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Potato Transformation by T-DNA Cytokinin Synthesis Gene

M. ONDŘEJ[3], IVANA MACHÁČKOVÁ[1], J. ČATSKÝ[2], J. EDER[1], M. HROUDA[3], JANA POSPÍŠILOVÁ[2], and HELENA SYNKOVÁ[2]

Institute of Experimental Botany, Czechoslovak Academy of Sciences, Praha 6 CS–160 00, [1]Vokovice, Ke dvoru 15/16 and [2]Na Karlovce 1, [3]CS–370 05 České Budějovice, Na sádkách 702, Czechoslovakia

Abstract. Potato plants were transformed by the *A. tumefaciens* vector which integrates into the plant genome two foreign genes only: pTi C58 T-DNA gene 4 (ipt) responsible for elevated synthesis of cytokinins and kanamycin resistance gene. Three teratoma clones studied showed approximately 3 times, 6 times and 9 times increased levels of zeatin riboside and zeatin in comparison with untransformed controls and slight increase of the IAA level. Shoots formed roots in agar cultures sporadically, if the increase of zeatin riboside and zeatin levels was not higher than 6 times the control level. The chlorophyll content and net photosynthetic rate decreased with increasing levels of zeatin and zeatin riboside.

Integration of *A. tumefaciens* Ti plasmid T-DNA into the plant genome causes dedifferentiation of the plant tissues and their tumor growth. This is the consequence of the increase of endogenous levels of both auxins and cytokinins and possibly also the results of interaction with the expression of some other T-DNA genes.

Different *A. tumefaciens* strains with engineered T-DNA, where either the cytokinin synthesis gene 4 (ipt) or one or both the auxin synthesis genes 1 (iaaM) and 2 (iaaH) were inactivated are available; as an example, see LEEMANS *et al.* (1982). All or most of other T-DNA genes in these constructions are left intact and could possibly interact in the modification of the resulting plant phenotype. To avoid possible impact of expression of the remaining T-DNA genes, we have constructed a series of vector plasmids which bring into the plant genome individual T-DNA genes 1,2 and 4 and their combinations (ONDŘEJ et al. 1989, 1990). Here we present the results with potato plants transformed with a member of this series, the vector plasmid pCB1334, which brings into the plant genome the T-DNA gene 4, together with the selectable kanamycin resistance gene.

The aim of experiments described here was to find the effects of the endogenous

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increase in cytokinin level on some morphological and photosynthetic characteristics of transformed potato plants.

MATERIAL AND METHODS

Potato (*Solanum tuberosum* L. cv. Oreb) was used throughout the experiments. The *A. tumefaciens* (pAL4404) (pCB1334) strain (ONDŘEJ *et al.* 1989, 1990) was used for transformation by the leaf disc method of ROGERS *et al.* 1986) without the use of feeder layer and with internode segments instead of leaf ones. The target segments were placed on MURASHIGE and SKOOG (1962) medium containing 2 % sucrose, 0.1 mg l^{-1} α-naphthylacetic acid (Lachema, Prague), 1 mg l^{-1} 5-benzylaminopurine (Fluka, Heidelberg), 500 mg l^{-1} Ticarcillin (Beecham Laboratories, England) and 200 mg l^{-1} kanamycin sulphate (Medexport, SSSR). The kanamycin resistant transformed tissues were subcultivated for the next three subcultivations, one month each, on the MURASHIGE and SKOOG (1962) medium with antibiotics but without phytohormones. The presence of T-DNA in the potato genome was detected by Southern blotting procedure, as described in MANIATIS *et al.* (1982). Plant DNA was isolated according to ROGER and BENDICH (1985). As a probe, ^{32}P-labelled pTi C58 T-DNA fragments 19 and 22 cloned in pCB1341 were used as described in ONDŘEJ *et al.* (1989).

Auxins were determined by high-performance liquid chromatography (HPLC) with fluorimetric detection after purification of a methanolic extract by means of either partitioning or polyvinylpyrrolidone chromatography (EDER *et al.* 1988). 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.* 1989). To evaluate losses, internal standards were added: 1–^{14}C–IAA (8 kBq) and ^{3}H–*m*–hydroxy–benzyladenine riboside (5 kBq). IAA analysis was performed at two different growth stages: at teratoma stage and later on after subcultivation, when some shoots already differentiated (Table 2).

Chlorophyll content and ratio of chlorophylls *a*/*b* were determined with spectrophotometer PU–8800 (Philips Scientific, Cambridge, U. K.) in 80 % acetone extracts of whole shoots by the standard method of ARNON (1949). Net photosynthetic rate was determined as a CO_2 influx in a calculator-assisted closed system with infra-red gas analyzer (Infralyt IV, Junkalor, Dessau, G.D.R) in a CO_2 concentration range from 80 to 400 mg m^{-3}, leaf temperature of 20 °C and saturating irradiance (400–700 nm) of 860 μmol m^{-2} s^{-1} (*cf.* ČATSKÝ and TICHÁ 1975, POSPÍŠILOVÁ *et al.* 1989).

RESULTS

Transformation of the internode segments resulted in teratoma growth. Generally, in the first subcultivation the transformed tissues showed extreme teratoma appearance (very short, dense and thick stems, dense clumps of very small and thick

TABLE 1

Cytokinin level in potato teratoma clones 5 weeks after subcultivation

Experimental variant	Zeatin riboside [μg kg^{-1} (fr. m.)]	[% increase]	Zeatin [μg kg^{-1} (fr. m.)]	[% increase]	Isopentenyl adenin [μg kg^{-1} (fr. m)]	[% increase]
Control	37.33		27.63		15.43	
Clone 1	335.61	899	212.35	769	39.6	257
Clone 4	209.35	561	164.80	696	19.8	128
Clone 13	117.41	315	122.76	444	34.1	221

leaves of modified shape). Later the teratoma tissues showed a tendency to normalization in different degrees, in dependence on the clone used (Fig. 1). After six months of cultivation on antibiotics, three independently transformed clones, designated 1, 4 and 13, representing different degrees of differentiation of teratoma clones, were used for further study. The clone 1 was formed by very compact, almost moss-like growing teratomas. It consisted of dense clumps of very short (less than 1 cm) and highly branched thick shoots with a small number of very small and thick leaves. The strain 13 consisted of long (up to 5 cm) shoots with strong abnormal, small leaves and a low degree of stem branching. In subcultivation intervals longer than one month, the shoots showed a tendency to resemble those of non-transformed plants and occasional root formation. The clone 4 was intermediate between 1 and 13 and its shoots also sometimes formed roots. Rooted shoots were transferred into the soil, where they formed plants with normal, branched stems with small, round leaves.

Southern blotting analysis showed the presence of introduced T-DNA sequences

TABLE 2

Free IAA level in teratoma clones

Clone	Interval after subcultivation (growth stage)	IAA [μg kg^{-1} (fr. m.)]	[% of control]
Control	2 weeks	27.4	
	5 weeks	9.7	
Clone 1	5 weeks (teratoma + some shoots)	16.9	174.2
Clone 4	2 weeks (teratoma)	31.8	116.0
Clone 13	2 weeks (teratoma)	33.0	120.4
	5 weeks (differentiated shoots)	19.0	195.9

TABLE 3

Chlorophyll content and net photosynthesis rate in 5 weeks old control and transformed plants *in vitro**. Mean of 9 repetitions, standard mean error in brackets

Experimental variant	Chlorophyll content [g kg^{-1} (dry m.)]		Net photosynthetic rate [mg (CO_2) kg^{-1} (dry m.) s^{-1}]	[g (CO_2) kg^{-1} (Chl. $a + b$) s^{-1}]
	$a + b$	$a : b$		
Control	4.69 (0.40)	3.18 (0.03)	3.040 (0.450)	0.603
Clone 13	4.26 (0.40)	3.01 (0.12)	2.439 (0.288)	0.572
Clone 4	3.89 (0.33)	2.92 (0.10)	1.621 (0.091)	0.440

* Clone 1 was not suitable for measuring photosynthesis due to its morphology

in clones 1 and 13 (Fig. 2). The clone 4 was not tested, but its phenotype deviations from controls were similar as in clones 1 and 13 and thus this clone can also be regarded as transformed.

Transformed potato tissues of all three clones showed considerable increase of zeatin riboside and zeatin levels (Table 1). The increase of isopentenyl adenin was much lower and isopentenyl adenosin was found only in small amounts. The clone 1 contained the highest levels of cytokinins, followed by clones 4 and 13. The IAA evels (Table 2) showed slight increase in relation to controls.

Teratoma clone 1 was not suitable for photosynthetic studies because of its extreme teratoma appearance and slow growth. Clones 13 and 4 showed a progressive decrease in the chlorophyll content, the ratio of chlorophylls *a/b* and net photosynthetic rate with increasing levels of cytokinins in comparison with untransformed controls (Table 3).

DISCUSSION

After the integration of T-DNA of the vector plasmid pCB1334, which brings into the plant genome the pTi C58 T-DNA gene 4 (ipt) for cytokinin synthesis, potato teratoma tissues were obtained. Three clones were selected, which have approximately a 3, 6 and 9times increased level of zeatin riboside in relation to untransformed controls. Teratoma tissues of the clones 4, with 5.6times increased zeatin riboside level and 13 with 3.2times increased level were able to form roots rarely, but those of the clone 1, with 9 times increased zeatin riboside level never rooted.

The increase of the level of zeatin riboside was not found as high as in our previous potato transformants with another plasmid construction (ONDŘEJ *et al.* 1989), where an 88fold increase of the level of zeatin riboside plus zeatin was found. The reason for this difference will be investigated.

In the present experiments a slight increase of IAA level with increase of the cytokinin level was observed. OOMS and LENTON (1985) have studied potato tissues transformed with *A. tumefaciens* octopine strain, the T-DNA of which was lacking the gene 2 (iaaH) for auxin synthesis. The teratoma shoot cultures derived showed a 200times higher level of cytokinins than controls with a simultaneous decrease of IAA concentration to about 1/4. Similar results with the octopine strain carrying T-DNA gene deletions inactivating the auxin genes were obtained in tobacco by AKIYOSHI *et al.* (1983). The decrease of IAA level in those cases can be attributed to the action of other T-DNA genes. On the other hand, VAN ONCKELEN *et al.* (1988) reported a slight increase in IAA level in tobacco accompanying a large decrease in the level of cytokinins after the transformation by gene 4 vectors. The increase of the endogenous cytokinin level may activate the pathway of auxin synthesis coded by the plant genome genes. In our experiments, a similar effect was found.

This is the first study of the effects of endogenous increase of the levels of cytokinins due to transgenosis by T-DNA gene 4 for cytokinin synthesis on the characteristics of photosynthesis. The cytokinin-stimulated plastid development and stimulation of ribulose-1,5-bisphosphate carboxylase activity by cytokinins is known from the literature (PARTHIER *et al.* 1985). The decline of parameters of photosynthesis here indicates that the increase of the level of cytokinins was already beyond the optimum. Transgenic plants with a still lower degree of expression of the gene 4 should be used in further studies.

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Figs. 1 and 2 at the end of the issue.