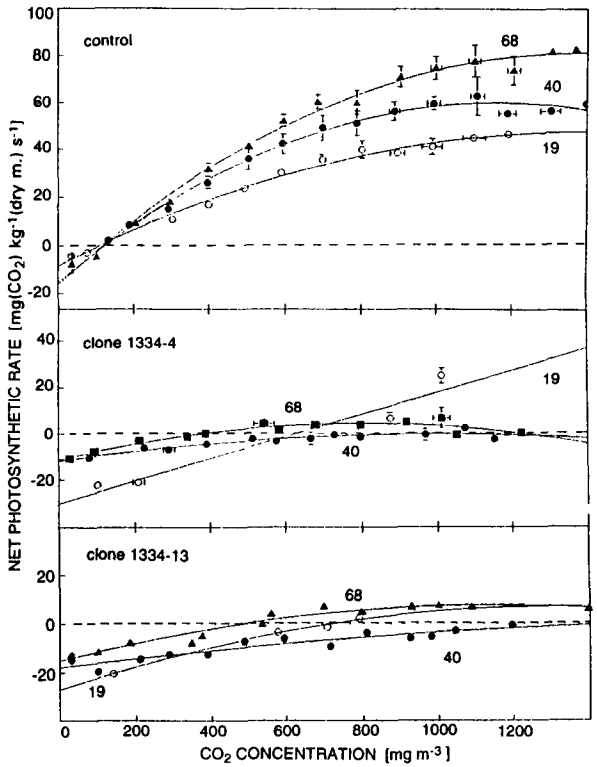

Fig. 1. Dependence of net photosynthetic rate on ambient CO_2 concentration during ontogeny of control and transgenic (clones 1334-4 and 1334-13) potato plants grown *in vitro*. Figures labelling individual curves denote plant age [d] starting from transplanting internode segments. Means of 5 replications. Vertical and horizontal bars denote standard mean errors. If not shown, the point size exceeds standard mean error.

The sum of cytokinins measured was negatively correlated with P_N. The ontogenetic variation of the two variables in control and in the two clones enabled to obtain an evident relation between the level of endogenous cytokinins and P_N (Fig. 2).

Respiration rate declined with plantlet age, but it was not much affected by increased cytokinin content (Table 2).

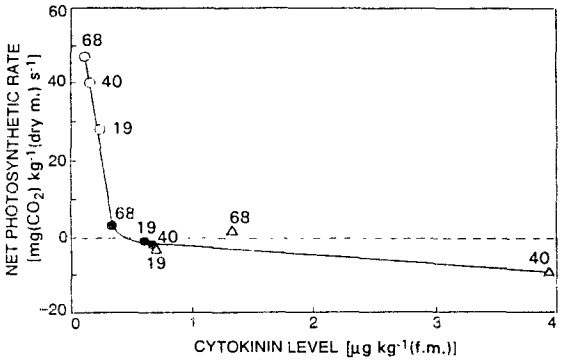

Fig. 2. Relationship between net photosynthetic rate and level of endogenous cytokinins (sum of zeatin, zeatin riboside, isopentenyladenine and isopentenyladenosine) in developing transgenic and control potato plants grown *in vitro*. *Empty circles*: control, *full circles*: clone 1334-4, *empty triangles*: clone 1334-13. The figures at individual points denote plantlet age [d].

Light respiration rate calculated from the curve relating P_N to CO_2 concentration increased during ontogeny of control plantlets, while in transgenic plantlets a decline was found (Table 2). A statistically significant increase in R_L against control was found only in 19-d plantlets. Γ did not differ considerably, during plantlet ontogeny, but it was much higher in transgenic than control plants (Table 2).

The considerable increase in cytokinin level was associated with reduced photochemical activities of both photosystems. In clone 1334-4 characterized by intermediate cytokinin content, the activities of PS1 and PS2 reduced more than in clone 1334-13 containing higher amounts of cytokinins. Nevertheless, activity of total electron transport chain from H_2O to ferricyanide was diminished appreciably in clone 1334-13. Thus that clone seems to contain some modification in electron carriers between PS1 and PS2. Contrary to the increase in chlorophyll $a + b$ content during control plantlet ontogeny, in transformed plants this value declined considerably with the age. The ratio of Chl a/b decreased in all plants (including control) during ontogeny.

The effects observed in transgenic potato suggest that higher concentration of endogenous cytokinins caused some changes in electron carriers between PS2 and PS1, probably near PS1, rather than in PS2 and PS1 reaction centres themselves. However, this hypothesis requires a more detailed analysis. Šiffel *et al.* (1992) support our results by findings of no changes in the emission spectrum of the reaction

centre complexes and in the contents of complex PS1 (CP1) and PS2 (CP2) found by SDS PAGE in similar transgenic potato. According to Pethadiya *et al.* (1989) kinetin application on developing cotyledons promoted photosystem activities, but did not change the ratio of PS2/PS1.

Table 2. Rates of net photosynthesis, P_N, per unit dry mass [mg kg^{-1}(d.m.) s^{-1}] and per unit chlorophyll *a+b* [g kg^{-1}(Chl) s^{-1}], photorespiration, R_L [mg kg^{-1}(d.m.) s^{-1}] and respiration, R_D [mg kg^{-1}(d.m.) s^{-1}], CO_2 compensation concentration, Γ [mg m^{-3}], content of chlorophylls (Chl) *a*, *b*, *a+b* [g kg^{-1}(d.m.)] and their ratio, shoot water content, W [%], and leaf water, ψ_w, osmotic, ψ_s, and pressure, ψ_p, potentials [MPa] in control and transgenic potato plantlets of different ages. P_N and R_D are given for 600 mg(CO_2) m^{-3} and 20 °C; P_N was measured under photon fluence rate of 800 μmol m^{-2} s^{-1}. Means ± S.E. n = 30 for CO_2 exchange rates, n = 9 for chlorophyll contents, and n = 10 for water potentials. *, ** = significant against respective controls at P = 5 or 1 % level.

Plant age [d]	Control			Clone 1334-4			Clone 1334-13		
	19	40	68	19	40	68	19	40	68
P_N per dry m.	43.5	53.5	59.5	-2.9**	-2.9**	3.3**	-2.5**	-2.9**	3.3**
	± 1.0	± 3.1	± 2.2	± 0.1	± 0.2	± 0.1	± 0.5	± 0.1	± 0.1
P_N per Chl	11.8	13.1	13.3	-1.6	-2.4	1.3	-1.4	-3.2	4.4
R_L	8.5	14.3	16.1	29.7**	11.8**	11.2*	27.9**	17.5**	14.4
	± 0.8	± 0.9	± 1.6	± 3.8	± 2.5	± 1.1	± 1.9	± 1.1	± 1.4
R_D	52.0	21.3	14.0	52.5	18.6	16.7	48.8	24.5	24.1*
	± 4.2	± 2.1	± 1.8	± 6.2	± 2.5	± 3.1	± 1.1	± 1.4	± 2.4
Γ	101.7	120.1	120.1	584.3**	876.9**	387.6**	717.1**	1430.3**	484.5**
	± 11.4	± 9.7	± 10.2	± 20.2	± 19.7	± 10.9	± 20.8	± 25.4	± 18.9
Chl *a*	2.82	3.15	3.43	1.38	0.95**	0.47**	1.39	0.68**	0.56**
	± 0.71	± 0.43	± 0.10	± 0.39	± 0.10	± 0.12	± 0.30	± 0.10	± 0.17
Chl *b*	0.87	0.96	1.13	0.46	0.30**	0.18**	0.44	0.22**	0.20**
	± 0.22	± 0.12	± 0.06	± 0.15	± 0.03	± 0.13	± 0.09	± 0.02	± 0.05
Chl *a+b*	3.69	4.10	4.48	1.88	1.25**	0.65**	1.83	0.90**	0.76**
	± 0.93	± 0.55	± 0.16	± 0.81	± 0.12	± 0.16	± 0.38	± 0.12	± 0.23
Chl *a/b*	3.24	3.30	3.00	3.03	3.11**	2.62	3.15	3.07**	2.84
	± 0.03	± 0.03	± 0.05	± 0.16	± 0.02	± 0.15	± 0.06	± 0.08	± 0.19
W	90.9	90.8	94.1	92.1	95.3	95.6	92.7	95.6	95.9
ψ_w	-0.85	-0.77	-0.67	-0.91	-0.78	-0.76	-0.94	-0.61	-0.80
	±0.07	±0.09	±0.12	±0.13	±0.07	±0.12	±0.11	±0.06	±0.09
ψ_s	-1.04	-0.96	-0.98	-1.18	-0.99	-0.88	-1.13	-0.94	-1.11
	±0.09	±0.08	±0.12	±0.10	±0.06	±0.12	±0.12	±0.07	±0.08
ψ_p	0.19	0.19	0.31	0.27	0.21	0.12	0.19	0.33	0.31
	±0.08	±0.03	±0.04	±0.03	±0.05	±0.07	±0.04	±0.05	±0.05

Shoot water, osmotic and pressure potentials were not expressively influenced by increased level of endogenous cytokinins. The water and osmotic potential slightly decreased with the age of both the control and transformed regenerants, and at each ontogenetic stage the water and osmotic potentials were usually more negative in transformants (Table 2). The transformed plants had usually higher water content and lower dry matter content (in % of fresh mass) than the control regenerants.

The described results show that increased, perhaps supraoptimal cytokinin level due to expression of gene 4 in morphologically changed plants (teratoma habitus) seems to be correlated with a decrease leaf net photosynthetic rate, decreased chlorophyll content and chlorophyll *a/b* ratio, reduced activities of both

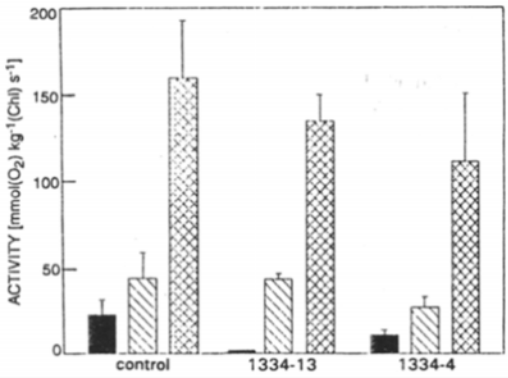

Fig. 3. Photochemical activities of the whole electron transport chain ($H_2O \rightarrow$ FeCy, *full columns*), PS2 ($H_2O \rightarrow$ p-PD, *hatched columns*) and PS1 ($TMPDH_2 \rightarrow$ MV, *cross-hatched columns*) of chloroplasts isolated from control (C) and transgenic (clones 1334-13 and 1334-4) potato. Data were obtained from 6 independent chloroplast isolations.

photosystems and increased dark and light respiration. This is exactly opposite to the effects brought about by exogenous cytokinin application (*cf.* Introduction). At our state of knowledge, it is very difficult to explain this contradiction. Only a detailed study of the effect of exogenous cytokinins on changes in the levels of endogenous cytokinins, effects on morphogenesis and photosynthetic activities can help to explain this contradiction. Such a study is under progress.

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