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PLANT GENETICS

Chromosome Variation in Ginseng Cells Transformed with the *rolC* Plant Oncogene

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Abstract—Chromosome numbers were studied in ginseng cell line 1c transformed with *Agrobacterium rhizogenes* strain A4, which carried plasmid pRiA4, and with *A. tumefaciens* strain GV3101, which carried vector pPCV002-35S*rolC*. As compared with the nontransformed cell line 1c, tumor cell cultures 1c-A4 and 1c-*rolC* and the tissues of *rolC* teratoma (excluding leaves) displayed higher polyploidy and aneuploidy. The 1c-A4 and 1c-*rolC* hairy-root cultures also had aneuploid and polyploid cells, but the chromosome variation was lower than in tumor cells or the initial culture 1c. Generally, an increase of chromosome variation in cultivated cells was the main effect of the integration of several oncogenes, which were in *A. rhizogenes* A4 T-DNA, or of the individual *rolC* gene in the ginseng genome. Another effect consisted in stabilization of the chromosome number in some differentiated transgenic tissues. Possible reasons for this effect are discussed.

INTRODUCTION

Transformation of plant cells with the aid of *Agrobacterium rhizogenes* (e.g., strain A4) results in the transfer of a fragment of agrobacterial plasmid DNA (T-DNA) in the plant genome; the tumor tissue thus obtained can be used to regenerate what is known as hairy roots [1]. According to published data, a stable diploid or euploid chromosome set is characteristic of in vitro cultivated hairy roots of various species, including *Nicotiana rustica, N. tabacum, N. africana, Datura stramonium, Catharanthus roseus, Beta vulgaris, Phaseolus vulgaris* [2], *Pisum sativim* [3], and *Panax ginseng* [4]. However, a significantly reduced chromosome number has recently been reported for *Onobrychis viciaefolia* transformed roots [5], suggesting for the first time instability of the chromosome set in hairy root cultures.

The Ri plasmids of *A. rhizogenes* A4 contain various oncogenes, e.g., *rolA*, which disturbs gibberellin [6] and polyamine [7] metabolism in plant cells; *rolB*, which codes for glycosidase hydrolyzing indole-3-acetic acid (IAA) conjugates [8]; and *rolC*, which codes for glycosidase hydrolyzing cytokinin–glycoside conjugates [9]. Tumor growth induced by *rolC* is associated with altered hormone balance and cell development [9, 10]. Such alterations are known to increase genome variation in plant cells [11, 12].

In our experiments, the *rolC* gene was isolated from pRiA4 [13] and inserted in the genome of a nondifferentiated ginseng callus tissue (line 1c). The insertion induced cell differentiation morphogenetically manifested in rhizogenesis, the formation of somatic embryoids, and the proliferation of teratoma structures, including callus, roots, and shoots [14]. Transgenic tissues displayed markedly changed contents of free and bound cytokinins and IAA [14]. In addition, the content of ginseng-specific biologically active substances (ginsenosides) significantly increased [15]. Since genetic stability is of principal importance for efficient use of cell cultures in biotechnology, we compared chromosome polymorphism in ginseng cultures carrying *rolC*, the initial line 1c, and cultures transformed with A4. Another objective of this work was to analyze possible factors responsible for a discrepancy between the actual stability of chromosome number in most hairy root cultures and the variation theoretically expected for transgenic tissues on the basis of their neoplastic transformation and hormone disbalance.

MATERIALS AND METHODS

Plants. We used *Panax ginseng* C.A. Mey plants from several sources. Three-year-old plants were grown from seeds of wild ginseng (Chuguevka population) on an experimental plantation of the Institute of Biology and Soil Sciences (Chuguevka raion, Primorskii krai). One-year-old plants were grown from seeds of cultivated ginseng (obtained from the Zhen'shen' state farm, Primorskii krai) in a ginseng farm of the Institute of Biology and Soil Sciences. Three-day-old seedlings were obtained from seeds of wild ginseng (Khasan population) and cultivated ginseng (Zhen'shen' state farm) and then stratified for eight months as described earlier [16], without phytohormone treatment.

Line 1c was obtained from a leafstalk of a two-month-old plant from a cultivated *P. ginseng* population in 1990 [17]. The line is stored in the collection; its number is VSKK (VR) 41.

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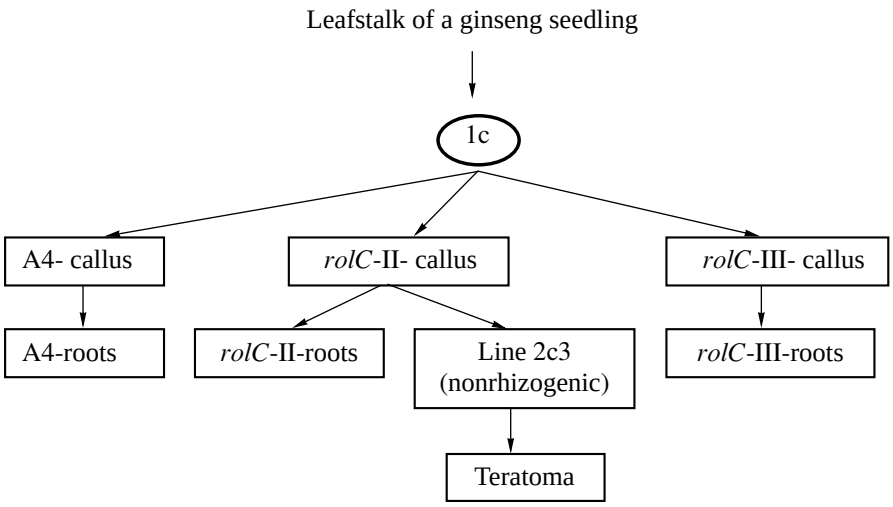

Fig. 1. The origin of transgenic ginseng cultures.

Transformation and origin of transgenic lines. Construction of the 1c-A4 and 1c-*rolC* callus and root cultures and verification of their transformant nature have been described previously [15]. Ginseng callus cells (line 1c) were transformed with *A. rhizogenes* strain A4 and *A. tumefaciens*, which carried plasmid pPCV002-CaMVC containing the *rolC* gene under the control of the 35S promoter of the cauliflower mosaic virus and the NPTII gene under the control of the promoter of the nopaline synthase gene. Primary 1c-A4 and 1c-*rolC* callus cultures (tumors) were used to regenerate roots. Roots were transferred to a liquid medium to produce 1c-*rolC* and 1c-A4 root cultures. Tumor and root cultures 1c-A4, 1c-*rolC*-II, and 1c-*rolC*-III were used in this work (Fig. 1).

The 1c-*rolC*-II line 2c3, which was incapable of forming roots, was obtained from the 1c-*rolC*-II callus culture via selection of root-free aggregates in several passages. Line 2c3 formed embryoids, which were used to obtain teratomas containing callus, roots, stems, and leaves [18] (Fig. 1).

Culture media and conditions. Cultures were grown in agar-containing (1c, primary tumors, teratoma) or liquid (roots) medium W_{2CPA} [19], which contained salts, vitamins, meso-inositol, peptone, sucrose, and 0.4 mg/l 4-chlorophenoxyacetic acid. Before autoclaving, the media had a pH factor of 5.6–5.8. Cultures were grown in 20 or 60 ml of medium (agar or liquid); in 100- and 250-ml Erlenmeyer flasks, respectively; at 25°C and relative humidity 50–70%; and in the dark. Teratomas were cultivated in the light (2500 lx for 8 h, dark period 16 h); the medium contained 4 mg/l 6-benzylaminopurine and 0.5 mg/l gibberelic acid (GA_3) but no 4-chlorophenoxyacetic acid. All cultures were grown for 30 days.

Analysis of chromosome numbers. Samples were taken after cell and tissue cultures were grown for 24 days. Preparations were fixed in the glacial acetic acid–ethanol mixture (3 : 1), treated with 0.1 M HCl for 15 min, and stained with acetocarmine [20]. Chromosomes were counted in metaphase plates on preparations obtained from at least three passages of each culture. In each passage, at least five samples were taken from different regions of a callus or a tissue. Preparations were also made from bases of leaf blades; meristem of seedlings and teratoma shoots 1 mm in length; and 1-mm apexes of seedling radicles, transgenic roots, and teratoma roots. The results are summarized in the table.

Cluster analysis by Ward's method with a consideration of percentage discrepancies was performed using the STATISTICA (Windows 95) program package.

RESULTS

Chromosome Numbers in Ginseng Plants

Different chromosome numbers ($2n$) have been reported for species of the genus *Panax*: 44 and 48 for *P. ginseng*; 24, 44, and 48 for *P. japonicus* C.A. Mey; 24 for *P. trifolius* L.; 48 for *P. quinquefolius* L.; and 22 and 24 for *P. fruticosus* L. [21–24]. On the strength of these data, I.V. Grushvitskii [16] assumed *P. ginseng* to be naturally tetraploid. This assumption was supported by data obtained in our laboratory: isozyme analysis showed that alleles of several enzyme-encoding loci in *P. ginseng* had tetrasomal inheritance (O.G. Koren', personal communication). In our calculations, we assumed the diploid chromosome number $2n = 24$ for *P. ginseng*. The data on ginseng leafs and shoots are given in the table. *Panax ginseng* plants proved to contain cells with chromosome number varying from $2n = 6$ to $2n = 72$; diploid ($2n = 24$) and tetraploid ($2n = 48$) chromosome sets were observed in 77.6% of cells. High chromosome mosaicism was observed in all ginseng organs, including root and leaf meristem of seedlings and leaves of adult plants. Cells with $2n = 48$ prevailed

Frequency of cells varying in chromosome number in *Panax ginseng* intact plants, line 1c, and transgenic tissue cultures

Tissue (number of cells examined)	Cell frequency, %													
	chromosome number													
	6	12	16	18	20	24	30	32	34	36	42	48	72	96
	ploidy													
	1*x*					2*x*				3*x*		4*x*	6*x*	8*x*
Intact plant (243)	2.8 ± 1.2	11.5 ± 2.0	–	–	–	39.9 ± 9.4	2.5 ± 0.6	–	–	8.1 ± 3.4	–	37.7 ± 9.3	2.0 ± 1.7	–
1c (173)	–	7.8 ± 0.8	–	–	–	30.8 ± 2.1	2.0 ± 0.7	–	–	4.0 ± 1.2	–	51.5 ± 2.4	2.8 ± 1.7	–
1c-A4 callus (79)	–	0.8 ± 0.5	0.8 ± 0.6	–	1.6 ± 1.1	45.0 ± 13	5.7 ± 0.7	–	–	7.3 ± 0.4	–	36.7 ± 11.7	6.5 ± 0.1	0.8 ± 0.6
1c-A4 roots (30)	–	–	–	–	–	9.0 ± 2.3	18 ± 2	–	–	18 ± 3	–	55 ± 5	–	–
1c-*rolC* callus (69)	–	–	–	–	–	25 ± 2.1	1.2 ± 0.8	–	1.2 ± 0.6	–	1.2 ± 0.8	55.5 ± 1.1	11.5 ± 0.4	4.3 ± 0.5
1c-*rolC* roots (103)	–	–	–	–	–	27.0 ± 5.9	7.1 ± 3.0	–	–	11.6 ± 3.2	–	53.0 ± 0.7	1.5 ± 1.1	–
1c-*rolC*-teratoma (280)	2.3 ± 1.0	8.8 ± 1.2	3.5 ± 1.9	0.25 ± 0.2	–	28.8 ± 2.6	2.8 ± 1.0	3.3 ± 1.5	–	4.5 ± 0.8	1.5 ± 0.8	44.3 ± 2.7	0.5 ± 0.25	–

Note: (–), cells with a given chromosome number were not found.

in seedlings, whereas most cells in adult plants had $2n = 24$ (Fig. 2). Aneuploid cells were found in all organs of intact plants. The proportion of aneuploid cells slightly varied with each organ, averaging 5.3%. Chromosome numbers did not differ in plants grown from seeds that were obtained from wild or cultivated plants (data not shown).

Chromosome Numbers in Initial and Transformed Cultures

We found no cells with $2n = 6$ in the initial 1c culture. The other chromosome numbers detected in 1c were the same as in intact plants, but the proportions of cells with $2n = 12$, 36, and 48 were different (table). In total, 82.3% of cells were diploid or tetraploid, and 2% of cells were aneuploid. The callus line 1c proved to be mosaic in chromosome numbers: phenotypically similar callus regions showed a prevalence of cells with either $2n = 24$ or $2n = 48$.

A higher variation in chromosome number was characteristic of callus cultures resulting from transformation of 1c cells with wild-type *A. rhizogenes* A4. In contrast to intact plants and culture 1c, A4 transformants had cells with 16, 20, and 96 chromosomes (table). The proportion of hypodiploid cells (carrying less than 24 chromosomes) in 1c-A4 calluses was markedly lower (3.2%) than in 1c callus cultures (7.8%) and intact plants (14.3%). The proportion of diploid and tetraploid cells was 81.7%; 8.1% of cells were aneuploid.

Cell ploidy tended to be higher in 1c-*rolC* callus cultures, which were obtained by transforming 1c cells with the individual oncogene. Hypodiploid cells were not detected; the proportion of hexaploid and octoploid cells was 15.8%, only 2.0 and 2.8% in intact plants and 1c callus cultures, respectively (table). Diploid and tetraploid cells together comprised 80.5%; aneuploidy was 3.6%. In addition, cells carrying 34 and 42 chromosomes were found in this tissue.

The lowest variation in chromosome number was observed in 1c-A4 and 1c-*rolC* roots regenerated from the corresponding callus tissues (table). Neither hypodiploid nor octoploid cells were found, and the proportion of hexaploid cells was insignificant (Fig. 3). Interestingly, we detected hexaploid cells in radicles of seedlings but not in the aboveground organs of adult plants (Fig. 2). The 1c-A4 and 1c-*rolC* root cultures displayed high aneuploidy (18 and 7.1%, respectively), which was explained by a high proportion of cells with $2n = 30$ (Fig. 3).

To study the chromosome number in transgenic shoots and leaves, we obtained the line 2c3, which was able to form embryoids, and selected growth conditions so that embryoids developed in teratoma including callus tissue, roots, shoots, and leaves (see Materials and Methods). In total, teratoma showed high aneuploidy (13.6%) and a high variation in chromosome numbers; cells carrying 18 or 32 chromosomes were found only in this tissue (table). However, the chromosome number was more stable in transgenic leaves, being nearly the same as in seedling leaves (Figs. 2, 4).

Cluster Analysis

Cluster analysis by Ward's method revealed three major clusters. The first one included various organs of intact plants and teratoma leaves (Fig. 5). The second cluster included transgenic root cultures displaying the lowest variation; the line 1c and *rolC*-II callus cultures proved to be closely associated with them. The *rolC*-III and, especially, A4 callus cultures were distant from the line 1c and intact plants. Teratoma organs were found at an even a greater distance from intact plants.

DISCUSSION

Our results showed that chromosome mosaicism was characteristic of ginseng plants. Mosaicism was observed in all organs tested, including leaves of adult plants and leaf and root meristem of seedlings. The difference was that cells carrying 24 chromosomes prevailed in leaves, whereas cells carrying 48 chromosomes were more common in leaf apexes and radicles of seedlings (Fig. 2). In intact ginseng plants, metaphase cells with six chromosomes were found, and chromosome number was a multiple of 6 in most cells (table). An ancestor of the Araliaceae family possibly had $2n = 6$; hence, Araliaceae species can be assumed to be paleotetraploid. Interestingly, $2n = 6$ in *Zosima korovinii* Pim., a representative of the Apiaceae family which is closely related to Araliaceae [22].

Long-term in vitro cultivation commonly increases chromosome polymorphism of plant cells [11]. However, the callus line 1c, which was obtained from a ginseng seedling ten years ago, proved to be relatively stable, as aneuploidy was low and the proportion of euploid cells was high in callus cell populations (table). For comparison, chromosome number varied from 41 to 136 in ginseng line IFRZh2 and from 28 to 400 in line IFRZh3 [25, 26].

The initial ginseng line 1c displays neither rhizogenesis nor the formation of embryoids and teratomas. Profound morphogenetic changes resulting from cell transformation with the *rolC* gene suggest a certain genome variation. We previously revealed such a variation by means of the polymerase chain reaction with arbitrary primers (RAPD–PCR) [27]. The data obtained in this work showed that transformation of the callus line 1c was associated with a higher chromosome variation. The transgenic callus cultures included cells with chromosome numbers that were not detected in intact plants and the initial line 1c; moreover, the proportions of aneuploid and polyploid cells were higher (table, Fig. 4). This was characteristic of both callus cultures transformed with the wild-type plasmid and with the plasmid carrying the *rolC* gene.

The range of variation in chromosome number in roots regenerated from transgenic tissues was lower than in the initial undifferentiated cultures, as was observed with both 1c-A4 and 1c-*rolC* hairy roots (table, Fig. 3). Two alternative explanations are possi-

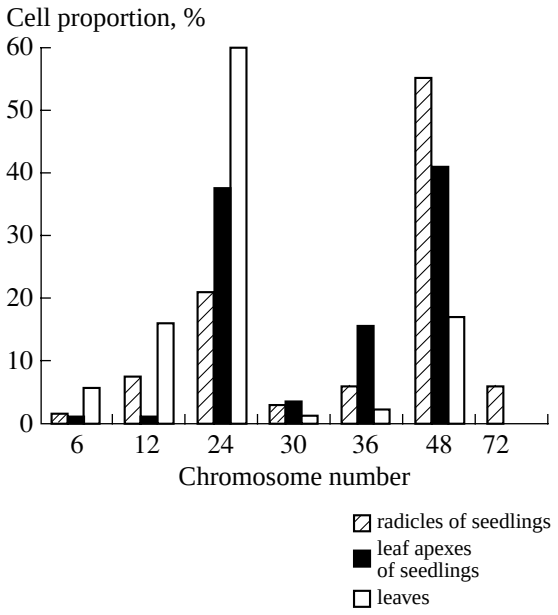

Fig. 2. Frequency distribution of chromosome numbers in intact ginseng plants.

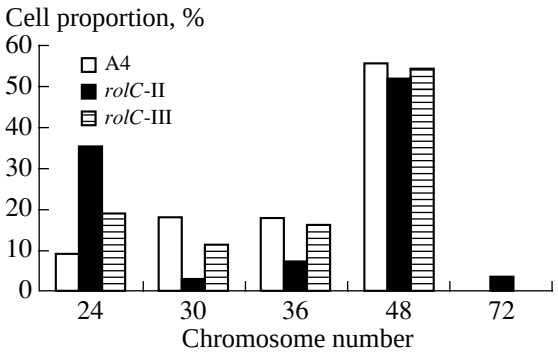

Fig. 3. Frequency distribution of chromosome numbers in transgenic ginseng roots.

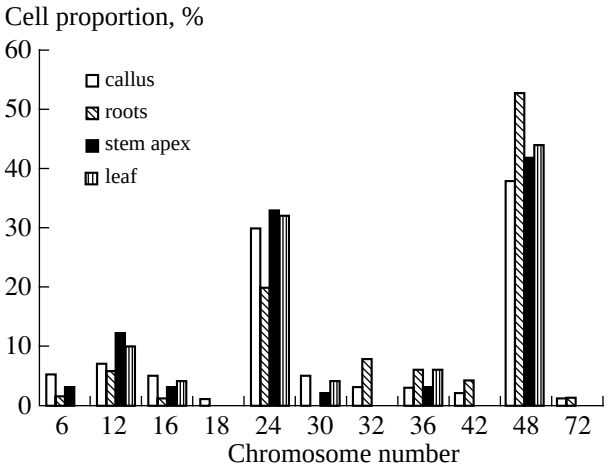

Fig. 4. Frequency distribution of chromosome numbers in teratoma tissues transformed with *rolC*.

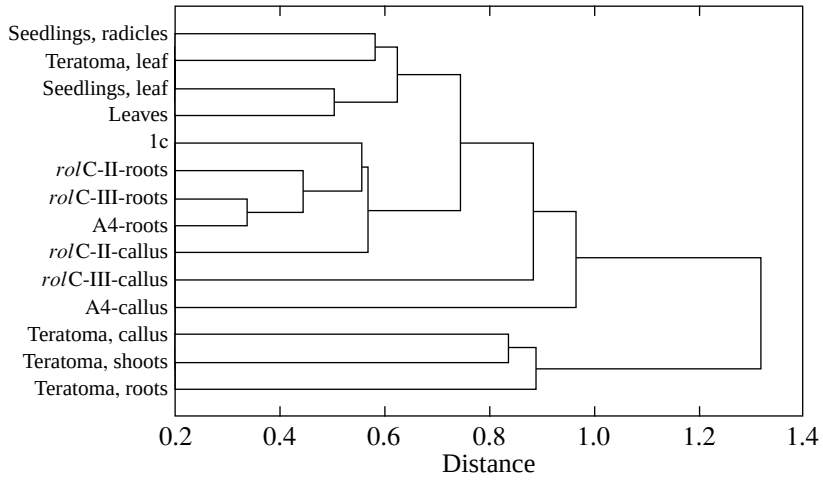

Fig. 5. Cluster analysis of chromosome number in ginseng plants and in initial and transgenic cultures.

ble. First, J. Bercetche *et al.* [3] assumed that roots regenerate from cells of certain ploidy. Second, chromosome number could be stabilized with an increasing level of culture organization (e.g., in rhizogenesis), since roots, but not undifferentiated tissues, possess some specific physiological factors. In addition, euploidy may be advantageous for growth and survival of roots, since in vitro viability of euploid tissues is higher than that of aneuploid tissues [28]. We think that all these factors may contribute to the formation of chromosome number in transgenic hairy roots of various species and, in particular, ginseng. However, it is hardly possible to reconstruct the events that lead to stable ploidy, mostly because the origin of hairy roots is still unclear.

Hairy roots are commonly believed to be a clone originating from a single transformed cell [29]. However, Bercetche *et al.* [3] noted that, although genetic and molecular analyses suggest the clonal origin of transgenic hairy-root cultures, hairy roots are always formed from groups of actively proliferating meristem cells. We observed chromosome polymorphism in transgenic ginseng hairy roots grown from callus tissue. K.M. Ko *et al.* [4] obtained transformed ginseng roots from root and stem plant segments; the resulting hairy-root cultures were monomorphic in chromosome number and, with only one exception, carried 48 chromosomes. To explain this discrepancy, transgenic roots can be assumed to originate not from one, but from several cells which are close together but have different chromosome sets. On the other hand, if the origin of each hairy-root line is clonal, chromosome number may diverge during the formation and proliferation of primary root meristem and further cultivation.

Next, we compared chromosome number in *rolC* and A4 cultures. As seen from the table and Fig. 3, the proportion of cells with $2n = 24$ was higher and that of cells with $2n = 20$ was lower in 1c-*rolC* roots than in 1c-A4 roots, though the difference was not significant (see also the results of cluster analysis). A cardinal difference between the *rolC* and A4 callus cultures was that the former had a higher proportion of polyploid ($6x$, $8x$) cells and were able to form embryoids and teratoma tissue. Cluster analysis showed that, as regards chromosome number, various teratoma tissues (excluding leaves) were the most distant from the initial line 1c and the organs of intact plants (Fig. 5). Teratoma roots and stem apexes displayed high aneuploidy (Fig. 4). Here, we report for the first time the formation of teratomas in *rolC* plant tissues; it remains unclear why *rolC* calluses produce either roots or teratomas. Further biochemical studies on teratomas will provide for a better understanding of the specific frequency distribution of their chromosome numbers.

We assume that the hormonal disbalance caused by the *rolC* gene is one of the factors determining a high chromosome variation in the *rolC* ginseng cultures. J.J. Estruch *et al.* [9] showed that this gene, which codes for glycosidase hydrolyzing cytokinin conjugates, can affect the ratio between free and bound cytokinins in transgenic plants. We previously found that bound cytokinins were accumulated in the 1c culture over a passage, whereas the content of cytokinin–glycosides decreased in the 1c-*rolC*-II culture [14]. The 21-kDa product of the *rolC* gene isolated from transgenic ginseng roots showed glycosidase activity in vitro [14]. Hence, the product of *rolC* possibly acted as glycosidase of cytokinin conjugates in ginseng cells. As compared with the initial culture, the content of free cytokinins and, in particular, auxins is changed in transgenic *rolC* cultures [14]. L.A. Lutova *et al.* [30] regarded the experiments in which the role of *rolC* in the cytokinin release from glycoside conjugates was revealed [9] as largely conditional and noted that their results did not reflect the actual biochemical function of this gene. We disagree with this. Explaining the effect of *rolC* only by the cytokinin-glycosidase activity of its product, rather than the reliable findings of Estruch

et al. [9], is questioned in the two most important recent works on the biochemical role of this gene [10, 31]. The assumption of V.A. Kunakh [11], that phytohormones play a key role in regulating the cytogenetic state of plant cells, seems appealing in light of the above data on the association between chromosome variation and hormones.

To sum up, chromosome number in ginseng cells transformed with the *rolC* plant oncogene was studied for the first time in this work. Transfer of this gene in the ginseng genome increased chromosome variation as compared with that in the initial cell culture, which is possibly explained by the hormone disbalance caused by the oncogene. Chromosome polymorphism in hairy roots regenerated from transformed cells was lower than in tumor cells carrying *rolC* and than in the initial cell line 1c.

In principle, similar cytogenetic changes were associated with the integration of T-DNA and of the *rolC* gene. The difference was that a higher ploidy was characteristic of cells transformed with *rolC*. The causes of the alteration in chromosome number in cells transformed with T-DNA or *rolC* are still unknown. The biochemical role of the oncogenes is also far from clear. Further studies on the association between the expression of individual oncogenes, the activity of their protein products, and cytogenetic variation of cells will contribute to a better understanding of this process.

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