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

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**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.

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**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 processes, 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 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 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,
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. Minoraki 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-chloroanoxenic 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 Sikkhote-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 (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.

2.3. 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 Milk Kit” Sileks, Russia) and subcloned into the pTZ5R/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 FEIRES 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 gene 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 immediately 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 callus 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>P. ginseng cell culture 1c</th>
<th>Plant # 1</th>
<th>Plant # 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERK</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 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.
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.

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

The asterisks indicate significant differences (p < 0.05, *; p < 0.01, **) between the P. ginseng 1c culture and the cultivated plants.
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 185–255 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.
The different types of nucleotide substitutions observed in the Actin, PAL, DDS, SERK, and nptII genes (Kiselev et al., 2009a) of the 1c culture and cultivated P. ginseng the percentage of the observed nucleotide substitutions.

<table>
<thead>
<tr>
<th>A → T</th>
<th>A → C</th>
<th>A → G</th>
<th>A → C</th>
<th>A → G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3 1.0</td>
<td>0.8 0.3</td>
<td>0.4 0.2</td>
<td>0.3 0.2</td>
<td>0.9 0.3</td>
</tr>
<tr>
<td>0.3 0.1</td>
<td>0.3 0.1</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
<td>0.0 0.0</td>
</tr>
<tr>
<td>1.0 0.8</td>
<td>0.8 0.6</td>
<td>0.4 0.4</td>
<td>0.3 0.3</td>
<td>0.9 0.7</td>
</tr>
<tr>
<td>0.5 0.5</td>
<td>0.5 0.5</td>
<td>0.3 0.3</td>
<td>0.3 0.3</td>
<td>0.5 0.5</td>
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<tr>
<td>0.5 0.5</td>
<td>0.5 0.5</td>
<td>0.3 0.3</td>
<td>0.3 0.3</td>
<td>0.5 0.5</td>
</tr>
</tbody>
</table>

Table 3 The percentages of nucleotide substitutions observed in the Actin, PAL, DDS, SERK, and nptII genes. The asterisks indicate significant differences (p < 0.01, **) between the averaged nucleotide substitutions observed in the Actin, PAL, DDS, SERK, and nptII genes.

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