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# The Effect of an Elevated Cytokinin Level Using the *ipt* Gene and $N^6$-Benzyladenine on Single Node and Intact Potato Plant Tuberization *in Vitro*

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**Abstract.** Two models of potato (*Solanum tuberosum* L.) tuberization in vitro (intact plants and single nodes) were used to study the role of cytokinins in this process. We applied hormone in two different ways. The *exogenous* addition of 10 mg · $L^{-1}$ $N^6$-benzyladenine (BA) into the tuberization medium resulted in advanced tuber formation in intact plants, and microtubers appeared 10–20 days earlier than in the experiments in which no cytokinin was supplied. Transformation with the *Agrobacterium tumefaciens ipt* gene provided potato clones with *endogenously* elevated cytokinin levels (3–20 times higher zeatin riboside content in different clones). The onset of tuberization in intact *ipt*-transformed plants with low transgene expression was advanced in comparison with control material, and exogenously applied BA further promoted the tuberization process. On the contrary, tuberization was strongly inhibited in *ipt*-transformed nodes, and an external increase of the cytokinin level caused complete inhibition of explant growth. In untransformed (control) nodes cytokinin application resulted in primary and secondary tuber formation, which depended on the BA concentration in cultivation media.

Tuberization in potato represents a transition of the stolon apex into the underground storage organ, the tuber. The same process can be induced in vitro, and so-called microtubers are formed on the aerial parts of the stems from both lateral and apical buds. Key factors involved in this process have been studied in detail: sucrose level (Ewing 1990); different kinds of inhibitors (Harvey et al. 1991, Stallknecht and Farnsworth 1982); photoperiod (Ewing 1978, Slimmon et al. 1989); temperature (Levy et al. 1993); and phytohormones, particularly cytokinins and gibberellins (Vreugdenhil and Helder 1992, Vreugdenhil and Struik 1989). Cytokinins are known to influence many physiologic processes such as cell proliferation, together with auxin shoot regeneration (Skoog and Miller 1957), apical dominance (Medford et al. 1989), and delay of senescence (Smart et al. 1991). The role of cytokinins ($N^6$-benzyladenine (BA), kinetin, $N^6$-($\Delta^2$-isopentenyl)adenine) has also been studied in connection with tuber induction (Levy et al. 1993, Pelacho and Mingo-Castel 1991, Slimmon et al. 1989). Typically, in these studies the hormones have been applied in an appropriate concentration directly to the cultivation medium. Such an approach encounters problems with poorly determined hormone uptake, distribution, and metabolism. Thus, new methods have been sought for a better characterized hormone treatment, and this search has led us to the *Agrobacterium tumefaciens* transformation system. This qualitatively new approach is based on the ability of the bacterial machinery to transfer and stably integrate a part of Ti plasmid into the plant genome. The genes for cytokinin (isopentenyltransferase, *ipt*) and auxin (tryptophan monoxygenase, *iaaM* and indoleacetamide hydrolase, *iaaH*) biosynthesis are localized in this fragment (T-DNA), and their expression leads to uncontrolled tissue proliferation and tumor formation (Kado 1991). Individual genes for the plant hormone biosynthesis pathways were isolated (*ipt, iaaM,* and *iaaH*), cloned, and vectors for transformation prepared. The whole

**Abbreviations:** BA, $N^6$-benzyladenine; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; NAA, α-naphthylacetic acid.

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spectrum of plants overproducing auxin (*iaaM* and *iaaH*) and cytokinins (*ipt*) has been reported, and these transgenics exhibit typical phenotypic traits resulting from the disturbed endogenous hormone balance (Hamill 1993).

We have used transgenic potato plants with an integrated *ipt* gene to study in vitro tuberization as a consequence of increased cytokinin levels.

## Materials and Methods

### *Plant Cultivation*

Potato plants (*Solanum tuberosum* L. cultivars Miranda, Kamýk, and Zvíkov) were cultivated in vitro on medium with M&S salts composition (Murashige and Skoog 1962) supplemented with 100 mg · $L^{-1}$ inositol, 0.1 mg · $L^{-1}$ thiamine, 0.5 mg · $L^{-1}$ nicotinic acid, 0.5 mg · $L^{-1}$ pyridoxin, 2 mg · $L^{-1}$ glycine, 3% sucrose, and 0.7% agar. The photoperiod was 16 h. All tuberization experiments were done in darkness.

### *Potato Transformation*

Stem segments were used for tissue transformation and plant regeneration (Horsch et al. 1985). The binary vectors *A. tumefaciens* LBA 4404 p(CB 1334) (or 1339) were used as described in Vlasák and Ondřej (1992). They contain an isopentenyltransferase gene in different orientations under the control of its native promoter and *nos*-NPT II chimeric sequence providing kanamycin resistance to the transformed tissue. Regenerated shoots were selected for kanamycin resistance and micropropagated in vitro.

### *Transgene Detection*

Genomic DNA from individual potato clones was isolated according to Dellaporta et al. (1983). The presence of the T-DNA *ipt* gene was tested by polymerase chain reaction (PCR) using primers CYT-1 (5′ GTC CAA CTT GCA CAG GAA AGA 3′) and CYT-2 (5′ ATC CTC CCT CAA GAA TAA GCC 3′), which amplify 273-base pairs fragment from the *ipt* coding sequence. To avoid false positive results caused by bacterial contamination, the DNA samples were in parallel subjected to PCR with another set of primers designed for amplification of an *Agrobacterium vir* region (primers VirA-7, 5′ AAT TCA CCG ACG CGG CAG GAT TTT AAG ACA G 3′, and VirA-8, 5′ AGC TTT GGT ACG AGA GAC TAT TTC GCG TAG 3′; the length of the product is 1093 base pairs). The PCRs were performed in 50 µl of the reaction mix (0.2 µM primers, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 1 × PCR buffer, and 2 units of *Taq* DNA polymerase (Stratagene). After initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min were carried out followed by final extension step at 72°C for 5 min, using Techne Cyclogene PCR apparatus. The reaction products were electrophoresed on 1% agarose gel.

### *Cytokinin Determination*

A modified method of Strnad et al. (1990) was used. Plant material (leaves and stems) was collected and stored in a deep freezer until measurement. It was ground in liquid nitrogen before extraction in 80% methanol, and internal standard ([$^3$H]zeatin, prepared in the Institute of Nuclear Biology and Radioisotopes of Academy of Sciences, Czech Republic, specific activity 1.6 TBq · $mmol^{-1}$) was added up to 0.5 kBq/sample. After centrifugation, supernatants were passed through a C-18 Sep-Pak cartridge (Waters, Millipore Corp.) and evaporated to water phase. Samples were diluted in 0.04 M ammonium acetate buffer, pH 6.5, and loaded onto coupled DEAE-Sephadex A25 (Pharmacia Biotech) C-18 cartridge columns. Retained cytokinins were eluted from C-18 with 80% methanol and evaporated to dryness. A Separon SGX C-18 column (Tessek Prague, CZ, 5 µM, 250 × 4.6 mm inner diameter) was used with a flow rate 0.8 ml · $min^{-1}$ for HPLC fractionation of the extracted cytokinins.

Collected fractions of zeatin, zeatin riboside, $N^6$-($\Delta^2$-isopentenyl)adenine, and $N^6$-($\Delta^2$-isopentenyl)adenosine were evaporated and subsequently measured with ELISA (antibodies and cytokinin conjugates with alkaline phosphatase were produced and kindly provided by M. Strnad, IEB ASCR Olomouc, CZ). The results were recalculated to 1 g of the fresh weight and 100% of the added internal standard.

### *Tuberization Systems*

In the *overlay experiments* we used intact plants grown for 30 days in culture (100-ml containers and 20 ml of solidified M&S medium). Then the top of agar was overlaid with 30 ml of liquid tuberization medium-M&S supplemented with 10% sucrose (medium S) or 10% sucrose and 10 mg of BA/L (medium B).

In the *single node segments system* we cut separate internodes with lateral buds and attached leaves from the middle parts of the 30-day-old in vitro plants. They were placed on Petri dishes (nine/plate) with 30 ml of the solid tuberization medium S or B. In later experiments we tested the medium with modified content of sucrose (5 and 8%) and BA (0.1; 1 mg · $L^{-1}$). For auxin treatment the media were supplemented with 0.1 or 0.2 mg · $L^{-1}$ α-naphthylacetic acid (NAA). Four plates were used for every treatment, and each experiment was repeated at least three times. The number of tubers was scored periodically.

The results presented in the graphs are from one individual experiment. The absolute numbers of the tubers differed in separate repetitions of the experiments, but relative differences between treatments (with and without BA) and experimental materials (transformed and control plants) did not change.

## Results

### *Transformation*

Several independent kanamycin-resistant clones of *ipt*-transformed potato plants were derived from two cultivars (Miranda and Kamýk). They exhibited different phenotypes ranging from typical teratomas lacking roots to plants with shortened internodes and poor rooting. During micropropagation we used preferably the less branching plants with more extended internodes to get plants with lower *ipt* expression. After a long term selection procedure we obtained stable phenotypes assigned MIR 1334-2 and KAM 1339-2 (original clones MIR 1334-1 and KAM 1339-1). The differences in Miranda phe-

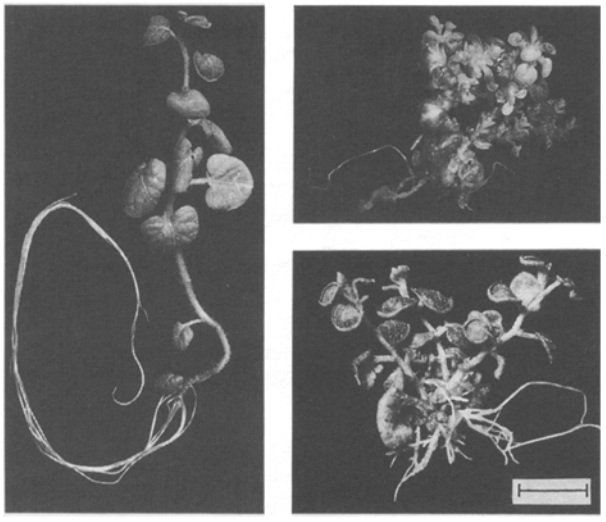

**Fig. 1.** In vitro potato plants transformed with *A. tumefaciens ipt* gene. *Left*, nontransformed control; *right upper*, clone 1334-1; *right lower*, clone 1334-2. *Size bar*, 1 cm in the whole separation.

notypes are shown in Figure 1; the levels of endogenous cytokinins are documented in Table 1. For the tuberization experiments we used clone MIR 1334-2, which is the most comparable in phenotype and growth parameters to nontransformed plants, and it has slightly increased levels of zeatin type cytokinins during the whole time of cultivation.

The presence of the T-DNA *ipt* gene in the genome of the transformed potato clones has been proven by PCR. Our primers amplified a fragment of the correct size (273 base pairs) from the isolated genomic DNA. No signal was detected in identical DNA samples with primers specific for the *vir* region of the *Agrobacterium*-derived vector (Fig. 2), confirming that the *ipt* fragment was not amplified from bacteria-contaminated tissues.

### *Tuberization in Intact Plants*

The transformed clone MIR 1334-2 was used to investigate tuberization in intact plants. Plants were cultivated for 30 days in vitro and then overlaid with sterile tuberization medium S or B. First microtubers appeared in the clone MIR 1334-2 (medium B) and then in MIR 1334-2 on medium S and in control Miranda on B. The last microtubers were observed in the control Miranda with only an increased sucrose level (S) (Fig. 3). Thus, exogenous application of cytokinins promoted the tuberization process in both control and transformed clones. Endogenous overproduction of cytokinins in transformed genotype had a stimulating effect in both media. The yield of the tubers is summarized in Table 2.

Similar results with regard to advanced tuber formation in the presence of BA were also obtained with other potato cultivars tested, Kamýk and Zvíkov (unpublished).

### *Tuberization in Nodal Segments*

Levy et al. (1993) studied the influence of $N^6$-($\Delta^2$-isopentenyl)adenine in a nodal tuberization system. They documented no changes in the tuber-forming capacity of the segments (derived from the different potato cultivars) after cytokinin application. In our experiments we obtained clear temporary differences that correlate with the presence of BA in the cultivation medium. Figure 4 demonstrates tuberization of the single nodes on media S and B. In transformed clones we found strong inhibition of tuber formation and inhibition of the explant growth, especially on medium B as compared with nontransformed lines. The onset of tuberization was earlier in controls without BA; but within 20–30 days of cultivation variants with supplemented cytokinin surpassed medium S. This kind of response correlates with the difference in tuber origin. The tubers on medium S were formed from the original lateral buds (primary tubers); in medium B primary microtubers developed rarely, but they formed on the sprouting shoots (secondary tubers) (Fig. 5).

The role of BA levels on the primary to secondary tuber ratio is illustrated in Figure 6. The ability of BA to suppress primary tuber formation rises with an increase in its concentration. The most efficient treatment was 10 mg of BA/L.

In another experiment the influence of a lower sucrose concentration (5 and 8%) on single node tuberization was investigated. Primary and secondary tuber formation was not influenced, and optimal levels were 8 and 10% sucrose (Fig. 7). The tuberization capacity in a transformed clone was not affected (Fig. 8).

We also tested the possible role of auxin in this system, especially with regard to primary tuber formation. The tuberization of transformants was not improved. Preliminary results showed that NAA treatment could support the primary tuber development if applied together with BA (data not presented).

## Discussion

We studied a typical physiologic response, tuberization of potato plants with the integrated gene for cytokinin biosynthesis. The gene presence in the

**Table 1.** Cytokinin content in Miranda clones.

| Clone | Description | Days in cult. | Cytokinin content (pmol/g fresh weight) | | | | |
|---|---|---|---|---|---|---|---|
| | | | ZR | ZEA | IPR | IP | Total |
| Miranda control | Normal roots | 8 | 14 | 9 | 9 | 91 | 123 |
| | no branching | 32 | 15 | 22 | 49 | 76 | 162 |
| Miranda 1334-2 | Poor rooting | 8 | 47 | 75 | 22 | 189 | 333 |
| | branching | 32 | 34 | 35 | 68 | 44 | 181 |
| Miranda 1334-1 | No rooting | 8 | 88 | 24 | 69 | 84 | 265 |
| | teratom | 32 | 309 | 63 | 41 | 14 | 427 |

Stems and leaves of the in vitro cultivated plants were harvested (8 or 32 days after propagation), purified, separated on HPLC, and measured by ELISA. Measurement was repeated two times. The determinations gave different absolute levels, but relative comparison in control to transformed material stayed constant. Then, one of the measurements was selected for presentation. ZR, zeatin riboside; ZEA, zeatin; IP, $N^6$-($\Delta^2$-isopentenyl)adenine; IPR, $N^6$-($\Delta^2$-isopentenyl)adenosine.

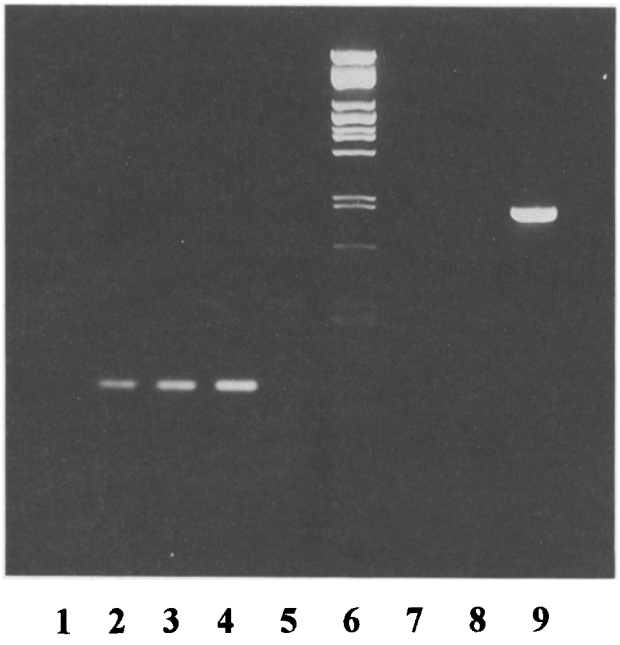


**Fig. 2.** Transgene detection. Genomic DNA was extracted from individual potato clones and subjected to PCR with primers specific for *ipt* and *virA* genes. As a positive control, the plasmids used for transformation were included. *ipt* detection: *lane 1*, Miranda control (not transformed); *lane 2*, Miranda 1334-2; *lane 3*, Miranda 1334-1; *lane 4*, plasmid DNA with *ipt* gene (pCB1334); *lane 5*, negative PCR control (without DNA); *lane 6*, *PstI*-digested $\gamma$-DNA (600 ng). *vir* detection; *lane 7*, Miranda 1334-2; *lane 8*, Miranda 1334-1; *lane 9*, plasmid DNA with *vir* target (pAL4404).

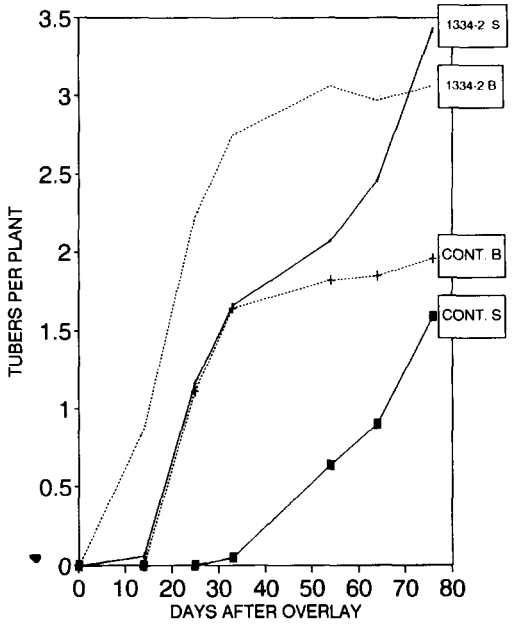


**Fig. 3.** Tuberization of the intact plants overlaid with liquid medium. In vitro potato plants (control Miranda and transformed clone MIR 1334-2) were cultivated for 30 days in 100-ml vessels with 20 ml of solid M&S medium. Then the cultures were overlaid with 30 ml of liquid tuberization medium S (10% sucrose) or medium B (10% sucrose and 10 mg · $L^{-1}$ BA).

genome was proven by PCR, and the resulting phenotypes were compared with regard to the endogenous levels of cytokinins. As demonstrated in Figure 1 and Table 1, the phenotype and cytokinin content correlated well, and a lower cytokinin content was found in a moderate phenotype of the transformants (poorly rooting plants) compared with the high cytokinin levels in teratomas. Rooting plants (clone MIR 1334-2) with modified leaves, weak apical dominance, and short internodes (Fig. 1) were selected during subcultivations of the original teratomas. This material has a hormonal content very close to that of the control, and the phenotype is stable for at least 3 years. The transgenic plants are kanamycin resistant, and they grow well in the presence of 200 mg · $L^{-1}$ kanamycin.

The most important cytokinins with respect to their effect on phenotype were zeatin and zeatin riboside. The $N^6$-($\Delta^2$-isopentenyl)adenine and $N^6$-($\Delta^2$-isopentenyl)adenosine levels fluctuated during

**Table 2.** Yield of the Miranda microtubers in the overlay tuberization experiment.

| | Tuber number per plant ± S.E. | Tuber fresh weight per plant (mg) ± S.E. |
|---|---|---|
| MIR control | | |
| Medium S | 1.72 ± 0.36 | 342 ± 112 |
| Medium B | 1.96 ± 0.50 | 578 ± 184 |
| MIR 1334-2 | | |
| Medium S | 3.69 ± 0.97 | 860 ± 326 |
| Medium B | 3.06 ± 1.03 | 602 ± 172 |

In vitro potato plants (control Miranda and transformed clone MIR 1334-2) were cultivated for 30 days in the 100-ml vessels with 20 ml of the solid MS medium. Then the cultures were overlaid with 30 ml of the liquid tuberization medium S (10% sucrose) or B (10% sucrose and BA 10 mg · $L^{-1}$). The amount and fresh weight of the tubers from 40 individual plants in every treatment were scored, and mean values with standard error (S.E.) were calculated.

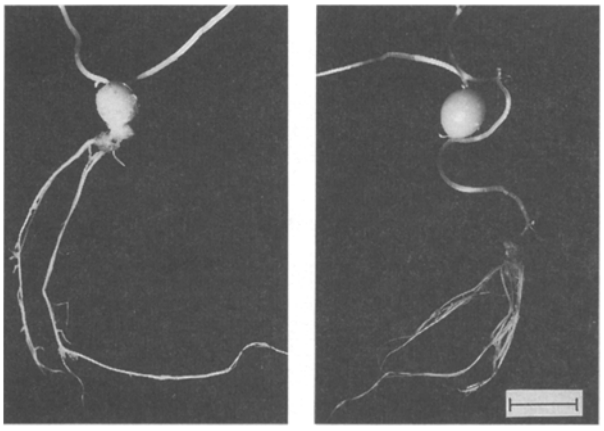

**Fig. 5.** Two categories of microtubers formed in the single node experiments: sprouting primary tuber (*left*), secondary tuber (*right*). *Size bar*, 1 cm in the whole separation.

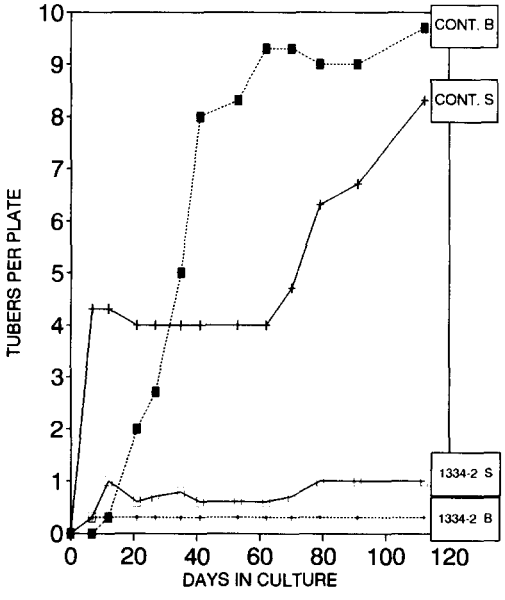


**Fig. 4.** Tuberization of the single nodes on the solidified media S and B. In vitro potato plants (control Miranda and transformed clone MIR 1334-2) were cultivated for 30 days in 100-ml vessels with 20 ml of solid M&S medium. Nine single node segments were cut from the middle part of the plants and placed on 30 ml of solid tuberization medium S (10% sucrose) or B (10% sucrose and 10 mg · $L^{-1}$ BA) in Petri dishes.

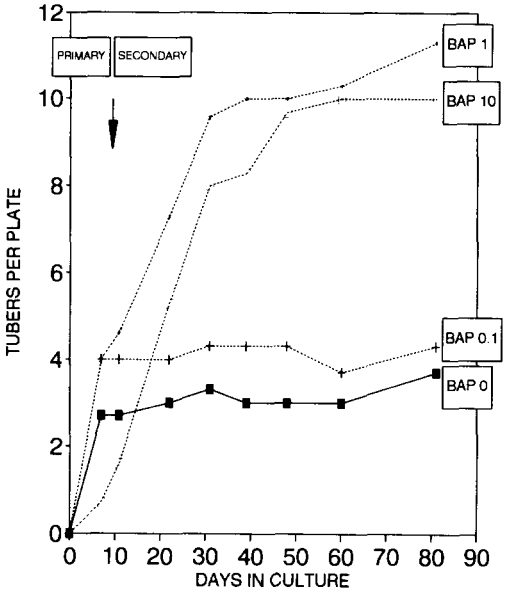


**Fig. 6.** Tuberization of the control nodes on M&S medium supplemented with 10% sucrose and different BA levels. In vitro potato plants (control Miranda) were cultivated for 30 days in 100-ml vessels with 20 ml of solid M&S media. Nine single node segments were cut from the middle part of the plants and placed on 30 ml of solid tuberization medium supplemented with 10% sucrose and BA (0, 0.1, 1, or 10 mg · $L^{-1}$). The *arrow* indicates the beginning of secondary tuber formation (10 days).

development and were sometimes even lower in the transformants than in control plants. The most abundant cytokinin in teratomic clones was zeatin riboside, which is a typical feature of the *ipt* transformed tissue (Hamill 1993).

The background of the teratoma transition into poorly rooting branching plant described above is not completely understood. Most probably it can be explained by a partial modification of the *ipt* gene promoter sequence by cytosine methylation (Amasino et al. 1984, Pavingerová and Hrouda 1991). Another interpretation can be found in the same promoter activity but increased metabolic capacity of the plants. The conversion of cytokinins into less active glucosidic forms was described for both nat-

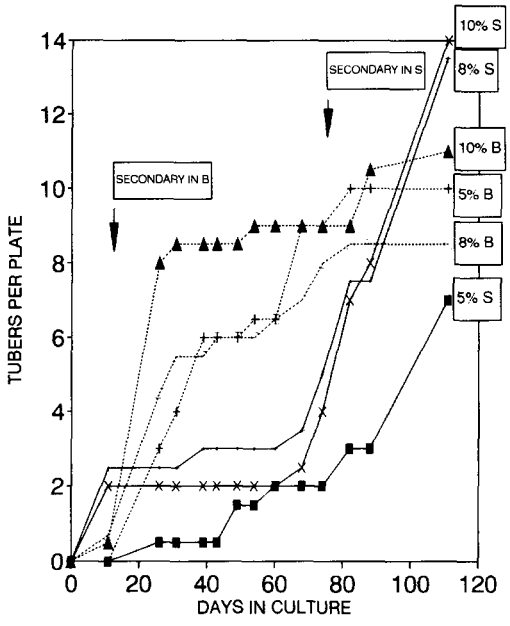


**Fig. 7.** Tuberization of control nodes in the presence of different concentrations of sucrose in media S and B. In vitro potato plants (control Miranda) were cultivated for 30 days in 100-ml vessels with 20 ml of solid M&S medium. Nine single node segments were cut from the middle part of the plants and placed on 30 ml of solid tuberization medium supplemented with 5, 8, or 10% sucrose. *Arrows* indicate the beginning of secondary tuber formation in media S and B.

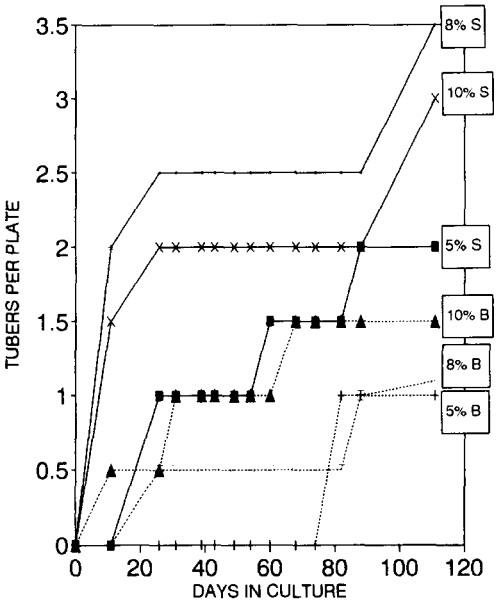


**Fig. 8.** Tuberization of *ipt*-transformed nodes in the presence of different concentrations of sucrose in media S and B. In vitro potato plants (clone MIR 1334-2) were cultivated for 30 days in 100-ml vessels with 20 ml of solid M&S medium. Nine single node segments were cut from the middle part of the plants and placed on 30 ml of solid tuberization medium supplemented with 5, 8, or 10% sucrose.

ural and synthetic phytohormones, whereas degradative cytokinin oxidase activity is well known to affect the level of natural cytokinins. All these metabolic pathways are normally present in plants, and they control hormone homeostasis very efficiently (Hare and Van Staden 1994, Van Staden et al. 1994).

In the first set of experiments we tested all material available, but teratomic clones MIR 1334-1 and KAM 1339-1 were excluded soon because of their heterogeneity in phenotype and because they showed no tuberization. We suppose that in this material the cytokinin content overstepped the physiologically acceptable level. As an optimal material we selected derived clones (assigned number 2), and, together with nontransformed control plants, they represented the key material for our studies.

In the overlay tuberization system the results were as expected. Application of the liquid tuberization medium with a high sucrose content (10%) resulted in advanced tuber formation in the transgenic clone (media S and B) and in controls when BA was added (medium B). Overall, tuberization on medium S was induced earlier, and the tuber yield was higher in a transformed clone than in control. Similar results with high tuberization capacity of the *ipt*-transformed plants were obtained also by Ooms and Lenton (1985). They grafted *ipt* shoots normally unable to form roots onto the untransformed stocks. Such chimeric plants formed aerial tubers that appeared very early in development. This observation is also in agreement with Levy et al. (1993), where the positive effect of $N^6$-($\Delta^2$-isopentenyl)adenine on tuberization of intact plantlets was documented.

We analyzed the overlay tuberization system in detail, and we considered the comparison between transformants and controls not to be precise. There are several factors that influence plants in a different manner: smaller transgenic plants are flooded more if the same amount of the medium is applied, there are more potential buds in branching transformants, and they have a less developed root system. Therefore, for better standardization of our experiments another approach using single nodes was included. Separate lateral buds with attached leaves were excised from the middle parts of the in vitro plants, and they were placed on solid M&S medium with identical parameters as in the overlay system. Surprisingly, high tuberization capacity of the *ipt* clones did not appear. Transgenic nodes rarely formed microtubers, and in the presence of BA they even stopped growing. Such an inhibitory effect was not observed in the intact plants, and this de-

velopment could not be reverted by a simple application of auxin (changing the unfavorable cytokinin-to-auxin ratio). This response is caused most likely by excision of the segment from plant organ correlations. Another important factor may be plant injury due to cutting, which may induce developmentally specific native *ipt* promoter expression (Neuteboom et al. 1993). Subsequent gene expression can lead to the unregulated increase in cytokinin content up to a physiologically toxic level (Ainley et al. 1993). Because of these uncontrolled factors we could not compare the reaction of control cuttings on medium B and transgenic nodes on medium S, which were expected to behave similarly.

The behavior of control (untransformed) nodes seems remarkable. They reflect very sensitively the addition of the plant hormones into the cultivation medium. As shown in Figure 4, there is a short delay in tuber initiation on medium B if compared with medium S. This difference is based on the different origin of the developing microtubers. We established two categories: primary and secondary tubers. The primary are formed directly from the original lateral buds on medium S; whereas in secondary tubers, the buds sprout initially, and subsequently lateral buds of the sprouts develop into microtubers (medium B). Depending on cultivation medium we could pursue the whole scale in forms of primary bud transition during the tuberization process. It begins with forming tuber-like organs due to swelling of the outgrowing stem and ends with regular tuber formation. The problem of the uncompleted tuber formation and subsequent second growth in one-leaf cuttings is discussed in Van Den Berg et al (1990). They explained these phenomena as a temporary reversion of the tuberization depending on the initial level of tuber induction. In our experiments the efficiency of BA in suppressing the inductive conditions and in the subsequent development of the primary tubers was correlated with high levels of the hormone, e.g., 10 $mg \cdot L^{-1}$ (Fig. 6).

Most of the primary tubers formed on medium S sprouted, and secondary tubers formed on the shoots, but much later in comparison with medium B. Approximately 60 days of cultivation was necessary to induce this process in media without cytokinin (Fig. 7). This time course corresponds in some aspects with tuberization of the intact plants (Fig. 3), probably at the late developmental stage of the cultures, high concentrations of inhibitors have already accumulated in the tissue, which might result in tuber formation. From our experience we know that very old potato cultures in vitro sometimes tuberize spontaneously without any treatment.

On the contrary, the addition of BA caused fast lateral bud sprouting (a typical cytokinin feature) and prevented primary tuber formation. Approximately 10 days later (plant organ correlation was restored), the promoting effect of BA on tuberization, proved in intact plants, provoked tuber formation on the sprouts (Fig. 4). To support this observation we tested the influence of synthetic auxin NAA on primary tuber formation. Preliminary results showed an increased apical dominance of the excised lateral buds and higher frequency of the primary tuber appearance.

In our study we found different abilities of the intact plants and single node segments to form microtubers in vitro. We also showed the influence of potato transformation and cytokinin overproduction on this process. It can be concluded that a moderate *ipt* expression under the control of its native promoter sequence positively influences the tuberization process in intact plants, whereas strong *ipt* activity prevents tuber formation and explant development. Exogenous cytokinin application may play an important but not a key role in the microtuberization process, BA in the tuberization medium advances the onset of tuber formation. This was confirmed in both systems used, but only in cases when intact plants were tested or plant integrity and hormonal correlations were reestablished (sprouts in the single nodes).

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