Journal of Plant Physiology 168 (2011) 1280–1285

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

Mutation of *Panax ginseng* genes during long-term cultivation of ginseng cell cultures

Konstantin V. Kiselev*, Olga A. Shumakova, Galina K. Tchernoded

Laboratory of Biotechnology, Institute of Biology and Soil Science, Far East Branch of Russian Academy of Sciences, Stoletija Str. 159, Vladivostok 690022, Russia

ARTICLE INFO

Article history:
Received 3 September 2010
Received in revised form 2 December 2010
Accepted 13 January 2011

Keywords:
Panax ginseng
Cell cultures
Mutagenesis
Nucleotide substitutions

ABSTRACT

It has previously been shown that the nucleotide sequences of the *Agrobacterium rhizogenes rolC* locus and the selective marker *nptII* developed mutations during the long-term cultivation of transgenic cell cultures of *Panax ginseng*. In the present report, we analyzed the nucleotide sequences of selected plant gene families in the 20-year-old *P. ginseng* 1c cell culture and in leaves of cultivated *P. ginseng* plants. We sequenced the *Actin* genes, which are a family of house-keeping genes; the phenylalanine ammonia-lyase (*PAL*) and dammarenediol synthase genes (*DDS*), which actively participate in the biosynthesis of ginsenosides; and the somatic embryogenesis receptor kinase (*SERK*) genes, which control plant development. We demonstrate that the plant genes also developed mutations during long-term cultivation. The highest level of nucleotide substitution was detected in the sequences of the *SERK* genes (2.00 ± 0.11 nt per 1000 nt), and the level was significantly higher when compared with the cultivated *P. ginseng* plant. Interestingly, while the diversity of *Actin* genes was similar in the *P. ginseng* cell culture and the cultivated plants, the diversity of the *DDS* and *SERK* genes was less in the 20-year-old cell culture than in the cultivated plants. In this work, we detail the level of nucleotide substitutions in different plant genes during the long-term culture of plant cells.

1. Introduction

Due to their ability to produce biologically active substances, plant cell cultures have attracted the attention of scientists in recent years (Dicosmo and Misawa, 1995; Gómez-Galera et al., 2007; Shih and Doran, 2009). Plant cell cultures are also important in experimental biology as suitable models for studying different fundamental cell process, e.g. programmed cell death in plants (Reape et al., 2008). It has been demonstrated that the *in vitro* culture of plant tissue induces various mutations, and genetic variation has been observed both in cultured cells and in plants regenerated from cultured cells. These mutations include cytological abnormalities, such as ploidy changes and chromosome rearrangements, single base substitutions, changes in the copy number of repeated sequences, and alterations in DNA methylation patterns (Rani and Raina, 2000; Kaeppler et al., 2000). Those mutations in the plant cell cultures are referred to somaclonal variation. Somaclonal variation has been described for many phenotypes, including plant height, plant biomass, grain yield, disease and insect resistance, acid and salt tolerance, and agronomic performance (Carver and Johnson, 1989; Dahleen et al., 1991; Duncan et al., 1997; Bregitzer et al., 1998; Veilleux and Johnson, 1998). The mechanisms producing both somatically and meiotically heritable variations can contribute to the decline in the vigor and regenerability of cultures over time (Kaeppler et al., 2000). Studying mutagenesis in plant cell culture is important because it improves our understanding of evolutionary processes and may help avoid losses when using plant cell cultures for commercial production of biologically active compounds.

It has been previously shown that the nucleotide sequences of the *Agrobacterium rhizogenes rolC* locus and the selective marker *nptII* developed mutations during the long-term cultivation of transgenic cell cultures of *Panax ginseng* (Kiselev et al., 2009a). In particular, 1–4 nucleotide substitutions were found in the complete *rolC* and *nptII* genes sequences. However, these nucleotide substitutions had no effect on *rolC* and *nptII* gene expression, and the *rolC* and *nptII* genes were expressed even after the 15 year cultivation of transgenic *P. ginseng* cell cultures (Kiselev and Bulgakov, 2009). Although we have previously described the nucleotide substitutions in the sequence of the transferred genes in plant cells (Kiselev et al., 2009a), there is little information regarding the single base substitutions present in plant genes that accumulate during long-term cultivation. For example, two tissue culture-derived mutant *Adh1* alleles were found to be to the result of two independent A → T transversions (Dennis et al., 1987). Through the comparison of sequences obtained from either the *P. ginseng* 1c cell culture,

Abbreviations: DDS, dammarenediol synthase; nt, nucleotides; PAL, phenylalanine ammonia-lyase; SERK, somatic embryogenesis receptor kinase.

* Corresponding author. Fax: +7 4232 310193.
E-mail address: kiselev@biosoil.ru (K.V. Kiselev).

0176-1617/$ – see front matter.
doi:10.1016/j.jplph.2011.01.028

which has been cultivated for more than 20 years, or cultivated plants, the aim of this study was to determine if plant genes undergo a similar rate of nucleotide substitution during long-term cultivation.

2. Material and methods

2.1. P. ginseng cell culture 1c

The 1c callus culture was established in 1988 from the stem of a 2-month-old plant of *Panax ginseng* var. Mimaki C.A. Meyer. Culture 1c was cultivated in the dark on solid W medium (Kiselev et al., 2009b) supplemented with 0.4 mg/l p-chlorophenoxyacetic acid (4-CPA) at 24–25 °C, with a 30 day subculture interval. We used these *P. ginseng* cell cultures (including transgene cell cultures cultivated in the same conditions), because they have been cultivated for the longest period of time in our lab (more than 20 years).

Importantly, the control 1c culture looks like an actively growing callus culture and did not show any signs of morphological differentiation. Using transplantation into initiating medium and exposure to light (Kiselev et al., 2008), we were not able to induce embryogenesis in the 1c culture.

2.2. Plant material

Wild *P. ginseng* plants were sampled from a non-protected natural population in Sikhote-Alin. The collected living plants were transferred to an open experimental nursery and kept under conditions that were similar to the natural ginseng habitat (Spassky District of the Primorsky Kray) for further investigation. The best negative control to study mutagenesis in plant cell cultures during long-term cultivation is using tissues of the plants from which the tested cell cultures have been established. Unfortunately, we do not have these plants. Therefore, we used the leaves of other *P. ginseng* plants growing in the same regions in Russia: the Spassky Region of Primorsky Kray and Sikhote-Alin. We reasoned that the leaves are applicable as negative controls in our case.

The experiments used leaves that were collected from 2-year-old plants originally collected in 2003 and stored in dry conditions. In the present work, we used leaves from two different plants: plant # 1 (PL1) and plant # 2 (PL2).

2.3. Analysis of the Actin, PAL, DDS, and SERK sequences

The isolation of total DNA was performed as described previously (Kiselev and Bulgakov, 2009), and the PCR analysis was performed as described previously (Kiselev et al., 2007; Dubrovina et al., 2009). We used a mix (1:6) of Pfu and Taq polymerases ("Silex M", Russia) (Kiselev and Bulgakov, 2009; Kiselev et al., 2009a). We sequenced a house-keeping gene (*P. ginseng Actin*), genes that actively participate in the biosynthesis of ginsenosides (phenylalanine ammonia-lyase, *PAL*; dammarenediol synthase gene, *DDS*), and a gene that controls plant development (somatic embryogenesis receptor kinase, *SERK*). The tested gene families are highly (*Actin*, *PAL*) or moderately (*SERK*, *DDS*) expressed in the *P. ginseng* cell cultures. Expression patterns of the analyzed genes in ginseng plant cell cultures were published previously (Kiselev et al., 2008; Kiselev and Tchernoded, 2009; Kiselev et al., 2009c).

The primers 5′-GAT GAC ATG GAA AAG ATT TGG CAT C-3′ and 5′-TGT TGT ACG ACC ACT AGC ATA CAG G-3′ were designed based on the *P. ginseng Actin* sequence (GenBank AY907207) and were used for the amplification of a 210 bp PCR product of the central coding part of the actin genes, with an annealing temperature of 55 °C and an elongation time of 20 s (Kiselev et al., 2006). The degenerate primers 5′-GAR GCY GCY GCY ATY ATG GA-3′ and 5′-GGR GTG CCY TGR AAR TT-3′ (Persiyanova et al., 2008; Kiselev et al., 2009b) were used for the amplification of a 266 bp PCR product of the central coding part of the *PAL* genes (PAL core domain), with an annealing temperature of 55 °C and an elongation time of 16 s. The primers 5′-AGT TAC AAC CGC TGT GAA GAA A-3′ and 5′-TAC TGA CCC AAT CAT CGT GCT G-3′ were designed based on the *P. ginseng DDS* sequence (GenBank AB122080) and were used for the amplification of a 718–721 bp PCR product of the central coding part of the *DDS* genes with 2 introns (about 110 and 320 bp), with an annealing temperature of 57 °C and an elongation time of 20 s. The degenerate primers 5′-ACT GGA GCA ATA GCK GGW GGA GT-3′ and 5′-GCC ATG TAA GGA TAM ACA AGC AA-3′ (Kiselev and Tchernoded, 2009) were used for the amplification of a 505–512 bp PCR product of the central coding part of the *SERK* genes (kinase domain) with 90 bp intron, with an annealing temperature of 55 °C and an elongation time of 41 s.

2.4. Screening of Actin, PAL, DDS, and SERK clones

The PCR products were isolated from gels with using a Glass Milk Kit (Sileks, Russia) and subcloned into the pTZ57R/T plasmid using the InsT/Aclone PCR Product Cloning Kit (FERMENTAS, Vilnius, Lithuania). The clones were amplified using M13 primers and sequenced, as described previously (Kiselev et al., 2006; Kiselev and Dubrovina, 2010), at the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEBRAS using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The BLAST program was used for sequence analysis. Multiple sequence alignments were performed using the BioEdit 7.0.8 program (www.mbio.ncsu.edu/BioEdit/bioedit.html).

For each cell culture, 91–120 *Actin*, *PAL*, *DDS*, and *SERK* clones were sequenced. Importantly, all of the examined genes are multi-gene families. The representative members of these multi-gene families have already been described (Persiyanova et al., 2008; Kiselev and Tchernoded, 2009). We categorized these multi-gene families according to the nucleotide sequences, and if a PCR product differed by two and more nucleotides from the previously described gene and if it was sequenced several times from clones obtained from different transformations, we considered it a novel sequence. In rare instances, we separated a new sequence from the previously described genes if it differed by only one nucleotide and if it was consistently detected in sequences from different transformations.

The designation of the sequenced genes depends upon the level of the differences in the nucleotides sequences from previously sequenced genes. The designation consists of three terms (e.g., 1a1). For example GENE1a1 differed slightly from GENE1a2 by 2–4 nucleotides, GENE1a1 was intermediately different from GENE1b1 by 4–10 nucleotides, and GENE1a1 strongly differed from GENE2a1, usually by more than 10 nucleotides.

The number of substitutions per 1000 nt was determined using the following formula: $(Ns \times 1000)/((G-P) \times Nc)$, where Ns is the general number of nt substitutions in all clones obtained from a certain cell culture; G is the length of the analyzed gene fragment; P is the length of the primers (in nt) used for the amplification of the analyzed gene; and Nc is the total number of analyzed clones for the analyzed gene.

The amino acid sequences of the ginseng fragments of Actin, PAL, DDS, and SERK were deduced from the nucleotide sequences with the Gene runner 3.05 program and compared with the earlier known Actin, PAL, DDS, and SERK sequences of *P. ginseng*, using the BioEdit 7.0.8 and BLAST software programs.

2.5. Statistical analysis

The total number of analyzed clones is the result of three collections of clones. Three independent amplifications of the *Actin*, *PAL*, *SERK*, and *DDS* genes from each cell culture were carried out. The

differences from the individual clones were used for the statistical analysis. Statistical analysis was carried out using the Statistica 8.0 program. The results are presented as the mean ± standard error and were tested using a paired Student's *t*-test. A *p* value less than 0.05 was considered statistically significant.

3. Results

Originally, we organized the information regarding the quantity of the *Actin*, *PAL*, *DDS*, and *SERK* sequences in accordance with the scheme described above. Overall, 11, 12, 7, and 8 sequences from the *Actin*, *PAL*, *DDS*, and *SERK* genes, respectively, amplified from the 1c culture contained nucleotide differences (Figs. 1 and 2). We isolated 10–11, 17–19, 8, and 9–11 *Actin*, *PAL*, *DDS*, and *SERK* sequences, respectively, amplified from cultivated plants containing nucleotide differences (Figs. 1 and 2). Therefore, the diversity of *Actin* sequences from the 1c cell culture and in cultivated plants was similar. In contrast, the diversity of *PAL*, *DDS*, and *SERK* sequences was greater in the cultivated plants than in the 1c culture. Furthermore, approximately 50% of nucleotide substitutions in the DNA of the analyzed genes of 1c culture did not alter the amino acid sequence of the genes products (silent mutations). There were not nonsense mutations. The number of the synonymous substitutions in the analyzed genes in the cultivated plants was 1.5 times higher ($p < 0.05$) than in 1c culture.

We also analyzed the rate of the nucleotide substitutions in the selected plant genes. The highest rate of nucleotide substitution was detected in the *Actin*, *PAL*, and *SERK* genes of the 1c culture (Table 1), while the lowest rate of nucleotide substitution was detected in the *DDS* gene of the cultivated plants (Table 1). In addition, we analyzed the variability of the *Actin*, *PAL*, *DDS*, and *SERK* genes clones from the 1c culture and the cultivated *P. ginseng* plants. The general rate of nucleotide substitution in the 1c culture and the cultivated plants was 1.09 ± 0.12 and 0.67 ± 0.11, respectively. The rate of nucleotide substitution was lower in the cultivated plants than the 1c culture (a 38% decrease, $p < 0.05$).

We also analyzed the types of nucleotide substitution that were detected (Table 2). Interestingly, the most frequent nucleotide substitutions in the *Actin*, *PAL*, *DDS*, and *SERK* genes from the 1c culture and the cultivated plants were similar. The most frequent nucleotide substitutions were A → G (11.5–55.2%, Table 2), T → C (11.5–41.1%), G → A (10.6–36.4%), and C → T (5.6–42.8%). There were no A → T, A → C, T → A, T → G, G → T, G → C, C → G, or C → A transversions, except for single instances (Table 2). 23.4% of the substitutions in the *Actin* gene from the 1c culture were T → A transversions.

Finally, we compared the type of nucleotide substitutions detected in the *Actin*, *PAL*, *DDS*, and *SERK* genes of ginseng plants and the 1c culture to the type of nucleotide substitutions detected in the *rolC* and *nptII* genes, which were transferred to genome of

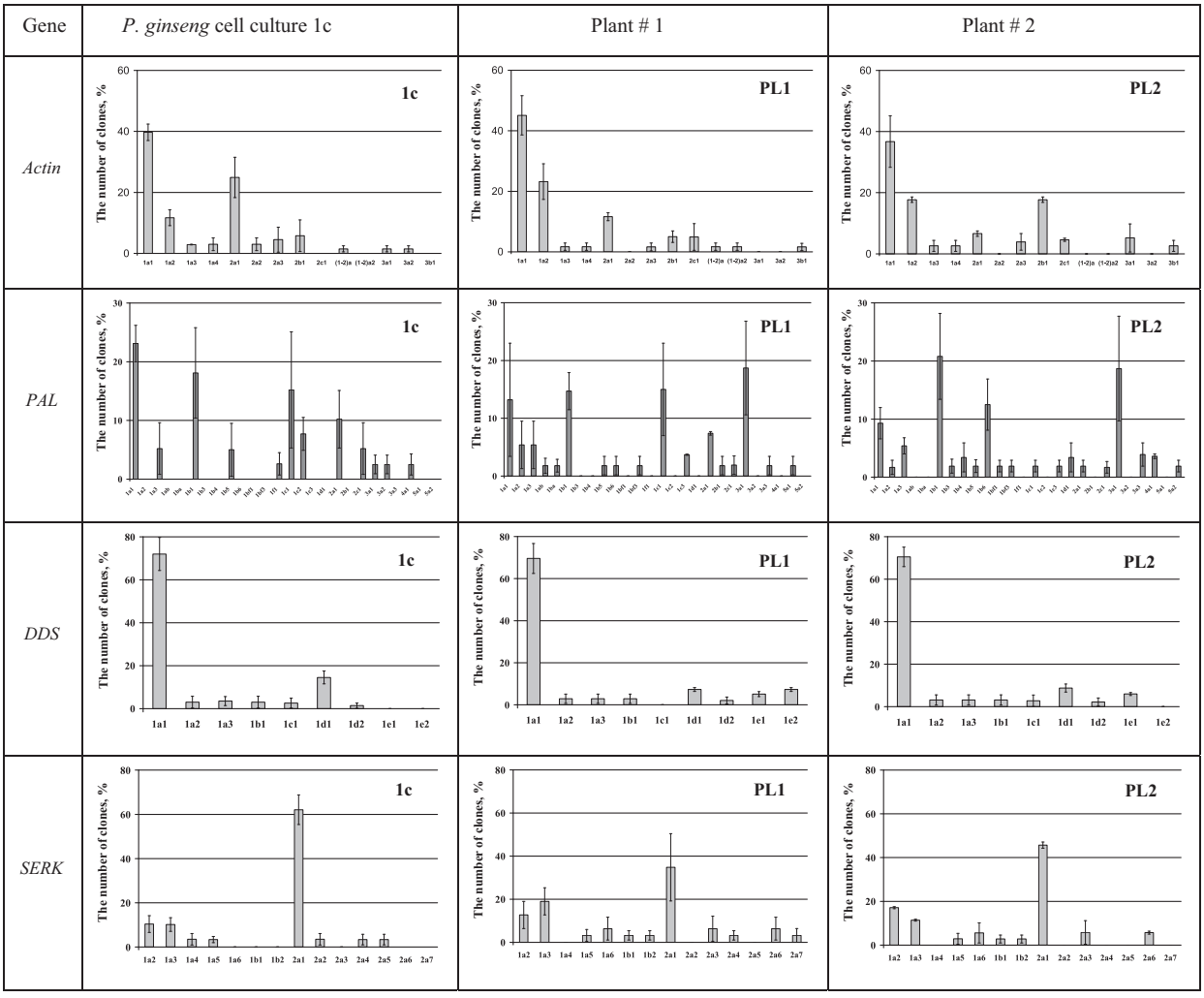

Fig. 1. The number of partial sequences of the *Actin*, *PAL*, *DDS*, and *SERK* genes obtained from DNA isolated from the 1c culture and cultivated *P. ginseng* plants. The data are presented as the percentage of *P. ginseng Actin*, *PAL*, *DDS*, and *SERK* genes in the analyzed sequences.

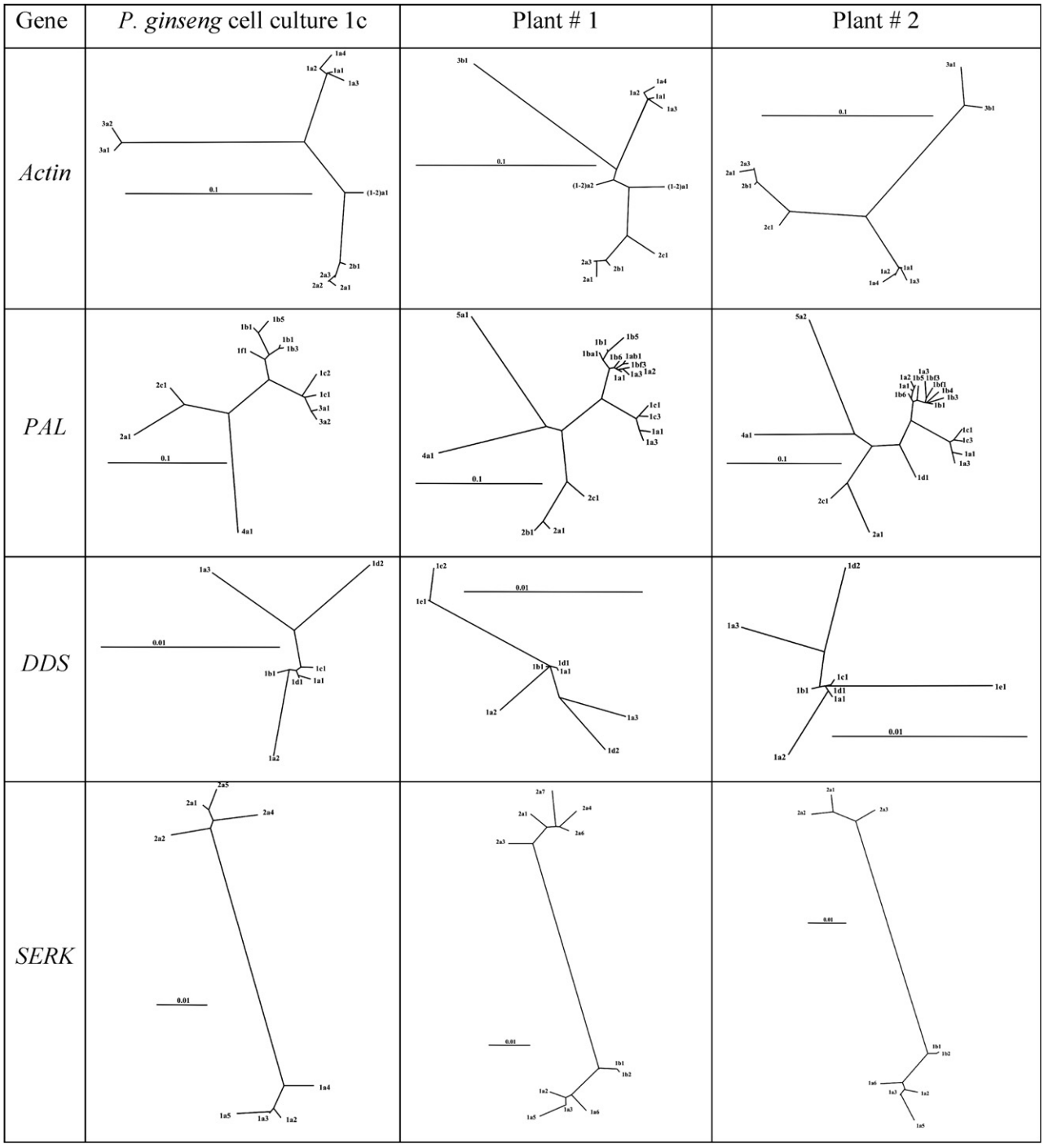

Fig. 2. Phylogenetic relationship of the *P. ginseng Actin*, *PAL*, *DDS*, and *SERK* genes obtained from DNA isolated from the 1c culture or cultivated plants.

several ginseng cell cultures and cultivated 15 years ago (Kiselev et al., 2009a). Overall, the type of nucleotide substitutions detected in the *Actin*, *PAL*, *DDS*, and *SERK* genes was similar to those detected in the *rolC* and *nptII* genes (Table 3). We observed only two differences: in the *rolC* and *nptII* genes, the number of T → G and T → C substitutions was significantly higher compared to the level of these substitutions in the *Actin*, *PAL*, *DDS*, and *SERK* genes. The total level of the N → G or N → C nucleotide substitutions in the *rolC*

Table 1
The rate of nucleotide substitutions in the partial *P. ginseng Actin*, *PAL*, *DDS*, and *SERK* sequences obtained from the 1c culture or cultivated *P. ginseng* plants. The data are presented as the frequency of nucleotide substitutions per 1000 nt.

	Actin	PAL	DDS	SERK
P. ginseng plant cell culture 1c	1.21 ± 0.29	1.07 ± 0.31	0.26 ± 0.06	2.00 ± 0.11
Cultivated *P. ginseng* plant # 1	0.94 ± 0.04	0.68 ± 0.08	0.07 ± 0.03*	1.02 ± 0.04**
Cultivated *P. ginseng* plant # 2	1.04 ± 0.07	0.61 ± 0.07	0.11 ± 0.04*	1.12 ± 0.05*

The asterisks indicate significant differences ($p < 0.05$, *; $p < 0.01$, **) between the *P. ginseng* 1c culture and the cultivated plants.

Table 2
The different types of nucleotide substitutions observed in the *Actin*, *PAL*, *DDS*, and *SERK* sequences obtained from the 1c culture or cultivated *P. ginseng*. The data are presented as the frequency (in percent) of the observed nucleotide substitutions and the total number of substitutions (in parentheses).

Nucleotide substitution	*Actin* from 1c	*PAL* from 1c	*SERK* from 1c	*DDS* from 1c	*Actin* from PL1	*PAL* from PL1	*SERK* from PL1	*DDS* from PL1
A → T	0 (0)	0 (0)	7.7 ± 0.1 (4)	0 (0)	0 (0)	0 (0)	6.4 ± 3.6 (2)	0 (0)
A → G	**17.2 ± 4.1 (4)**	**14.0 ± 7.1 (6)**	**11.5 ± 2.2 (6)**	**47.8 ± 7.9 (6)**	**55.2 ± 2.9 (10)**	**29.3 ± 0.4 (10)**	**19.8 ± 3.1 (6)**	**43.8 ± 6.3 (4)**
A → C	6.3 ± 3.3 (2)	0 (0)	27.0 ± 2.2 (14)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
T → A	23.4 ± 0.8 (6)	4.7 ± 2.4 (2)	3.8 ± 2.2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	6.3 ± 6.3 (1)
T → G	0 (0)	0 (0)	0 (0)	5.6 ± 5.6 (1)	0 (0)	0 (0)	0 (0)	0 (0)
T → C	**17.2 ± 4.1 (4)**	**14.0 ± 7.1 (6)**	**11.5 ± 2.2 (6)**	**41.1 ± 4.9 (5)**	**32.8 ± 4.3 (6)**	**17.3 ± 1.7 (6)**	**27.4 ± 8.9 (8)**	**20.9 ± 4.2 (3)**
G → T	0 (0)	13.2 ± 10.2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
G → A	**17.2 ± 4.1 (4)**	**36.4 ± 1.6 (12)**	**11.5 ± 2.2 (6)**	**0 (0)**	**0 (0)**	**10.6 ± 5.8 (4)**	**19.2 ± 10.8 (6)**	**14.6 ± 2.1 (2)**
G → C	6.3 ± 3.3 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
C → T	**6.3 ± 3.3 (2)**	**17.8 ± 7.9 (4)**	**7.7 ± 0.1 (4)**	**5.6 ± 5.6 (1)**	**12.0 ± 7.2 (2)**	**42.8 ± 7.9 (14)**	**21.0 ± 12.4 (6)**	**14.6 ± 2.1 (2)**
C → G	6.3 ± 3.3 (2)	0 (0)	15.4 ± 1.1 (8)	0 (0)	0 (0)	0 (0)	6.4 ± 3.6 (2)	0 (0)
C → A	0 (0)	0 (0)	3.8 ± 2.2 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The types of nucleotide substitutions that provided the major part of the 12 possible substitutions are shown in bold.

and *nptII* genes was 8% higher compared to the *Actin*, *PAL*, *DDS*, and *SERK* genes.

4. Discussion

The *P. ginseng* 1c cell culture has been passed onto fresh nutrient medium monthly for more than 20 years, which is approximately 250 passages. The variability of the partial *PAL*, *DDS*, and *SERK* sequences from the 1c culture was lower compared to cultivated *P. ginseng* plants, with the exception of the *Actin* sequences. The same change of genetic diversity of cell cultures growing on solid media was demonstrated for 18S–25S rDNA (Andreev et al., 2005). We propose that during the 20 year cultivation, there was a reduction in the *PAL*, *DDS*, and *SERK* gene copy number in the 1c culture compared to the cultivated *P. ginseng* plants. Additionally, the obtained results show that the analyzed genes contain nucleotide substitutions that cannot be attributed to polymerase errors because the detected nucleotide substitutions were more frequent than the polymerase error rate. Previously, we also examined the rate of nucleotide substitution in the *rolC* and *nptII* genes obtained from plasmid DNA; however, the substitution rate was not higher than 0.08 nt per 1000 nt (Kiselev et al., 2009a). We interpreted this variability (0.08 nt per 1000 nt) to be the result of the error rate of the polymerase. Moreover, the nucleotide substitutions cannot be attributed to new copies of the genes because the sequences were not reproducible in independent transformations. It is noteworthy that a specific number of nucleotide substitutions were found in the DNA of cultivated *P. ginseng* plants; however, it was significantly lower compared to the number detected in the 1c culture. These results indicate that the number of mutations has increased during the long-term cultivation of *P. ginseng* callus cultures and cultivated plants.

Previously, we demonstrated that the frequency of nucleotide substitution in two foreign genes transferred to plant cells 15 years ago (*rolC* and *nptII* genes) was 1.21–1.37 nt per 1000 nt (Kiselev et al., 2009a). Therefore, the rate of nucleotide substitution in ginseng genes of either 2-year-old cultivated ginseng plants or the 20-year-old 1c cell culture was lower than rate of substitution in the *rolC* and *nptII* genes, which were transferred to ginseng cells 15 years ago. Also, we analyzed the types of nucleotide substitutions in the sequences of the transferred *rolC* from *A. rhizogenes* and *nptII* genes in *P. ginseng* cells (Kiselev et al., 2009a), and these data can be compared with the present results. It appears that the transgenes and the *Actin*, *PAL*, *DDS*, and *SERK* genes were exposed to similar mutational processes both in the *P. ginseng* cell culture and plants: most of the substitutions were A ↔ G or T ↔ C transitions. The potential reason of the observed substitutions is the increasing of the variety in the protein products of the analyzed genes. We show that approximately 30% of substitutions in the 1c culture are missense mutations which alter the amino acid sequences of the genes products.

However, in the *Actin*, *PAL*, *DDS*, and *SERK* genes, the nucleotide substitutions were more regularly distributed between the 12 possible variants compared to the *rolC* and *nptII* transgenes (Table 3). However, we observed 23.4% and 27.0% of the substitutions in the *Actin* and *SERK* genes from the 1c culture were T → A and A → C substitutions, these substitutions were uncommon among the other genes in the cultivated ginseng plants and the 1c culture. These particular substitutions in the sequenced fragment of *Actin* and *SERK* genes in the 1c culture may be a local mutational process in the *Actin* and *SERK* genes family of the cultivated *P. ginseng* cells.

Perhaps, the distribution of the different types of nucleotide substitutions between *rolC* and *nptII* genes and plant genes has a functional meaning because the increased number of G and C nucleotides results in an increased number of possible methylation sites (GC; GNC, where N is any nucleotide). The increased number of methylation sites likely results in the silencing of a transgene (Dieguez et al., 1998).

This study significantly extends our current understanding of the number and types of nucleotide substitutions that occur in plant genes during the long-term cultivation of a plant cell culture. After 20 years of cultivation, we observed a considerable increase in the number of nucleotide substitutions and the absence of some *PAL*, *DDS*, and *SERK* gene copies in the 1c culture. It is possible that these processes are the main reasons underlying the decline in the vigor and regenerability of cell cultures over time. For example, the ginseng cell culture 1c accumulates only small amounts of ginsenosides (less than 0.01% dry wt) and is not capable of regenerating ginseng plants, only single leaf-like and embryo-like structures (Kiselev et al., 2008).

Importantly, if we do not elucidate the mechanisms underlying the increased mutation rate in plant cell cultures, their use for various purposes requiring long-term cultivation will not be efficient. Specific mutations may be the result of the activity of certain types of transposons, the infidelity of DNA synthesis or repair mechanisms, or base instability. It is known about culture-induced activation of transposons (Okamoto and Hirochika, 2000), also synthesis or repair mechanisms may have less stability in culture. Phillips et al. (1994) suggested the hypothesis that duplicate sequences in the genome, which peacefully coexisted under normal cellular conditions, begin to interact under the cultivation *in vitro* inducing a mutagenic process. Therefore, understanding the mechanisms responsible for the changes observed following long-term tissue culture will be useful in defining the cellular mechanisms that act during evolution and in elucidating the mechanism(s) by which plants respond to stress.

Table 3
The different types of nucleotide substitutions observed in the *Actin*, *PAL*, *DDS*, and *SERK* sequences and the *rolC* and *nptII* genes (Kiselev et al., 2009a) of the 1c culture and cultivated *P. ginseng* plants. The data are presented as the percentage of the observed nucleotide substitutions.

	A→T	A→G	A→C	T→A	T→G	T→C	G→T	G→A	G→C	C→T	C→G	C→A
Plant genes, present work	2.02 ± 1.3	29.5 ± 6.22	4.71 ± 3.81	4.52 ± 3.23	0.8 ± 0.8	23.01 ± 3.51	1.82 ± 1.65	13.4 ± 4.11	0.9 ± 0.9	15.11 ± 4.32	4.01 ± 2.2	0.53 ± 0.45
rolC and *nptII* genes (Kiselev et al., 2009)	0.18 ± 0.08	18.89 ± 8.39	0.18 ± 0.07	0.48 ± 0.32	3.44 ± 0.88*	44.99 ± 2.71**	2.94 ± 0.88	8.88 ± 1.15	5.85 ± 1.67	13.51 ± 1.67	0.71 ± 0.51	0

The asterisks indicate significant differences ($p < 0.05$, *; $p < 0.01$, **) between the averaged nucleotide substitutions observed in the *rolC* and *nptII* genes and the *Actin*, *PAL*, *DDS*, and *SERK* genes.

Acknowledgments

The authors express their thanks to Alexandra S. Dubrovina for helpful comments on the manuscript. This work was supported by grants from the Russian Foundation for Basic Research (10-04-00189-a), the Far East Division of the Russian Academy of Sciences, and the Russian Science Support Foundation.

References

Andreev IO, Spiridonova KV, Solovyan VT, Kunakh VA. Variability of ribosomal RNA genes in Rauwolfia species: parallelism between tissue culture-induced rearrangements and interspecies polymorphism. Cell Biol Int 2005;29:21–7.
Bregitzer P, Halbert SE, Lemaux PG. Somaclonal variation in the progeny of transgenic barley. Theor Appl Genet 1998;96:421–5.
Carver BF, Johnson BB. Partitioning of variation derived from tissue culture of winter wheat. Theor Appl Genet 1989;78:405–10.
Dahleen LS, Stuthman DD, Rines HW. Agronomic trait variation in oat lines derived from tissue culture. Crop Sci 1991;31:90–4.
Dennis ES, Brettell RIS, Peacock WJ. A tissue culture induced ADH1 null mutant of maize results from a single base change. Mol Gen Genet 1987;210:181–3.
Dieguez MJ, Vaucheret H, Paszkowski J, Scheid OM. 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 1998;259:207–15.
Dicosmo F, Misawa M. Plant-cell and tissue-culture – alternatives for metabolite production. Biotechnol Adv 1995;13:425–53.
Dubrovina AS, Kiselev KV, Veselova MV, Isaeva GA, Fedoreyev SA, Zhuravlev YN. Enhanced resveratrol accumulation in *rolB* transgenic cultures of *Vitis amurensis* correlates with unusual changes in *CDPK* gene expression. J Plant Physiol 2009;166:1194–206.
Duncan RR. Tissue culture-induced variation and crop improvement. Adv Agron 1997;58:201–40.
Gómez-Galera S, Pelacho AM, Gené A, Capell T, Christou P. The genetic manipulation of medicinal and aromatic plants. Plant Cell Rep 2007;26:1689–715.
Kaeppler SM, Kaeppler HF, Rhee Y. Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 2000;43:179–88.
Kiselev KV, Kusaykin MI, Dubrovina AS, Bezverbny DA, Zvyagintseva TN, Bulgakov VP. The *rolC* gene induces expression of a pathogenesis-related beta-1,3-glucanase in transformed ginseng cells. Phytochemistry 2006;67:2225–31.
Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN. The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. J Biotechnol 2007;128:681–92.
Kiselev KV, Gorpenchenko TY, Tchernoded GK, Dubrovina AS, Grishchenko OV, Bulgakov VP, Zhuravlev YN. Calcium-dependent mechanism of somatic embryogenesis in *Panax ginseng* cell cultures expressing the *rolC* oncogene. Mol Biol 2008;42:243–52.
Kiselev KV, Bulgakov VP. Stability of the *rolC* gene and its expression in 15-years old cell cultures of *Panax ginseng*. Appl Biochem Microbiol 2009;45:252–8.
Kiselev KV, Tchernoded GK. Somatic embryogenesis in the *Panax ginseng* cell culture induced by the *rolC* oncogene is associated with increased expression of *WUS* and *SERK* genes. Russ J Genet 2009;45:445–52.
Kiselev KV, Turlenko AV, Tchernoded GK, Zhuravlev YN. Nucleotide substitutions in rolC and nptII gene sequences during long-term cultivation of Panax ginseng cell cultures. Plant Cell Rep 2009a;28:1273–8.
Kiselev KV, Dubrovina AS, Bulgakov VP. Phenylalanine ammonia-lyase and stilbene synthase gene expression in rolB transgenic cell cultures of Vitis amurensis. Appl Microbiol Biotechnol 2009b;82:647–55.
Kiselev KV, Turlenko AV, Zhuravlev YN. *CDPK* gene expression in somatic embryos of *Panax ginseng* expressing *rolC*. Plant Cell Tiss Organ Cult 2009c;99:141–9.
Kiselev KV, Dubrovina AS. A new method for analysing gene expression based on frequency analysis of RT-PCR products obtained with degenerate primers. Acta Physiol Plant 2010;32:495–502.
Okamoto H, Hirochika H. Efficient insertion mutagenesis of Arabidopsis by tissue culture-induced activation of the tobacco retrotransposon Tto1. Plant J 2000;23:291–304.
Persiyanova EV, Kiselev KV, Bulgakov VP, Timchenko NF, Chernoded GK, Zhuravlev YN. Defense response mechanisms of ginseng callus cultures induced by *Yersinia pseudotuberculosis*, a human pathogen. Russ J Plant Physiol 2008;55:748–55.
Phillips RL, Kaeppler SM, Olhoft P. Genetic instability of plant tissue cultures: breakdown of normal controls. Proc Natl Acad Sci USA 1994;91:5222–6.
Rani V, Raina SN. Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol – Plant 2000;36:319–30.
Reape TJ, Molony EM, McCabe PF. Programmed cell death in plants: distinguishing between different modes. J Exp Bot 2008;59:435–44.
Shih SM, Doran PM. Foreign protein production using plant cell and organ cultures: advantages and limitations. Biotechnol Adv 2009;27:1036–42.
Veilleux RE, Johnson AT. Somaclonal variation: molecular analysis, transformation interaction, and utilization. Plant Breed Rev 1998;16:229–68.