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ORIGINAL PAPER

CDPK gene expression in somatic embryos of *Panax ginseng* expressing *rolC*

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Abstract The 2c3 embryogenic culture was obtained as a result of transferring the *rolC* oncogene from *Agrobacterium rhizogenes* to the callus cells of *Panax ginseng*. Calcium-dependent protein kinases (CDPKs) are known to play a role in the development of somatic embryos in the 2c3 cell culture. Ten *CDPK* genes with altered expressions in the 2c3 embryogenic cell culture have previously been described. In this study, the importance of the ginseng *CDPK* gene for stimulation of somatic embryogenesis was investigated. Frequency analysis of RT-PCR products and real-time PCR were used to analyze *CDPK* gene expression in the 2c3 callus at different stages of somatic embryo development. Our results suggest that members of the *PgCDPK2d* subfamily (*PgCDPK2d*, *PgCDPK2ds*, and *PgCDPK2dL*) play a role in the initialization and development of somatic embryos. It was also found that the kinase domain of these genes was subjected to insertion and deletion modifications. The observed transcriptional and post-transcriptional modifications (alternative splicing, RNA editing or nonsense-mediated mRNA decay) of the *PgCDPK2d* genes could contribute to the formation of somatic embryos initiated by the *rolC* oncogene.

Keywords *Agrobacterium rhizogenes* · *CDPK* · Genes *rol* · *Panax ginseng* · *rolC* · Somatic embryogenesis

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Abbreviations

CDPK	Calcium-dependent protein kinases
RT-PCR	Reverse transcription PCR

Introduction

The expression of *rolC*, a gene from *Agrobacterium rhizogenes* T-DNA in plants causes dramatic morphological and biochemical changes and plays an important role in the hairy root formation (Spena et al. 1987; Faiss et al. 1996; Nilsson et al. 1996). The *rolC* is capable of inducing root development in tobacco cell cultures (Spena et al. 1987; Schmulling et al. 1988) and *Atropa belladonna* leaf explants (Bonhomme et al. 2000). The mechanism underlying the *rolC*-induced rhizogenesis is unknown. We were the first to describe the embryogenic effect of *rolC* on ginseng cells (Gorpenchenko et al. 2006). The morphology and histology of the *rol*C-transgenic embryogenic callus have been studied in detail, and *rolC* expression has been determined to induce somatic embryogenesis in the ginseng 2c3 embryogenic cell culture. A weak *rolC* expression did not induce embryogenesis in ginseng cell cultures, while higher *rolC* expression induced root development (Gorpenchenko et al. 2006). Embryogenesis in the 2c3 cell culture was associated with calcium signaling, and calcium-dependent protein kinases (CDPKs) were shown to play a role in the development of somatic embryos (Kiselev et al. 2008). The primary objective of this work was to study the association between somatic embryogenesis of the *P. ginseng* and *CDPK* expression in *rolC* transgenic ginseng calluses. The received information will be useful for ginseng breeding; furthermore *P. ginseng* is one of the most valuable Chinese traditional medicinal herbs (Yue et al. 2007).

Complete genome sequencing of the taxonomically distant plants, *Oryza sativa* (monocot) and *Arabidopsis thaliana* (dicot), has identified 27 and 32 *CDPK* genes, respectively (Cheng et al. 2002; Asano et al. 2005). Without doubt different CDPK perform different functions, and are involved in different signaling pathways. The number of known CDPK targets continues to increase, and include membrane transport proteins (plasmalemma H+-ATPase, potassium and anion channels of guard cells, aquaporins, etc.), transcription factors, enzymes (sucrose phosphate synthase, nitrate reductase, and phenylalanine ammonia-lyase), and cytoskeleton proteins. Thus, CDPKs are involved in transmitting the Ca^{2+} signals associated with the membrane potential, regulation of carbohydrate and nitrogen metabolism, stoma movements, stress response, cell architecture, and embryo development (Cheng et al. 2002; Ludwig et al. 2004; Harper and Harmon 2005; Lecourieux et al. 2006).

Ginseng *CDPK* genes have not been as intensively studied as *CDPKs* of *Oryza sativa* and *A. thaliana.* Our first attempt to study the expression of ginseng *CDPK* genes using degenerate PCR primers resulted in the discovery of 17 *CDPK* gene transcripts (Kiselev et al. 2008). The expression levels of ten of these ginseng *CDPK* genes were altered in the 2c3 embryogenic culture. Therefore, it was impossible to determine the precise role of each ginseng *CDPK* gene in the process of somatic embryogenesis. In the present study, we assigned putative embryogenic roles for the proteins encoded by the ginseng *CDPK* genes based on the spatiotemporal correlation of their transcripts with stages of embryonic development in the 2c3 cell culture. As a result, we define *CDPK* genes that are expressed during somatic embryogenesis. We propose that members of the *PgCDPK2d* subfamily (*PgCDPK2d*, *PgCDPK2ds*, and *PgCDPK2dL*) play a key role in the initialization and further somatic embryo development in the 2c3 ginseng cell culture. Furthermore, we detected modifications (insertions, deletions) in the kinase domains of these genes. We intend to determine the significance of such modifications and the resulting post-transcriptional changes in future studies.

Materials and methods

Panax ginseng cell cultures

The *rolC* transgenic cell cultures were established previously (Gorpenchenko et al. 2006) by transformation of the 1c callus with *A. tumefaciens* strains GV3101/pMP90RK containing plasmid vector pPCV002-CaMVC (Spena et al. 1987). In the construct, the *rolC* gene was under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The construct also carried a gene for kanamycin resistance (*nptII*) under the control of the nopaline synthase promoter. The 1c-vector culture (designated as GV) was obtained by co-cultivation of the 1c callus with *A. tumefaciens* GV3101/pPCV002 (empty vector).

The phenotypic features of the used cell cultures were described earlier (Gorpenchenko et al. 2006). The appearance of hairy roots was registered within a year after transformation. The CII hairy root cell culture was obtained by excision the adventitious roots from the 1c-CII tumors and placing into liquid medium. The 2cR2 callus cell culture was obtained from the root explants of the CII cell culture. Two callus cultures (2c2 and 2c3) were established from the non-root-forming 1c-CII calli by selection of vigorously-growing cell clusters. After 11-month cultivation, the 2c3 calli spontaneously began to form single leaf-like and embyo-like structures while the 2c2 and 2cR2 cultures continued to grow as undifferentiated calli. All embryonic and shoot developmental stages are present simultaneously in the 2c3 culture. Embryogenesis in this cell culture does not reach the late stages.

The ginseng cultures 1c, GV, 2c3, and 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 30–35-day subculture intervals. The CII hairy root culture was cultivated in the dark on W_{4CPA} liquid medium supplemented with 0.4 mg/l 4-CPA at 24–25°C, with 25-day subculture intervals.

RT-PCR

Total RNA isolation and semiquantitative RT-PCR analyses of the *rolC* transcripts were performed as described previously (Bulgakov et al. 2005; Kiselev et al. 2007). To amplify sequences corresponding to the *CDPK* genes, the degenerate primers 5′-GTKCAYYTWGTKATGGAR and 5′-TTCKGCCCAAAADGGWGG were designed according to GenBank amino acid sequences of CDPK (Kiselev et al. 2008). These primers were used for amplification of a *CDPK* transcript (~390 bp); Ta = 53°C, elongation time = 25 s.

In semiquantitative RT-PCR reactions, PCR products were collected after 25, 30, 35, 40, and 45 cycles, and the linearity of the PCR was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). The linearity of the PCR was determined to be between 40 and 45 cycles for the *CDPK* genes. The identity of all RT-PCR products was confirmed by DNA sequencing. Quantitative analysis of mRNAs was performed using a microchip technology with a DNA 1000 LabChip® kit and the Agilent 2100 Bioanalyzer, following the manufacturer's protocol and recommendations. The data are presented as relative

fluorescent units normalized to the expression of the *P. ginseng* actin gene (Kiselev et al. 2006).

Screening of *CDPK* clones

The cDNAs obtained after reverse transcription of mRNA in RT-PCR reactions 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). Sequencing of each gene was performed at least three times. 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 the ginseng *CDPK* were deduced from the nucleotide sequences with the Gene runner 3.05 program, and compared with the known *CDPK* sequences of other organisms using the BioEdit 7.0.8 and BLAST software programs. For each cell culture, 47–118 clones of *CDPK* were sequenced. Quantity of analyzed clones was summarized from two clones collections. Clones collections differed in PCR product subcloning in the pTZ57R/T plasmid: PCR products of *CDPK* genes for each cell culture were received twice in the independent PCR reactions. The interval between the first and the second sample collections of the CDPK clones was 2 months. The differences in clone collections were used for the statistical analysis.

These results and the data on the total *CDPK* expression assayed with the degenerate primers were used to estimate the expression level in relative units (r.u.) for each of the *CDPK* genes. The relative expression was estimated as the total expression normalized to the expression of the ginseng actin gene × % clones of each gene/100 (Kiselev et al. 2008; Kiselev and Tchernoded 2009). Sequenced fragments of *CDPK* transcripts were deposited to GenBank (Kiselev et al. 2008).

Real-time PCR

The specific primers and TaqMan probes for real-time PCR were designed on the basis of the sequencing results, using the Primer Premier 5.0 program. Quantitative real-time PCR was performed using an established protocol (Giulietti et al. 2001). Real-time PCR was used to assay the *CDPK* genes that were most interesting in the context of somatic embryogenesis: *PgCDPK1a*, *PgCDPK2b*, *PgCDPK2d*, and *PgCDPK3a.* It was rather difficult to select the specific primers because of the high homology of some *PgCDPK* genes. Primers and probes specific to *PgCDPK1a* could not be selected, because their sequences were almost identical to those for *PgCDPK1b* and *PgCDPK1c.* The primers and probes for *PgCDPK2b* coincided with the primers for *PgCDPK2a.* Only the primers and probes for *PgCDPK2d*, *PgCDPK2dL*, and *PgCDPK3a* were specific to the corresponding genes. The gene-specific primer pairs and probes are presented in Table 1. For real-time quantitative-PCR, cDNAs were amplified using a Real-time PCR Kit (Syntol, Russia) with an iQ5 thermal cycler (Bio-Rad Laboratories Inc., USA) supplied with Optical system software v.2.0. Expression was normalized to actin using highest scaling

Table 1 Primers and TaqMan probes used in real-time PCR for the *P. ginseng CDPK* and actin genes

	Primer	TaqMan-probe
PgCDPK1a	5′-CGATTTTGGTCTGTCTGTCTTCA	5′-CGTGATATAGTTGGTAGTGCTTACTATGTTGCTC
PgCDPK1b	5′-CCATAACTACGCCGCAACACTTC	
PgCDPK1c		
PgCDPK2a	5′-TTCTGAAATTGTTGGGAGTCCAT	5′-ATTTCTGGTCCATAGTTTCGCTTGAGCACCT
PgCDPK2b	5′-GAATGACTCCAGCACTCCATATA	
PgCPK2dL	5′-ATGACATGGTCTTGAATTATGCTT	5′-TTTTCTGTGTGTTTTATTGCTTTTCCTTCTTTATGACA
	5′-AAAACACTGAATAAATATCATACC	
PgCDPK2d	5′-CATTATAGCGAGAGAGCGGC	5′-TGACCACTTCCGCCACCGTCCTCGCAA
	5′-CAAGAAATTCTCGGGCTTCAAGT	
PgCDPK3a	5′-CATTCAGAGGGGACATTACAGTG	5′-ACCTACAATAATCTTTGTCAATTCAGCAGCCTT
	5′-ACCAACAAGAAATTCTCAGGCTT	
P. ginseng actin gene	5′-GTATGTTGCTATTCAAGCCGATC	5′-ACCTGTTGTACGACCACTAGCATACAGGGA
	5′-ACCATCACCAGAATCCAGCACA	

options. The data were summarized from five independent experiments.

Statistical analysis

Statistical analysis employed the Statistica 8.0 program. The results were represented as mean ± standard error and tested by paired Student's *t*-test. $P = 0.05$ was selected as the point of minimal statistical significance in all analyses.

Results

Phenotype of ginseng cell cultures

The 2c3 cell culture naturally presents heterogeneous calluses containing several cellular aggregates with various degrees of differentiation. In the present experiment, the 2c3 cell culture was divided into groups of cells with different stages of somatic embryogenesis:

2c3-DD—friable dedifferentiated callus presented as small meristematic cells with a typical structure described earlier (Gorpenchenko et al. 2006).
2c3-ES—early stages of somatic embryogenesis. Somatic embryos in the early stages of development have several forms: globule, heart-shaped and torpedo-shaped constructs, as described earlier (Gorpenchenko et al. 2006).
2c3-LS—somatic embryogenesis usually does not reach late stages in the 2c3 culture cultivated on solid W_{4CPA} medium. Using transplantation to initiating medium (Chang and Hsing 1980) and light, we were able to stimulate the development of the late stages of embryogenesis.

It is important that the GV control cell culture did not show any signs of morphological differentiation. This observation strengthens the conclusion that the appearance of roots and somatic embryos in the *rolC*-transgenic ginseng cell cultures was the consequence of *rolC* gene expression.

rolC gene expression in the ginseng cell cultures

According to earlier data, *rolC* gene expression in the *rolC*-transgenic cell cultures differs in intensity (Gorpenchenko et al. 2006; Kiselev et al. 2006). The highest level of *rolC* expression has been shown in the CII root and 2cR2 callus cell cultures, which were obtained from the CII culture. The lowest level of *rolC* expression was identified in the 2c2 callus culture, and a medium level of expression was shown for the 2c3 culture (Gorpenchenko et al. 2006). New results obtained by means of real-time PCR confirmed the results obtained by RT-PCR (Fig. 1a). Furthermore, we analyzed the *rolC* gene expression in different aggregates of the 2c3 cell culture, containing somatic embryos in the different developmental stages described above. Different aggregates of the 2c3 cell culture showed different levels of *rolC* gene expression. The level of *rolC* expression in 2c3-DD and 2c3-ES cell aggregates was not significantly different from that in the whole 2c3 cell culture. The level of *rolC* expression in 2c3-LS was 1.9 times significantly higher than that in the 2c3, 2c3-DD, or 2c3-ES cultures (Fig. 1a). It was recently shown that gene expression under the 35s promoter may be regulated in various ways in different plant species and tissues (Benfey and Chua 1990). The differences in *rolC* expression may be attributed to the different tissues of the cell aggregates in the 2c3 cell culture.

CDPK gene expression in the different cell aggregates of the 2c3 cell culture

Results obtained with the Ca^{2+} channel blockers and protein kinase inhibitors implicate the Ca^{2+} signaling pathway in embryogenesis in the 2c3 cell culture (Kiselev et al. 2008). Since the *CDPK* genes play an important role in the

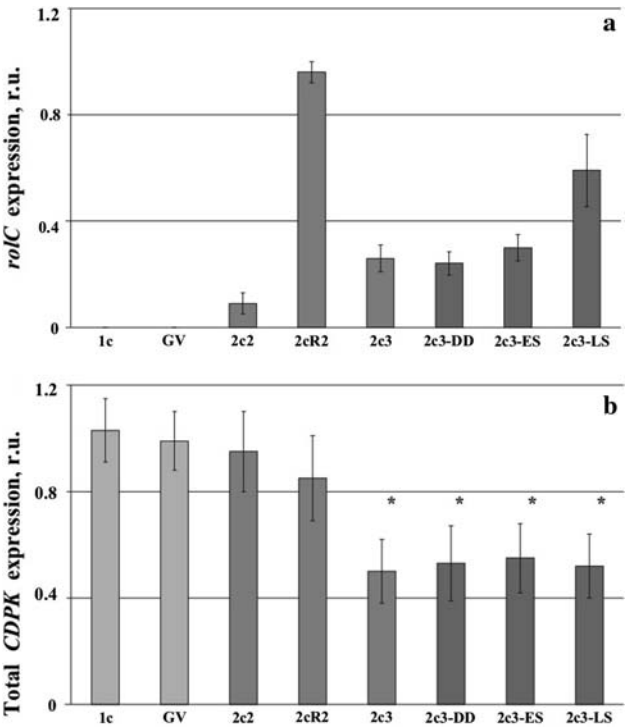

Fig. 1 Expression of the *rolC* (**a**) and *CDPK* (**b**) genes in the 1c, GV vector, 2c2, 2cR2, 2c3, 2c3-DD, 2c3-ES, and 2c3-LS cell aggregates and cultures. *rolC* expression obtained by real-time PCR, *CDPK* expression were obtained with the degenerate primers. See text for comments. * Denotes the statistical significance ($P < 0.05$) between the expression of *CDPK* genes in GV cell culture from *CDPK* expression in other cell cultures

Ca^{2+} signaling pathway (Cheng et al. 2002), we studied *CDPK* gene expression in the 1c, GV and 2c3 cell cultures. We divided the embryonic 2c3 culture into three groups of cellular aggregates by its developmental stages of somatic embryogenesis (2c3-DD, 2c3-ES, and 2c3-LS). Total RNA was extracted from each group, and expression of the *CDPK* genes was investigated using frequency analysis of RT-PCR products and real-time PCR.

PCR with the degenerate primers yielded fragments of the expected sizes for cDNA in the 1c, GV, 2c3-DD, 2c3-ES, and 2c3-LS cultures. Isolated fragments were cloned and sequenced. The fragments were verified to encode a part of the kinase domain of several *CDPKs*. The kinase domain fragments were highly similar to known plant CDPKs. Deduced amino acid sequences of the *CDPK* transcripts were aligned, using the BioEdit program, and they formed four subfamilies (Kiselev et al. 2008), as is the case with *A. thaliana CDPKs* (Cheng et al. 2002). The gene subfamilies were designated accordingly as *PgCDPK1*, *PgCDPK2*, *PgCDPK3*, and *PgCDPK4*. Each subfamily was represented by several genes. A total of 17 genes were identified as follows: a difference of more than one residue in the amino acid sequence suggested a new gene (Kiselev et al. 2008). It is known there are *CDPK* genes in some organisms that give rise to the same protein but differ in nucleotide sequence, in our case there were no such genes. The nucleotide sequences established by three independent sequencing experiments were deposited in GenBank (Kiselev et al. 2008).

Total expression of the *CDPK* genes in a mixed 2c3 culture and in the different aggregates of this culture was 1.8–2.0 times lower than that in the control 1c or GV cell culture (Fig. 1b). The expression of certain *CDPK* genes was calculated using total *CDPK* expression data and qualitative composition of the *CDPK* as described in "Screening of *CDPK* clones" in "Materials and Methods".

CDPKs expression in the 1c and GV cell cultures

PgCDPK1 and *PgCDPK3* gene expressions were highest in the control 1c and GV cultures. The *PgCDPK1* subfamily contained four transcript variants, with *PgCDPK1a* being the predominant transcript (Fig. 2a, b). The *PgCDPK3* subfamily contained two transcript variants, with *PgCDPK3a* as the predominant transcript (Fig. 2a, b). The expression levels of the *PgCDPK2a* and *PgCDPK3b* transcript variants were low (Fig. 2a, b). Since *CDPK* gene expression did not significantly differ in 1c and GV cultures, the *CDPK* gene expression in GV culture was used as a control for comparison with an *CDPK* expression in *rolC* transgenic cultures.

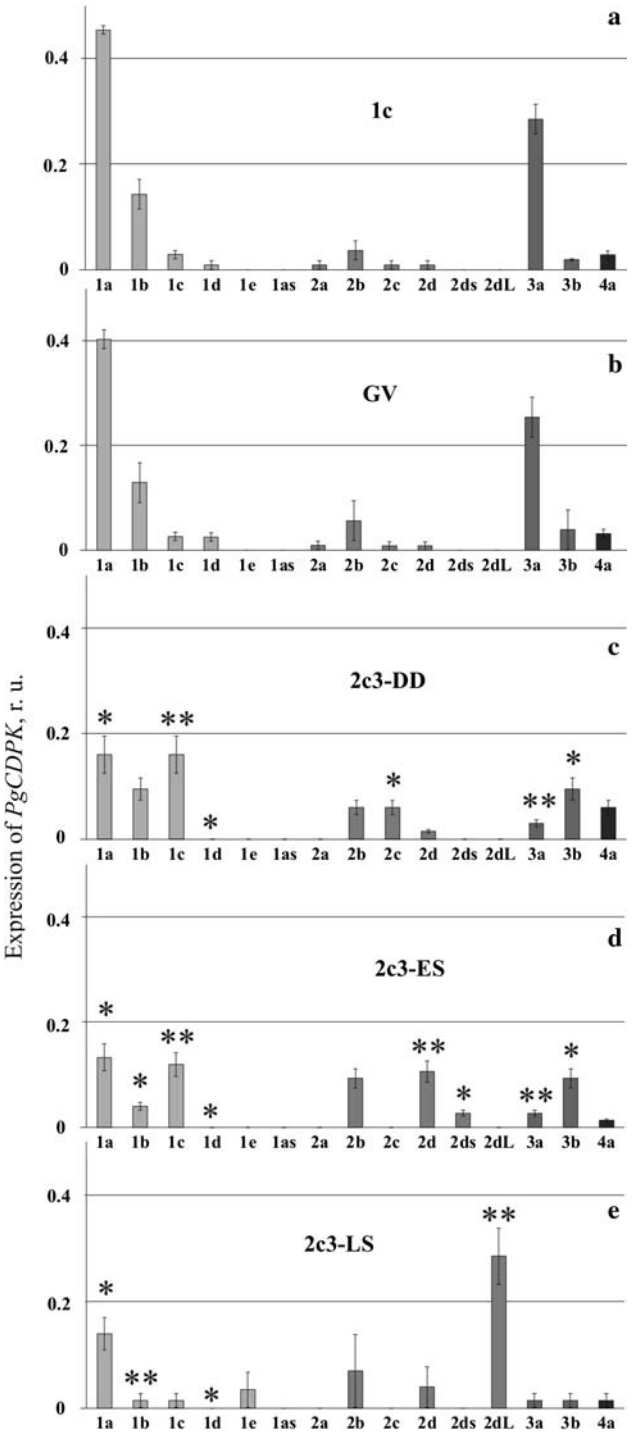

Fig. 2 Expression of the individual *CDPK* genes in the 1c (**a**) and GV (**b**) control and 2c3-DD (**c**), 2c3-ES (**d**), 2c3-LS (**e**) cell aggregates of the 2c3 *rolC* transgenic embryogenic cell culture. Signals were obtained using degenerate primers and were calculated as described in Material and Methods. See explanation in text. * Denotes the statistical significance ($P < 0.05$) between the expression of *CDPK* genes in GV cell culture from *CDPK* expression in other cell cultures, ** ($P < 0.01$)

CDPKs expression in 2c3-DD

We analyzed friable dedifferentiated calluses obtained from the 2c3 embryogenic cell culture. *PgCDPK1a* and *PgCDPK3*a expression levels were significantly lower than in the control cells (Fig. 2c). However, we observed a significant increase in the expression of other members of the *PgCDPK1* and *PgCDPK3* subfamilies (*PgCDPK1c* and *PgCDPK3b*) as well as *PgCDPK2c* (Fig. 2c).

CDPKs expression in 2c3-ES

We did not find any reliable changes in the expression of the *PgCDPK1* and *PgCDPK3* subfamilies in comparison with the 2c3-DD calluses, but there were some important changes in the expression of the *PgCDPK2* subfamily (Fig. 2d). The expression of the *PgCDPK2d* increased by 7-fold compared with the 2c3-DD, whereas expression of the *PgCDPK2c* dramatically increased compared with the 2c3-DD.

In 2c3-ES cDNA we found the "short" *CDPK* transcript, *PgCDPK2ds(2d-3b)* (Fig. 3). The *PgCDPK2ds(2d-3b)* transcript differs from the *PgCDPK2d* transcript by the absence of 102 nucleotides, including the VI catalytic subdomain (Fig. 3). We have previously described similar "short" transcripts in *rolC*-transformed ginseng cell cultures (Kiselev et al. 2008). However, the *PgCDPK2ds1* and *PgCDPK2ds2* transcripts lack 132 and 15 nucleotides, respectively (Fig. 3). The *PgCDPK2ds1* transcript was found in cDNA of the 2c3 cell culture, and the *PgCDPK2ds2* transcript was found in cDNA of the 2cR2 cell culture. The first part of the *PgCDPK2ds(2d-3b)* transcript is identical to the first part of *PgCDPK2d*, whereas the second part is identical to the second part of *PgCDPK3b*. Thus, *PgCDPK2ds(2d-3b)* is a "chimeric" transcript.

Expression of *CDPKs* in 2c3-LS

CDPK gene expression in somatic embryos in late developmental stages in the 2c3 culture differed significantly from the *CDPK* gene expressions in other aggregates of the 2c3 cell culture. In 2c3-LS, all *CDPKs* described above had lower levels of expression with the exception of *PgCDPK1a* (Fig. 2e). The predominant *CDPK* transcript was *PgCDPK2dL* (Fig. 2e). The nucleotide sequence of *PgCDPK2dL* was close to that of *PgCDPK2d*, the only difference being the insertion of 53 nucleotides within the VIII catalytic subdomain (Fig. 4). These insertions disrupt the reading frame, and create premature stop codons (Fig. 4).

Real-time PCR of the ginseng *CDPK* genes in different stages of morphological differentiation

Real-time PCR based on the known nucleotide sequences was used to verify the results of the *CDPK* gene expression obtained with the degenerate primers. We have shown that both methods have similar outcomes but the amplitude of changes of *CDPK* expression varied (Table 2), which could be explained by measurement errors or the fact that the PCR signals were obtained with different cDNA preparations and a 3-month interval between RNA isolations. Thus, expression of the *PgCDPK1* subfamily showed a decreasing trend in all cultures, compared to the control (Table 2). Expression of *PgCDPK2a* and *PgCDPK2b* significantly increased by 3.0–3.1 fold in the early and late stages of somatic embryogenesis, compared to the control (Table 2). Expression of *PgCDPK2d* increased considerably, by 5.3–12.6 fold, in the early and late stages of somatic embryogenesis compared to the control. *PgCDPK2dL* expression dramatically increased in 2c3-LS, compared to the control (Table 2).

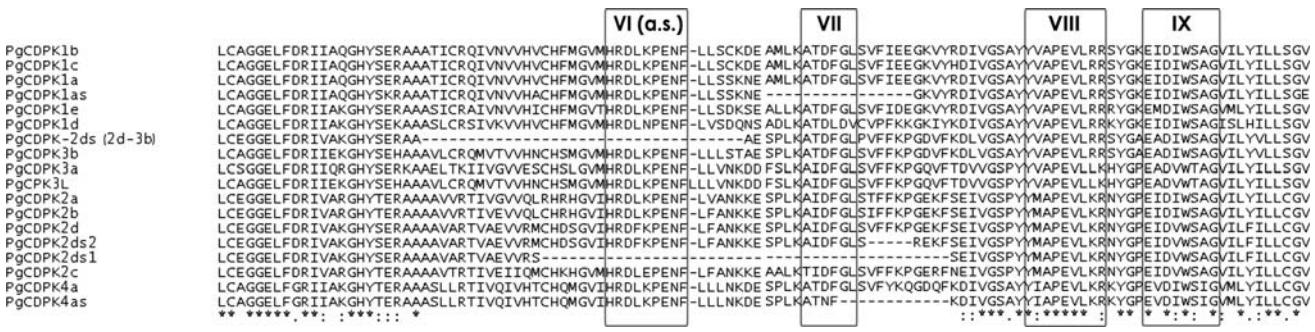

Fig. 3 Alignment of the deduced amino acid sequences of the partial kinase domains of the *CDPK* genes isolated from *P. ginseng* control and *rolC*-transformed calluses. *Dashes* indicate the absent residues. *Asterisks* indicate the conserved residues. The VI-IX catalytic subdomains are separated by *boxes*

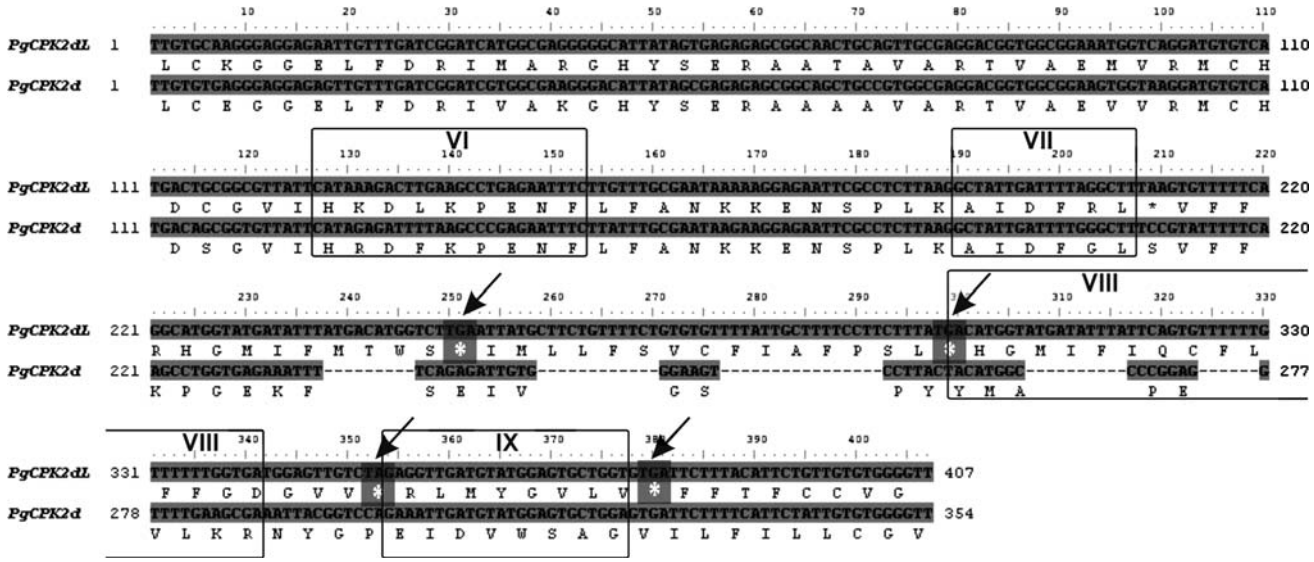

Fig. 4 Alignment of the deduced amino acid and nucleotide sequences of the partial kinase domains of the *PgCDPK2d* and *PgCDPK2dL* genes, isolated from *P. ginseng*. *Dotted arrow* indicates a 53 bp-insertion site. *Arrows* indicate stop codons. The VI–IX catalytic subdomains are separated by *boxes*

Table 2 Expression of the *P. ginseng CDPK* genes obtained by real-time PCR and degenerate primers

	1c	GV	2c3-DD	2c3-ES	2c3-LS	GV//2c3-DD/2c3-ES/2c3-LS
Real-time PCR						
PgCDPK1a	0.914 ± 0.098	0.902 ± 0.089	0.265* ± 0.008	0.405* ± 0.061	0.526* ± 0.035	↓ 3.4/↓ 2.2/↓ 1.7
PgCDPK1b						
PgCDPK1c						
PgCDPK2a	0.275 ± 0.045	0.298 ± 0.023	0.325 ± 0.041	0.911* ± 0.047	0.895* ± 0.035	↑ 1.1/↑ 3.1/↑ 3.0
PgCDPK2b						
PgCDPK2d	0.021 ± 0.010	0.027 ± 0.011	0.031 ± 0.011	0.142* ± 0.068	0.341** ± 0.091	↑ 1.2/↑ 5.3/↑ 12.6
PgCDPK2dL	0.009 ± 0.004	0.012 ± 0.006	0.014 ± 0.008	0.017 ± 0.007	0.703** ± 0.191	↑ 1.2/↑ 1.4/↑ 58.6
PgCDPK3a	0.931 ± 0.034	0.883 ± 0.053	0.627 ± 0.211	0.139** ± 0.055	0.479 ± 0.115	↓ 1.4/↓ 6.4/↓ 1.8
Degenerate primers						
PgCDPK1a	0.626 ± 0.044	0.555 ± 0.064	0.420 ± 0.181	0.292 ± 0.062	0.195 ± 0.052*	↓ 1.2/↓ 1.9/↓ 2.9
PgCDPK1b						
PgCDPK1c						
PgCDPK2a	0.046 ± 0.026	0.065 ± 0.046	0.063 ± 0.04	0.093 ± 0.025	0.075 ± 0.051	∼/↑ 1.4/↑ 1.2
PgCDPK2b						
PgCDPK2d	0.009 ± 0.008	0.011 ± 0.008	0.014 ± 0.010	0.107 ± 0.021**	0.041 ± 0.021*	↑ 1.3/↑9.7/↑ 3.8
PgCDPK2dL	0	0	0	0	0.285 ± 0.055	∼/∼/↑
PgCDPK3a	0.285 ± 0.028	0.254 ± 0.038	0.101 ± 0.028*	0.027 ± 0.012**	0.095 ± 0.021*	↓ 2.5/↓ 9.4/↓ 2.7

CDPK expression in the 2c3-DD, 2c3-ES or 2c3-LS cultures was (↓) lower, (↑) higher or (∼) the same in comparison with the control GV culture

* $P < 0.05$; ** $P < 0.01$

Discussion

A correlation between the expression of the *CDPK* genes and the developmental stage of somatic embryogenesis in a 2c3 cell culture was determined.

We have recently shown that blockage of calcium influx and CDPK activity prevents somatic embryogenesis in the 2c3 cell culture (Kiselev et al. 2008). Moreover we have shown significant changes in the expression of approximately 10 *CDPK* genes in the 2c3 embryogenic cell culture

compared to the control culture. However, it was difficult to determine which genes had the most important role for somatic embryogenesis. In the present investigation, we proposed embryogenic roles for the proteins encoded by the *CDPK* genes based on a spatiotemporal correlation of their respective transcripts with the stages of embryonic development in the 2c3 ginseng cell culture.

A comparison of *CDPK* gene expression in 2c3-DD and in 2c3-ES cell aggregates showed the most interesting results. 2c3-DD and 2c3-ES cells have similar profiles of *CDPK* expression, but the principal difference is the presence of *PgCDPK2d* and *PgCDPK2ds(2d-3b)* transcripts in the cDNA samples from early developmental stages of the somatic embryos (Fig. 3). *PgCDPK2d* and *PgCDPK2ds(2d-3b)* transcripts were predominant in 2c3-ES cell aggregates. *PgCDPK2dL* transcripts (the most similar to the *PgCDPK2d*) appear in the late developmental stage in 2c3-LS cells. Therefore, we consider changes in expression of the *PgCDPK2d* subfamily as the most important during the appearance of ginseng somatic embryos and their development.

Hypothetical functions of the "short" and "long" *CDPK* transcripts

The presence of the "short" (e.g. *PgCDPK2ds(2d-3b)*) and "long" (*PgCDPK2dL*) *CDPK* transcripts in the cDNA from the different aggregates of the 2c3 cell culture is of special interest. Information about RNA editing or alternative splicing of the *CDPK* transcripts is very limited. Nishiyama et al. (1999) reported the only known case of alternative splicing of a *CDPK* gene, where sequences corresponding to EF hands were modified. In contrast, several examples of the alternative splicing modifications of Ser/Thr kinase domains of other protein kinases are known. Xiong and Yang (2003) reported an alternative splicing modification to the Ser/Thr kinase sub domains of a rice MAPK gene. A "short" MAPK transcript was generated by the excision of a 312-bp sequence corresponding to the III–VI subdomains of the catalytic Ser/Thr kinase domain, leading to the production of a protein lacking kinase activity. Park et al. (2006) reported that an alternatively spliced transcript of the interferon-induced dsRNA-dependent Ser/Thr protein kinase, PKR, was lacking the exon 12 (159 bp) in the kinase domain. This alternative protein, named PKR-ΔE12, was incapable of auto-phosphorylation and phosphorylation of eIF2-α, indicating that the kinase subdomains III and IV, embedded in exon 12, are indispensable for catalytic function.

We are not aware of any examples of multiple changes in plant or animal kinase genes/transcripts similar to the modifications we described for the *PgCDPK2dL* transcripts. However, several studies have reported changes resembling some elements of the *PgCDPK2dL* modifications. For example, Wei et al. (2007) described three alternatively spliced variants of the mouse testis-specific Ser/Thr kinase 5. The isoforms have an insertion of ten amino acid residues in the region VIb. The splicing variants were shown to have lost kinase activity. The stop codons present in the "long" *P. ginseng CDPK* transcripts might result in the expression of "short" CDPK proteins with new protein functions. Alternatively, *PgCDPK2dL* transcripts might be the targets of a nonsense-mediated mRNA decay or NMD (Baker and Parker 2004) leads to mRNA degradation. It is known from '50 nucleotide rule' (Nagy and Maquat 1998) if a termination codon is more than about 50 nucleotides upstream of the final exon (as in *PgCDPK2dL*), it is a premature termination codons and the mRNA that harbors it will be degraded.

Similar "short", "long" or "chimeric" transcripts of *CDPK* were previously reported for *Vitis amurensis CDPK*, although their physiological significance is unknown (Dubrovina et al. 2009). Further study is needed to investigate functions of these transcripts. We speculate that the *PgCDPK2ds* and *PgCDPK2dL* transcripts, whose kinase domains lack some important catalytic subdomains, lack kinase activity but may have other important functions.

Hypothetical mechanism of somatic embryogenesis caused by the *rolC* oncogene in the 2c3 cell culture

Studies concerning the biochemical functions of the Rol proteins have been reviewed by several authors (Costantino et al. 1994; Nilsson and Olsson 1997). Early studies suggested that the RolB and RolC proteins are glucosidases that liberate auxin and cytokinins, respectively, from their bound forms (Estruch et al. 1991). However, other investigations have not provided evidence to support this hypothesis; it was proposed instead that changes in the levels of free hormones observed in some *rolC*- and *rolB*-transformed plants reflect non-specific effects of the oncogenes (Nilsson et al. 1996; Schmulling et al. 1993; Delbarre et al. 1994). The present investigation showed that the expression of the *rolC* gene in the *P. ginseng* cells resulted in changes of the expression of different *CDPK* genes, and provoked the generation of new transcripts with modified sequences corresponding to catalytic Ser/Thr kinase subdomains. The function of each *CDPK* gene which expression was modified in *rolC*-expressing cell cultures requires further examination. It is likely that *rolC* affects multiple biochemical processes (including somatic embryogenesis in *P. ginseng*) in transformed cells by changing the expression of various *CDPK* genes and generating new *CDPK* transcripts.

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