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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.

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.

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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