J Plant Growth Regul (2004) 23:37–43
DOI: 10.1007/s00344-004-0004-8

J P G R
Journal of Plant Growth Regulation

Hormonal Control of Tumor Formation in Radish

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Abstract

The role of phytohormones in genetic tumor formation on radish crop-roots was investigated using the collection of inbred *Raphanus sativus* lines as a model system. The genetic analysis showed that the trait <<tumor formation>> was recessive and monogenic in some crossings. The spectrum of main phytohormones in tumor and non-tumor radish lines has shown that at the initiation of tumor formation (30 days old plants) the amounts of main cytokinins in the lower part of plants from the tumor line were dramatically increased. The transformation of the non-tumor line by the *ipt* gene of *Agrobacterium tumefaciens* resulted in tumor formation in plants of the T_1 progeny. We propose that increasing the cytokinin/auxin ratio may lead to tumor formation on radish crop roots.

Key words: *Raphanus sativus*; *Agrobacterium tumefaciens*; Inbred lines; Auxins; Cytokinins; Tumorgenesis

Introduction

Dissection of the genetic control of the differentiation process is one of the central problems in modern plant biology. Recently, significant progress has been achieved for animals and fungi. However in plants, which have particular specificity compared to other kingdoms of eukaryotes, the precise mechanisms of differentiation are still unknown. It is widely accepted that phytohormones, especially auxins and cytokinins, play key roles in the regulation of plant differentiation (Kende and Zeevaart 1997; Schmuelling 2000; Ulrich and Aloni 2000). But the details of such control remain unclear. In this case it is extremely useful to analyze plant forms possessing some genetic deviations in their morphogenesis. One of the most widely used deviations is genetic tumors of plants.

Genetic tumors represent relatively undifferentiated tissues, which spontaneously appear in plants of defined genotypes. Bayer and others (1989) have demonstrated hormone independent tumor growth in plant tissues, similar to tumors induced by *Agrobacterium* transformation in which hormone independence has clearly been shown (Veselov and others 2003; Azmi and others 2001; Xu Xiu and others 2000).

Genetic tumors have been described in a number of species and interspecific hybrids. Such tumors have been intensively studied in the genus *Nicotiana*, for example in hybrids *N. glauca x N. langsdorffii* (Ahuja 1968). Tobacco genetic tumors were the first

Received: 8 January 2003; accepted: 6 February 2004; Online publication: 1 June 2004
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plant tissues maintained in prolonged culture in vitro on artificial growth medium (White 1939, cited by Ichikava and Syono 1991). Explants of tumorous hybrids grow well on hormone-free medium and are capable of regeneration (Bayer and others 1989). A number of authors have investigated the role of phytohormones in tobacco tumor growth. Increased auxin levels in tumor plants and a correlation between auxin content and tumor size have been shown (Bayer and others 1989). Some authors indicated no changes in auxin content, but rather increased auxin sensitivity (Fujita and others 1991) in tumor hybrids compared to parental species. At the same time there are some data indicating that changes in cytokinin level are also important for tumor formation: tumor tobacco hybrids have increased levels of cytokinin-nucleotides (Ichikava and Syono 1991). On the other hand, exogenous treatment with cytokinins of the non-tumor mutant obtained from amphydiploid *N.glauca x N. langsdorffi* as well as its transformation with the ipt gene, leads to the restoration of the tumor phenotype (Feng and others 1990). Investigation of phytohormone content dynamics in tobacco tumors has shown increased cytokinin/auxin ratios during tumor initiation (Ames and others 1974, cyted by Ichikava and Syono 1991). Additional evidence in favor of the hormonal nature of tumors in tobacco hybrids was obtained by White and others in 1982. They found sequences that were 80% homologous with *A. rhizogenes* T-DNA in untransformed *Nicotiana glauca*. Detailed investigations of these sequences have shown that they contain genes, homologous to rolB, rolC, ORF13 and to 14 oncogenes of *A. rhizogenes* (Aoki and others 1994). Tumor formation in the hybrid plants was induced by the interaction of some factors originating from the *N. langsdorffii* genome with these oncogene-like sequences. These findings support the hypothesis of a hormonal role in tobacco hybrid tumor formation.

Another available model suitable for studying plant genetic tumor formation is a unique collection of highly inbred radish lines developed at the Department of Genetics, St.-Petersburg State University, Russia (Narbut 1967). This collection has been derived from single plants of Saxa and Virovsky Bely cultivars by selfing for more than 30 generations. At present, the collection includes about 30 self-compatible inbred radish lines possessing different morpho-physiological deviations such as dwarfism/gigantism, agravitropic growth, vivipary, wiltyness, intensive crop-root cracking, and non-terminal development of the flower meristem. Some of these lines are also able to produce large genetic tumors arising on the crop-root and/or lower part of the stem during flowering (Narbut and others 1985) (Figure 1).

Figure 1. Large genetic tumors arising on the crop-root of the tumor radish line during flowering.

Tissues derived from the tumor line can produce hormone independent growth under in vitro conditions whereas the non-tumor lines do not possess this ability (Buzovkina and others 1991). As in other species, radish tumor formation is a complicated process, that includes several steps: tumor induction, tumor growth, and tumor senescence. Flowering is the stage when radish tumors can be observed. Because radish tumors become visible at the flowering stage we proposed that tumor induction should take place just prior to flowering. This stage corresponds to the calendar age of 30 days. Ames and others (1974, cited by Ichikava and Syono 1991) reported that during tumor induction, hormonal content changes leading to an increased cytokinin/auxin ratio. The aim of this study was to investigate whether such changes take place during tumor induction in radish. The objectives of our work were to

- genetically analyze tumor formation in radish
- measure phytohormone content in tumor and non-tumor lines
- investigate phenotypic response of the non-tumor line upon agrotransformation with the gene for cytokinin biosynthesis.

Materials and Methods

Plant Material

We used highly homozygous inbred lines of radish *(Raphanus sativus var. Radicola* Pers.) derived from two cultivars after selfing for more than 30 generations. The radish lines derived from ''Saxa'' cultivar included

- lines 18, 30 - no tumor formation in vivo
- lines 19, 21-100% tumor formation in vivo the radish lines derived from ''Virovsky Bely'' cultivar included
- line 8, 6-no tumor formation in vivo
- lines 10, 16-100% tumor formation in vivo

The *Agrobacterium tumefaciens* strain, containing the pGV3850 transformation vector with P35ScaMW-ipt gene and Pnos-nptII plant selectable marker was kindly supplied by Dr. T. Schmuelling (Zambryski and others 1982).

Methods

The F_1 and F_2 hybrids

The F_1 and F_2 hybrids for genetic analysis were produced according to the procedure described by Narbut and others (1985).

Phytohormone Analysis

Leaves, crop, and roots of 30-day old radish plants were collected, immediately frozen in liquid nitrogen, and stored at −70°C until further phytohormone analysis. Lyophilized plant samples were transferred into Bieleski solution (Bieleski 1964), mixed and extracted overnight at −20°C.

Cytokinin Analysis

For each cytokinin (CK) compound determined, 20 pmol of the corresponding deuterated standard (APEX Int., Honiton, Devon, UK) was added before centrifugation (20,000 rpm, 15', 4°C). The pellet was resuspended for 1 h at 4°C in 80% methanol and centrifuged once more. The supernatants of both fractions were collected and cytokinins were purified by a combination of solid phase and immuno-affinity chromatography as previously described by Redig and others (1996). CK's were quantified by LC-(ES+)MS/MS in MRM mode (Prinsen and others 1998). The chromatograms obtained were processed by means of Masslynx software (Micromass). The method of isotope dilution allowed calculation of concentrations expressed in pmol/gram dry weight.

IAA Analysis

Free indolilacetic acid (IAA) was analyzed by a combined solid phase extraction procedure based on methods presented by Prinsen and others (1995). $^{13}C_6$-IAA (100 ng) (Cambridge Isotope Laboratories Inc., Andover, Massachusetts, USA) was used for isotope dilution purposes. After pentafluorobenzylation (Epstein and Cohen 1981), the PFB ester of IAA (PFB-IAA) was analyzed by GC-MS SIR CI^- (HP 5890 series II coupled to a TRIO 2000 quadruple Mass Spectrometer) (VG-Micromass). Diagnostic ions for SIR were 180 (PFB−^{13}C-IAA); 174 (PFB-IAA) (Netting and Milborrow 1988). All data were processed by Labbase (VG Micromass) software.

In Planta Transformation of Radish and Production of the Transformants

''Overnight culture'' suspension of the agrobacterial strain was 10 times dissolved and plotted on the stigma of pistil of unrevealed knops. After that the T_0 seeds were obtained by self-pollination and the T_1 progeny were germinated in the greenhouse. PCR-positive plants were transferred to the field for morphological characterization.

Molecular-Genetics Methods

DNA preparation and PCR analysis were performed according to laboratory manuals (Draper and others 1991; Maniatis and others 1989). T_1 progeny were analyzed by PCR with the following primers to the nptII gene:

nptII (1): GTCGTCTGGTCGGTCATTTCG
nptll (2): GTGATCTCACCTTGCTCCTGCC

Statistics

In the genetic analysis experiment, Pearson criteria (χ^2) were used to compare experimental and theoretically expected results. In the analyses of phytohormone content of radish tissues, standard deviations were calculated for the quantity of each hormone (Lakin 1990).

Results and Discussion

Genetic Analysis of Tumor Formation

The genetic analysis was carried out using a number of interline F_1 and F_2 hybrids. F_1 hybrids were

non-tumorous in all hybrid combinations of tumor and non-tumor lines. F_1 hybrids between two tumor lines were tumorous, which indicates allelism of the tumor formation locus found in different inbreeds.

The distribution of the F_2 generation is presented in Table 1. In the hybrid combination between two tumor lines (19 and 21) all F_2 plants were tumorous. In all studied hybrid combinations between tumor and non-tumor lines the distributions we found did not contradict the 3:1 ratio. Therefore, there are monogenic differences in the ability to form tumors between the studied lines and tumor formation is recessive.

Our data on genetic control of tumor formation is one of the first examples of intra-species variation due to tumor formation. In most other cases this phenomena was described in the interspecific hybrids and amphydiploids. In such forms it is difficult to study the genetic control of tumor formation, especially when researchers deal with interspecific hybrids. *Raphanus sativus* is a diploid species and tumor formation may be involved in hybridological analysis.

Table 1. Distribution of the F_2 Generation with regard to Tumor Formation in Radish

Cross	Tumor+	Tumor−	χ^2 (1:3)
18 × 10*	9	15	2,00
18 × 16*	6	19	0,01
18 × 19*	67	171	1,26
18 × 21*	11	50	1,41
6 × 10*	14	46	0,09
8 × 16*	12	24	1,33
19* × 21*	11	0	-

Radish lines derived from ''Saxa'' cultivar: lines 18, 30 - no tumor formation in vivo; lines 19, 21 - 100% tumor formation in vivo; Radish lines derived from ''Virovsky Bely'' cultivar: lines 6, 8 - no tumor formation in vivo; lines 10, 16 −100% tumor formation in vivo; tumor <<>>-line; <<−>>-segregation was not detected; χ^2 (Pearson criteria) - statistical criteria of consent between empiric and theoretic function of distribution*

Role of Cytokinin in Tumor Formation

It is widely accepted that endogenous plant hormones are responsible for tumor growth. In our work we used two different approaches to study the involvement of cytokinins in radish tumor formation. First was the direct measurement of hormonal content in roots, crop-roots and leaves of 30-day-old radish plants in tumor and non-tumor lines. Because radish tumors appear at the flowering stage we proposed that hormonal levels should change just before this stage; in our experiment this stage corresponds to 30-day old plants.

The second approach was transformation of non-tumor lines with the cytokinin biosynthesis gene ipt.

Analysis of Phytohormone Content

Phytohormone contents were studied in 2 radish lines: 8-non-tumor line and 10-tumor line. The lines differed significantly by cytokinin spectrum (Table 2). These differences were less in the leaves. The most dramatic differences were described in the lower parts of the plants, roots and crop roots. The levels in tumor line 10 of the major groups of cytokinins that is, zeatin (Z), zeatin riboside (ZR), dihydrozeatin (DHZR), and zeatin riboside phosphate (ZR-P), were 10 times higher than in line 8. Such accumulations of phytohormones in the lower part of the plant where cytokinin synthesis takes place might be caused by the presence of a cytokinin transport inhibitor. The possibility exists that these differences reflect the ratio of synthesis and degradation of cytokinins in roots and other tissues. For example, a high level of biosynthesis of CK and its weak degradation in roots together with high degradation in leaves would result in the same picture, as could be observed in the case of transport inhibitor activity.

It is important to stress that the induction of tumor formation processes depends not on the particular hormone, but on the hormone ratio. That is why the free IAA level was measured. We did not note big differences in IAA content in leaves and crop-roots of tumor and non-tumor lines (data not shown). Measurement of the auxin level in the root showed a higher content of IAA in the non-tumor line 8. Cytokinin and auxin physiologically active ratios are shown in Figures 2 and 3. The cytokinin/auxin ratio was much higher in the tumor line.

So the most dramatic differences in the phytohormone ratios were detected in the lower part of the tumor line plants. Increased levels of cytokinins at the organs where tumors appear and at the stage when tumor induction takes place, may be interpreted as involvement of cytokinins in radish tumor induction. At the later stages no correlation was detected between tumor formation on the crop-root and hormonal content (Smets, personal communication).

Agrotransformation of Line 30

To support the hypothesis about the role of cytokinin in tumor induction we transformed the radish plants in vivo with a vector containing the cytokinin

Table 2. Amounts of Major Cytokinins in Tumor and Non-tumor Radish Lines

	Amount of Cytokinins, pmol/g dr.w.					
	Line 8 (non-tumor)			Line 10 (tumor)		
Main Cytokinins	Leaves	Crop roots	Roots	Leaves	Crop roots	Roots
Zeatin	173 ± 45	12.1 ± 3.1	<66.6	<14.3	<11.8	315 ± 82
DHZ	39.8 ± 10.4	<7.5	<66.6	<14.3	<11.8	<61.8
ZR	479 ± 56	237 ± 28	891 ± 104	305 ± 36	131 ± 15	4050 ± 474
DHZR	13.2 ± 1.5	9.76 ± 1.14	<17.1	10.0 ± 1.2	<2.2	115 ± 14
Z-N-G	11.6 ± 1.4	5.00 ± 0.85	<37.8	<7.6	<4.1	49.3 ± 5.8
DHZ-N-G	<7.3	<2.4	<37.8	<7.6	<4.1	<30.8
IP	364 ± 30	<30.1	<402.3	<62.3	<91.1	793 ± 151
IPA	30.0 ± 1.4	22.1 ± 1.8	<94.5	33.4 ± 2.7	32.4 ± 2.6	<47.5
IP-G	<6.91	<4.1	<54.0	<6.4	<8.5	<21.6
ZR-P	4570 ± 535	6720 ± 786	2180 ± 255	335 ± 39	106 ± 12	3280 ± 384
DHZR-P	90.7 ± 10.6	187 ± 22	32.0 ± 3.8	9.31 ± 1.1	2.52 ± 0.3	66.3 ± 7.8
IPA-P	31.0 ± 5.8	31.6 ± 5.9	73.0 ± 13.5	21.4 ± 4.0	10.0 ± 1.9	119 ± 22

Abbreviations: DHZ – dihydrozeatin; ZR – zeatin riboside; DHZR – dihydrozeatinriboside; Z-N-G – zeatin – N9 – glucoside; DHZ-N-G – dihydrozeatin – N9 – glucoside; IP – isopentenyl; IPA – isopentenyl adenoside; IP-G – isopentenylglucoside; ZR-P – zeatinriboside – phosphate; IPA-P – isopentenyladenoside – phosphate; DHZR-P – dihydrozeatinriboside-phosphate

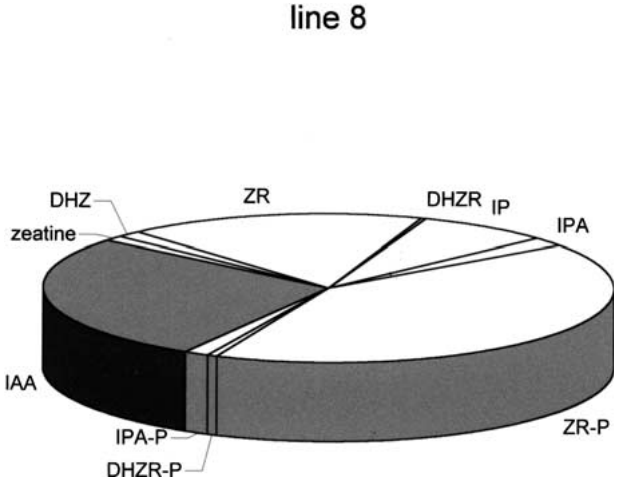

Figure 2. Amounts of major cytokinins and IAA in non-tumor radish line 8. DHZ – dihydrozeatin; ZR – zeatin riboside; DHZR – dihydrozeatinriboside; *(Z-N-G – zeatin – N9 – glucoside*; *DHZ-N-G - dihydrozeatin – N9 – glucoside*; IP – isopentenyl; IPA – isopentenyl adenoside; *IP-G – isopentenylglucoside*; *ZR-P – zeatinriboside-phosphate*; IPA-P-isopentenyladenoside-phosphate; DHZR-P-dihydrozeatinriboside–phosphate; IAA-indolilacetic acid.

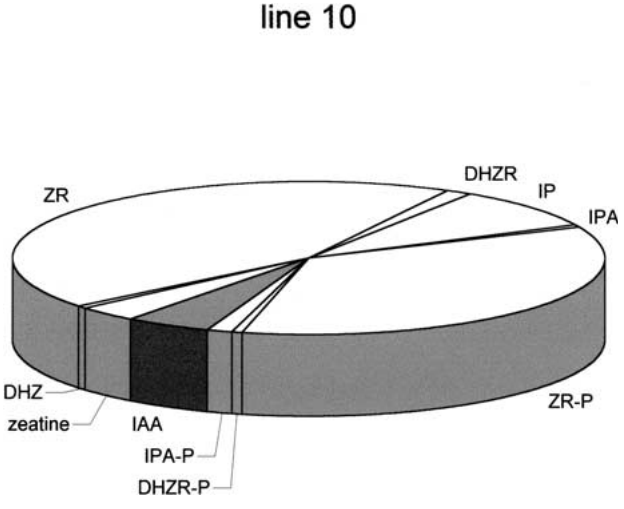

Figure 3. Amounts of major cytokinins and IAA in tumor radish line 10. DHZ-dihydrozeatin; ZR-zeatin riboside; DHZR-dihydrozeatinriboside; *Z-N-G – zeatin – N9 – glucoside; DHZ-N-G – dihydrozeatin – N9 glucoside* IP – isopenteny IPA – isopentenly adenoside; *IP-G – isopentenylglucoside:* ZR-P – zeatinriboside-phosphate; IPA-P-isopentenyladenoside-phosphate; DHZR-P – dihydrozeatinriboside-phosphate; IAA-indolilacetic acid.

biosynthesis gene ipt. For the transformation experiment, the non-tumor line 30 with a high ability to form seeds (compared to other lines) was used. Transformed in vivo plants were self-pollinated and seeds of To were obtained, DNA from the plants of the next generation (T_1) was analyzed by PCR using primers to the selective marker gene npt II. Fragments (0.5 kb) were shown to be present as PCR products in 12 plants (Figure 4). Several transformants, derived from transformed line 30, demonstrated some morphological deviations from control such as white color of flowers instead of

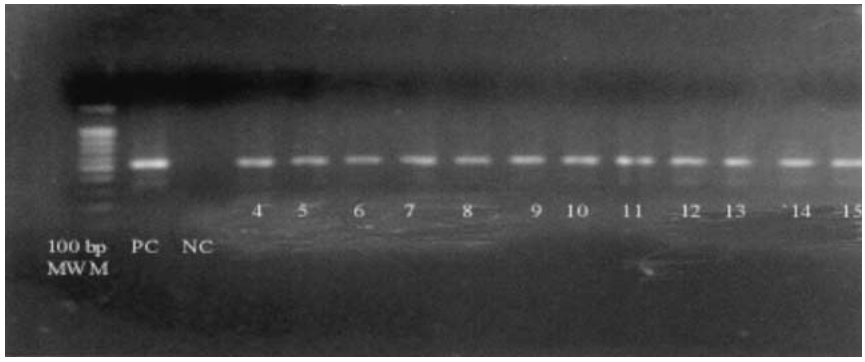

Figure 4. Results of PCR on the template DNA of T_1 plants with primers to the nptII gene. line 1-100 bp MWM (molecular weight marker), Sibenzyme; line 2-PC – positive control (PCR on the template DNA of agrobacterium with nptll gene); line3- NC - negative control (PCR on the template DNA of intransformed *untransformed* radish plant with npt gene); line 4-15 - PCR products on the template DNA of T_1 plants.

Figure 5. Phenocopy of tumor formation on the crop-root of radish plant transformed with the gene for cytokinin biosynthesis ipt (isopentenyl transferase).

pink, and white-pink color of the crop-root instead of red. It is difficult to give an accurate explanation of such alterations. All the alterations may not be connected to the effect of the ''cytokinin gene'' itself, but may be induced by the process of transformation. The most notable fact is that eight of twelve transformed plants demonstrated tumor formation on the crop root (Figure 5). Line 30 itself does not produce tumors.

Because we used the ipt gene under the control of constitutive promoter 35S, we expected expression of the gene in different plant organs at different stages of morphogenesis. Therefore, the level of cytokinin at the stage of tumor induction is increased (Azmi and others 2001), such increases could induce tumor formation.

The fact that only eight of the twelve plants formed tumors should be discussed. It is well known that the expression of T-DNA genes depends on the site of insertion, number of insertions, physiological status of the particular plant and so on. Besides, the insertion event itself may disrupt a plant gene that is involved somehow in the same process as the inserted gene. In our case, the hormonal content of radish plants is under the control of many endogenous factors (including radish genes for hormone metabolism and sensitivity) along with the transferred agrobacterial ipt gene for cytokinin biosynthesis. Changes in the ratio of total (plant and T-DNA insertion caused) cytokinins to total auxins are critical for induction of tumor formation. If the expression of the ipt gene in some particular case is lower, or the inactivation of synthesized cytokinin is higher, then the total level of active hormone could be lower than the threshold value for tumor induction. Nevertheless, appearance of tumors on the majority of transformed plants leads us to the conclusion that the reason for such phenotypic changes is the inserted transgene. The induction of tumorigenesis in this non-tumor line after transformation with the ipt-containing vector supports our hypothesis concerning the role of cytokinin in tumorigenesis.

Summarizing our results we can say that in our experiments 1) we have shown inheritance of tumor formation in radish, the recessive character of the studied trait as well as possible monogenic control of this trait in studied lines.

2) our data support the hypothesis about the role of increased cytokinin/auxin ratio in the induction of tumor formation on the crop roots of radish lines

from the genetic collection of St. Petersburg State University.

ACKNOWLEDGMENTS

The research described in this publication was made possible in part by Award No.ST-012-0 of the U.S. Civilian Research & Development Foundation for the Independent States of the Former Soviet Union (CRDF), Russian Foundation for Basic Research (project no. 99-04-49108) and INTAS (project no. 96-1674). The authors are grateful to Professor Igor A. Tikhonovich for critical reading of the manuscript.

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