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Influence of *Agrobacterium tumefaciens ipt* and *Agrobacterium rhizogenes rolC* Genes on Spontaneous Tumor Formation and Endogenous Cytokinins Content in Radish (*Raphanus sativus*) Inbred Lines

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ABSTRACT

Several inbred lines from a radish (*Raphanus sativus* var. *Radicula* Pers.) genetic collection spontaneously form tumors on the crop roots during flowering. Here we studied the influence of the *Agrobacterium tumefaciens ipt* gene, which controls cytokinin biosynthesis, and the *Agrobacterium rhizogenes rolC* gene, which influences cytokinin metabolism, on tumor formation in the radish lines. Two *in planta* transformation methods – transformation of ovaries via pollen-tube pathway and transformation of seedling apexes – were firstly used to obtain transgenic radish plants. Both methods showed high efficiency for radish. Transformation of radish lines by both *ipt* and *rolC* genes induced tumor formation in non-tumorous lines. We noted a several-fold increase of free zeatin concentration in *ipt*-transgenic radish plants while *rolC*-transgenic radish plants showed an increase in zeatin riboside concentration comparing with the untransformed line. We suppose that the tumorigenic effect of *ipt* and *rolC* genes on radish lines is likely to be due to change of phytohormonal balance in the tissues of transgenic plants.

Keywords: *in planta* transformation, *ipt*, plant tumor formation, radish (*Raphanus sativus* var. Radicula Pers.)
Abbreviations: CK, cytokinins; **ELISA**, enzyme-linked immuno sorbant assay; **IAA**, indole-3-acetic Acid; **PCR**, polymerase chain reaction; **RT-PCR**, reverce transcription - polymerase chain reaction; **Z**, zeatin; **ZR**, zeatin riboside

INTRODUCTION

Tumor formation is a phenomenon caused by disturbance in the balance of cells proliferation and differentiation. Therefore, tumorigenesis is a suitable model to study the mechanisms involved in the control of coordinated cell division. In higher plants, tumorigenesis may be caused by several pathogens (pathogen-induced tumors) or develops spontaneously in the plant of specific genotype (spontaneous tumors) (for review see: Dodueva *et al.* 2007). Among plant pathogens which induce tumor formation, two species of *Agrobacterium* genus are best known. *Agrobacterium tumefaciens* causes formation of rapidly growing undifferentiated tumor (the so called crown gall) mainly on the base of stems of infected plants and *Agrobacterium rhizogenes* induces development of numerous adventitious roots at the infection site (the so called hairy root disease). Expression of certain *Agrobacterium* T-DNA genes called oncogenes cause the local shift of phytohormonal balance in host plant tissues which leads to an increased rate of cell division and development of hyperplasia. Products of several *A. tumefaciens* oncogenes were shown to catalyze cytokinins or IAA biosynthesis (Barry *et al.* 1984; Kemper *et al.* 1985); the expression of *A. rhizogenes* genes does not cause cytokinins or IAA overproduction, but seems to increase the sensitivity of plant tissues to cytokinins and auxins (Schmülling *et al.* 1988). The most known example of spontaneous tumorigenesis in higher plants is tumor formation in *Nicotiana* interspecific hybrids (Ahuja 1998). Tumors which develop in certain tobacco hybrids resemble crown gall tumors induced by *A. tumefaciens* in their appearance and anatomy. Moreover, common mechanisms were shown for spontaneous tumorigenesis in tobacco hybrids and *Agrobacterium*-induced tumorigenesis. Increased cytokinin concentration (Nandi *et al.* 1990) and increased tissue sensitivity to this hormone (Qu *et al.* 2006) are typical characters of tumor-producing tobacco hybrids. Genes homologous to *A. rhizogenes* T-DNA oncogenes were found in the genomes of several *Nicotiana* species (Intrieri and Buiatti 2001). Rapid increase of "agrobacterial" genes' expression levels after the induction of spontaneous tumors in *Nicotiana* hybrids testify the participation of these genes in the control of tumorigenesis (Aoki and Syono 1999).

One suitable model for investigation of plant tumorigenesis is spontaneous tumor formation in radish inbred lines. A genetic collection of radish (*Raphanus sativus* var. *Radicula* Pers.) inbred lines was created at the department of Genetics and Breeding of Saint-Petersburg State University, Russia, in 1960s by selfing of the individual plants from three radish cultivars of different origin (Narbut 1967). At present, the collection includes 33 high inbred (34-35th inbred progeny) lines. Some radish lines demonstrate different morphological abnormalities, most interest among them is spontaneous tumor formation (Narbut 1967; Matveeva *et al.* 2004). Tumors (**Fig. 1**) appear on the radish roots and lower part of stem at the beginning of the flowering stage and highly resemble crown gall induced by *A. tumefaciens* and spontaneous tumors on *Nicotiana* interspecific hybrids. We have previously demonstrated that several tumor-producing radish lines have increased levels of cytokinins, mainly zeatin and zeatin riboside in their leaves, stems and roots as compared with related non-tumorous lines (Matveeva *et al.* 2004) and display high sensitivity to cytokinins *in vitro* (Buzovkina *et al.* 1993; Matveeva *et al.* 2004; Ilyina *et al.* 2006). The supply of exogenous cytokinins *in vivo* or *in vitro* can also induce tumor formation in several non-

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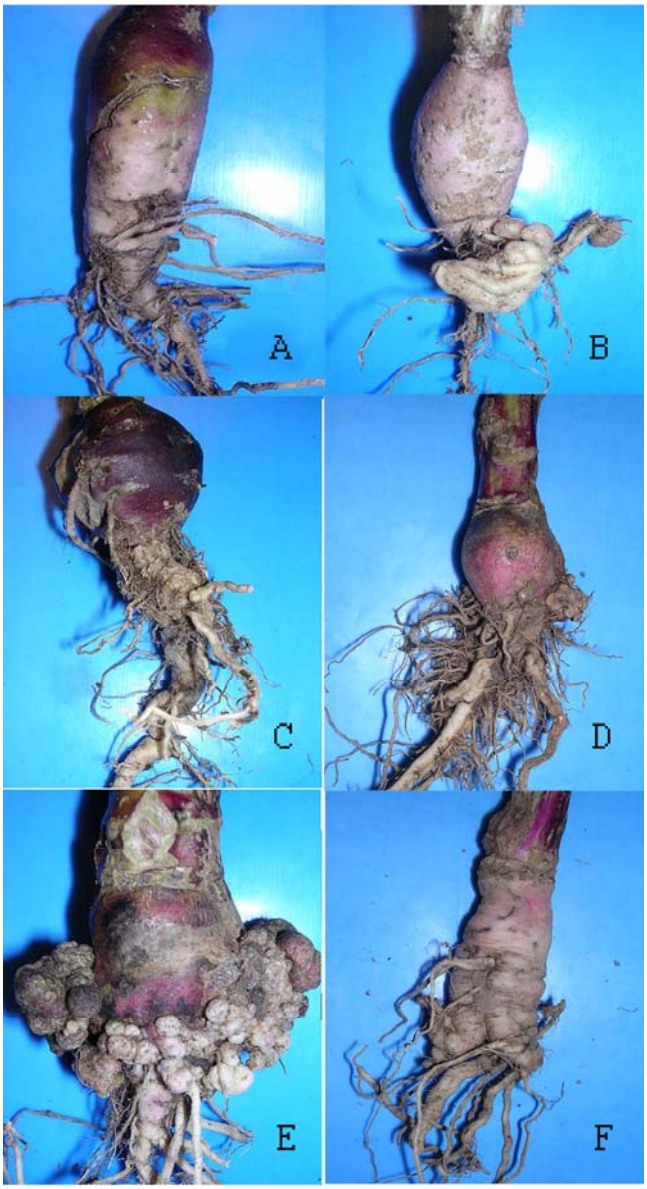

Fig. 1 Tumors on the crop-roots of radish lines and T_1 transgenic plants. (**A**) Crop-root of non-tumorous line 3; (**B**) tumor on the crop-root of T_1 plant of line 3 transformed by *rolC* gene; (**C**) crop-root of non-tumorous line 30; (**D**) tumor on the crop-root of T_1 plant of line 30 transformed by *ipt* gene; (**E**) numerous tumors on the crop-root of tumorous line 19; (**F**) absence of tumors on the crop-root of T_1 plant of line 19 transformed by *ipt* gene.

tumorous radish lines (Buzovkina *et al.* 1993; Ilyina *et al.* 2006). These data suggest an increase in the level of cytokinins in the tissues to be the main cause of tumor formation in radish inbred lines.

In the present work, we investigated the influence of single T-DNA genes of *A. tumefaciens* and *A. rhizogenes* on spontaneous tumorigenesis in radish inbred lines. Transformation of different plants species by single T-DNA genes causes many morphogenetic abnormalities and shifts in phytohormonal balance but never leads to tumor formation (Von Schwartzenberg *et al.* 1994; Nilsson *et al.* 1996; Schmülling *et al.* 1988; Casanova *et al.* 2004; Grishunina *et al.* 2005; Khodakovskaya *et al.* 2005). On the other hand, *Nicotiana* homologues of *A. rhizogenes* T-DNA genes seem to play a role in the development of spontaneous tumors in tobacco interspecific hybrids (Aoki and Syono 1999). So, we expected that transformation of radish inbred lines by *Agrobacterium* T-DNA genes might influence the ability to tumor formation and endogenous phytohormone content. This work is the first attempt to investigate the action of *Agrobacterium* T-DNA genes on the background of spontaneous tumorigenesis.

In our experiments we used two *Agrobacterium* strains carrying the *ipt* gene of *A. tumefaciens* and the *rolC* gene of *A. rhizogenes* under a constitutive 35S CaMV promoter. The *ipt* gene encodes isopentenyl transferase (*IPT*), a key enzyme of cytokinin biosynthesis (Barry *et al.* 1984). The unknown function of the *rolC* gene is probably connected with the regulation of β-glucosidases that hydrolyse the inactive cytokinin *N*-glucosides: the product of the *rolC* gene has β-glucosidase activity against cytokinin glucosides *in vitro* (Estruch *et al.* 1991) but seems to be unable to hydrolyse them *in planta* (Faiss *et al.* 1996). We tried to find out if the function of both genes under investigation would increase the amounts of active cytokinins in radish plants.

Raphanus sativus is a plant species that cannot be transformed by any *in vitro* method due to very low frequency of plant regeneration from embryogenic calli (Jeong *et al.* 1995). Thus, only *in planta* transformation methods allow to create transgenic radish plants. The essence of *in planta* methods is the transformation of generative meristem on an intact plant leading to the obtaining of transgenic seeds. High effectiveness of several *in planta* transformation methods was demonstrated for several plant species (Trieu *et al.* 2000; Kojima *et al.* 2000, 2004; Supartana *et al.* 2005; Kojima *et al.* 2006). The first successful method of radish *in planta* transformation was the floral-dip method which consists in the inoculation of inflorescence by liquid *Agrobacterium* culture supplied with surfactants – substances which increase *Agrobacterium* adhesion on plant cells. Transgenic Korean radish (*Raphanus sativus* var. *longipinnatus* Bailey) plants with delayed flowering caused by the antisense-suppression of *GIGANTEA* photoperiodic gene were created by floral-dip method. However, transformation efficiency of floral-dip for radish did not exceed 1.4% (Curtis and Nam 2001; Curtis *et al.* 2002). The second *in planta* transformation method which was successful for radish was vacuum infiltration of *Agrobacterium* liquid culture into sonificated radish seeds (Park *et al.* 2005). Transgenic radish (*Raphanus sativus* var. *radicula*, cultivar Kosena) plants carrying the *LEA* gene encoding the osmoprotector protein were obtained by this method and the percentage of transgenic plants in T_1 progeny reached 2-4% (Park *et al.* 2005).

Here we examine two *in planta* transformation methods which have never been used for radish. The first method is ovary transformation via the pollen-tube pathway which represents a modification of the floral-dip method and consists of the inoculation of *Agrobacterium* culture into immature flower pistils (Chen *et al.* 1998). Another method was the inoculation of *Agrobacterium* culture into seedling apexes. In experiments with different plant species very high transformation efficiency (27.6-70%) was demonstrated (Trieu *et al.* 2000; Kojima *et al.* 2000, 2004; Supartana *et al.* 2005; Kojima *et al.* 2006).

MATERIALS AND METHODS

Plant material and *Agrobacterium* strains

Experiments were performed with highly inbred lines from radish (*Raphanus sativus* var. *Radicula* Pers.) genetic collection (Narbut 1967). In this article we show the results obtained for non-tumorous radish lines 3 and 30 and tumorous line 19. We used two *Agrobacterium* strains carrying the *ipt* gene from *A. tumefaciens* or the *rolC* gene from *A. rhizogenes* genes under the control of the *Cauliflower mosaic virus* (CaMV) 35S constitutive promoter and the neomycin phosphotransferase gene *nptII* driven by a nopaline-synthase (*nos*) promoter. For control transformation we used an *Agrobacterium* strain carrying only the *nptII* gene (**Table 1**). *Agrobacterium* strains were kindly provided by Professor T. Schmülling (Institute of Biology, Applied Genetics, Freie Universitat Berlin, Berlin, Germany) and Professor M. Ondřej (Institute of Plant Molecular Biology, Czech Republic).

Table 1 Vectors for radish *in planta* transformation.

Vector	Selective marker gene	*Agrobacterium* T-DNA gene(s)	Source
pGV3850	*pnos-nptII*	*p35S-ipt*	Zambryski *et al.* 1982
pCV002	*pnos-nptII*	*p35S-rolC*	Schmulling *et al*. 1988
pCB1346	*pnos-nptII*	-	Vlasak and Ondřej 1992

Transformation procedures

Agrobacterium strains were cultivated on solid LB medium supplied with carbenicillin (100 mg/l). *Agrobacterium* overnight culture diluted with liquid LB medium (1:10) was used for inoculation. We did not use any surfactants in the radish transformation experiments.

Transformation of ovaries

Flower buds of 4 month-old, actively flowering radish plants under field conditions were used for ovary transformation experiments. All open flowers and slightly immature flower buds were cut from the inflorescence. We opened flower buds and inoculated approximately 5 µl of *Agrobacterium* overnight culture onto the stigma of pistils with a sterile needle. Approximately 500 flowers of each line were inoculated. After inoculation we surrounded inflorescences with isolators made from parchment paper for self-pollination.

Seedling apex transformation

Seeds of radish inbred lines (2-3 seeds per pot, totally 40 seeds of each line) were germinated in pots with soil ("Terra vita Universal", ZAO MNPP «Phart», Russia). Apical meristems of 6 day-old seedlings were pricked by a sterile needle and inoculated with approximately 5 µl of *Agrobacterium* overnight culture ($OD_{600\ nm}$ = 0.5-0.6). When plants reached the flowering stage we placed their inflorescences into isolators made from parchment paper for self-pollination.

Seeds of plants transformed by both methods were harvested. T_0 plants which were transformed by seedling apexes inoculation method yielded numerous seeds (no less than 50 seeds per plant), T_0 plants which were transformed via pollen-tube pathway yielded 5-10 seeds per plant. We sew 10 seeds from each T_0 plant; T_1 transformants were analysed for phenotype, and the presence of T-DNA insertion and expression of genes of interest was assessed. T_2 progeny was obtained by the self-pollination of individual T_1 plants.

DNA extraction and PCR analysis

Total DNA from radish T_1 and T_2 plants and also untransformed lines was extracted by the STAB method (Rogers and Bendich 1985). Plants were analysed for the presence of T-DNA insertion using PCR with specific primers sets for *nptII*, *ipt* and *rolC* genes. We have used PCR of plasmid DNA of *Agrobacterium* strains which was used in our transformation experiments as the positive control and PCR of DNA of untransformed radish lines as the negative control. Primers were designed by PrimerQuest Software (Integrated DNA Technologies) and provided by Syntol (Russia): *nptII*: 5′-GTCGTCTGGTCGGTCATTTCG-3′ (forward), 5′-GTG ATCTCACCTTGCTCCTGCC-3′ (reverse); *ipt*: 5′-TATTCGCCA CAAGTTACCCGACCA-3′ (forward), 5′-CTGCCACAACAGGA CAAAGCCAAA-3′ (reverse); *rolC*: 5′-CCATTAGCCGATTGCA AACTTGCA-3′ (forward), 5′-CATGGCTGAAGACGACCTGTG TTC-3′ (reverse).

To test putatively transgenic plants for the presence of *Agrobacterium* in their tissues we applied PCR with specific primers for the *A. tumefaciens VirD2* gene.

VirD2: 5′-ACGTACGCTAGCATGCCCGATCGCGCTCAA-3′ (forward), 5′-TCCCCCCGGGGGTCCCCCCGCGCCCATCGT-3′ (reverse).

PCR reactions for all four genes consisted of 30 cycles with 30 sec at 95°C (denaturation), 30 sec at 55°C (annealing) and 1 min at 72°C (extension). We have used "Tercik" thermocycler (DNA-technology, Russia). Amplified DNA was analysed by gel electrophoresis on a 1% agarose gel and stained with ethidium bromide (1 mg/l).

RNA extraction and RT-PCR analysis

Total RNA was extracted from the upper leaves of radish lines and transgenic plants by the guanidine thiocyanate method (Suzuki *et al.* 2001). After processing twice with DNAse (20 units per 50 µl reaction mix) cDNA was obtained from equal amounts (5 µg) of each sample RNA using kit "RT-PCR version with oligo-dT-primers" (Sileks-M, Russia). Expression of transferred genes was evaluated by RT-PCR with specific primers for *ipt* and *rolC* genes, as shown above; RT-PCR conditions were the same as for PCR of genomic DNA. For the control, we use the RT-PCR with primers to ubiquitin gene: 5′-ATGCAGAT(C/T)CTTGTGAAGAC-3′ (forward); 5′-ACCACCCCG(G/A)AGACGGAG-3′ (reverse).

Cytokinin analysis

For cytokinin determination by ELISA young upper leaves were collected from T_2 plants from individual progenies obtained from tumorous and non-tumorous T_1 plants and also untransformed lines at the beginning of the flowering stage (about 80 days old). Leaves were immediately frozen in liquid nitrogen, homogenized and extracted by incubation overnight at 4°C in 80% methanol. After filtration through paper filter and vacuum evaporation of extracts under air flow in the laminar box to remove all traces of methanol, cytokinins were dissolved in 80% ethanol, and loaded on to precoated 0.25-mm-thick silicagel 60 F-254 thin-layer plates (Merck, Darmstadt, Germany). The solvents 2-butanol, 14 M NH_3 and distilled water were used (6:1:2, v/v, upper phase). Zones were identified by cytokinin (zeatin and zeatin riboside) standards (Sigma) on each TLC plate. The zones were identified under UV-light (260 nm), removed from the plate, mixed with 500 µl of 0.1 M phosphate buffer (pH 7.4) and sonicated for 30 min. After centrifugation, the supernatant itself and its aliquot diluted 10 times were subjected to ELISA using rabbit polyclonal antibodies raised against zeatin riboside/bovine serum albumin (BSA) conjugates, which were highly specific for *trans*-zeatin and zeatin riboside (Kudoyarova *et al.* 1990). Cross-reactivity of these antibodies was described earlier (Kudoyarova *et al.* 1998). A calibration curve was constructed by optical density of zeatin/zeatin riboside standard dilution. The linear section of calibration curve was from 0.1 to 8 ng/microwell.

Statistics

For determine the transformation efficiency we calculated the percent of PCR-positive plants and the standard errors. For determine the frequency of plants with altered phenotype we calculated the percent of plants with altered phenotype and the standard errors. Segregation of the *npt2* gene in T_2 plants was determined by Chi-square analysis. For cytokinin measurement we choose 3 tumorous and 3 non-tumorous T_2 plants in each variant of transformation (certain line/certain gene). Cytokinin measurement was carried out in triplicate. In each variant, we calculated the arithmetical means and standard errors for tumorous and non-tumorous plants using "Statistica" software.

RESULTS

Transformation efficiency

In planta transformation efficiency was estimated by PCR of DNA from T_1 plants with specific primers for either the selective marker gene *nptII* or genes of interest (*ipt* or *rolC*) (**Fig. 2A-C**). The absence of *Agrobacterium* in the tissues of transgenic plants was established by negative results of PCR with the *VirD2* gene (**Fig. 2D**).

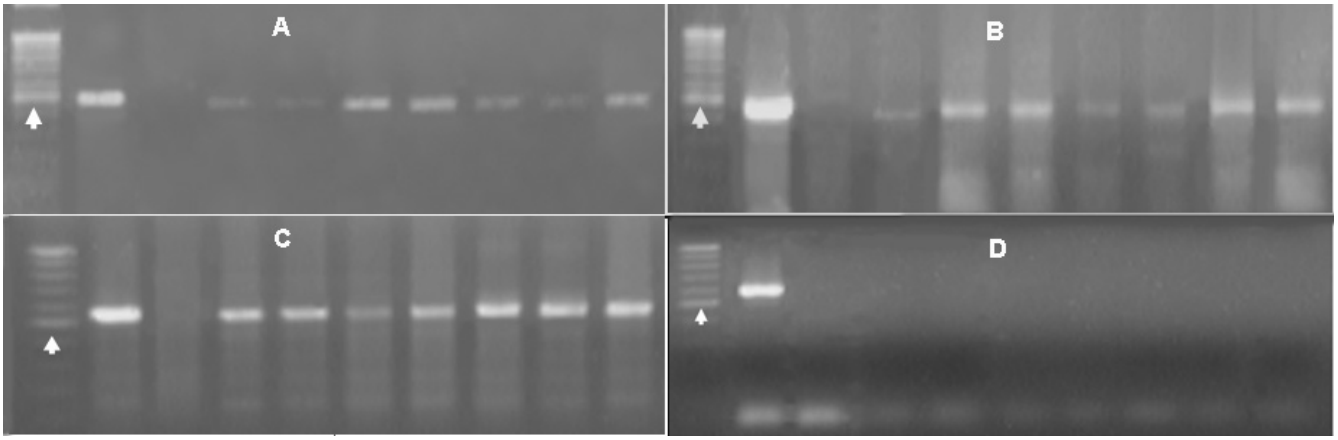

Fig. 2 PCR-analysis of T_1 transgenic radish plants with primers for *nptII* (A), *ipt* (B), *rolC* (C) and *VirD2* (D) genes. (**A**) line 30 transformed with the *ipt* gene, PCR with primers to *npt2*; (**B**) line 19 transformed with the *ipt* gene, PCR with primers to *ipt*; (**C**) line 3 transformed with the *rolC* gene, PCR with primers to *rolC*; (**D**) line 3 transformed with the *rolC* gene, PCR with primers to *VirD2*. Lane 1: 100 bp marker; lane 2: PCR of *Agrobacterium* strain used for transformation; lane 3: PCR of untransformed radish line; lanes 4-10: PCR of T_1 radish plants. Arrow indicates the position of 500 bp marker.

Table 2 Efficiency of *in planta* transformation methods for radish inbred lines.

Radish line	Transformation method	Transformation efficiency
3	Ovary transformation	57.14 ± 13.23
	Apex transformation	83.87 ± 6.60
19	Ovary transformation	32.26 ± 8.39
	Apex transformation	58.33 ± 14.25
30	Ovary transformation	34.29 ± 8.02
	Apex transformation	41.67 ± 14.25

Data represented as means ± standard errors.

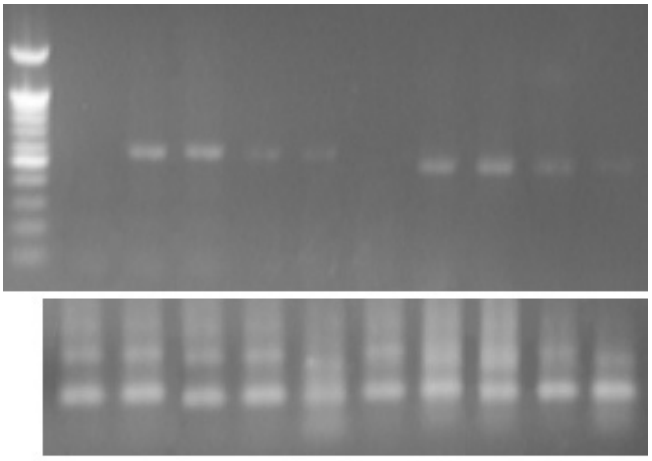

Fig. 3 Expression of *ipt* and *rolC* genes in the upper leaves of transgenic radish plants. Lane 1: 100 bp marker; lanes 2-6: RT-PCR with primers for *rolC*: untransformed line 3 (lane 2), tumorous *rolC*-transgenic T_2 plants (lanes 3-4) and non-tumorous *rolC*-transgenic T_2 plants (lanes 5-6). Lanes 7-11: RT-PCR with primers for *ipt*: untransformed line 30 (lane 7), tumorous *ipt*-transgenic T_2 plants (lanes 8-9) and non-tumorous *ipt*-transgenic T_2 plants (lanes 10-11). Lower gel: RT-PCR of the same RNA probes with primers for the ubiquitin gene.

Among T_1 plants from different lines, 32-57% of plants obtained by ovary transformation and 42-84% of plants obtained by apex transformation amplified bands of expected length: 495 bp for *npt2*, 547 bp for *rolC* and 445 bp for *ipt*, but no T_1 plant amplified 600 bp band with primers to *VirD2* gene (**Table 2**). However, PCR-analysis of segregations in many individual T_2 progenies revealed that both *in planta* transformation methods in half of the cases resulted in two or more T-DNA insertions. So, further work with the aim of improvement of transformation procedures is required. RT-PCR analysis revealed that transferred genes were expressed in the tissues of transgenic radish plants (**Fig. 3**).

Phenotype of transgenic radish plants

Transgenic radish plants carrying only the *nptII* gene did not differ from parent lines. At the same time, a significant part of transgenic plants carrying either *ipt* or *rolC* genes demonstrated certain phenotypic alterations.

An alteration of the ability to form tumors was the most intriguing characteristic of transgenic radish plants carrying the *ipt* or *rolC* genes. Both *ipt*-transgenic and *rolC*-transgenic radish plants from non-tumorous lines acquired the ability to form tumors (**Fig. 1B, 1C**). Transformation of non-tumorous line 30 by the *ipt* gene yielded 66.67% tumor-producing plants; transformation of non-tumorous line 3 by the *rolC* gene yielded 62.5% of tumor-producing transgenic plants (**Table 3**). The ability to form tumors was inherited in the T_2 generation of both *ipt*-transgenic and *rolC*-transgenic radish plants. We observed segregation of the tumor-formation trait in individual T_2 progenies from tumorous T_1 plants. Furthermore, no tumors formed in T_2 from non-tumorous T_1 plants (**Table 3**). Totally, the percentage of tumor-producing T_2 plants reached 68.75% for line 19 transformed with *ipt*, 27.03% for line 30 transformed with *ipt* and 25.00% for line 3 transformed with *rolC*.

At the same time, among *ipt*-transgenic plants from line 19 which normally forms tumors at a high frequency (97-99%) (Narbut *et al.* 1995) tumor formation was observed only in 60% of T_1 plants (**Fig. 1F, Table 3**). In the T_2 generation, segregation of the ability to form tumors was observed only in the progenies from non-tumorous T_1 plants (**Table 3**).

Besides tumor formation, several *ipt*- and *rolC*-transgenic T_1 plants had a wavy and flattened stem (**Fig. 4**). No T_2 plants however, inherited these characteristics.

Numerous *ipt*- and *rolC*-transgenic radish plants from different lines showed altered anthocyanin coloring of crop-roots and petals (**Table 4, Fig. 5**); at the same time, transgenic plants which carry only the *npt2* gene did not differ from the parent lines in their coloring. Interestingly, in our experiments transformation by the *ipt* gene seems to have had a negative influence on anthocyanin accumulation: for example 25.0% of *ipt*-transgenic T_1 plants of line 30 had light-colored or spotty crop-roots instead of dark-red (**Fig. 5B**), and white petals instead of pink (**Fig. 5D**). Amongst *ipt*-transgenic T_1 plants of line 19, 10.00% also had light-colored crop-roots instead of dark-red ones. At the same time, the *rolC* gene seems to have had a positive influence on the accumulation of anthocyanins: for example, 37.5% of T_1 *rolC*-transgenic plants of line 3 had crimson petals instead of light-pink (**Fig. 5E**). We observed segregation of anthocyanin coloring in individual T_2 plants obtained from T_1 plants with altered coloration (**Table 4**). In total, percentage of T_2 plants with altered coloration reached 28.13% for

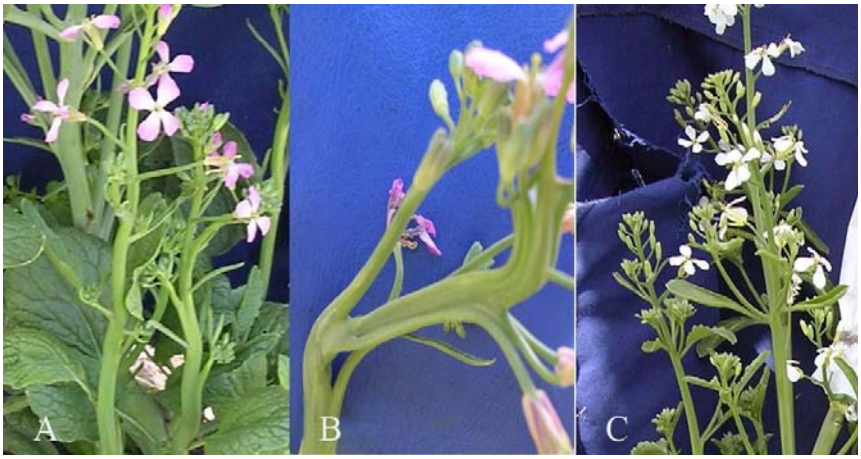

Fig. 4 Altered stem morphology in the transgenic radish plants. (**A, B**) wavy and flattened shoots of T_1 plant of line 3 transformed by the *rolC* gene; (**C**) normal shoots of untransformed line 3.

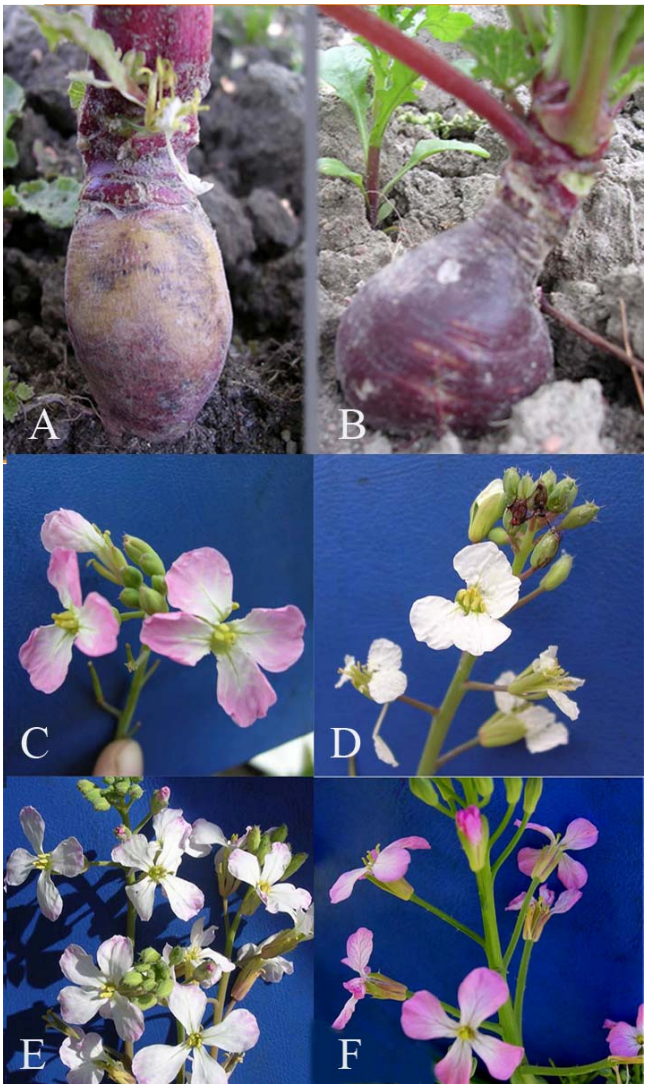

Fig. 5 Altered anthocyanin coloring in transgenic T_1 radish plants. (**A**) Crop-root of untransformed line 30; (**B**) spotty crop-root of T_1 plant of line 30 transformed by *ipt* gene; (**C**) flowers of untransformed line 30; (**D**) flowers with delayed anthocyanin coloring of T_1 plant of line 30 transformed by *ipt* gene; (**E**) flowers of untransformed line 3; (**F**) flowers with enhanced anthocyanin coloring of T_1 plant of line 3 transformed by the *rolC* gene.

line 19 transformed with *ipt*, 18.92% for line 30 transformed with *ipt* and 50.00% for line 3 transformed with *rolC*. Segregations on tumor formation and anthocyanin coloring in the individual T_2 families showed that altered anthocyanin coloring was not linked with tumor formation (**Table 5**).

Cytokinin content in transgenic radish plants

We measured the zeatin (Z) and zeatin riboside (ZR) concentration in young leaves of tumorous and non-tumorous transgenic radish plants and also their parent lines (**Fig. 6**). Transgenic radish plants carrying the *ipt* gene had a higher concentration of Z than their parent lines. Tumorous *ipt*-transgenic plants demonstrated a several-fold higher Z concentration (on average 195.8% of Z level in the parent line for tumorous transgenic plants of line 30, on average 363.6% for tumorous transgenic plants of line 19) while non-tumorous transgenic plants had a practically unchanged Z level (on average 106.1% of the parent level line for non-tumorous transgenic plants of line 30 and 94.8% for non-tumorous transgenic plants of line 19) (**Fig. 6A, 6C**). The concentration of ZR in the leaves of both tumorous and non-tumorous *ipt*-transgenic plants of line 30 remained practically unchanged. Among *ipt*-transgenic plants of line 19 tumorous T_2 plants demonstrated an increase in the level of ZR (on average 112.4% ZR level in the parent line), while the ZR content of non-tumorous T_2 plants of line 19 did not differ from the parent line (**Fig. 6B, 6D**).

All *rolC*-transgenic radish plants demonstrated a several-fold increase in the concentration of ZR (on average 324.7% of parent line's level) and a decrease in the concentration of Z (on average 58.6% of parent line's level) (**Fig. 6E, 6F**). However, Z and ZR concentration was higher in tumorous *rolC*-transgenic plants.

Surprisingly, untransformed tumorous line 19 had a lower level of Z than both non-tumorous lines (on average 76.33% of line's 30 Z level and 48.81% of line's 3 Z level) and also lower level or ZR than non-tumorous line 30 (on average 37.20% of line's 30 ZR level).

DISCUSSION

One of the aims of the present work was to examine two *in planta* transformation methods which have never been used before to create transgenic radish plants. In our experiments both ovary transformation and seedling apex transformation methods were shown to be effective for radish. Our data are in agreement with very high transformation efficiencies that were obtained for several other plant species using the same *in planta* transformation methods (Trieu *et al.* 2000; Kojima *et al.* 2000, 2004; Supartana *et al.* 2005; Kojima *et al.* 2006). Thus, the efficiency of both transformation methods which were tested for radish in our work was higher than the efficiency of floral-dip (Curtis and Nam 2001) and vacuum infiltration of *Agrobacterium* into sonicated seeds (Park *et al.* 2005). In recent years, radish has come under renewed interest as a source of many biologically active compounds such as peroxidases, glucosinolates and isothiocianates (Guttierez and Perez 2004). New effective *in planta* transformation methods might be used for radish transformation with the aim of improving of its agricultural characteristics and production of medical compounds within a shorter

Table 3 Tumor formation frequency in the transgenic radish plants.

Line	Untransformed	*nptII*		*ipt*			*rolC*		
		T_1	T_2	T_1	T_2		T_1	T_2	
					tum+*	tum-**		tum+*	tum-**
3	0.00 + 10.00	0.00 + 16.66	-	-	-		62.50 ± 17.17	36.36 ± 14.49	0 + 16.66
19	100.00 - 6.66	100.00 - 12.50	-	60.00 ± 15.82	100 -11.11	58.33 ± 10.06	-	-	
30	0.00 + 9.09	0.00 + 7.69	-	66.67 ± 13.62	34.48 ± 8.83	0 + 14.29	-	-	

Data represented as means ± standard errors.
*tum+: T_2 from tumor-producing T_1 plants
**ant- : T_2 from non-tumorous T_1 plants

Table 4 Percentage of transgenic radish plants with altered anthocyanin coloring.

Line	Untransformed	*nptII*		*ipt*			*rolC*		
		T_1	T_2	T_1	T_2		T_1	T_2	
					ant+*	ant-**		ant+*	ant-**
3	0.00 + 10.00	0.00 + 16.66	-	-	-		37.50 ± 17.11	50 ± 12.50	0+16.66
19	0.00 + 6.66	0.00 + 12.50	-	10.00 - 9.49	75.00 ± 9.69	0 + 4.76	-	-	
30	0.00 + 9.09	0.00 + 7.69	-	25.00 ± 12.51	53.85 ± 13.81	0 + 4.34	-	-	

Data represented as means ± standard errors.
*ant+ T_2 from T_1 plants with altered anthocyanin coloring
**ant-: T_2 from T_1 plants with normal anthocyanin coloring

Table 5 Segregation on tumor formation and altered anthocyanin coloring in several T_2 families of *ipt*- and *rolC*-transgenic radish.

T_2 families	n	Segregation			
		tum+* ant+**	tum+ ant-**	tum-* ant+	tum- ant-
Line 30-*ipt* tumor+, altered coloration	13	3	2	4	4
Line 19-*ipt* tumor-, altered coloration	12	7	1	2	2
Line 3-*rolC* tumor+, altered coloration	11	1	3	7	0

*tum+: tumor producing plants, tum-: non-tumorous plants
**ant+: altered anthocyanin coloration, ant-: normal anthocyanin coloring

time-span.

The second aim of our study was to assess the effects of *A. tumefaciens ipt* and *A. rhizogenes rolC* genes on spontaneous tumorigenesis in radish inbred lines. We hypothesized that the *ipt* gene coding for cytokinin biosynthesis and which plays a major role in the control of *A. tumefaciens*-mediated tumorigenesis (Garfinkel *et al.* 1981), and the *rolC* gene which also influences cytokinin metabolism (Estruch *et al.* 1991), might be able to induce tumors when transferred into plants with a specific genetic background, e.g. radish inbred lines.

Increase of cytokinin level plays a crucial role in the formation of tumors of different origin in higher plants or stimulate the growth of plant tumors that were not initially caused by increase of endogenous cytokinin content (for review see: Ahuja 1998; Dodueva *et al.* 2007). Transformation of different plant species with *ipt* and *rolC* genes leads to an increased levels of all cytokinin forms in the case of *ipt* (von Schwartzenberg *et al.* 1994; Faiss *et al.* 1997; Khodakovskaya *et al.* 2005) or certain cytokinin compouns in the case of *rolC* (Nilsson *et al.* 1996; Casanova *et al.* 2004; Grishunina *et al.* 2005). So, we expected that the alteration of cytokinin content in *ipt*- and *rolC*-transgenic radish plants might lead to the alteration of the ability to tumor formation. In the present work we measured Z and ZR content in tumorous and non-tumorous radish lines and also *ipt*- and *rolC*-transgenic plants. According to the available data (Gordon *et al.* 1974; Bukhov *et al.* 1996; Matveeva *et al.* 2004) Z and ZR are main cytokinins in the radish tissues, so alteration in their content might have a great influence on radish plant development.

In our experiment, *ipt* and *rolC* genes exerted different influence on Z and ZR content in the radish lines: transformation with *ipt* led to an increase in Z and ZR concentration while transformation with *rolC* gene caused an increase in ZR and a decrease in Z content. In the experiments with different plant species, transformation with the *ipt* gene under its native promoter (von Schwartzenberg *et al.* 1994), *CaMV35S* (Faiss *et al.* 1997) or inducible promoters (Khodakovskaya *et al.* 2005) caused a dramatic increase in concentration of many cytokinin metabolites, including Z and ZR. An increase in ZR concentration was demonstrated for *rolC*-transgenic aspen (Nilsson *et al.* 1996), carnation (Casanova *et al.* 2004) and potato (Grishunina *et al.* 2005) plants. Thus, changes in cytokinin content in *ipt*- and *rolC*-transgenic radish plants correspond to results that were obtained in other plant species.

At the same time, no typical characteristics of the effect of cytokinin such as dwarfism, a reduced root system, decreased apical dominance and dark-green leaves (von Schwartzenberg *et al.* 1994; Faiss *et al.* 1997) were observed in *ipt*-transgenic radish plants. Similarly, transgenic radish plants carrying the *rolC* gene did not demonstrate reduced internode length, small pale green leaves and small flowers that are typical for *rolC*-transgenic plants of different species (Schmülling *et al.* 1988; Nilsson *et al.* 1996; Casanova *et al.* 2004).

The only effect of transformation of radish by either *ipt* or *rolC* genes was an alteration of the ability to tumor formation: we observed formation of tumors in the *ipt*- and *rolC*-transgenic plants from non-tumorous radish lines 3 and 30 and a decrease in tumor formation frequency in the *ipt*-transgenic plants of tumorous line 19. Regrettably, we have no valuable explanation for these facts, but we can suppose that the radish crop-root can play a role as a "buffer" that detains a part of cytokinins and reacts to their abundance by increasing the rate of cell divisions leading to tumor formation. In fact, it was reported that the accumulation of Z and ZR plays an important role in the formation and growth of the radish crop-roots (Bukhov *et al.* 1996). Accumulation of great amounts of cytokinins was reported for tumors induced by *Agrobacterium tumefaciens* (Veselov *et al.* 2003) as well as in the spontaneous tumors in the interspecific hybrids of *Nicotiana* (Nandi *et al.* 1990) and radish inbred lines (Matveeva *et al.* 2004) while non-tumorous tissues of the same plants were shown to have normal levels of cytokinin (Nandi *et al.* 1990; Matveeva *et al.* 2004). Since acropetal transport of zeatin-type cytokinins carry out mainly in the xylem sap (Radin and Loomis 1974; Hirose *et al.* 2007), cytokinin accumulation in tumor tissues is likely to be due to the development of circular vascular bundles that are typical for plant tumors (Ullrich and Aloni 2000). Formation of circularly and spirally oriented vessels was detected also in the tumors on radish crop-roots (Ilyina *et al.* 2006).

Another effect of transformation of radish lines by the

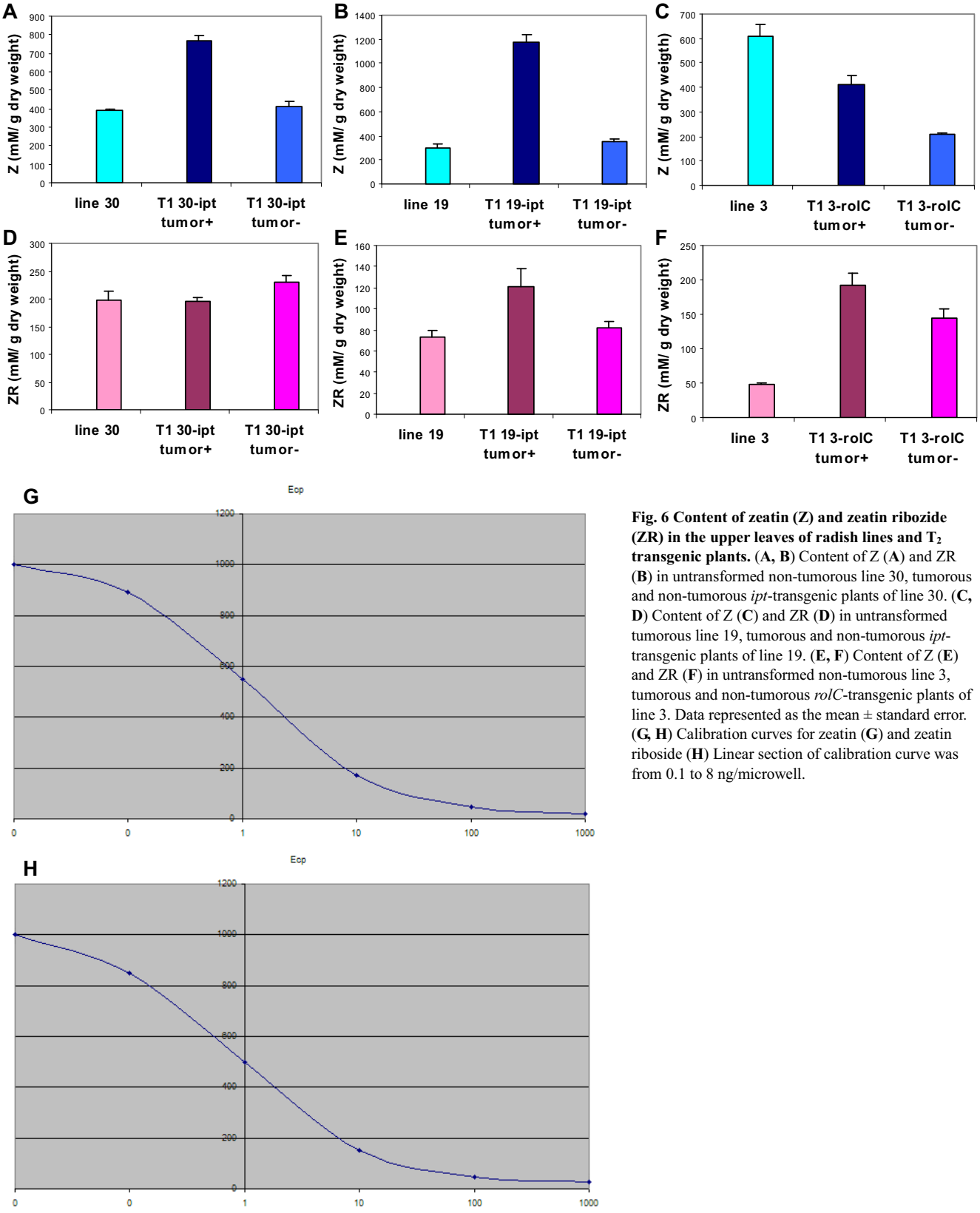

Fig. 6 Content of zeatin (Z) and zeatin ribozide (ZR) in the upper leaves of radish lines and T_2 transgenic plants. (**A, B**) Content of Z (**A**) and ZR (**B**) in untransformed non-tumorous line 30, tumorous and non-tumorous *ipt*-transgenic plants of line 30. (**C, D**) Content of Z (**C**) and ZR (**D**) in untransformed tumorous line 19, tumorous and non-tumorous *ipt*-transgenic plants of line 19. (**E, F**) Content of Z (**E**) and ZR (**F**) in untransformed non-tumorous line 3, tumorous and non-tumorous *rolC*-transgenic plants of line 3. Data represented as the mean ± standard error. (**G, H**) Calibration curves for zeatin (**G**) and zeatin riboside (**H**) Linear section of calibration curve was from 0.1 to 8 ng/microwell.

ipt and *rolC* genes - an alteration of anthocyanin coloring – might also related with the alteration in cytokinin content. A high percentage of transgenic plants with altered coloring was obtained by independent transformation events and inherited in the T_2 generation allowing us to suppose that T-DNA genes influence the metabolism or distribution of anthocyanins. This effect is likely to be due to an alteration in cytokinin level caused by expression of the *ipt* and *rolC* genes. In fact, exogenous cytokinins were shown to stimulate anthocyanin accumulation in the tissues of different plant species (Sakurai *et al.* 1997; Chen *et al.* 2006). Deikman and Hammer (1995) established that BA treatment of *Arabidopsis* seedlings positively influenced the expression of several genes acting in the anthocyanin biosynthesis pathway.

One of the aims of the present work was examination of hypothesis about the relation between cytokinin content and tumor formation in radish lines. A cytokinin assay showed that transgenic radish plants with the tumorous phenotype had increased Z+ZR content as compared with their parent lines while non-tumorous transgenic plants had only a slight increase (in the case of *ipt*-transgenic plants) or decrease (in

the case of *rolC*-transgenic plants) in the Z+ZR level. So, increase of Z and ZR content caused by transformation with *ipt* or *rolC* stimulate tumor formation in radish.

On the other hand, data obtained in the present work show the absence of the direct correlation between Z and ZR level and tumor formation in the radish lines and transgenic plants. Particularly, lower level of Z in 100% tumorous line 19 as compared with non-tumorous lines 3 and 30 argue against the crucial role of cytokinins, at least Z and ZR, in tumor formation in radish lines. Similarly, non-tumorous transgenic radish plants which were obtained as a result of transformation of tumorous line 19 with the *ipt* gene showed a slight increase in Z and ZR concentration as compared with tumorous plants from the parent line.

Transformation of radish lines with *ipt* and *rolC* genes did not obligatory lead to the increase of Z and ZR level. We assume that normal cytokinin content in the tissues of *ipt*- and *rolC*-transgenic radish plants might be a result of work of the regulatory mechanisms which control phytohormonal homeostasis in the plant tissues. In fact, change in the levels of other phytohormones (especially auxin and abscisic acid) was detected for the cytokinin-overproducing transgenic *Nicotiana tabacum* plants that overexpress isopentenyl transferase and β-glucosidase genes (Kiran *et al.* 2006; Polanska *et al.* 2007), indicating a complex interaction between phytohormones which is important for survival of the plant organism. Regulation of phytohormonal balance might cause the repression of tumor formation which we observed in certain part of *ipt*-transgenic plants from tumorous line 19.

Thus, we have shown the absence of direct correlation between Z and ZR content and tumor formation in radish lines and also *ipt*- and *rolC*-transgenic plants, however, this problem needs further investigations. The main outcome of the present work is a new effective method for radish *in planta* transformation.

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