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# Phenotypes of tobacco plants expressing genes for the synthesis of growth regulators

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## Abstract

The expression of genes for synthesis of auxin (*iaaM* and *iaaH*) and cytokinins *(ipt)* was studied in tobacco plants transformed by two *Agrobacterium tumefaciens* strains C 58 and LBA 4404. The strain LBA 4404 carried binary vector plasmid pCB 1334 (*ipt* gene) and plasmid pCB 1349 *(iaaM, iaaH* and *ila* genes). Both plasmids carried reportered gene for npt II. Obtained plants expressed incorporated genes. New proteins with molecular masses of about 74, 40, 26, 25, 21 and 17 kDa for wild plasmid pTi C58; 60, 36, 31.5, 27, 26 and 17 kDa for binary vector plasmid pCB 1334 and 74, 49, 36, 31.5, 26 and 25 kDa for binary vector plasmid pCB 1349 were found in the patterns of soluble proteins. Significant changes in the content of chlorophylls, especially chlorophyll *a*, were detected in the plants carrying *ipt* gene and in plants transformed by the wild strain C58 of *A. tumefaciens*. Tobacco plants expressing *ipt* gene and genes from T-DNA of pTi C58 plasmid were dwarf, and in comparison to the controls, they had thicker stems, and the surface of the leaf blades was reduced to 20 - 50 %. Adventitious roots, growing from the stem, were typical for transformants overproducing auxins. Regenerants and transformants expressing genes from T-DNA of plasmid pTi C58 differed in the shape of the flowers and their fertility.

*Additional key words*: *Agrobacterium tumefaciens*, auxin, chlorophylls, cytokinin, *Nicotiana tabacum*, proteins.

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*Abbreviations:* NAA - α-naphtalene acetic acid; BAP - benzylaminopurine; IAA - indole-3-acetic acid; CBB - Coomassie Brilliant Blue; m.m. - molecular mass; SDS - sodium dodecylsulphate; PAGE - polyacrylamide gel electrophoresis.

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## Introduction

Under natural conditions many strains of *Agrobacterium tumefaciens* induce crown gall disease in wounded stems of most dicotyledonous plants. Chilton *et al.* (1977) found that this disease is caused by the incorporation of plasmid part DNA (T-DNA, transferred DNA) into the plant genome. The common 7.5 kb part of T-DNA pTi C58 plasmid contains genes *iaaM* (coding tryptophan-2-monooxygenase) , *iaaH* (coding indolylacetamide hydrolase), *ila* (coding indolyl-3-lactate synthase) and *ipt* (coding isopentenyl-transferase) for the synthesis of growth regulators (Willmitzer *et al.* 1983). Their expression in the transformed cells can modify several pathways of biosynthesis. The incorporation of *iaaM* and *iaaH* genes into plant genome complemented original indolylpyruvate pathway of IAA biosynthesis by indolylacetamide pathway (Rovenská *et al.* 1988, Kutáček 1991). Isopentenyl-transferase as a product of *ipt* gene influenced the first step of the conversion of 5′-AMP-isopentenylpyrophosphate into zeatin and zeatin riboside in the transformed cells (Morris and Powell 1986, Motyka and Kamínek 1989, Schmülling *et al.* 1993). The expression of main morphoregulatory genes in various combination changed both qualitative and quantitative content of soluble proteins, which influenced the phenotype of transformed plants (Tepfer 1984, Beinsberger *et al.* 1991, Estruch *et al.* 1991, 1993, Martin-Tanquy *et al.* 1991, Hlinková and Ondřej 1994). The higher level of auxins in tobacco plants, transformed with unmodified *iaaM* gene, extends the apical dominance and leaf epinasty (Romano *et al.* 1993). On the other hand, transformants expressing *ipt* gene with unmodified promotor, have a typical dwarfed growth and slow leaf senescence (Van Onckelen, personal communication). Plants have reduced sensitivity to auxin and their inhibitors.

The aim of our work was to find some phenotype and molecular characteristics of the tobacco plants transformed with the two strains of *Agrobacterium tumefaciens* (wild strain C58 and strain LBA 4404 carrying binary vector plasmid pCB 1334/pCB 1349, respectively) in the $T_0$ generation.

## Materials and methods

**Chemicals used:** NAA, BAP, TRIS, SDS, amonium persulphate, kanamycin, Coomassie Brilliant Blue R-250, G-250 and low melt agarose were purchased from *Serva* (Heidelberg, Germany); TEMED, acrylamide, bisacrylamide and low molecular mass protein standard were from *LKB Pharmacia* (Uppsala, Sweden). The nylon membrane for DNA blot was obtained from *Schleicher* and *Schuell* (Dassel, Germany). The probe was labeled with [$\alpha$-$^{32}$P]dCTP from *Amersham* (Little Chalfont, UK). Ticarcillin was from *Beacham* (Singapore, UK). Other chemicals of p.a. purity were from *Lachema* (Brno, Czech Republic).

**Bacterial strains and plasmids:** For co-cultivation of tobacco leaf discs cv. Virginia 57 we used two strains, C 58 and LBA 4404 of *Agrobacterium tumefaciens.* The wild strain C58 carried big ~ 220 kb Ti-plasmid, T-DNA of which contains 13 genes including the morphoregulatory genes *iaaM* (~ 2.41kb), *iaaH* (~ 1.65kb), *ila* (~ 1.05kb) and *ipt* (~ 1.3kb) (Depicker *et al.* 1983, Willmitzer *et al.* 1983). Strain LBA 4404 carried helper plasmid pAL 4404 with completely eliminated T-DNA region (Hoekema *et al.* 1983) and small vector plasmid pCB 1949 (22.3 kb) containing in T-DNA region gene marker for kanamycin resistance (*nptII*) and genes: *iaaM*, *iaaH* and *ila*. Alternative plasmid pCB 1334 (19.3 kb) contained in the T-DNA region *ipt* and *nptII* genes (Ondřej *et al.* 1991, Vlasák and Ondřej 1992). The bacterial strain C58 was cultivated at the temperature 28 °C on a complete LK medium (Longley and Kado 1972). *Agrobacterium tumefaciens* strains carrying binary vector plasmids were cultivated with a complement of 5 mg dm$^{-3}$ tetracycline in LK medium.

**Plants:** *Nicotiana tabacum* L. cv. Virginia 57 leaf discs with removed lower epidermis were cultivated in bacterial suspension with absorbance ~ 0.8 for 30 h at the temperature 28 °C according to Horsch *et al.* (1985). The affected leaf discs were then cultivated on the MS medium (Murashige and Skoog 1962) containing 0.1 mg dm$^{-3}$ of NAA, 1 mg dm$^{-3}$ of BAP and 500 mg dm$^{-3}$ of ticarcillin. Media for selection of transformants modified with *A. tumefaciens* carrying binary vector plasmids contained 100, 150 and 200 mg dm$^{-3}$ of kanamycin consecutively. Leaf discs and transformants were grown at temperature of 24 ± 1 °C and irradiance of 200 W m$^{-2}$ (16-h photoperiod). Antibiotics were eliminated after 4 subcultivation. One group of transformants was prepared for *in vivo* cultivation and the another group was used for analyses. To reduce stress factors during the transfer to the soil, regenerants and transformants were cultivated in sterile *Mavicell* (*Magyar Viscosagyar*, Nyugesújfalu, Hungary) saturated with the liquid MS medium containing the half concentration of macroelements for at least 14 d.

**Nopalinsynthetase assay:** The nopalinsynthetase was detected in the extracts from the leaves of one month old transformants carrying T-DNA pTi C58 according to Otten and Schilperoort (1978).

**Protein isolation and quantification:** Proteins were extracted to 0.1 M Na-phosphate buffer (pH 7) as described previously Hlinková and Ondřej (1994). Concentration of soluble proteins from the leaf extracts was determined according to Bradford (1976). Samples for 1-SDS-PAGE were prepared according to Laemmli (1970). SDS-PAGE of denaturated proteins was done in 12.5 and gradient 10/20 % discontinuous gel system according to Smith (1988). Staining of the gel slabs was carried out by standard method with CBB R-250. Molecular mass of proteins and polypeptides was determined from calibration curve for the low molecular mass protein kit of *LKB Pharmacia* (Uppsala, Sweden). Densitometrical scanning was done on the *Beckman DU-65 spectrophotometer* (*Beckman Instruments*, Fullerton, USA) for gradient gel and *Ultra Scan XL* (*Labsystems*, Helsinki, Finland) was used for nongradient 12.5 % slab gel.

**Chlorophyll isolation and quantification:** Chlorophyll *a/b* isolation was performed according to Hlinková and Ondřej (1996). Determination of chlorophyll *a/b* content was done as described by Vernon (1960).

**Auxin bioassay:** Second leaves from the control plants, regenerants, and transformants cultivated four months *in vitro* were cut off in sterile conditions and incubated in sterile double distilled $H_2O$ in Petri dishes in the same conditions as plants during 16 d. The colour of the leaves and root forming from the leaf peduncle was observed.

**Plasmid and plant DNA isolation:** Low molecular DNA from the *A. tumefaciens* strains was isolated according to Maniatis *et al.* (1982). The digestion of plasmid DNA pCB1334 and 1349 was done with the restriction enzymes *PstI* and *Hind III* (*Strata Gene*, Heidelberg, Germany) (Fig. 1). Isolation and separation of total plasmid DNA was made by "bloc" method using pulsed field electrophoresis according to Smith *et al.* (1988). Pulsed field electrophoresis was done in 0.7 % agarose and TBE buffer at 95 V with the 11-s pulse on *Pulsaphor* (*LKB Pharmacia*, Uppsala, Sweden). Total plant DNA was isolated from the leaves of transgenic and nontransformed tobacco plants according to Lin and Whitter (1994). Plant DNA was blotted by a semidry method to the nylon membrane and hybridised with radiolabelled probe for the chimeric *nptII* gene (1.2 kb) according to *Amersham* protocol. Membrane was exposed at -70 °C for 2 d using *Kodak XR-5* X-ray film. Experiments and analyses were repeated 3 times.

## Results

Depending on the leaf position from the apex, the location of the leaf disc on the leaf blade, and *A. tumefaciens* strains used, we obtained 0 - 5 shoots per leaf disc after one month cultivation on the MS medium with kanamycin (Fig. 2). After 3 passages 10 regenerants, 7 transformants positive to nopalinsynthetase, 6 transformants carrying *nptII* and *ipt* gene and 4 transformants carrying *nptII* and some of auxin genes were selected from 158 shoots. One half of regenerants and transformants was used for cultivation *ex vitro*, the other half was used for analyses. Teratomas produced by transformation with T-DNA of pTiC58 and pCB1349 plasmids were ommited from the analyses.

Tobacco clones 1334/1-4 carrying *ipt* gene for overproduction of cytokinin had reduced leaf blade area and leaf dry mass when compared to control plants (Table 1). The leaves were wrinkled. The lenght-to-width ratio oscilated around 1 (Table 1). Plants were dwarfed and no roots were differentiated (Fig. 3*b*). Stems were thicker and formed compact yelow calli in the contact with the medium. Chlorophyll *a* and *b* content in the leaves was significantly lower, especially chlorophyll *a* content, in comparison to the control value (Table 1). Protein analyses showed both quantitative and qualitative changes in the protein patterns (Table 2, Fig. 4*a*, *lane 5*). Three new proteins with molecular mass ~ 60, 36 and 27 kDa were observed. Quantitative

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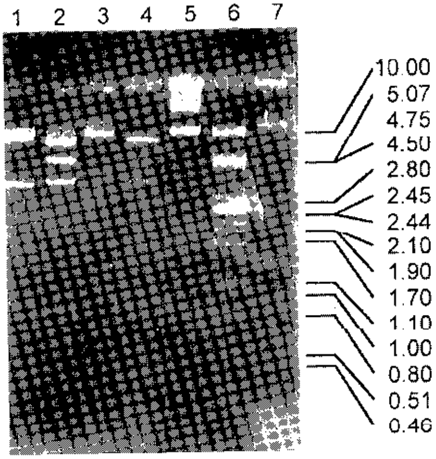

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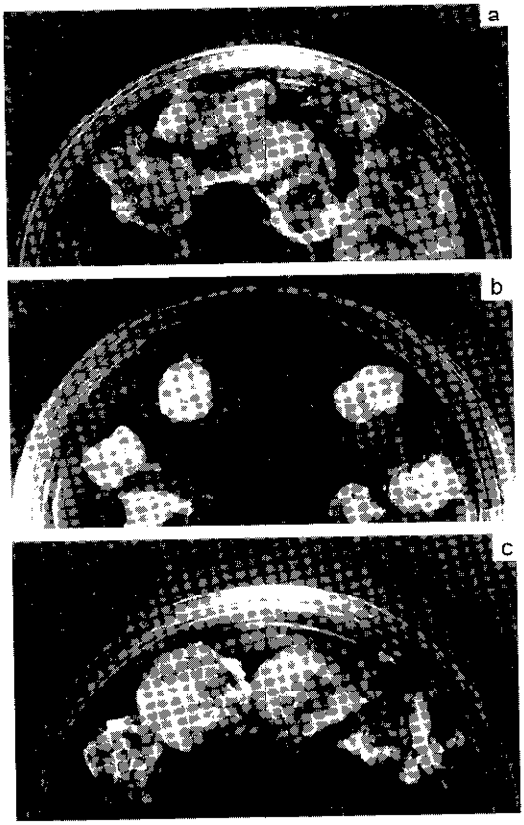

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Fig. 1. Profile plasmid DNA digested with various restriction enzymes. *1* - pCB1334 plasmid DNA digested with *Hind III; 2* - pCB1334 plasmid DNA digested with *Pst I; 3* - pCB1349 plasmid DNA digested with *Hind III; 4* - pCB1349 plasmid DNA digested with *Pst I; 5* - pCB1334 plasmid DNA; *6* - λ-phage DNA digested with *Pst I* - nucleic acid molecular markers (kb: 10.0, 5.077, 4.749, 4.5, 2.838, 2.459, 2.443, 2.140, 1.986, 1.7, 1.159, 1.093, 0.803, 0.594); *7* - pCB1349 plasmid DNA.

Fig. 2. Tobacco leaf disc segment treated with various *Agrobacterium tumefaciens* strains after one month cultivation on the MS medium with 100 mg dm$^{-3}$ of kanamycin (*a* - strain C58, *b* - strain LBA 4404/pCB1349, *c* - strain LBA 4404/pCB1334).

Fig. 3. Tobacco transformants expressing genes for overproduction of growth regulators after 4-month cultivation *in vitro* (*a* - plants carrying genes for overexpression of auxins, clone 1349-2; *b* - plants carrying genes for overexpression of cytokinins, clone 1334-2; *arrow* - adventitious roots).

Table 1. Phenotype characteristics of tobacco transformants cultivated *in vitro* (means ± SE; $n = 4$). *- difference significant at $P > 0.05$; **- difference highly significant at $P > 0.01$; f.m. - fresh mass.

| Parameter | Control-1 | Regenerant-1 | Clone C58-1 | Clone 1334-1 | Clone 1349-1 |
|---|---|---|---|---|---|
| Leaf area [$cm^2$] | 5.80± 1.50 | 4.80± 2.30 | 3.90±0.80 | 2.00±0.50 | 2.25±0.60 |
| Dry mass [mg $g^{-1}$(f.m.)] | 128.70±13.15 | 123.60±21.18 | 89.75±9.15 | 68.74±7.19* | 75.10±10.19 |
| length/width | 1.49± 0.01 | 1.49± 0.11 | 1.35±0.22 | 1.07±0.13 | 2.23±0.11 |
| Chl *a* [mg $g^{-1}$(f.m.)] | 0.29± 0.02 | 0.25± 0.02 | 0.20±0.03** | 0.18±0.02** | 0.25±0.01 |
| Chl *b* [mg $g^{-1}$(f.m.)] | 0.16± 0.01 | 0.14± 0.02 | 0.19±0.02 | 0.11±0.02* | 0.19±0.02 |
| Chl *a/b* | 1.816 | 1.776 | 1.083 | 1.629 | 1.364 |

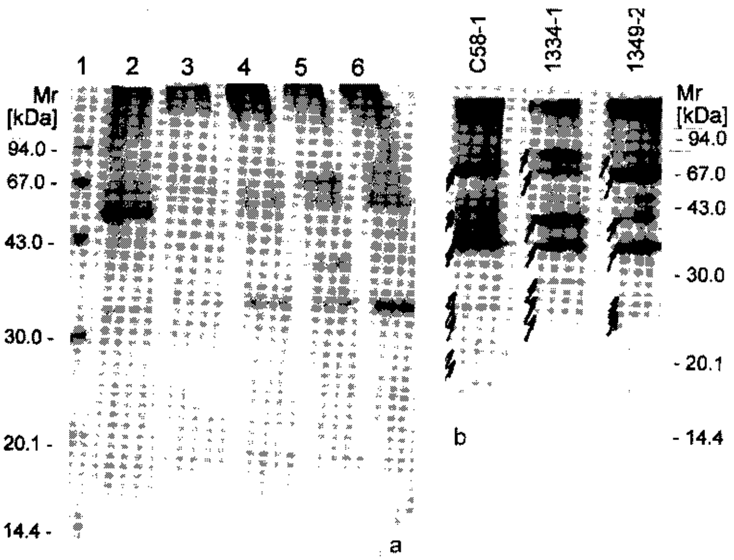

Fig. 4. SDS-PAGE soluble protein patterns of tobacco plants transformed with various strains of *Agrobacterium tumefaciens* (*a* - low voltage electrophoresis on the gradient 10/20 % slab gel: *lane 1* - *LKB* low molecular mass standards, *lane 2* - control plant, *lane 3* - regenerant clone R-1, *lane 4* - transformant clone C58-1, *lane 5* - clone 1334-1, *lane 6* - clone 1349-1. Protein mass separated per lane was 13 ± 0.5 μg; *b* - high voltage electrophoresis on 12.5 % slab gel.

differences of proteins (52, 32.5, 21 and 17 kDa) and polypeptides were detected (Table 2). Leaf senescence was reduced when compared to control leaves (Fig. 5). The transferred plants did not grow *ex vitro*. Transformants carrying genes from T-DNA plasmid pCB1349 had the lowest ability to produce viable plants. The leaves had finer structure and were longer. Length-to-width ratio oscillated around 2. Chlorophyll *a/b* content did not differ from control plants (Table 1). Transformed plants were characterised by longer internodes and thinner stem from which adventitious roots originated (Fig. 3*a*). SDS-PAGE and densitometrical analyses of soluble proteins showed both, quantitative and qualitative differences in the protein content which indicate the changes in the gene expression (Table 2, Fig. 4*a*, *lane 6*, 4*b*). New proteins and polypeptides (74, 49, 26 and 25 kDa) were found. Quantitative changes were detected for ~ 36, 32 and 21 kDa polypeptides. Auxin bioassay test showed that leaves from transformants carrying genes for overproduction of auxin began differentiating roots on the leaf peduncle after 10 d of cultivation in sterile water. Roots were completely differentiated after 16 d. Epinasty was observed in the leaves (Fig. 5). The controls did not differentiated roots, they had yellow colour as a result of senescence, degradation of chloroplast membranes and chlorophylls. Transgenic plants carrying genes for the overproduction of auxins in their genome were growing in soil only 3 months.

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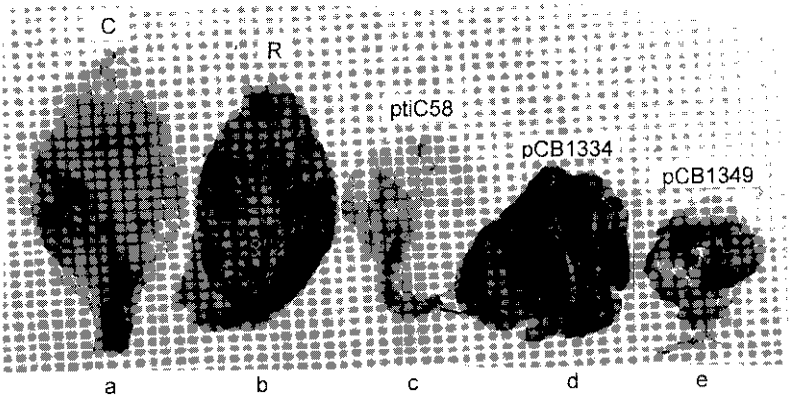

Fig. 5. Response of the tobacco leaves detached from plants transformed with various *Agrobacterium tumefaciens* strains to 16-d of incubation in sterile water (*a* - control leaf, *b* - regenerant clone R-1, *c* - clone C58-1, *d* - clone 1334-1, *e* - clone 1349-1).

The highest variability in the phenotype was acquired by transformants carrying genes from T-DNA region of pTiC58 plasmid. Clones C58/1-7, positive to the

Table 2. Percentage of soluble proteins of different molecular mass in the protein patterns of transformed tobacco plants cultivated *in vitro*.

| [kDa] | Clone C-1 | Clone C58-1 | Clone 1334-2 | Clone 1349-1 |
|---|---|---|---|---|
| 74 | - | 0.1 | - | 0.5 |
| 60 | 4.5 | 1.5 | 7.5 | 0.7 |
| 50-49 | 3.7 | 5.1 | 4.1 | 13.5 |
| 40 | - | 0.8 | - | - |
| 36 | 0.9 | 1.1 | 6.1 | 4.6 |
| 33-32 | 5.2 | 10.1 | 8.6 | 20.0 |
| 27 | 0.2 | 2.1 | 4.6 | 2.1 |
| 26 | 1.7 | 3.1 | 2.0 | 3.8 |
| 25 | 1.2 | 2.0 | 2.0 | 2.9 |
| 23 | 0.5 | 1.9 | 2.1 | 2.1 |
| 21 | 0.1 | 2.9 | 2.1 | 1.1 |
| 18-17 | 1.5 | 2.5 | 1.8 | 1.5 |

nopalinsynthetase (four identical 40 kDa subunit), showed reduced leaf blade area and shorter internodes compared to control plants. Leaves were smaller and thicker. Their Chl $a+b$ content was lower compared to control plants (Table 1). The pattern of soluble proteins contained new polypeptides (50, 36, 33, 26 and 25 kDa) (Fig. 4*a*, *lane 4*). Auxin bioassay test showed that leaves of transformants started to differentiate roots after 12 d. The leaf senescence and Chl degradation was slower as in the control leaf (Fig. 5). From 4 transformants transferred into *ex vitro* conditions, three plants flowered. Plants were smaller and they had significantly lower leaf dry mass and Chl $a+b$ content (Table 3). Flowers, compared with control plants, had darker colour and their size was reduced more than 38 %. Floral organs were unordered and two had sterile flowers. One plant with fertile flowers (clone C58-6) did not complete its ontogeny after the first flowering but during the next 5 months it produced floral buds and flowering occurred again. Transformants expressing genes from T-DNA of plasmid pTiC58 represented wide variability among obtained plants. This variability reflected genetical as well as epigenetical factors of transformation. DNA dot-blot analyses for tobacco plants clone 1334-1 and clone 1349-1 showed positive signals in hybridization with *nptII* gene and confirmed so that the gene for kanamycin resistance was incorporated. DNA from the leaf of control plant as well as the transformant clone C58-1 had negative signal (Fig. 6). Regenerants differed from control plants in both *in vitro* and *in vivo* conditions (Table 1, 2, 3). Differences were found in leaf blade area, dry mass and content of

Table 3. Phenotype characteristics of tobacco clones cultivated *in vivo* (means ± SE; $n = 4$). *- difference significant at $P > 0.05$; **- difference high significant at $P > 0.01$.

| Parameter | Control | Regenerant-3 | C58-3 |
|---|---|---|---|
| Height [mm] | 930 ±15.1 | 910 ± 35.7 | 523 ±25.8** |
| Leaf area [cm$^2$] | 73 ± 1.5 | 92 ± 2.0 | 45 ± 3.5* |
| Length/width ratio | 1.39 | 1.48 | 1.56 |
| Leaf dry mass [mg g$^{-1}$(f.m.)] | 153.80±14.13 | 131.68±18.75 | 98.17± 4.28* |
| Soluble proteins [mg g$^{-1}$(f.m.)] | 5.82± 0.037 | 10.15± 0.08* | 5.37± 0.16 |
| Chl *a* [mg g$^{-1}$(f.m.)] | 0.31± 0.01 | 0.27± 0.01 | 0.17± 0.02** |
| Chl *b* [mg g$^{-1}$(f.m.)] | 0.25± 0.01 | 0.26± 0.02 | 0.19± 0.01* |
| Chl *a/b* | 1.198 | 1.022 | 0.861 |

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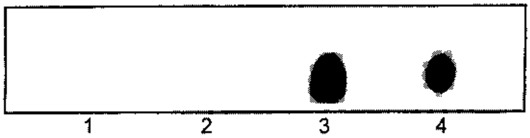

Fig. 6. Dot-blot of plant DNA of transgenic tobacco plants modified with T-DNA of *Agrobacterium tumefaciens* strains and hybridized with the probe for the *nptII* gene. DNA (10 µg) was isolated from the tobacco leaves and bloted on the membrane hybridized for the *nptII* gene (*1* - control plant, and *2* - clone C58-1, *3* - clone 1334-1, *4* - clone 1349-1).

soluble proteins in compared with control characteristics. Quantitative differences were found in polypeptides with m.m. ~ 60, 50, 46 and 33 kDa (Fig. 4*a*, *lane 3*). Plants growing *in vivo* conditions had in the period of flowering floral-head ordered. Flowers, compared to control, were lighter and floral-tubes had conspicuous light-green nervature. Leaf senescence was delayed. Phenotypical differences between regenerants showed typical somaclonal variability. Mature capsules from fertile tobacco plants (clone R-4 and clone C58-5) differed in their mass, colour and form of sepala leaves comparing to the control capsules.

## Discussion

Obtained results showed that variability in phenotype of transformants depended not only on the length of incorporated region of T-DNA and on the expression of new incorporated genes but also on the efficient targeting of the T-DNA to the plant cell nucleus. The efficiency of incorporation is dependent on the nuclear localisation of signal in the C-terminal part of Vir D2 protein (site-specific endonuclease) and preserved function of Vir E2 protein of *A. tumefaciens* (Rossi *et al.* 1996). Matzke and Matzke (1990) found that the incorporation of new genes did not occur in the same place. The expression of the new genes incorporated into the plant genome is influenced by both genetic and epigenetic factors. In case of *ipt* gene, its expression in the transgenic tobacco plants is regulated by the concentration of saccharose in the medium and the duration of cultivation. Changes in the protein patterns of transformants expressing *ipt* gene (Fig. 4*a*, *lane 5*) or their combination with *6b* gene (plasmid pCB 3029), *ila* and *6b* gene (plasmid pCB 3016) (Hlinková and Ondřej 1994) showed several quantitative and qualitative changes for polypeptides with M.m. ~ 60, 55, 48, 27, 26, 21 and 17 kDa, which affected the plant phenotype. Klee *et al.* (1987) proved that the overproduction of cytokinin inhibited the root formation in *Petunia* plants. Their results were complemented by Ainly *et al.* (1993) who found that the high content of cytokinin in the transgenic plants was associated with delayed leaf senescence, reduced leaf blade area and thicker stem. Our results correspond well with the results of the above mentioned authors. In the case that *ipt* gene was under the transcriptional control of hsp 70 promotor from *Drosophila melanogaster*, plants rooted and showed no differences in phenotype compared to control plants (Ainly *et al.* 1993). Estruch *et al.* (1993) incorporated *ipt* gene under transcriptional control of CaMV35 promotor with *Ac* transposon insert into tobacco genome. Transgenic tobacco plants were characterised with somatic mosaics, viviparous leaves and epiphyllous buds. The problem of influence of *ipt* gene on the content of Chl *a*+*b* content in the leaves is not completely clear. Results, obtained for transgenic tobacco (cv. White burley) and potato (cv. Oreb), were not statistically significant (Šesták *et al.* 1989, Šiffel *et al.* 1988, 1992). *In vitro* cultivated tobacco clones (cv. Samsun) carrying unmodified *ipt* gene significantly differed in the Chl content compared to control plants (Hlinková and Ondřej 1996). The decrease of Chl *a* content was observed in legumes transformed by the same plasmid (Hlinková 1994). Fladung *et al.* (1993) described the decrease of the Chl content in the leaves of transgenic potato transformed with the *rolC* gene. This gene controls cytokinin-N-glucosidase synthesis and has very similar effects as *ipt* gene (Estruch *et al.* 1991). Low gain of viable transformants carrying genes *iaaM* (74 kDa subunit) and *iaaH* (49 kDa subunit) for auxin overproduction was obtained also by Rüdelsheim *et al.* (1987). These authors showed that the IAA content increased 10-times compared to control plants as a result of the expression of both genes in the transformed tobacco cells. Romano *et al.* (1993) found changes in the content of IAA as well as in ethylene production in transformated tobacco and *Arabidopsis thaliana* carrying *iaaM* gene. Morphogenetic changes in *Petunia*, growth of adventitious roots and changes in apical dominance induced by overproduction of auxin was reported by Klee *et al.* (1987). It seems that the influence of genes from T-DNA pTiC58 for overproduction of growth regulators with unmodified promotor, have very similar effect in *Solanaceae.* Phenotype differences reflected changes in the level of gene expression. Baker *et al.* (1983) identified 74, 49 and 27 kDa proteins as the products of genes *iaaM, iaaH* and *ipt.* Körber *et al.* (1991) identified the 26 kDa protein for the *ila* gene. The expression of *ila* gene correlated with the content of 46 and 36 kDa proteins. Polypeptides with this molecular mass were detected in the protein patterns of the transformants expressing adequate genes (Table 3, Fig. 4*a*, *lanes* 5 and 6; Fig. 4*b*). Quantitative differences in the content of proteins with m.m. ~ 60, 50, 32, 25, 21, and 17 kDa could reflect changes induced by the indolylacetamide pathway in the transformed plants. We suppose that the increase of amount of 50 kDa protein is due to calmodulin and by 32 kDa β-1,3-glucanase. However, this assumption can be verified only by 2D-SDS-PAGE and immunoblot.

The light-harvesting Chl complex (LHC I) could participate in the portion of proteins with M.m. ~ 20.5 - 25 kDa (Ikeuchi *et al.* 1991, Glaser and Melis 1987). Mature 25 kDa protein (233 amino acids) was assigned for LHC I complex of transformed tobacco plants by Nap *et al.* (1993). In the protein patterns (Fig. 4*a*, *lanes 4, 5, 6*; Fig. 4*b*) we can see that transformants expressing genes from T-DNA plasmids pTiC58, pCB1334 and pCB1349 differ in the content of these proteins and polypeptides (m.m. ~ 25, 21.5 and 17 kDa). Patterns indicated that quantitative differences of proteins with this molecular mass might be caused by the LHC I complex. The results of Parthier *et al.* (1985) with pumpkin suggest that the content of cytokinins controlled the gene expression of ribulose-1,5-biphosphate carboxylase. We observed a disproportion between the content of polypeptides synthesized for large and small subunits (~ 52 and 15 kDa) of this enzyme in the transformed plants (Fig. 4). This problem is intensively studied.

We can conclude that changes in the phenotype of tobacco plants carrying genes for overproduction of growth regulators depended on the level of expression of newly introduced genes, their type, the place of incorporation and gene dose. The influence of somaclonal variability on the phenotype of plants in the $T_0$-generation should also be considered.

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