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Photosynthesis and water relations in transgenic tobacco plants with T-DNA carrying gene 4 for cytokinin synthesis

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Abstract

Expression of the gene 4 for cytokinin synthesis in two clones of transgenic tobacco plants resulted in slightly higher (*ca.* 10 - 20 %) levels of endogenous cytokinins - zeatin, zeatin riboside and isopentenyladenosine. This was associated with changes in photosynthetic processes on chloroplast and leaf level. In comparison with non-transformed plantlets a higher degree of grana stacking was found in both clones of transgenic plants which indicated a higher proportion of photosystem 2 light-harvesting complex in thylakoids. This was supported by a decreased chlorophyll *a/b* ratio in the separated grana fraction. The rate of leaf net photosynthetic rate (measured as CO_2 uptake) was *ca.* 25 % higher in both clones than in non-transformed plants. Also the rates of dark and light respiration, and CO_2 compensation concentration were higher in transgenic plants. On the other hand, parameters of water relations (water, osmotic and pressure potentials, stomatal and cuticular transpiration rates) were not significantly affected in transgenic tobacco plants.

Introduction

Expression of gene 4 for cytokinin synthesis in transgenic plants leads to increased levels of endogenous cytokinins associated with changes in biomass production and distribution, and in plantlet morphology (*e.g.* Ooms 1987, Ondřej *et al.* 1989, 1991).

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

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It has been shown (Čatský *et al.* 1993) that the clear expression of gene 4 in transgenic potato induced considerable changes in photosynthetic activities (chlorophyll content, photochemical activities of both photosystems, rates of CO_2 uptake and evolution) and water relations (water content, water, osmotic and pressure potentials). This study has therefore been aimed to characterize photosynthetic characteristics and water relations in tobacco transgenic plants in which the expression of gene 4 is weaker as in our previous studies with potato, and where the plant morphology allows a more detailed analysis.

Materials and methods

Transgenic plants of tobacco (*Nicotiana tabacum* L. cv. Samsun) carrying gene 4 for cytokinin synthesis were obtained by Ondřej *et al.* (1989, 1990) 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, 0.1 g m^{-3} (mg l^{-1}) naphthyl acetic acid, 1 g m^{-3} 5-benzylaminopurine, 500 g m^{-3} Ticarcillin and 200 g m^{-3} kanamycin sulphate in an air-conditioned box at temperature of 25 °C and irradiance of 200 μmol(photon) m^{-2} s^{-1} (400-700 nm). Further subcultivation was on MS medium with 2 % of saccharose but without growth regulators. 19- to 23-d old plants were used for experiments.

Membrane stacking estimation: Chloroplasts were isolated from leaves of about 3-week old plants by the method of Melis and Anderson (1983). About 5 g of cut leaves were ground with a *Ultra-Turax* homogenizer (*Janke & Kunkel,* Staufen, Germany) in 50 cm^3 of isolation medium (50 mM Tricine, 10 mM NaCl, 5 mM $HgCl_2$, 0.33 M sorbitol with addition of 1 % polyvinylpyrrolidone, pH 7.8). After filtration through a gauze, the homogenate was centrifuged at 2 000 × *g* for 10 min. The pellet was resuspended in incubation medium (50 mM Tricine, 150 mM KCl, pH 7.3).

Digitonin fragmentation: Chloroplasts were treated with 0.3 % digitonin (Dg) (Dg:Chl = 3:1) for 30 min at 4 °C in the dark. The grana (pellet) and intergranal thylakoid vesicles (supernatant) were separated after centrifugation at 10 000 × *g* for 30 min.

Chlorophyll contents both in chloroplast suspension and in leaf extract were determined in 80 % acetone with spectrophotometer *PU-8740* (*Philips Scientific,* Cambridge, U.K.) according to Arnon (1949).

CO_2 exchange: Rates of net photosynthesis (P_N) and respiration (R_D) were determined as CO_2 influx in a PC-assisted closed gas exchange system with an infrared 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 (photoflood

incandescent lamps) of 860 μmol(photon) 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.

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

Transpiration rate was determined from water loss curves measured gravimetrically on leaves originally fully turgid. Irradiance was 860 μmol(photon) m^{-2} s^{-1}, temperature 25 °C, and air humidity 35 %. Stomatal and cuticular transpiration rates were calculated as first derivatives of water loss curves at the beginning and at the end of measurement, respectively.

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 matter was determined after killing the samples in a microwave oven and drying them at 90 °C to constant mass.

Results and discussion

In transgenic plants of *Nicotiana tabacum* cv. Samsun the expression of T-DNA carrying gene 4 for cytokinin synthesis was weak and the level of endogenous cytokinins (zeatin, zeatin riboside, isopentenyladenosine) in plantlet leaves was only sligtly increased, whereas the level of isopentenyladenine was not changed (Table 1). On the other hand, in comparable transgenic plants of other cultivar of tobacco (cv. Maryland Mammoth) the levels of zeatin and isopentenyladenine were considerably increased, but not the level of zeatin riboside (Ondřej *et al.* 1991). However, in comparable transgenic potato plants where the expression of gene 4 was high, a manifold increase in level of all endogenous cytokinins was observed (Ondřej *et al.* 1990, Čatský *et al.* 1993).

Table 1. Levels of endogenous cytokinins in control and two clones of 21-d old transgenic tobacco plantlets cultivated *in vitro*.

Clone	Cytokinin content [μg kg^{-1}(f.m.)]				
	Z	ZR	iP	iPA	Sum
Control	14.7	24.0	11.9	6.9	57.5
1334-3	17.5	28.9	9.8	7.1	63.3
1334-5	18.8	27.9	11.8	9.7	68.2

Even the slightly elevated cytokinin level in transformed clones influenced the inner structure of photosynthetic membrane system (Table 2). A higher degree of grana stacking was observed in both transgenic plant types than in control plants.

Table 2. Comparison of membrane stacking level (granal:intergranal membrane ratio) in chloroplasts of transgenic plants of tobacco. Grana were isolated after digitonin treatment and differential centrifugation. Change in Chl *a/b*: in comparison with whole chloroplasts. S - stromal (intergranal) fraction; G - granal fraction; G/S - ratio of Chl content in granal and stromal fractions. ** - significant against control at $P = 0.01$ level.

Clone	Type of fraction	Chl content [%]	Chl *a/b*	Change in Chl *a/b* [%]	G/S
Control	S	13.85 ± 0.28	6.66 ± 0.15	228.08 ± 5.32	6.25 ± 0.12
	G	86.15 ± 0.28	2.68 ± 0.02	91.96 ± 0.60	
1334-3	S	12.40 ± 1.01	5.25 ± 0.13	173.32 ± 4.29	7.19 ± 0.07**
	G	87.60 ± 1.01	2.72 ± 0.01	90.06 ± 2.48	
1334-5	S	11.65 ± 0.02	6.04 ± 0.04	201.02 ± 1.34	7.58 ± 0.01**
	G	88.35 ± 0.02	2.65 ± 0.01	88.40 ± 0.38	

Table 3. Net photosynthetic rate, P_N and P_{Nsat}, per unit leaf area [$\mu g(CO_2)\ m^{-2}\ s^{-1}$], P_N per unit chlorophyll *a+b* [$g\ kg^{-1}(Chl)\ s^{-1}$], photorespiration, R_L and dark respiration, R_D [$mg(CO_2)\ m^{-2}\ s^{-1}$], CO_2 compensation concentration, Γ [$mg\ m^{-3}$], chlorophyll (Chl) *a+b* content per fresh mass [$g\ kg^{-1}$(f.m.)] and per leaf area [$mg\ m^{-2}$], Chl *a/b* ratio, stomatal, E_s, and cuticular, E_c, transpiration rates [$g(H_2O)\ kg^{-1}(H_2O)\ s^{-1}$], and leaf water, ψ_w, osmotic, ψ_s, and pressure, ψ_p, potentials [MPa] in control and transgenic tobacco plantlets. P_N and R_D are given for 600 $mg(CO_2)\ m^{-3}$ and 20 °C; P_{Nsat} was measured under saturating CO_2 concentration, optimum temperature and near-saturating irradiance. Means ± S.E. $n = 30$ for CO_2 exchange rates, $n = 9$ for chlorophyll contents, and $n = 10$ for water potentials. *, ** = significant against control at $P = 0.05$ or 0.01 level, respectively.

	Control	Clone 1334-3	Clone 1334-5
P_N per leaf area	236.9 ± 14.1	314.5 ± 10.3**	307.5 ± 11.0*
P_{Nsat} per leaf area	324.6 ± 11.4	426.7 ± 14.8**	412.5 ± 13.3*
P_N per Chl	7289	11113	9984
R_L	82.5 ± 2.26	111.7 ± 9.8**	106.3 ± 7.5**
R_D	31.7 ± 2.5	34.3 ± 1.8	33.9 ± 2.8
P_N/R_D	7.47	9.17	9.07
Γ	122.1	116.8	122.5
Chl *a+b* per fr.m.	1.744 ± 0.246	1.417 ± 0.201	1.555 ± 0.118
Chl *a+b* per leaf area	0.325 ± 0.044	0.283 ± 0.031	0.308 ± 0.015
Chl *a/b*	3.176 ± 0.096	2.989 ± 0.102	3.069 ± 0.026
E_S	0.425 ± 0.011	0.321 ± 0.038**	0.522 ± 0.017**
E_C	0.009 ± 0.002	0.019 ± 0.002**	0.013 ± 0.002
ψ_w	-0.85 ± 0.06	-0.82 ± 0.03	-0.83 ± 0.06
ψ_s	-1.05 ± 0.08	-0.97 ± 0.03	-0.95 ± 0.07
ψ_p	0.20 ± 0.02	0.15 ± 0.04	0.12 ± 0.08

This could be related to higher proportion of photosystem 2 content, namely of the light-harvesting complex in thylakoids of transgenic plants. This is supported by decreased Chl *a/b* ratio in separated grana fraction in comparison with whole unfragmented chloroplasts. Also diminution of the Chl *a/b* ratio in whole leaves of transgenic plants was found (Table 3). This finding is in agreement with results of Ondřej *et al.* (1990) and Čatský *et al.* (1993) on transgenic potato plants.

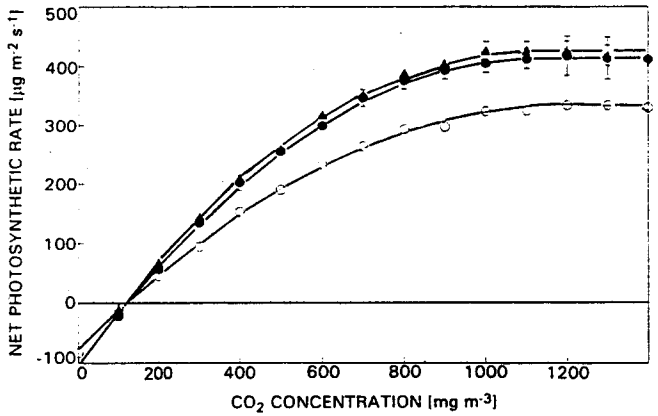


Fig. 1. Dependence of net photosynthetic rate on ambient CO_2 concentration in control and transgenic (control - *open circles*, clone 1334-3 - *closed triangles*, clone 1334-5 - *closed circles*) tobacco plants grown *in vitro*. Irradiance 860 μmol m^{-2} s^{-1}, temperature 22 °C. Means from 30 repetitions. Vertical and horizontal bars denote S.E. If not shown, the point size exceeds standard mean error.

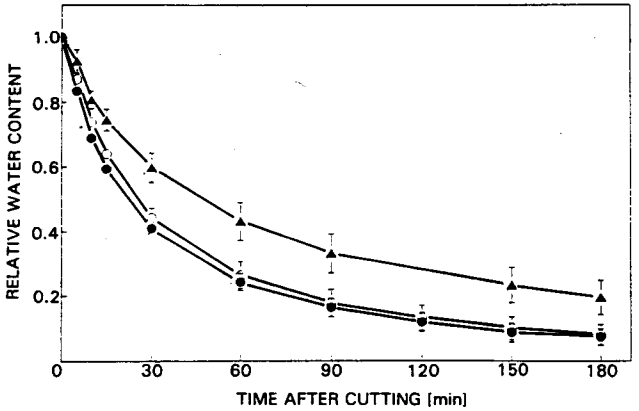


Fig. 2. Transpiration curves (changes in relative water content after cutting) in control and transgenic (control - *open circles*, clone 1334-3 - *closed triangles*, clone 1334-5 - *closed circles*) tobacco plantlet leaves (irradiance 860 μmol m^{-2} s^{-1}, temperature 21.5 °C, air humidity 32 %). Means from 10 repetitions, vertical bars denote S.E. If not shown, the point size exceeds standard mean error.

The effect of increased cytokinin levels on membrane arrangements may be attributed to retardation of their senescence, which is expressed among other factors in grana deterioration.

In comparison with non-transformed plantlets, in both clones with increased cytokinin content P_N and R_L were considerably higher (by *ca.* 25 and 30 %, respectively) whereas R_D and Γ were not affected (Table 3). Thus, the P_N/R_D ratio in transgenic plants was considerably increased.

Leaf water, osmotic and pressure potentials were not significantly changed in transgenic plantlets. The ψ_w values were very similar and the slightly less negative ψ_s and lower ψ_p in trangenic plants were not statistically different from those in control (Table 3). The transformed plants usually had higher water content and lower dry matter content than the control plantlets. E_s was slightly lower in clone 1334/3 and slightly higher in clone 1334/5 and E_c was slightly higher in both clones of transgenic plants in comparison with E_s and E_c in control plants (Table 3). The slightly lower E_s in clone 1334-3 and thus the slower rate of water loss (Fig. 3) may be useful for the acclimation of these plantlets to lower air humidity after transplanting *ex vitro*.

References

Arnon, D.I.: Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Čatský, J., Pospíšilová, J., Macháčková, I., Synková, H., Wilhelmová, N., Šesták, Z. : High level of endogenous cytokinins in transgenic potato plantlets limits photosynthesis. - Biol. Plant. **35**: 191-198, 1993.

Čatský, J., Tichá, I: A closed system for measurement of photosynthesis, photorespiration and transpiration rates. - Biol. Plant. **17**: 405-410, 1975.

Kaše, M., Čatský, J.: Calculator-assisted measurements of photosynthetic, respiration and photorespiration rates in a closed gas exchange system. - Biol. Plant. **25**: 139-146, 1983.

Macháčková, I., Krekule, J., Eder, J., Seidlová, F., Strnad, M.: Cytokinins in photoperiodic induction of flowering in *Chenopodium* species. - Physiol. Plant. in press, 1993.

Melis, A., Anderson, J.M.: Structural and functional organization of the photosystems in spinach chloroplasts.Antenna size, relative electron transport capacity, and chlorophyll composition.- Biochim. biophys. Acta **724**: 473-484, 1983.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Ondřej, M., Bavrina, T.V., Dudko, N., Hrouda, M., Krekule, J., Lozhnikova, V.N., Macháčková, I., Seidlová, F., Vlasák, J. : Transgenic tobacco plants with T-DNA phytochrome synthesis genes. - Biol. Plant. **32**: 40 - 48, 1991.

Ondřej, M., Hrouda, M., Karavajko, N.N., Matoušek, J., Mikulovič, T.P., Pavingerová, D., Vlasák, J.: Transformation of *Agrobacterium* vectors and the study of functions of plant hormones. - In: Krekule, J., Seidlová, F. (ed.): Signals in Plant Development. Pp. 73-89. SPB Academic Publishing, The Hague 1989.

Ondřej, M., Macháčková, I., Čatský, J., Eder, J., Hrouda, M., Pospíšilová, J., Synková, H.: Potato transformation by T-DNA cytokinin synthesis gene. - Biol. Plant. **32**: 401-406, 1990.

Ooms, G.: Controlling differentiation and endogenous growth regulator content by means of genetic transformations. - In: Advances in the Chemical Manipulation of Plant Tissue Cultures. (Monograph No. 16.) Pp. 1 - 17. British Plant Growth Regulator Group, Bristol 1987.

Strnad, M., Vaněk, T., Binarová, P., Kamínek, M., Hanuš, J.: Enzyme immunoassays for cytokinins

and their use for immunodetection of cytokinins in alfalfa cell culture. - In: Kutáček, M., Elliott, M.C., Macháčková, I.: Molecular Aspects of Hormonal Regulation of Plant Development. Pp. 41-54. SPB Academic Publishing, The Hague 1990.

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