ISSN 1022-7954, Russian Journal of Genetics, 2014, Vol. 50, No. 6, pp. 598–605. © Pleiades Publishing, Inc., 2014.
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PLANT GENETICS

Regulation of Somatic Embryogenesis in *Panax ginseng* C. A. Meyer Cell Cultures by *PgCDPK2DS1*

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Received November 12, 2013

Abstract—We isolated the full-length cDNA of *PgCDPK2DS1* gene whose expression was significantly increased at early stages of embryo development in cell cultures of ginseng *P. ginseng* 2c3. Interest in this gene also was supported by its nonstandard structure: the amino acid sequence of the *PgCDPK2DS1* gene contained only the N-terminal domain and 80% of the kinase domain. Overexpression of the *PgCDPK2DS1* gene in nonembryonic calli 1c resulted in the appearance of embryonic structures in the *PgCDPK2DS1*-transgenic ginseng cell culture 1c-2d. Also, expression of the plant embryogenesis marker genes *WUS* and *SERK* significantly increased in cell culture 1c-2d. The observed embryo-like structures were at early stages of embryo development; attempts to obtain adult plants from these embryo-like structures were unsuccessful. Overexpression of *PgCDPK2DS1* gene in the embryonic cell culture PG resulted in a decrease of embryonic structures in the *PgCDPK2DS1*-transgenic ginseng cell culture PG-2d. Moreover, expression of plant embryogenesis marker genes *WUS* and *SERK* and expression of the endogenous *PgCDPK2DS1* significantly decreased in the cell culture PG-2d. Thus, for the first time it was shown that the *PgCDPK2DS1* gene is involved in the regulation of somatic embryogenesis in *P. ginseng* cell cultures.

DOI: 10.1134/S1022795414060106

INTRODUCTION

The ginseng *Panax ginseng* C. A. Meyer is one of the most well-known, unique, and valuable medical plants. The increased interest in ginseng biological active compounds raises the question of the possible existence of this relict plant under natural conditions and its use as raw material. One quickly supplied method of whole-plant development is the biotechnological approach of ginseng derivation, which is based on the formation of somatic embryos. The process of somatic embryogenesis is poorly studied to date. However, it is known that the somatic embryogenesis of plants depends on a number of internal and external factors. For example, more than a hundred genes, the expression of which affects somatic embryogenesis, are presently known [1]. One of the most important elements in the system of the intracellular signaling of plants is Ca^{2+} [2, 3]. Calcium as the secondary messenger is the effective regulator of metabolic processes in all cells where there are the systems reacting to change of its concentration. The major intracellular targets of Ca^{2+} are Ca^{2+}-binding proteins, some of which change their own activity, while others trigger the effects of this cation on different molecular triggers [4]. It is believed that the main portion of plant enzymatic activity, which is stimulated by calcium, is associated with calcium-depended protein kinases (CDPK) [5]. CDPKs belong to one of the groups of Ser/Thr-protein kinases of eukaryotic cells. A CDPK protein molecule contains a protein kinase domain and a regulatory (autoinhibitory) domain, which is similar to that of calmodulin and usually contains Ca^{2+}-binding "EF"-hands that suppress the activity of the enzyme in the absence of Ca^{2+}. The binding of calcium to CDPK ceases autoinhibition and leads to activation of the enzyme [6]. Hence, CDPKs are considered to be a special type of a Ca^{2+}-sensor, which not only binds Ca^{2+} but also possesses protein kinase activity.

It was previously shown that the Ca^{2+}-signaling system is involve in the regulation of somatic embryogenesis [7]. However, it is not clear how this system can regulate such a great deal of processes. Full sequencing of the genomes of two taxonomically remote plant species, *Oryza sativa* and *Arabidopsis thaliana*, showed them to contain 27 and 34 *CDPK* genes respectively [8, 9]. Apparently, this may be the evidence of the multifunctionality of CDPKs.

The target proteins of CDPKs are the membrane associated transporting proteins (membrane bound H^+-ATPase, potassium and anion channels, aquaporins, etc.), enzymes (sucrose phosphate synthetase, nitrate reductase, phosphatidylinositol kinase, phenylalanine-ammonia lyase, etc.), transcription factors, proteins of cytoskeleton, and other targets [10, 11]. CDPKs may regulate growth, development, membrane transportation, stress-inducible gene expres-

sion, and the system of protective reactions against pathogens [6, 8].

It was previously shown that the gene subfamily *PgCDPK2D* (genes *PgCDPK2D* and *PgCDPK2DL*) underwent significant changes in their expression during the development of somatic embryos in the embryogenic ginseng cell line 2c3. The expression of *PgCDPK2D* increased at early stages of somatic embryogenesis, and transcripts, the kinase domain of which lacked several amino acids (*PgCDPK2DS*), occurred. The late stages were characterized by occurrence of the the dominating transcript *PgCDPK2DL*, the kinase domain of which contained signs [12]. The aim of present work is the obtainment of the full sequence of the ginseng gene of the *PgCDPK2DL* subfamily, the expression of which considerably increased at early stages of embryogenesis, and the study of its role in somatic embryogenesis of *P. ginseng*.

MATERIALS AND METHODS

To model somatic embryogenesis, we used two cell lines of ginseng, line 1c (Fig. 1e) and the young line PG (Fig. 1b), which were obtained from the collection of the laboratory of biotechnology of the Institute of Biology and Soil Science, the Far Eastern Branch of the Russian Academy of Sciences. The embryogenic culture PG was obtained in 2010 from the stem of a two-month old plantation ginseng *P. ginseng*. The callus ginseng culture 1c was obtained in the laboratory of biotechnology of the Institute of Biology and Soil Science, the Far Eastern Branch of the Russian Academy of Sciences in 1988 from the stem of a two-month old *P. ginseng* [12]. It is noteworthy that cell line 1c has lost its ability to form embryogenic structures because of the long period of in vitro cultivation [12].

The binary vector was obtained on the basis of pSAT1 and included gene *PgCDPK2DS1*. A part of the kinase domain of gene *PgCDPK2DS1* (390 bp) was obtained using the degenerate primers of the following structure: 5'-GTK CAY YTW GTK ATG GAR and 5'-TCK GCC CAA AAD GGW GG [12]. Further, the full-length cDNA of the gene *PgCDPK2DS1* was obtained on the basis of this region in accordance with the manufacturer's recommendations for the 5'/3' RACE Kit (Roche Diagnostics GmbH, Roche Applied Science, Germany). In the first cycle of RACE, the specific primer with the following structure was used: 5'-GCT TTC AGT GTT TTT CAG G; in the second cycle, the following specific primer was used: 5'-GGA AGT CCT TAT TAC ATG G. In the 5'-RACE, the specific primer with the following structure was used: 5'-GCT CTC TCG CTA TAA TGC C. To obtain the full-length sequence of the *PgCDPK2DS1* gene (the GenBank acc. number EU073200), we used a pair of primers (5'-ATG GGT ATT GTT GTC GCA CGT and 5'-TTC AGT TTC TGC CCA AAA TG) in order to amplify the ~ 789 bp PCR product (T_a = 55°C, elongation time 55 s). The obtained amplicon of the gene *PgCDPK2DS1* was isolated from the gel with the Cleanup Mini kit (Evrogen, Russia) and cloned into the vector pTZ57R/T in accordance with the manufacturer's protocol (Fermentas, Lithuania). Further, we amplified the full-length sequence of the *PgCDPK2DS1* gene using the pair of primers (5'-GCT AAG CTT ATG GGT ATT GTT GTC GCA CGT and 5'-AGC GGA TCC CTA TTC AGT TTC TGC CCA AAA TGG), which contained in their sequences the restriction sites *Hin*dIII and *Bam*HI respectively. The obtained genetic construction pTZ57-*PgCDPK2DS1* was used as a template. The obtained PCR product was transferred into the vector pSAT1 by the restriction sites *Hin*dIII and *Bam*HI [13], in which the gene *PgCDPK2DS1* was placed under the control of the double promoter 35S of the cauliflower mosaic virus (CaMV 35S). Then, the cassette, which contained the gene *PgCDPK2DS1*, was transferred from the vector pSAT1 into the vector pZP-RCS2-*nptII* (Fig. 2) [13] using the restriction site *Pal*AI (SibEnzim, Russia). The genetic construction pZP-RCS2-*nptII*-*PgCDPK2DS1* (Fig. 2) was transferred into the *Agrobacterium tumefaciens* GV3101:pMP90.

To obtain the *PgCDPK2DS1*-transgenic cell lines of ginseng, we used the agrobacterial transformation in accordance with the protocol described in [14, 15]. After transformation callus cell cultures were cultivated in the presence of 250 mg/L cefotaxime for 3 months in order to suppress the growth of agrobacteria. The selection of transgenic cells was performed on a medium containing 40–70 mg/L kanamycin for 5 months. The transgenic nature of the obtained lines was proved by the presence of the insertion of the gene *nptII* using the primers of the following structure: 5'-GAG GCT ATT CGG CTA TGA CTG and 5'-ATC GGG AGC GGC GAT ACC GTA, as was previously described in [15]. Absence of the signal after PCR-analysis for the presence of the gene *virB2* was considered to be evidence of the absence of agrobacteria in the analyzed samples. The following primers were used for the experiments: 5'-AAT GCG CGT GAT ATC GAG CTG CG and 5'-ATA CTA CCG CCA GTG AGC GTT TGG.

To perform quantitative analysis of the endogenous intracellular expression of the gene, the transgenic expression of the additional insertion of the gene, and the total expression of the intracellular and additional insertion of the gene *PgCDPK2DS1*, we used PCR with real-time detection of results (real-time-PCR) as described in [16]. Total RNA was isolated as described in [17]. cDNA was obtained using the specially designed kit for the synthesis of the first chain of cDNA with oligo(dT)-primers (Silex, Russia) with modifications [18]. Total expression of the gene *PgCDPK2DS1* was assessed using a pair of primers complementary to the sequence of the kinase domain of *PgCDPK2DS1*. Endogenous expression was assessed using a pair of primers complementary to the end of the kinase domain and to the sequence of the 3'-non-

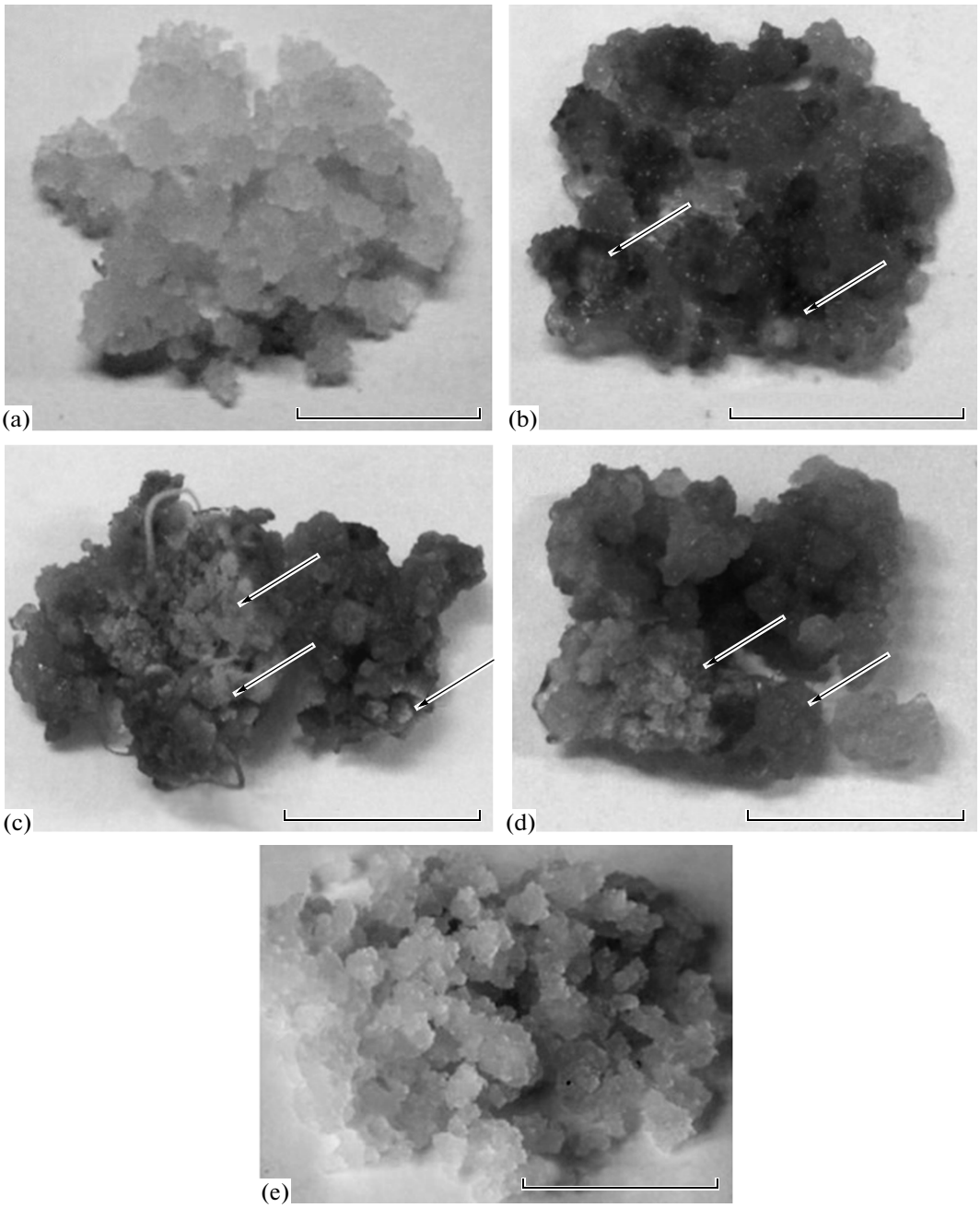

Fig. 1. The appearance of *P. ginseng* cell lines: (a)—the GV cell line, carrying the kanamycin resistance gene *nptII* only; (b)—*PgCDPK2DS1*-transgenic cell line 1c-2d; (c)—young embryogenic cell culture PG; (d)—*PgCDPK2DS1*-transgenic cell culture PG-2d; (e)—the long-term cell culture 1c. Arrows indicate embryo-like structures.

coding region (3'UTR) of the known cDNA of the gene. The expression of additional insertion of the *PgCDPK2DS1* gene was assessed using a pair of primers complementary to the end of the kinase domain of the gene *PgCDPK2DS1* and to the sequence of the CaMV 35S-terminator, which was located in the binary construction pZP-RCS2-*nptII*-*PgCDPK2DS1* (Fig. 2). The gene specific primer pairs are shown in Table 1. The primers for RT-PCR were designed in the program Primer Premier 5.0. cDNA was amplified with the reagent EvaGreen (Biotium, United States) using the Real-time PCR Kit (Sintol, Russia). To perform the amplification, a thermocycler equipped with a function for the real-time data detection was used (DNA Technology, Russia). The gene expression level was estimated as $2^{-\Delta\Delta CT}$. The highest level of expression obtained for an individual sample was considered to be a unit with respect to the amount of mRNA. Genes *VaActin1* (GenBank acc. number DQ517935) and *VaGAPDH* (GenBank acc. number GU585870)

were used as the housekeeping genes for the normalization of the amount of cDNA in each RT-PCR reaction. The data of RT-PCR analysis were obtained for each reaction independently [19, 20].

The number of embryo-like structures was assessed visually in the control and transgenic lines of *P. ginseng*. The development of embrioids in the ginseng cell lines was estimated by the level of plant embryogenesis marker gene expression, i.e. transcription factors *WUSHEL* (*WUS*) and somatic embryogenesis receptor kinases (*SERK*). It was previously shown that the level of ginseng somatic embryo formation correlated with the increase in expression of *WUS* and *SERK* [21]. The total expression of genes *WUS* and *SERK* was assessed by RT-PCR using primers designed for the homeobox sequence and the sequence encoding the kinase domain, respectively [21, 22].

The obtained data were processed in the program package Statistica, 10.0. All data are presented as mean values ± standard error (SE). The confidence of the obtained results was assessed using the paired Student test. A confidence level of 0.05 was chosen as the minimal value of statistical difference in all experiments.

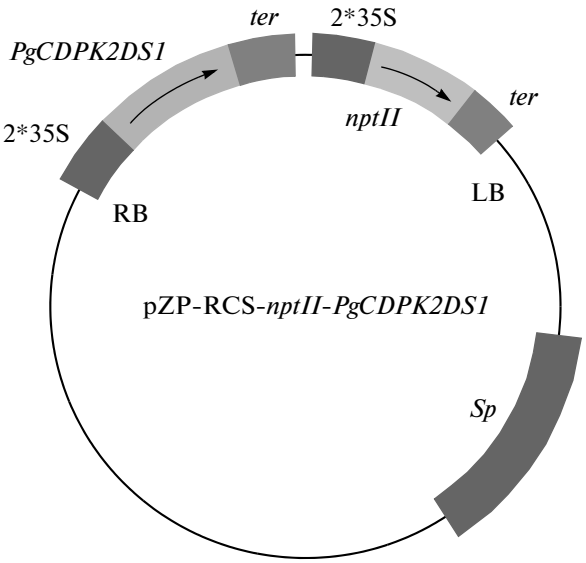

Fig. 2. The schematic image of the structure of the pZP-RCS2-*nptII* vector carrying the gene *PgCDPK2DS1*: RB and LB correspond to the sequences of the right and left borders of T-DNA; 2*35S corresponds to the double promoter of CaMV 35S; *ter*—CaMV 35S terminator; *nptII*—kanamycin resistance gene; *Sp*—spectinomycin resistance gene.

RESULTS

It was previously shown that the expression of the gene *PgCDPK2D* (GenBank acc. number EU073200) increased significantly at early stages of embrioid development. Obtainment of the full-length sequence of *PgCDPK2D* by RACE PCR revealed that this gene is characterized by an unconventional structure. The obtained sequence of *PgCDPK2D* lacked a sequence corresponding to the autoinhibitory domain and EF-hand domains. The primary structure of the protein obtained on the template of the obtained nucleotide sequence contained the N-terminal domain and 80% of the kinase domain only. The homology of the kinase domain of *PgCDPK2D* was higher than that of the formerly described CDPK of plants. However, the gene structure lacked a sequence encoding a part of the functionally important protein. Therefore, the corresponding gene was named *PgCDPK2DS1*. It is noteworthy that this truncated gene was sequenced on the basis of ginseng DNA (*P. ginseng*), which might provide evidence that the obtained cDNA was not formed by alternative splicing. We compared the obtained amino acid sequence of *PgCDPK2DS1* with sequences of well-known proteins isolated from *A. thaliana*. It was shown that the protein PgCDPK2DS1 demon-

Table 1. Primers used for RT-PCR of the ginseng *PgCDPK2DS1*

The pair of primers used for analysis of total expression of the *PgCDPK2DS1* gene	
PgCPK2ds-F	5'-CAT TAT AGC GAG AGA GCG GC
PgCPK2ds-R	5'-CAA GAA ATT CTC GGG CTT CAA GT
The pair of primers used for analysis of endogenous expression of the *PgCDPK2DS1* gene	
PgCPK2ds-F	5'-CAT TAT AGC GAG AGA GCG GC
PgCPK2ds-kon1	5'-TCA TAC ACT TCA TAA ACA CTT
The pair of primers used for analysis of the *PgCDPK2DS1* transgene expression	
PgCPK2ds-F	5'-CAT TAT AGC GAG AGA GCG GC
pSAT-term-a	5'-GAG AGA CTG GTG ATT TTT GCG

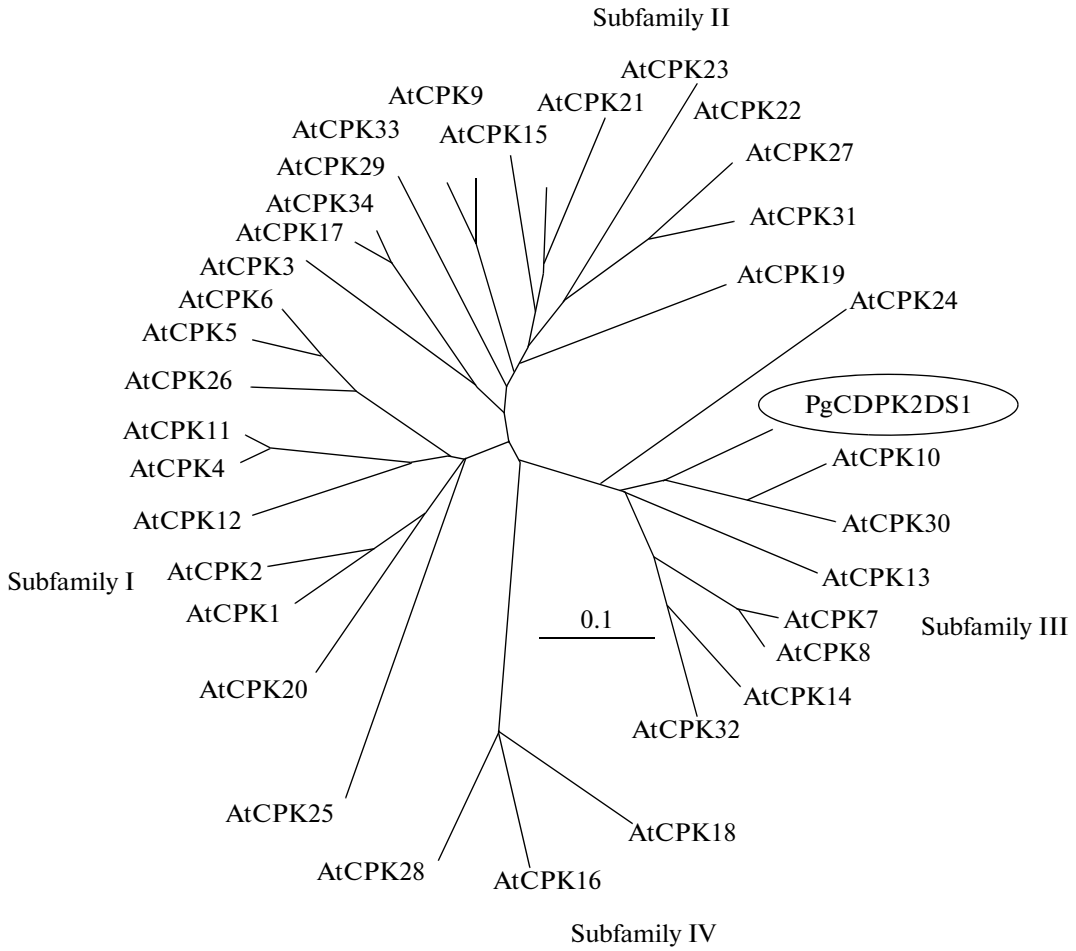

Fig. 3. Phylogenetic tree composed on the basis of CDPK representatives of *A. thaliana* by the neighbor-joining method.

strated the highest homology with the protein AtCPK10 of *A. thaliana* (Fig. 3).

Agrobacterial transformation of the long-term ginseng cell culture 1c and the young ginseng cell culture PG provided the *PgCDPK2DS1*-transgenic cell lines 1c-2d (Fig. 1b) and PG-2d (Fig. 1d). It is also noteworthy that we previously obtained a vector ginseng cell line *P. ginseng* GV (Fig. 1a) by agrobacterial transformation with a binary vector, which carried the kanamycin resistance gene only. This cell line was used for the control experiments. The obtained cell line demonstrated the same phenotype as the line 1c.

It was shown that the transgenic lines GV and *PgCDPK2DS1* 1c-2d and PG-2d contained the kanamycin resistance gene *nptII*. This was considered to be evidence of the successful transference of T-DNA. The absence of the signal of ginseng cDNA for the gene *virB2* proved the insertion of *nptII* gene into the ginseng genome (Fig. 4).

It was shown that in the cell line 1c-2d the total, endogenous and transgenic expressions of the gene *PgCDPK2DS1* were definitively higher than the expression of this gene in the long-term ginseng cell culture 1c (Fig. 5). Moreover, it was shown that expression of the marker genes of plant embryogenesis *SERK* and *WUS* in the obtained *PgCDPK2DS*-transgenic ginseng cell line 1c-2d was definitively higher than that in the control ginseng cell culture GV (Fig. 6). In the transgenic cell line PG-2d, a definite decrease in expression of the gene *PgCDPK2DS1* in comparison with that in the embryogenic cell line PG was observed (Fig. 5). We also showed that expression of the embryogenesis markers *SERK* and *WUS* was suppressed in the ginseng cell culture PG-2d in comparison with the embryogenic cell culture PG (Fig. 6).

We observed the occurrence of stem buds in the obtained *PgCDPK2DS1*-transgenic cell line of the long-term ginseng cell culture 1c-2d (Table 2, Fig. 1b). Apparently, overexpression of the gene *PgCDPK2DS1* in the cell line 1c led to the formation of embryogenic structures, which was followed by a definite increase in the expression of genes *WUS* and *SERK* (Table 2, Fig. 6). At the same time, it was shown that a definite decrease in the number of somatic embryos in the *PgCDPK2DS1*-transgenic young embryogenic ginseng cell culture PG-2d (Table 2, Fig. 1d) was followed by a decrease in

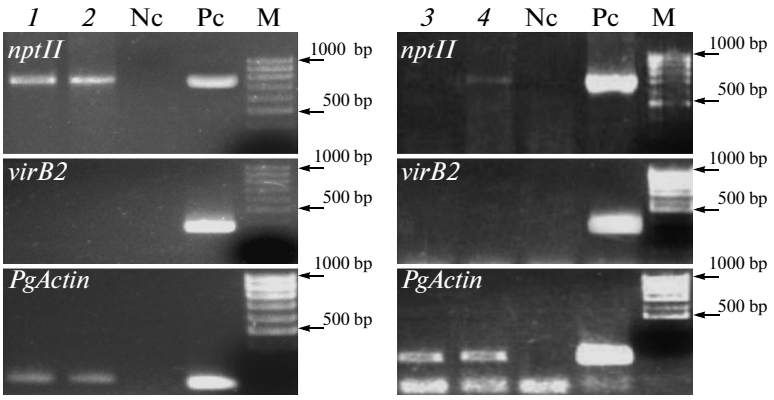

Fig. 4. The expression of genes *nptII*, *virB2*, and *PgActin* in ginseng cells: (*1*)—vector culture GV; (*2*)—*PgCDPK2DS1*-transgenic callus 1c-2d; (*3*)—embryogenic culture PG; (*4*)—*PgCDPK2DS1*-transgenic callus PG-2d; Pc—positive control; Nc—negative control.

the expression of genes *WUS* and *SERK* (Table 2, Fig. 6) and the expression of the endogenous gene *PgCDPK2DS1*, which was normally effectively expressed in the PG-cells (Fig. 5).

DISCUSSION

It is common knowledge that the calcium signaling system plays one of the key roles in the regulation of the majority of physiological processes in plant cells [23, 24], including embryogenesis [7]. Formerly, nothing was known about the *CDPK* genes in *P. ginseng*. The first studies attempted by our team showed that several *CDPK* transcripts are expressed in ginseng cells [12]. Gene *PgCDPK2D* attracted special attention, because its expression increased considerably at the early stages of somatic embryogenesis. Study of the full-length sequence of mRNA of this gene revealed that it is characterized by an unconventional structure. For example, it lacked the sequences encoding functionally significant parts of the protein. Therefore, it was decided to name this gene *PgCDPK2DS1*. The special feature of *PgCDPK2DS1* is that it lacks the sequence that correspond to the autoinhibitory domain and EF-hand domains. We succeeded in obtaining this nucleotide sequence from the DNA of *P. ginseng* cell line 1c. This may be considered evidence that this truncated gene is present in the ginseng genome.

It was shown that the full sequence of the *PgCDPK2DS1* gene is similar to that of the gene *ACPK10* of *A. thaliana*, which belongs to subfamily III (Fig. 3). A group of scientists from China previously showed [25]that expression of the *ACPK10* gene

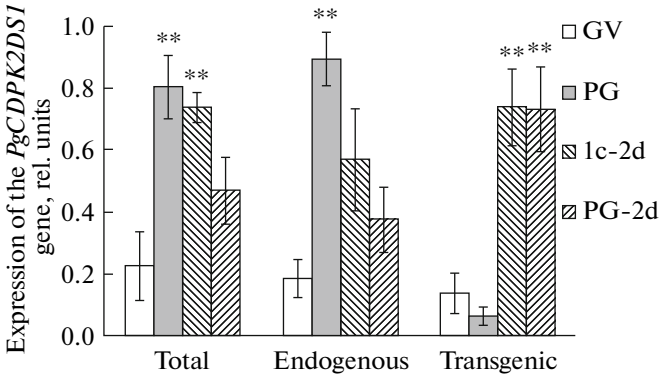

Fig. 5. Expression of *PgCDPK2DS1* in the vector and transgenic cell lines of ginseng assessed by RT-PCR: GV—cellular line GV carrying the kanamycin resistance *nptII* gene only; PG—young embryogenic cell culture PG; 1c-2d—*PgCDPK2DS1*-transgenic cell line 1c-2d; PG-2d—*PgCDPK2DS1*-transgenic cell line PG-2d; Total—total expression of intracellular and additional insertion of *PgCDPK2DS1* gene; Endogenous—intracellular expression of *PgCDPK2DS1* gene; Transgenic—expression of the additional insertion of *PgCDPK2DS1* gene; **—$p < 0.01$; the GV cell culture was used as a reference for expression level assessment.

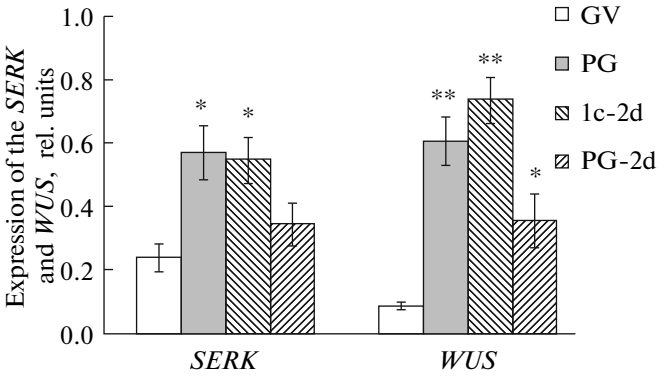

Fig. 6. Total expression of *PgSERK* and *PgWUS* genes in cell lines GV, PG, 1c-2d, and PG-2d assessed by RT-PCR; *—$p < 0.05$, **—$p < 0.01$; the GV cell culture was used as a reference for expression level assessment.

Table 2. The number of embryogenic structures per 1 g of fresh biomass of ginseng cells

Number of embryogenic structures in cell lines of ginseng			
GV	1c-2d	PG	PG-2d
0	5.1 ± 0.8**	122.5 ± 6.5	102.1 ± 5.6*

* $p < 0.05$, * the PG cell culture used as reference;
** $p < 0.01$, ** the GV cell culture used as reference.

homolog *OsCPK3/16* of *O. sativa* was increased in the inflorescences. It was also shown that these forms of CDPK are involved in the processes of seed development and zygotic embryo formation. We suggest that PgCDPK2DS1, like OsCPK3/16, possesses similar functions and may be involved in the process of embryogenesis. The results of our study demonstrate a connection between the occurrence of embryogenic structures in *PgCDPK2DS1*-transgenic cell culture 1c-2d and the increase in the total expression of the gene *PgCDPK2DS1* and marker genes of embryogenesis *WUS* and *SERK* (Figs. 1b, 5, 6). On the other hand, we revealed the decrease in the number of somatic embryos in the *PgCDPK2DS1*-transgenic cell culture PG-2d, which was followed by a decrease in the expression of *WUS*, *SERK* and the endogenous *PgCDPK2DS1* gene (Figs. 1d, 5, 6). Unfortunately, we failed to induce root formation of the observed embryogenic structures because of the early stage of their development. An attempt to transplant the embryogenic structures into different conditions (variations in phytohormones, the amount of macrosalts, and illumination) did not lead to considerable changes, and the embrioids remained at the early stages of their development. This again confirms that although PgCDPK2DS1 induces formations of somatic embryos in the cell culture, their further development apparently requires changes in the expression of other genes, namely, *PgCDPK2DL*, another representative of the PgCDPK2 CDPK subfamily of ginseng. Its expression increased considerably exactly at the later stages of embrioid development [12].

It was previously suggested that a mechanism of interaction between the protein products of *WUS* and *SERK* and formerly silent *CDPK* genes may possibly exist [21]. Several examples are known in which membrane proteins or transcription factors may serve as the targets for CDPKs [8, 24, 26]. Probably, WUS or SERK are proteins targets for new ginseng CDPKs. This suggestion was indirectly confirmed in our study, because the expression of *PgCDPK2DS1* was significantly increased in the embryogenic cell culture [12], while in the obtained cell lines a direct positive correlation between the expression of *PgCDPK2DS1*, *WUS*, *SERK*, and a number of embryogenic structures was observed.

The present study is important from a fundamental point of view, because the effect of the overexpression of the regulatory gene of the Ca^{2+}-signaling system on the process of somatic embryogenesis was demonstrated for the first time. Hence, it was shown for the first time that the gene *PgCDPK2DS1* is involved in the regulation of the initial stages of somatic embryogenesis in the ginseng cell culture. In the future, it will be interesting to study the mechanism of PgCDPK2DS1 activity via screening for the partner proteins of this CDPK.

ACKNOWLEDGEMENTS

This work was supported by the Russian Foundation for Basic Researches, project no. 12-04-33069-mol_ved and by the Far East Branch of the Russian Academy of Sciences.

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Translated by M. Bibov