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ORIGINAL PAPER

Structure and expression profiling of a novel calcium-dependent protein kinase gene *PgCDPK1a* in roots, leaves, and cell cultures of *Panax ginseng*

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Abstract Calcium-dependent protein kinases (CDPKs) are proposed to play an essential role in plant defense responses. In this study, we aimed to define the full sequence of a *CDPK* gene of *Panax ginseng* and analyze its expression in roots, leaves, and cell cultures of *P. ginseng*, one of the most valuable Chinese traditional medicinal herbs. We isolated the full-length cDNA of a *P. ginseng CDPK* gene, which was designated *PgCDPK1a*. *PgCDPK1a* shares high sequence identity at the amino acidic level with previously reported *CDPK* sequences for other plant species. We analyzed *PgCDPK1a* expression in the leaves of wild-growing *P. ginseng* plants, and in the roots and leaves of cultivated *P. ginseng* plants growing in an open experimental nursery at a natural ginseng habitat. *PgCDPK1a* was more actively expressed in the young leaves of cultivated *P. ginseng* plants than in that of wild-growing ones. Finally, we analyzed the expression of the gene in control GV and five *rolC* and *rolB* transgenic callus cultures of *P. ginseng* with different levels of fresh biomass accumulation, pathogen-related gene expression, and ginsenoside production. We observed a strong positive correlation between fresh biomass accumulation of *P. ginseng* cell cultures and expression of the *PgCDPK1a* gene. There was a less clear negative correlation between the expression of pathogen-related genes and the content of ginsenosides with the *PgCDPK1a* expression in cell cultures of *P. ginseng*. Perhaps, *PgCDPK1a* is involved in ginseng growth, as a positive regulator.

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Abbreviations

4-CPA	p-chlorophenoxyacetic acid
CDPK	Calcium-dependent protein kinases
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

Calcium-dependent protein kinases (CDPKs) are known to be of great importance for the activation of defense reactions in plants (Cheng et al. 2002). 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, transcription factors, enzymes, 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; Lecourieux et al. 2006).

Ginseng *CDPK* genes have not been as intensively studied as CDPKs of *O. sativa* and *A. thaliana*. Our first attempt to study the expression of ginseng *CDPK* genes using degenerate PCR primers resulted in the discovery of

17–20 *CDPK* gene transcripts (Kiselev et al. 2008, 2009). In the present study, we aimed to sequence the full-length cDNA of the first *CDPK* gene from *Panax ginseng* C.A. Meyer (Araliaceae), one of the most valuable Chinese traditional medicinal herbs (Yue et al. 2007; Kim et al. 2009). We isolated the full cDNA of a *P. ginseng CDPK* gene, which was designated *PgCDPK1a*. *PgCDPK1a* shares high sequence identity at the amino acidic level with previously reported *CDPK* sequences for other plant species. By real-time polymerase chain reaction (PCR), we analyzed *PgCDPK1a* expression in the leaves, roots, and plant cell cultures of *P. ginseng* with different levels of ginsenoside production, expression of pathogen-related genes, and resistance to salt stress (Kiselev et al. 2006, 2010; Persiyanova et al. 2008), thus, acquiring information on the full-length cDNA sequence of the first *P. ginseng CDPK* gene: *PgCDPK1a*. Possibly, this information will be useful for ginseng breeding, the regulation of stress tolerance, and ginsenosides content in *P. ginseng* cells.

Materials and methods

Plant material

Wild-growing *P. ginseng* plants were sampled from a non-protected natural population in Sikhote-Alin. Collected living plants were transferred to an open experimental nursery in the natural ginseng habitats (Spassky District of the Primorsky Kray) for further investigations. The experiments used the leaves and roots from 2- and 3-year-old plants.

P. ginseng cell cultures

The *rolC* transgenic cell cultures were established previously (Gorpenchenko et al. 2006) by transformation of the 1c callus with *Agrobacterium tumefaciens* strains GV3101/pMP90RK containing plasmid vector pPCV002-CaMVC (Spena et al. 1987). The 1c-vector culture (designated GV) was obtained by co-cultivation of the 1c callus with *A. tumefaciens* GV3101/pPCV002 (empty vector). The *rolB* transgenic cell culture (designated GB) was obtained by co-cultivation of the 1c callus with *A. tumefaciens* pPCV002-CaMVB, as described previously (Kiselev et al. 2007).

The phenotypic features of the used cell cultures were as described earlier (Gorpenchenko et al. 2006). The CII hairy root cell culture was obtained by excision of 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 the selection of vigorously growing cell clusters (Gorpenchenko et al. 2006).

Based on previously obtained results (Kiselev et al. 2006, 2010; Persiyanova et al. 2008), we divided the *P. ginseng* cell cultures into several groups as follows:

(a) Plant cell cultures with high level of expression of pathogen-related genes (phenylalanine ammonia-lyase, beta-1,3-glucanase) and ginsenosides content—CII, 2cR2.
(b) Plant cell cultures with moderate level of expression of pathogen-related genes and ginsenosides content—2c2, GB, 2c3.
(c) Plant cell cultures with low level of expression of pathogen-related genes and ginsenosides content—GV.

The ginseng cultures GV, 2c3, GB, and 2cR2 were cultivated in the dark on W_{4CPA} solid medium (Gorpenchenko et al. 2006) supplemented with auxin 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 25–30-day subculture intervals. The calluses were cultivated in test tubes with 15 ml of the nutrient medium. The inoculum biomass was 0.2 g (each callus was weighed using an electronic balance).

Salt stress was induced in GV cell culture by the addition of 60, 100, and 200 mM NaCl to nutrition medium (Kiselev et al. 2010). The growth of the GV culture was completely inhibited in the 200 mM NaCl medium, and, therefore, we analyzed the *PgCDPK1a* expression only in *P. ginseng* cells treated with 60 and 100 mM NaCl. Total RNA was isolated from 35-day-old cell cultures. We used 100 mM NaCl to expose the GV cell culture in the presence of 4-CPA to salt stress for 2, 4, and 24 h.

Isolation and sequence analysis of *PgCDPK1a*

The 354-bp cDNA fragment of *PgCDPK1a* (GenBank accession number DQ421785) obtained by RT-PCR (Kiselev et al. 2009) was used as a template. A cDNA of the *PgCDPK1* gene was amplified based on sequences of its kinase domain and known sequences of *LeCDPK1* (AF363784) and *NtCDPK1* (AF072908). Primers 5′ATG GGW GGW TGY GCT WSW AA and 5′GCA GCA GCT CTC TCC GAA TAA TGA CC were used to recover the 5′ end of the gene; primers 5′GCW CCW SWW CKC ATC AT and 5′ACC GAT TTT GGT CTG TCT GTC TTC ATA G allowed the amplification of the remaining 3′ end. Then, 5′ and 3′ RACE amplifications were performed using 5′/3′ RACE Kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) following the manufacturer's

protocol and recommendations. A gene-specific primer 5′GGA AGC TGC TGA TGT GGA TGG and the anchoring primer were used to perform the first step of 3′ RACE. Then, the nested primer 5′TAT TTC GAC AAG GAT AGT AGY GGC and the anchoring primer were used to perform the second step of 3′ RACE and the resulting 250-bp fragment was subcloned into a pTZ57R/T plasmid and sequenced. Specific primers (5′TGT GGC TGA GTT TGG TGG GGT CTT T and the nested primer 5′GTC GGG TTG TAT TGT GTT GAT GGT TT) were used for 5′ RACE.

RT-PCR products were subcloned into a pTZ57R/T plasmid using InsT/Aclone PCR Product Cloning Kit (FERMENTAS, Vilnius, Lithuania) and sequenced using a Big Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Biosystems, Forster City, CA, USA) following the manufacturer's protocol and recommendations (Kiselev et al. 2006). After purification with ethanol, the sequences were identified on an ABI 3130 Genetic Analyzer (Perkin-Elmer Biosystems). The sequences of plant *CDPK* genes were accessed from GenBank: *AtCPK1* (D21805), *AtCPK30* (NP_177612), *MsCK1* (AAB34237), *LeCDPK1* (AF363 784), *NtCDPK1* (AF072908), *MsCK2* (AAB34238), *NtCDPK2* (AJ344154), *OsCPK7* (AB042550), and *ZmCD PK1* (D84408). Multiple sequence alignments and a phylogenetic tree based on pairwise alignment were done with the ClustalX program (Altschul et al. 1990).

Real-time PCR

Total RNA isolation and real-time PCR analyses of the *PgCDPK1a* transcripts were performed as described previously (Bulgakov et al. 2005; Kiselev et al. 2007). Total RNA was isolated from 35-day calli (linear phase of growth) except during the experiment, in which GV cell culture grown in the presence of 4-CPA are exposed to salt for 2, 4, and 24 h, where the total RNA was isolated promptly after 2, 4, and 24 h.

For TaqMan real-time RT-PCR, cDNAs were amplified in 20-μl reaction mixtures containing 1× TaqMan Buffer B, 2.5 mM $MgCl_2$, 250 μM of each deoxynucleotide, 1 U Taq DNA polymerase, 0.5 μl cDNA sample, and 0.25 μM of each primer and probe (Real-Time PCR Kit, Syntol, Russia). Quantitative real-time PCR was performed using an established protocol (Giulietti et al. 2001). The amplification conditions consisted of one cycle of 2 min at 95°C followed by 50 cycles of 10 s at 95°C and 25 s at 62°C. The TaqMan PCR essays were performed in an iCycler thermocycler supplied with the iQ5 Multicolor Real-Time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and data were analyzed with the iQ5 Optical System Software v.2.0 according to the manufacturer's instructions (Dubrovina et al. 2009), normalized expression (relative quantification by the $2^{-\Delta\Delta CT}$ method) and scaling options: highest (the highest expressing sample accrued the value 1 in the relative mRNA calculation). *P. ginseng* actin gene (GenBank accession number AY907207) was used as an endogenous control to normalize variance in the quality and the amount of cDNA used in each real-time RT-PCR experiment. A no-template control was included in every assay, and no-cycle threshold (Ct) values were consistently obtained after 50 cycles of PCR. Each TaqMan probe for *P. ginseng* actin gene was labeled with an FAM reporter dye at the 5′-end and a RTQ-1 quencher dye at the 3′-end, and TaqMan probe for the *PgCDPK1a* gene was labeled with an ROX reporter dye at the 5′-end and a BHQ-2 quencher dye at the 3′-end (Syntol, Russia). The data were summarized from five independent experiments.

The primers 5′GGA TCA ATT ACG AGG AGT TCT, 5′AGA TTA GTG CCG CAT TGT TAA G, and probe 5′TCC TTG ATG TTG TGT TGT TCC GCT TCT CAT CA were used for the expression analysis of a *PgCDPK1a* gene. For *P. ginseng* actin gene, the primers 5′GTA TGT TGC TAT TCA AGC CGA TC and 5′ACC ATC ACC AGA ATC CAG CAC A were used, and probe 5′ACC TGT TGT ACG ACC ACT AGC ATA CAG GGA.

Statistical analysis

Statistical analysis was carried out using the Statistica 8.0 program. The results are 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

Analysis of cDNA of *PgCDPK1a*

Using 5′ and 3′ RACE amplifications, we obtained the full-length cDNA of *PgCDPK1a* (GenBank accession number GU137295). A strong homology between *PgCDPK1a* and *MsCK2* (91% identity, Table 1), *LeCDPK1* (80%), and *NtCDPK1* (80%) was revealed at the protein level. Unfortunately, the full sequence of *MsCK2* is not available at this time, so only the central part of *MsCK2* kinase domain was sequenced, and, therefore, it is not possible to compare the full sequences of *MsCK2* and *PgCDPK1a*. Alignments of the deduced amino acid sequences of *PgCDPK1a* with *NtCDPK1* and *LeCDPK1* are presented in Fig. 1. These kinases share a common structure: the amino terminus contains the catalytic Ser/Thr kinase domain and the carboxy terminus contains a calmodulin-like domain with four putative Ca^{2+}-binding EF hands. Between the

Table 1 Comparison of the deduced amino acid sequence encoded by the *PgCDPK1a* gene and some known plant CDPKs (amino acid identities)

CDPK	*PgCDPK1a*	References
MsCK2	91	Monroy and Dhindsa (1995)
NtCDPK1	80	Yoon et al. (1999)
LeCDPK1	80	Chico et al. (2002)
MsCK1	64	Monroy and Dhindsa (1995)
OsCPK7	63	Saijo et al. (2000)
ZmCDPK1	63	Berberich and Kusano (1997)
AtCPK1	55	Urao et al. (1994)
NtCDPK2	54	Romeis et al. (2001)

kinase and calmodulin-like domain lies the junction domain. The catalytic subdomains (I–IX), junction domain, and calmodulin-like domain of *PgCDPK1a* share 90–95% sequence identity, respectively, with the corresponding domains of *NtCDPK1* (Fig. 1), indicating a high conservation of these kinases between such different families as Nicotiana and Araliaceae. *PgCDPK1a* and *NtCDPK1* contain a homologous (45% identity) Pro-Gln-rich sequence in the N-terminus. In contrast to these kinases, *LeCDPK1* contains no obvious Pro-Gln-rich sequence (Fig. 1).

Phylogenetic relationships of these kinases with several representative members of defense-related CDPKs and with CCaMKs known from the plant–rhizobium studies (Lévy et al. 2004; Tirichine et al. 2006) are presented in Fig. 2. *PgCDPK1a*, *NtCDPK1*, *LeCDPK1*, and *MsCK2* comprise a separate cluster in the phylogenetic tree. *PgCDPK1a* is distantly related to CCaMKs involved in the plant–rhizobium interaction.

PgCDPK1a expression in *P. ginseng* cells

We analyzed *PgCDPK1a* expression in 2-year-old leaves and roots of the wild-growing plants and plants growing on an open experimental nursery in the natural ginseng habitats. Leaves were collected in July 2009 (actively growing

Fig. 1 Comparison of the *PgCDPK1a* amino acid sequence with those of homologous CDPKs. The CDPKs aligned with *PgCDPK1a* are: tomato *LeCDPK1* (AF363784) and tobacco *NtCDPK1* (AF072908). The *dashes* indicate absent residues and the *asterisks* indicate conserved residues. I-IX—catalytic subdomains. The junction domain and the calmodulin-like domain containing four putative EF hands are indicated in the figure

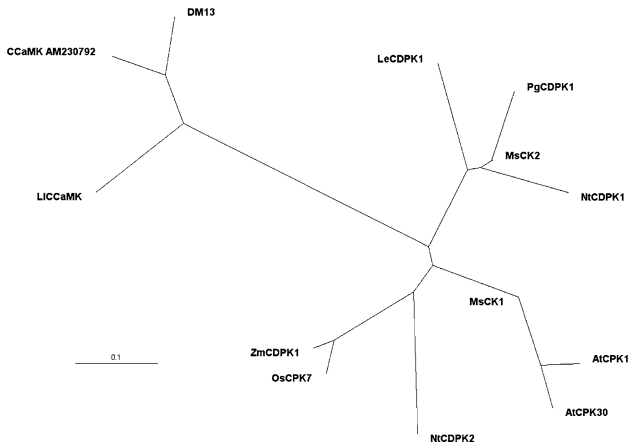

Fig. 2 Phylogenetic relationships of *P. ginseng PgCDPK1a* with defense-related plant CDPKs and CCaMKs involved in plant–rhizobium interaction. The branch lengths are proportional to divergence, with the scale "0.1" representing a 10% change

leaves) and in September 2009 (leaves had completed active growing). The roots from plants growing on an open experimental nursery were divided into two groups: slow-growing basic roots and actively growing young thin roots.

The highest *PgCDPK1a* expression was detected in actively growing young leaves and roots (Fig. 3). Expression in the leaves of the wild-growing ginseng plants was 1.3–1.9 times lower than the expression in leaves growing on an open experimental nursery (Fig. 3a).

Also, we analyzed the *PgCDPK1a* expression in *P. ginseng* plant cell cultures cultivated in our laboratory. We showed that the highest *PgCDPK1a* expression was detected in highly and moderately growing plant cell cultures GV, 2c2, and GB (Fig. 4). *PgCDPK1a* expression in CII and 2cR2 cell cultures was 1.7–1.9 times lower than the expression in GV cell culture (Fig. 4). The lowest *PgCDPK1a* expression was detected in the low-growing embryogenic 2c3 cell culture, which was 3.7 times significantly lower compared to the expression in the control GV culture (Fig. 4).

We used control culture GV to analyze *PgCDPK1a* expression in different growth conditions (different auxin concentrations) and under salt stress (different NaCl concentrations). Salt stress was induced by 60 mM and 100 mM NaCl. We showed that the biomass accumulation and the *PgCDPK1a* expression in GV cell culture positively correlates with auxin concentrations in the nutrient medium: the highest level of *PgCDPK1a* expression was in the GV cell culture growing on the medium with 1 mg/l 4-CPA, and the *PgCDPK1a* gene expression significantly reduced in the presence of lower 4-CPA concentrations (Fig. 5). We also observed a positive correlation between biomass accumulation, *PgCDPK1a* expression, and auxin concentrations for the 2cR2 cell culture of *P. ginseng* (Fig. 6). Interestingly, *PgCDPK1a* gene expression was significantly decreased under salt stress conditions in GV cell culture (Fig. 5).

GV cell culture was treated with 100 mM NaCl, and we analyzed the *PgCDPK1a* expression after 2, 4, and 24 h of cultivation, as well as at the end of the subculture period, on the 35th day of cultivation (Fig. 7). We observed a decrease in *PgCDPK1a* gene expression under salt stress after 4 and 24 h of cultivation and at the end of the subculture period (Fig. 7). We observed no significant difference in *PgCDPK1a* gene expression between control and NaCl-treated *P. ginseng* cells after 2 h of cultivation in the presence of 100 mM NaCl. Therefore, the decrease in *PgCDPK1a* expression in response to salt stress is not a rapid response. It is likely that the mechanical damage to *P. ginseng* cells caused by the cell culture transfer on fresh medium induced the increase in *PgCDPK1a* gene expression which was observed after 2 h of cultivation.

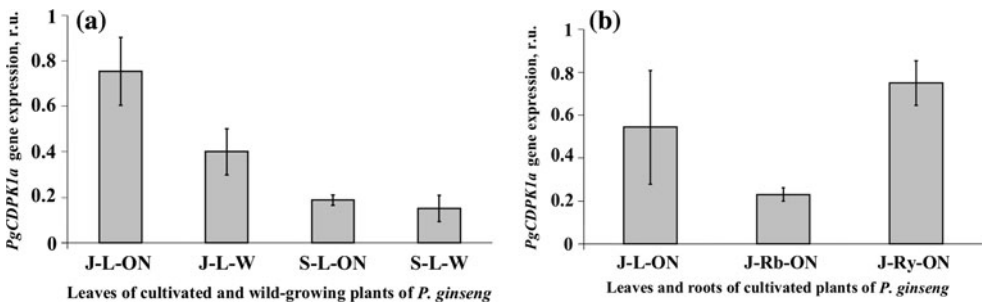

Fig. 3 Expression of the *PgCDPK1a* gene in *P. ginseng*. Abbreviations: *J-L-ON* RNA was extracted from leaves collected in July 2009 from plants growing on an open experimental nursery in the natural ginseng habitats, *J-L-W* leaves collected in July from wild-growing plants, *S-L-ON* leaves collected in September 2009 from plants growing on an open experimental nursery, *S-L-W* leaves collected in September from wild-growing plants, *J-Rb-ON* slow-growing basic roots collected in July from plants growing on an open experimental nursery, *J-Ry-ON* actively growing young thin roots collected in July from plants growing on an open experimental nursery

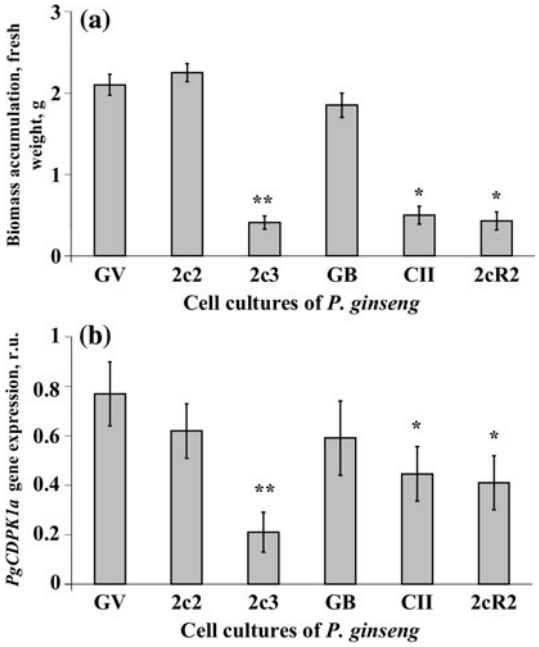

Fig. 4 Biomass accumulation (**a**) and expression of the *PgCDPK1a* gene (**b**) in the GV, 2c2, 2cR2, CII, 2c3, and GB cell cultures. *Statistical significance ($P < 0.05$) between the expression of *PgCDPK1a* in GV cell culture from *PgCDPK1a* expression in other cell cultures, **$P < 0.01$

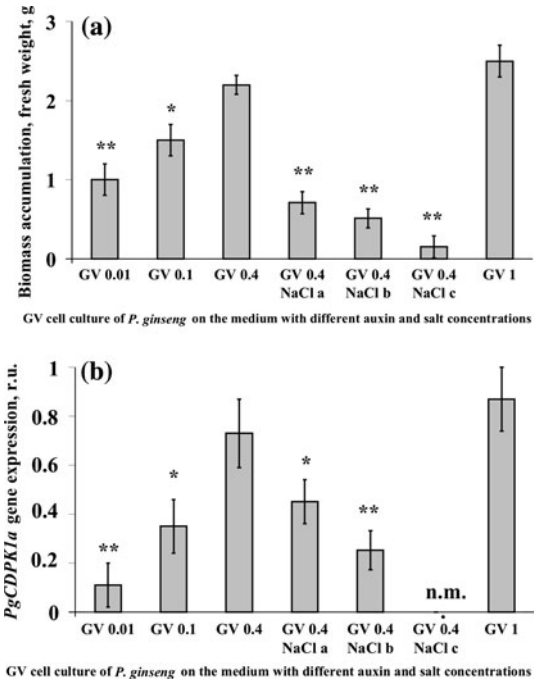

Fig. 5 Effect of auxin (4-CPA) and NaCl treatment on biomass accumulation (**a**) and expression of *PgCDPK1a* gene (**b**) in *P. ginseng* GV cell culture. Abbreviations: *GV 0.01* GV cell culture growing on medium with 0.01 mg/l 4-CPA, *GV 0.1* on medium with 0.1 mg/l 4-CPA, *GV 0.4* on medium with 0.4 mg/l 4-CPA, *GV 0.4 + NaCl a* on medium with 0.4 mg/l 4-CPA and 60 mM NaCl, *GV 0.4 + NaCl b* on medium with 0.4 mg/l 4-CPA and 100 mM NaCl, *GV 0.4 + NaCl c* on medium with 0.4 mg/l 4-CPA and 200 mM NaCl, *GV 1* on medium with 1 mg/l 4-CPA, *nm* not measured. *Statistical significance ($P < 0.05$) between the *PgCDPK1a* expression in GV cell culture in standard conditions (0.4 mg/l 4-CPA, without NaCl) and *PgCDPK1a* expression in cells growing on the media with different 4-CPA concentrations and in NaCl-treated cells, **$P < 0.01$

Discussion

In the present work, we obtained the full-length cDNA sequence of the *CDPK1a* gene of *P. ginseng* and analyzed its expression patterns in different growth conditions and under salt stress. We found a strong positive correlation between fresh biomass accumulation of *P. ginseng* cell cultures and the expression of *PgCDPK1a* gene. We also observed a less clear negative correlation between the expression of pathogen-related genes and content of ginsenosides with the *PgCDPK1a* expression. Perhaps, the *PgCDPK1a* is involved in ginseng growth, as a positive regulator.

Recently, Lee et al. (2003) employed virus-induced gene silencing of a native *NtCDPK1* gene in tobacco plants to generate several lines of transgenic plants with differing levels of *NtCDPK1* inhibition. In this study, a correlation between the degree of gene silencing and the degree of induction of defense-related genes was observed: the more severely suppressed lines exhibited higher expression of defense genes (Lee et al. 2003). Similar correlations were observed also in our case where *PgCDPK1a* mRNA levels were negatively correlated with the expression of defense-related genes (*PAL*, beta-1,3-glucanase) in 2c2, 2cR2 cell cultures and the presence of somatic embryos in 2c3 cell culture (Kiselev et al. 2006; Gorpenchenko et al. 2006; Persiyanova et al. 2008). Although *CDPK* genes involved in plant defense against biotic and abiotic stresses are generally recognized as inducible genes (Ludwig et al. 2004), there are several examples of *CDPK* genes whose inhibition is associated with activated defense reactions. In addition to *NtCDPK1* and *PgCDPK1a*, another member of this group appears to be the *MsCK2* gene from alfalfa plants. This plant contains the *MsCK1* gene, the expression of which was upregulated during cold stress and the *MsCK2* gene, the expression of which was downregulated under the same conditions (Monroy and Dhindsa 1995). Considering the high homology of the *NtCDPK1*, *PgCDPK1*, and *MsCK2* genes (Fig. 2), one can suggest the existence of a highly conserved group of CDPKs which perform a similar function in plants. The function may represent a link between cell growth and defense controls.

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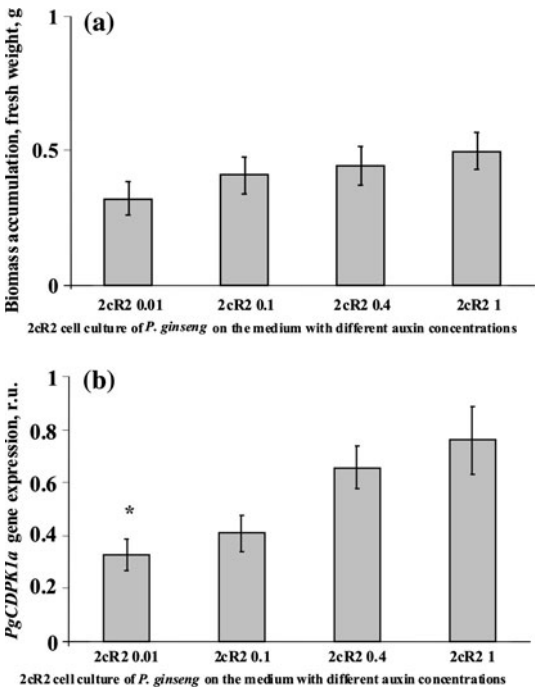

Fig. 6 Effect of auxin (4-CPA) on biomass accumulation (**a**) and expression of *PgCDPK1a* gene (**b**) in 2cR2 cell culture of *P. ginseng*. Abbreviations: *2cR2 0.01* 2cR2 cell culture growing on medium with 0.01 mg/l 4-CPA, *2cR2 0.1* on medium with 0.1 mg/l 4-CPA, *2cR2 0.4* on medium with 0.4 mg/l 4-CPA, *2cR2 1* on medium with 1 mg/l 4-CPA. *Statistical significance ($P < 0.05$) between the expression of *PgCDPK1a* in 2cR2 cell culture in standard conditions (0.4 mg/l 4-CPA) and *PgCDPK1a* expression in cells growing on the media with different 4-CPA concentrations

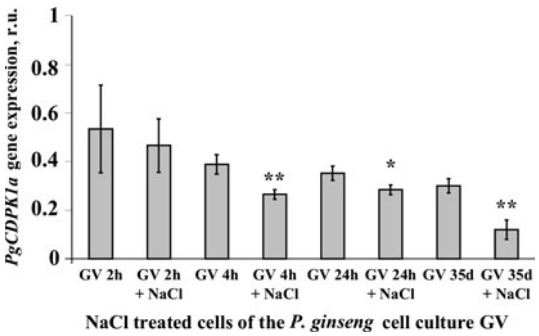

Fig. 7 Expression of the *PgCDPK1a* gene in the GV cell culture of *P. ginseng*. Abbreviations: *GV 2h* GV cell culture after growing on the standard medium for 2 h, *GV 2h + NaCl* after growing on the standard medium with 100 mM NaCl for 2 h, *GV 4h* after growing on the standard medium for 4 h, *GV 4h + NaCl* after growing on the standard medium with 100 mM NaCl for 4 h, *GV 24h* after growing on the standard medium for 24 h, *GV 24h + NaCl* after growing on the standard medium with 100 mM NaCl for 24 h, *GV 35d* after growing on the standard medium for 35 days, *GV 35d + NaCl* after growing on the standard medium with 100 mM NaCl for 35 days, *Statistical significance ($P < 0.05$) between the expression of *PgCDPK1a* in GV cell culture growing on the medium without NaCl and *PgCDPK1a* expression in GV cell culture growing on the medium with NaCl under the same conditions of cultivation, **$P < 0.01$

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