Plant Cell Rep (2009) 28:1273–1278
DOI 10.1007/s00299-009-0728-9

ORIGINAL PAPER

Nucleotide substitutions in *rolC* and *nptII* gene sequences during long-term cultivation of *Panax ginseng* cell cultures

Konstantin V. Kiselev · Anna V. Turlenko · Galina K. Tchernoded · Yuri N. Zhuravlev

Received: 11 April 2009 / Accepted: 29 May 2009 / Published online: 16 June 2009

Abstract It has been shown previously that the *rolC* gene from *Agrobacterium tumefaciens* gene was stably and highly expressed in 15-year-old *Panax ginseng* transgenic cell cultures. In the present report, we analyze in detail the nucleotide composition of the *rolC* and *nptII* (neomycin phosphotransferase) genes, which is the selective marker used for transgenic cell cultures of *P. ginseng*. It has been established that the nucleotide sequences of the *rolC* and *nptII* genes underwent mutagenesis during cultivation. Particularly, 1–4 nucleotide substitutions were found per sequence in the 540 and 798 bp segments of the complete *rolC* and *nptII* genes, respectively. Approximately half of these nucleotide substitutions caused changes in the structure of the predicted gene product. In addition, we attempted to determine the rate of accumulation of these changes by comparison of DNA extracted from *P. ginseng* cell cultures from 1995 to 2007. It was observed that the frequency of nucleotide substitutions for the *rolC* and *nptII* genes in 1995 was 1.21 ± 0.02 per 1,000 nucleotides analyzed, while in 2007, the nucleotide substitutions significantly increased (1.37 ± 0.07 per 1,000 nucleotides analyzed). Analyzing the nucleotide substitutions, we found that substitution to G or to C nucleotides significantly increased (in 1.9 times) in the *rolC* and *nptII* genes compared with *P. ginseng actin* gene. Finally, the level of nucleotide substitutions in the *rolC* gene was 1.1-fold higher when compared with the *nptII* gene. Thus, for the first time, we have experimentally demonstrated the level of nucleotide substitutions in transferred genes in transgenic plant cell cultures.

Keywords *Agrobacterium rhizogenes* · *Panax ginseng* · *rolC* · *nptII* · Cell cultures · Mutagenesis

Communicated by D. Somers.

K. V. Kiselev (✉) · A. V. Turlenko · G. K. Tchernoded · Y. N. Zhuravlev
Laboratory of Biotechnology, Institute of Biology and Soil Science, Far East Branch of Russian Academy of Sciences (FEB RAS), Stoletija Str. 159, 690022 Vladivostok, Russia
e-mail: kiselev@biosoil.ru

Introduction

Transgenic plant cell cultures and transgenic plants attract the attention of scientists in contemporary biotechnology due to their ability to produce biologically active substances (Gómez-Galera et al. 2007). Problems related to host defense mechanisms against alien DNA in plant cells are known to appear during long-term growth and cultivation. Several aspects of such defense mechanisms are currently known. Methylation of promoter and encoding regions are the most commonly recognized defense mechanisms against alien DNA (Fagard and Vaucheret 2000). The methylation of promoter regions is related to expression silencing, while methylation of the encoding regions causes mRNA degradation of transferred genes. Therefore, knowledge of the mechanisms of transgene inheritance in plant cells is necessary for obtaining valuable bioactive substances from plant cell cultures.

In the current literature, the issue of inheritance of stability and expression of transgenes in plant cell cultures in vitro remains poorly understood. However, it has been shown that after 4–5 years of culturing of *Aesculus hippocastanum* and *Catharanthus roseus* transgenic cell lines, the *rol* and green fluorescent protein reporter genes were integrated into the DNA of these cells and expressed (Zdravkovic-Korac et al. 2003; Peebles et al. 2007).

It has also been shown earlier that the *rolC* gene is stably expressed in several 15-year-old *P. ginseng* transgenic cell cultures (Kiselev and Bulgakov 2009). The main goal of our present research was to analyze the rate and level of mutagenesis of the *rolC* oncogene and *nptII* selectable marker gene in *P. ginseng* cell cultures during long-term cultivation.

Materials and methods

Panax ginseng CA Meyer cell cultures

The *P. ginseng rolC* transgenic cell cultures were established from 1992 to 1995 by transformation of the 1c callus with *Agrobacterium tumefaciens* strains GV3101/pMP90RK containing plasmid vector pPCV002-CaMVC (Spena et al. 1987). The scheme of the transgenic cell cultures selection which were used in the present research is presented in Fig. 1. The 1c callus culture was established in 1988 from the stem of a 2-month-old plant of *P. ginseng* var. Mimaki C.A. Meyer. The phenotypic features of the cell cultures and the protocol for *Agrobacterium*-mediated transformation were described earlier (Gorpenchenko et al. 2006; Kiselev et al. 2008). The appearance of hairy roots was registered within a year after transformation. The CII hairy root cell culture was obtained in 1995 by excision the adventitious roots from the 1c-CII tumors and placing into liquid medium. The 2cR2 callus cell culture was obtained in 1996 from the root explants of the CII cell culture.

The *ginseng* culture 2cR2 was cultivated in the dark on W_{4CPA} solid medium (Gorpenchenko et al. 2006) supplemented with 0.4 mg/l *p*-chlorophenoxyacetic acid (4-CPA) at 24–25°C, with 30-day subculture intervals. The CII hairy root culture was cultivated in the dark on W_{4CPA} liquid medium (Gorpenchenko et al. 2006) supplemented with 0.4 mg/l 4-CPA at 24–25°C, with 25-day subculture intervals.

In pPCV002-CaMVC, the *rolC* gene was under control of the cauliflower mosaic virus (CaMV) 35S promoter (Spena et al. 1987). The construct also carried a gene for kanamycin resistance (*nptII*) under control of the nopaline synthase promoter.

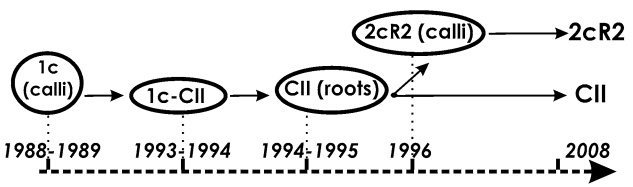

Fig. 1 Selection of *P. ginseng* cultures after transformation of the 1c callus culture with *rolC* and *nptII* genes

Analysis of the *rolC* and *nptII* DNA sequences

Total DNA isolation was performed as described previously (Bulgakov et al. 2005; Kiselev and Bulgakov 2009), and the PCR analysis was performed as described previously (Kiselev et al. 2007; Dubrovina et al. 2009). For this, we used a mix (1:6) of Pfu and Taq polymerases ("Silex M", Russia). The primers 5′-ATG GCT GAA GAC GAC CTG TT and 5′-TTA GCC GAT TGA AAA CTT GCA C were designed on the basis of the *rolC* gene nucleotide sequence (GenBank K03313), and were used for amplification of 540 bp *rolC* transcripts, $T_a = 58$°C, elongation time = 35 s.

The primers 5′-ATG TGG ATT GAA CAA GAT GG and 5′-TCA GAA GAA CTC GTC AAG AA were designed on the basis of the *nptII* gene nucleotide sequence (GenBank AJ414108). These primers were used for amplification of 798 bp *nptII* transcripts, $T_a = 58$°C, elongation time = 50 s. It was not possible to use primers annealing with the T-DNA region out of the *rolC* or *nptII* gene borders. The main problem was that target amplicons produced with the chosen primers were strongly contaminated by non-target amplicons.

The primers 5′-GAT GAC ATG GAA AAG ATT TGG CAT C and 5′-TGT TGT ACG ACC ACT AGC ATA CAG G were designed on the basis of *P. ginseng actin* gene nucleotide sequence (GenBank AY907207), and were used for amplification of 210 bp transcripts, $T_a = 55$°C, elongation time = 20 s.

Screening of DNA (*rolC*, *nptII*) clones

The PCR products were isolated from gels with a Glass Milk kit (Sileks, Russia), and subcloned into a pTZ57R/T plasmid using the InsT/Aclone PCR Product Cloning Kit (FERMENTAS, Vilnius, Lithuania). The clones were amplified with M13 primers and sequenced as described earlier (Kiselev et al. 2006) at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEBRAS using an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The BLAST search program was used for sequence analysis. Multiple sequence alignments were performed using the BioEdit 7.0.8 program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

The amino acid sequences of *rolC* and *nptII* were deduced from the nucleotide sequences with the Gene runner 3.05 program and compared with the known sequences using the BioEdit 7.0.8 and BLAST software programs. For each cell culture, 76–122 clones of *rolC* and *nptII* were sequenced (Table 1). The number of substitutions per 1,000 nucleotides for *rolC* was counted by using the following formula: $(X \times 1{,}000)/((540 - 41) \times Y)$, where *X*—general number of nucleotides substitutions in

Table 1 The number of *rolC* and *nptII* gene clones obtained from the DNA of cell cultures and from the plasmid, pPCV002-CaMVC

The type of analyzed probe	The number of clones with new *rolC* or *nptII* genes	The sum of analyzed clones	New *rolC*, %
nptII gene from pPCV002-CaMVC	4	76	5.3 ± 2.1
rolC gene from pPCV002-CaMVC	4	89	4.9 ± 1.4
nptII from the DNA of the CII cell culture, 1995	64	122	52.4 ± 3.8
nptII from the DNA of the CII cell culture, 2007	56	103	54.4 ± 5.5
nptII from the DNA of the 2cR2 cell culture, 1995	44	96	45.8 ± 2.9
nptII from the DNA of the 2cR2 cell culture, 2007	42	81	54.8 ± 7.7
rolC from the DNA of the CII cell culture, 1995	42	90	45.0 ± 5.0
rolC from the DNA of the CII cell culture, 2007	56	106	52.8 ± 0.4
rolC from the DNA of the 2cR2 cell culture, 1995	47	101	46.6 ± 3.2
rolC from the DNA of the 2cR2 cell culture, 2007	44	79	55.7 ± 7.9

all clones obtained from a certain cell culture; 540 bp is a length of the *rolC* gene part; 41 is a length of primers in nucleotides used for the amplification of *rolC* gene; and *Y* is the total number of analyzed clones of the *rolC* gene. The number of substitutions per 1,000 nucleotides for the *nptII* gene was counted by using an analogous method.

Statistical analysis

Quantity of analyzed clones was summarized from two clones' collections. Clones collection differed in PCR product subcloning in the pTZ57R/T plasmid: PCR products of *rolC* and *nptII* genes for each cell culture were received twice in the independent PCRs. The differences in clone collections were used for the statistical analysis. Statistical analysis was employed using the Statistica 8.0 program. The results are represented as the mean ± standard error and were tested by paired Student's *t* test. The 0.05 level was selected as the point of minimal statistical significance in all analyses.

Results

To begin, we have studied the level of nucleotides substitutions in the sequences of the *rolC* and *nptII* genes, which were obtained from plasmid DNA; however, the level of this variability was not higher than 0.09 nucleotides per 1,000 analyzed. These data are more than 14-fold lower than the nucleotide substitutions for the *rolC* and *nptII* genes from the DNA of the CII and 2cR2 cell cultures. Thus, we are inclined to interpret this variability as part of the polymerase's error rate.

Clones obtained from *rolC*-transgenic calli were heterogeneous, and approximately half of all clones had differences in 1–4 nucleotides from the sequences for *rolC* (K03313) and *nptII* (AJ414108) deposited in Genbank. The length of the *rolC* gene was 540 bp and the length of the *nptII* gene was 798 bp. More than 90 altered nucleotide sequences in the *rolC* and *nptII* genes were found. The majority of these substitutions were sequenced only once, although four altered sequences repeated in independent bacterial transformations by PCR products of the *rolC* and *nptII* genes subcloned to pTZ57R/T, were deposited in Genbank, and named, respectively, *rolC1-Pg* (EU642406), *rolC2-Pg* (EU642407), *rolC3-Pg* (EU642408), *rolC4-Pg* (EU642409).

The highest level of nucleotide substitutions was detected for the *rolC* gene from the *P. ginseng* CII cell culture cultivated in 2007 (Table 2). The lowest level of nucleotide substitutions was shown for the *nptII* gene from the 2cR2 cell culture cultivated in 1995 (Table 2). Furthermore, we have summarized the variability in clones of the *rolC* and *nptII* genes obtained from DNA of the *P. ginseng* cell cultures CII and 2cR2 cultivated in 1995 and 2007. Thus, the general level of nucleotide substitutions of

Table 2 The level of nucleotide substitutions for *rolC* and *nptII* obtained from plasmid DNA and DNA from *P. ginseng* cell cultures, CII and 2cR2

	Plasmid	CII, 1995	CII, 2007	2cR2, 1995	2cR2, 2007
nptII	0.09 ± 0.01	1.24 ± 0.07	1.35 ± 0.13	1.18 ± 0.06	1.23 ± 0.09
rolC	0.09 ± 0.02	1.25 ± 0.05	1.62 ± 0.11	1.18 ± 0.02	1.26 ± 0.07

Data are represented as the frequency of nucleotide substitutions per 1,000 analyzed nucleotides

genes from cell cultures cultivated in 1995 was 1.21 ± 0.02 and in 2007 was 1.37 ± 0.07, which reflects a 13% significant increase ($P < 0.05$). After this, we summarized the nucleotide substitutions in the sequences of the *rolC* and *nptII* genes. The general level of nucleotide substitutions in the sequences of the *rolC* gene was 7% higher than that of *nptII*, but this difference was not significant (1.33 ± 0.07 for *rolC* and 1.24 ± 0.04 for *nptII*, respectively).

Next, we analyzed the type of nucleotide substitutions in the sequences of *rolC* and *nptII* obtained from plasmid DNA and from *P. ginseng* cell cultures CII and 2cR2 DNA (Table 3). As such, nucleotide substitutions in the sequences obtained from plasmid DNA were quite rare (Table 3). The majority of nucleotide substitutions in the sequences of the *rolC* and *nptII* genes obtained from DNA of *P. ginseng* cell cultures CII and 2cR2 belonged to the replacement of thymine to cytosine (36–56%, Table 3). In addition, there were rather large numbers of substitutions of adenine for guanine or guanine for adenine (5–29%, Table 3). There were no A → T, A → C, T → A, A → T, C → G, C → A transitions, except for single instances (Table 3).

Using a similar approach, we then determined the nucleotide substitutions of a *P. ginseng* house-keeping gene (*actin*). For this, 102 clones of regions of the *ginseng actin* gene from DNA from the *P. ginseng* cell culture CII cultivated in 2007 were analyzed. Using detailed analysis of nucleotide sequences, it has been established that four various PCR products were amplified in defined areas of the sequenced region and these products were referred to as gene parts *Pg-act-1*, *Pg-act-2*, *Pg-act-3* and *Pg-act-4*. Unlike *rolC* and *nptII*, for the *actin* gene the initial sequences for purposes of comparison were not known. Therefore, we used *Pg-act-1* as initial sequence, because approximately half of analyzed sequences were identical to *Pg-act-1*. It is obvious from Table 4 that the transitional direction of the *actin* genes from *P. ginseng* versus *rolC* and *nptII* is generally alike (the main aspect of the substitutions reflects the changes T → C, C → T, G → A, and A → G). However, some significant differences between transgenes and house-keeping gene were apparent. For example, the quantity of T → A and C → T replacement was significantly low in *rolC* and *nptII* genes compared with *actin* (in 6.2 and 2.6 times accordingly). No one C → A replacement was detected in *rolC* and *nptII* genes while 4.1 ± 0.1% of C → A replacement was detected in *actin* (Table 4). The quantity of T → C replacements was 1.8 times significantly above in *rolC* and *nptII* genes compared with *actin* (Table 4). Thus, the substitution to G or to C nucleotides significantly increased in the *rolC* and *nptII* genes compared with *P. ginseng actin* gene (in 1.9 times).

Table 3 Different types of nucleotide substitutions in the sequences of *rolC* and *nptII* obtained from plasmid DNA and the *P. ginseng* cell cultures, CII and 2cR2

Nucleotides substitutions	*nptII* (plasmid)	*rolC* (plasmid)	*nptII* (CII, 1995)	*nptII* (CII, 2007)	*nptII* (2cR2, 1995)	*nptII* (2cR2, 2007)	*rolC* (CII, 1995)	*rolC* (CII, 2007)	*rolC* (2cR2, 1995)	*rolC* (2cR2, 2007)
A → T	0	0	0	0	0	0	1.4 ± 1.4 (1)	0	0	0
A → G	25 (1)	25 ± 25 (1)	17.7 ± 2.0 (20)	12.0 ± 5.9 (13)	14.4 ± 1.6 (13)	5.8 ± 0.6 (6)	21.1 ± 4.5 (13)	28.1 ± 3.1 (24)	23.6 ± 2.9 (14)	28.5 ± 1.8 (14)
A → C	0	0	0	0	0	0	0	1.4 ± 1.4 (1)	0	0
T → A	0	25 ± 25 (1)	0	3.9 ± 0.3 (4)	0	0	0	0	0	0
T → G	0	0	3.5 ± 3.5 (5)	4.9 ± 1.3 (5)	3.6 ± 1.0 (3)	1.2 ± 1.2 (1)	5.4 ± 0.3 (3)	2.8 ± 2.8 (2)	6.3 ± 6.3 (3)	0
T → C	0	0	55.9 ± 11.6 (62)	50.3 ± 10.9 (52)	43.4 ± 0.2 (36)	54.2 ± 4.2 (49)	45.1 ± 6.2 (27)	36.5 ± 3.2 (31)	31.6 ± 6.6 (19)	43.1 ± 3.6 (20)
G → T	25 (1)	0	3.9 ± 2.5 (4)	1.8 ± 1.8 (2)	4.7 ± 2.1 (4)	0	5.4 ± 0.3 (3)	0	7.7 ± 4.8 (4)	0
G → A	0	0	5.7 ± 1.4 (7)	7.9 ± 4.4 (8)	7.2 ± 0.5 (6)	10.8 ± 1.7 (10)	5.4 ± 0.3 (3)	13.6 ± 5.3 (12)	10.9 ± 6.7 (7)	9.4 ± 2.8 (5)
G → C	50 (2)	25 ± 25 (1)	5.0 ± 5.0 (7)	8.9 ± 8.9 (10)	3.8 ± 3.8 (3)	5.2 ± 5.2 (5)	8.3 ± 8.3 (3)	1.1 ± 1.1 (1)	6.3 ± 6.3 (3)	8.1 ± 5.2 (3)
C → T	0	25 ± 25 (1)	8.3 ± 6.0 (11)	10.5 ± 0.3 (11)	22.9 ± 0.2 (19)	22.9 ± 2.1 (21)	8.1 ± 3.0 (4)	12.5 ± 4.2 (10)	13.6 ± 1.1 (8)	9.4 ± 2.8 (5)
C → G	0	0	0	0	0	0	0	4.1 ± 4.1 (3)	0	1.5 ± 1.5 (1)
C → A	0	0	0	0	0	0	0	0	0	0

The data are represented as a percentage for the frequency of nucleotide substitutions and the total number of substitutions in parentheses

Table 4 Different types of nucleotide substitutions in the sequences of *P. ginseng actin*, *rolC* and *nptII* genes obtained from CII cell cultures

Nucleotides substitutions	*P. ginseng actin* (CII, 2007)	*rolC*, *nptII* (CII, 2007)
A → T	0.2 ± 0.2 (1)	0
A → G	10.8 ± 0.3 (124)	20.1 ± 8.1 (37)
A → C	3.0 ± 0.1 (34)	0.7 ± 0.7 (1)
T → A	12.4 ± 0.3 (140)	2.0 ± 2.0 (4)*
T → G	3.8 ± 0.2 (43)	3.9 ± 1.1 (7)
T → C	23.6 ± 0.6 (271)	43.4 ± 5.9 (83)*
G → T	0.2 ± 0.2 (1)	0.9 ± 0.9 (2)
G → A	12.4 ± 0.3 (142)	10.8 ± 2.9 (20)
G → C	0 (0)	5.0 ± 3.9 (11)
C → T	29.7 ± 0.3 (337)	11.5 ± 1.0 (21)**
C → G	0 (0)	2.0 ± 2.0 (3)
C → A	4.1 ± 0.1 (46)	0**

Data are presented as a percentage for the frequency of the nucleotide substitutions and the total number of substitutions in parentheses. The nucleotide substitutions in *rolC* and *nptII* genes were calculated from Table 3. See text for comments

* $P < 0.05$; ** The statistical significance ($P < 0.01$) between the frequencies of the nucleotide substitutions of *P. ginseng actin* from *rolC* and *nptII* genes

Discussion

Identification of the rate of nucleotide substitutions in transferred genes in the genome of transgenic plant cell cultures has important scientific meaning. Therefore, for the first time in our research, the level and rate of nucleotide substitutions in the sequences of *rolC* and *nptII* transferred to the plant cell genome have been established experimentally. It has been determined that the general level of nucleotide substitutions for *rolC* and *nptII* from the DNA of *P. ginseng* cell cultures cultivated in 1995 was 1.21 ± 0.02 per 1,000 analyzed nucleotides, and in 2007 was 1.37 ± 0.07, which is a 13% significant increase ($P < 0.05$). Thus, during these 13 years the level of nucleotide substitutions increased and the difference was 0.16 nucleotides per 1,000 analyzed.

High variability in nucleotide composition of *rolC* and *nptII* from the DNA of *P. ginseng* cell cultures cultivated in 1995 is not clear. Transgenic cell cultures of *P. ginseng* used in our research were at the generation stage in 1992, while the selection of transgenic cells and the description of obtained transgenic cell cultures were carried out until 1995. That is why analysis of these cell cultures earlier than 1995 is impossible. Therefore, this high variability in nucleotide composition suggests integrative mistakes and variability during the two-first years of obtaining cell cultures. Perhaps, long-term storage of these cultures in dry conditions and at room temperature has provoked an increase in variability of the *rolC* and *nptII* sequences in samples obtained from 1995. It is known that in certain conditions, such as treatment with chemical reagents (for example with sodium bisulphate), a certain direction of change in DNA sequences may be observed (Olek et al. 1996). This statement can be strengthened by the presence of an increasing number of G → T transitions in samples from 1995, but this proposal requires a special independent study.

About 50% of the cases of nucleotide composition change did not have an impact on protein products from *rolC* and *nptII* (synonymous substitutions); in other cases, protein products of these genes had one to two amino acids changed. As such, analysis of nucleotide substitutions revealed a path of regularity: there were 11 nucleotide substitutions per 12 possible variants detected, whereas T → C transitions were the main part of nucleotide substitutions in the *rolC* and *nptII* genes. Possibly, this regularity has biological meaning, because increasing the number of G and C nucleotides is correlated with an increasing number of possible sites for methylation (GC; GNC, where N is any of four nucleotides). This is very important for further silencing of the transgene (Dieguez et al. 1998). In addition, the variability was greater than 7% in *rolC* gene sequences compared to the selective marker, *nptII*. Perhaps, at the initial stage of transgenic plant cell selection, where *nptII* provides resistance to the antibiotic, the most meaningful genes responsible for the activation of a gene's product are selected out naturally.

It is known that the *Agrobacterium* gene, *rolC*, is contained in non-transgenic tobacco plants (Intrieri and Buiatti 2001). There is a supposition that these genes have been brought into the tobacco genome by means of natural *Agrobacterium*-mediated transformation during tobacco species formation inside the genus several millions years ago, and this event has significantly influenced speciation (Intrieri and Buiatti 2001). Undoubtedly, the level of nucleotide substitutions in the sequences of the *rolC* gene in *P. ginseng* cell cultures is significantly inferior to differences between the *rolC* (K03313) gene and other known *rolC* genes. We found 1.18–1.62 substitutions per 1,000 nucleotides, while *rolC* (K03313) differs from the *rolC* gene from *N. tabacum* in 270 positions per 1,000 nucleotides. The high rate of nucleotide substitutions of such changes and substitutions in the *rolC* gene during such a short period of cultivation (13 years) is astonishing. However, there is information that suggests the meaning of the rate of evolution for various genes may be significantly different. In addition, the rate of substitutions increases as a result of gene duplication and during adaptive radiation as well (Goodman 1981). Data on the rate of nucleotide substitutions in genes transferred into the plant genome are lacking, so the results of our study have a certain value.

Acknowledgments This work was supported by grants from the President of Russia (MK-714.2008.4), the Far East Division of the Russian Academy of Sciences, and of the Russian Science Support Foundation.

References

Bulgakov VP, Veselova MV, Tchernoded GK, Kiselev KV, Fedoreyev SA, Zhuravlev YN (2005) Inhibitory effect of the *Agrobacterium rhizogenes rolC* gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures. Planta 221:471–478

Dieguez MJ, Vaucheret H, Paszkowski J, Scheid OM (1998) Cytosine methylation at CG and CNG sites is not a prerequisite for the initiation of transcriptional gene silencing in plants, but it is required for its maintenance. Mol Gen Genet 259:207–215

Dubrovina AS, Kiselev KV, Veselova MV, Isaeva GA, Fedoreyev SA, Zhuravlev YN (2009) Enhanced resveratrol accumulation in *rolB* transgenic cultures of *Vitis amurensis* correlates with unusual changes in *CDPK* gene expression. J Plant Physiol doi: 10.1016/j.jplph.2009.01.006

Fagard M, Vaucheret H (2000) (Trans) gene silencing in plants: how many mechanisms? Annu Rev Plant Physiol Plant Mol Biol 51:167–194

Gómez-Galera S, Pelacho AM, Gené A, Capell T, Christou P (2007) The genetic manipulation of medicinal and aromatic plants. Plant Cell Rep 26:1689–1715

Goodman M (1981) Decoding the pattern of protein evolution. Prog Biophys Mol Biol 38:105–164

Gorpenchenko TY, Kiselev KV, Bulgakov VP, Tchernoded GK, Bragina EA, Khodakovskaya MV, Koren OG, Batygina TB, Zhuravlev YN (2006) The *Agrobacterium rhizogenes rolC*-gene-induced somatic embryogenesis and shoot organogenesis in *Panax ginseng* transformed calluses. Planta 223:457–467

Intrieri MC, Buiatti M (2001) The horizontal transfer of *Agrobacterium rhizogenes* genes and the evolution of the genus Nicotiana. Mol Phylogenet Evol 20:100–110

Kiselev KV, Bulgakov VP (2009) Stability of the *rolC* gene and its expression in 15-years old cell cultures of *Panax ginseng*. Appl Biochem Microbiol 45:252–258

Kiselev KV, Kusaykin MI, Dubrovina AS, Bezverbny DA, Zvyagintseva TN, Bulgakov VP (2006) The rolC gene induces expression of a pathogenesis-related beta-1,3-glucanase in transformed ginseng cells. Phytochemistry 67:2225–2231

Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN (2007) The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. J Biotechnol 128:681–692

Kiselev KV, Gorpenchenko TY, Tchernoded GK, Dubrovina AS, Grishchenko OV, Bulgakov VP, Zhuravlev YN (2008) Calcium-dependent mechanism of somatic embryogenesis in *Panax ginseng* cell cultures expressing the *rolC* oncogene. Mol Biol 42:243–252

Olek A, Oswald J, Walter J (1996) A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res 24:5064–5066

Peebles CA, Gibson SI, Shanks JV, San KY (2007) Long-term maintenance of a transgenic *Catharanthus roseus* hairy root line. Biotechnol Prog 23:1517–1518

Spena A, Schmulling T, Koncz C, Schell JS (1987) Independent and synergistic activity of *rol-A*, *rol-B* and *rol-C* loci in stimulating abnormal growth in plants. EMBO J 6:3891–3899

Zdravkovic-Korac S, Calic D, Druart PH, Radojevic L (2003) The horse chestnut lines harboring the *rol* genes. Biol Plant 47:487–491