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CDPK gene expression in salt tolerant *rolB* and *rolC* transformed cell cultures of *Panax ginseng*

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Abstract

CDPKs (calcium-depended protein kinases) are of great importance for the activation of defense reactions in plants. In this study, we aimed to find a connection between *CDPK* expression and increased salt tolerance in *Panax ginseng*. Treatment of *P. ginseng* cell cultures with W7 (CDPK protein inhibitor) showed that CDPK proteins were necessary for salt tolerance. Expression of *PgCDPK1c*, *PgCDPK2c* and *PgCDPK4a* was significantly increased in the cells treated with 60 mM NaCl compared to control cells, whereas expression of *PgCDPK1b* and *PgCDPK3a* was decreased. In the NaCl-treated cells, new *CDPK* transcripts also appeared (*PgCDPK3c*, *PgCDPK4as*). We also used *rolC* and *rolB* transformed cultures and the effects of the *rol* genes on *CDPK* expression were similar to the effects of salt stress: they caused a significant increase in the expression of *PgCDPK1c*, *PgCDPK2c*, and *PgCDPK4a* and decreased expression of *PgCDPK3a*, in addition to the appearance of the "short" *CDPK* transcripts.

Additional key words: *Agrobacterium rhizogenes*, calcium-dependent protein kinases, NaCl.

Introduction

High salinity is one of the common stresses, therefore, cellular and molecular responses to high salinity have been intensively studied in various plant species (Xiong *et al.* 2002, Radyukina *et al.* 2007, López-Carrión *et al.* 2008, Melgar *et al.* 2008, Su and Bai 2008). Despite the efforts of many research groups, the mechanisms of plant salt tolerance remain incompletely understood. Calcium-dependent protein kinases (CDPKs) are known to be of great importance for the activation of defense reactions in plants (Xiong *et al.* 2002). A simple scheme of defense reactions to salt stress in plants involves special receptors in the cell membrane that recognize high salt concentrations and use secondary signaling molecules (e.g. Ca^{2+}, ROS) to transmit this signal to regulator proteins [*e.g.* mitogen-activated protein kinases, (MAPK), CDPK and salt overly sensitive calcium binding proteins (SOS3)]. These regulator proteins then work through transcription factors to increase the expression of ion transporters and enzymes in pathways leading to the synthesis of osmolytes, antioxidants and LEA-like proteins (Xiong *et al.* 2002).

Calcium is a universal mediator in complex signal transduction networks: no other mediator responds to as great number of stimuli as free cytosolic Ca^{2+} does. CDPKs have been implicated as the primary calcium sensors in plant cells (Cheng *et al.* 2002). Complete genome sequencing of taxonomically distant species such as *Oryza sativa* and *Arabidopsis thaliana* has revealed 27 and 34 *CDPK* genes, respectively (Asano *et al.* 2005, Cheng *et al.* 2002), suggesting that different *CDPK* genes perform distinct functions and are involved in different signaling pathways. Four CDPK families have been recognized (Cheng *et al.* 2002). A number of studies have shown that *CDPK* expression is activated by abiotic stresses, suggesting that they may be involved in abiotic stress signaling (Urao *et al.* 1994, Tähtiharju *et al.* 1997, Hwang *et al.* 2000). In rice plants, a membrane-associated CDPK was found to be activated by cold

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Abbreviations: CDPK - calcium-dependent protein kinase; MAPK - mitogen-activated protein kinase; SOS3 - salt overly sensitive calcium binding proteins.

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treatment (Martin and Busconi 2001). In addition, over-expression of *OsCDPK7* resulted in increased cold and osmotic stress tolerance in rice (Saijo *et al.* 2000). Thus, CDPKs seem to play roles in the development of stress tolerance.

Recently, it has been shown that *rol* oncogenes significantly increase salt tolerance in *Panax ginseng* cell cultures (Kiselev and Grishchenko 2008). Therefore, we used *rolC* and *rolB* transgenic cell cultures of *P. ginseng* as a model system for the investigation of plant cell tolerance mechanisms to salt stress. The plant oncogenes *rolB* and *rolC* are carried by plasmids of *Agrobacterium rhizogenes*. Over the past decade, novel features of plant cells transformed with *rolB* and *rolC* have been revealed such as increased secondary metabolite production (Palazón *et al.* 1998, Kiselev *et al.* 2007, Veena and Taylor 2007, Tiwari *et al.* 2008), expression of plant pathogenesis-related (PR)-2 proteins (Kiselev *et al.* 2006) and promotion of the formation of teratoma-like structures (Bulgakov *et al.* 2006). RolB has been shown to have tyrosine phosphatase activity (Filippini *et al.* 1996), and it was recently found that RolB could interact with protein 14-3-3 and modulate its activity (Moriuchi *et al.* 2004). These authors reported nuclear localization of RolB, which increases the probability that it can act as a transcription co-activator/mediator. As CDPKs are commonly accepted as molecules that mediate cross-talk between signaling pathways (Cheng *et al.* 2002), modulation of their expression and/or activity could explain numerous unrelated and largely unexplained effects seen in the *rol* transformed cultures.

In the present study, we describe a connection between *CDPK* expression and increased salt tolerance. Moreover, we showed that the effects of the *rol* genes on *CDPK* expression were similar to the effects of salt stress.

Materials and methods

We used cultures of ginseng (*Panax ginseng* C.A. Meyer) obtained by transformation of 1 c callus culture (Kiselev *et al.* 2008) with *A. tumefaciens* strain GV3101/pMP90RK containing plasmid vector pPCV002-CaMVC (with *rolC* gene) or pPCV002-CaMVB (with *rolB* gene) (Spena *et al.* 1987). GV was a ginseng vector culture transformed by empty vector, pPCV002 (Kiselev *et al.* 2008). The 2c2 culture was a primary tumor line with low *rolC* expression. The 2cR2 line was obtained from *rolC* transformed roots by selection of root tip-derived callus tissue and represents a culture of clonal origin with high expression of *rolC* (Kiselev *et al.* 2008). The GB culture was a primary tumor line with low *rolB* expression. The cultures were cultivated in test tubes with 15 cm^3 W_4 CPA (Kiselev *et al.* 2009) medium (supplemented with 4-chlorophenoxyacetic acid) in the dark at 24 - 25 ^oC, with 30-d subculture intervals as previously described (Kiselev *et al.* 2008). Inoculum biomass was 0.2 g. GV, 2c2, 2cR2, and GB cultures were exposed to the following treatments: *a*) control, *b*) 60 mM NaCl, *c*) 5 or 30 μM CDPK inhibitor [N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7), *Sigma-Aldrich*, St. Louis, MO, USA], *d*) 60 mM NaCl and 5 or 30 μM W7. Inhibitors, NaCl and dimethyl sulfoxide (DMSO) were obtained from *ICN Biomedicals* (Eschwege, Germany). Sterile aqueous solutions of NaCl and DMSO solutions of W7 were aseptically added to autoclaved medium at necessary concentrations (NaCl: 0 - 200 mM, W7: 0 - 100 μM). Equivalent amounts of DMSO were added to control flasks. The differences in fresh biomass and the expression of individual *CDPK* genes were analyzed in all experiments.

Total RNA isolation and semiquantitative RT-PCR analysis of *rolC* transcripts was performed as described Bulgakov *et al.* (2005). In order to amplify sequences corresponding to kinase domains of *P. ginseng CDPK* genes, the degenerate primers 5'GTKCAYYTWGTK ATGGAR and 5'TTCKGCCCAAAADGGWGG were designed according to GenBank sequences of *AtCPK1* (D21805), *AtCPK2* (D21806), *LeCDPK1* (AF363784), *NtCDPK1* (AF072908), *NtCDPK2* (AJ344154), *OsCPK7* (AB042550) and *ZmCDPK1* (D84408). RT-PCR analysis was performed as described previously (Bulgakov *et al.* 2005). The resulting 390 bp fragment corresponded to the V through IX catalytic subdomains of plant CDPKs. Annealing temperature (T_a) was 53 ^oC, and elongation time was 25 s. Analysis of the *rolC* gene expression was performed as described previously (Kiselev *et al.* 2006). The primers 5'AGGTTGTGAACACGGAGCATCTC and 5'CATCCTCCTATGTTTGTTAGTCAGC were designed to flank a 200-bp *rolB* gene fragment. T_a was 60 ^oC, and elongation time was 15 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*, Waldbronn, Germany). The linearity of the PCR was determined to be between 35 and 40 cycles for *rolC* and *rolB* and between 40 and 45 cycles for *P. ginseng CDPK* genes. RNA expression profiles were normalized to the expression of the *P. ginseng* actin 1 gene (GenBank accession no. AY907207). The identities of all RT-PCR products were confirmed by DNA sequencing. Quantitative analysis of mRNAs was performed using microchip technology with a DNA *1000 LabChip*® kit and an *Agilent 2100* bioanalyzer according to the manufacturer's protocol and recommendations. The data are represented as relative fluorescent units normalized to the expression of the corresponding actin genes.

RT-PCR products were sequenced as described

previously (Kiselev *et al.* 2006) in the Instrumental Centre of Biotechnology and Gene Engineering of IBSS FEBRAS using an *ABI 310* genetic analyzer (*Applied Biosystems*, Forster City, USA). Sequencing of each gene was performed at least three times. The *BLAST* search program was used for sequence analysis, and multiple sequence alignments were done using the *BioEdit 7.0.8* program: http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

The PCR products were isolated from gels with a *Glass Milk* kit (*Sileks*, Moscow, 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.

The amino acid sequences of the fragments of CDPK were deduced from the nucleotide sequences using *Gene Runner 3.05* and compared with the known CDPK sequences in other organisms using the *BioEdit 7.0.8* and *BLAST* software programs. For each cell culture, 87 - 250 clones of *CDPK* were sequenced. All sequences were numbered in order of sequencing. These results and data on total *CDPK* expression assayed with degenerate primers were used to estimate the expression (in relative units) of each gene. The relative expression was estimated as the total expression normalized to the expression of the actin gene (Kiselev *et al.* 2009, Dubrovina *et al.* 2009). The data on *P. ginseng CDPK* gene expression were obtained from two independent experiments.

Quantitative real-time PCR was performed according to Giulietti *et al.* (2001). The gene-specific primer pairs and probes are presented in Table 1. It was difficult to select the specific primers due to the high homology of some *PgCDPK* genes. Primers and probes specific to *PgCDPK1a* could not be selected, because their sequences were near identical to those for *PgCDPK1b* and *PgCDPK1c.* The primers and probes to *PgCDPK2b* coincided with the primers to *PgCDPK2a.* The suitability of the primer sequences in terms of annealing efficiency was evaluated using the *Primer Premier 5.0* program. For real-time quantitative-PCR, cDNAs were amplified using a real-time PCR kit (*Syntol*, Russia) with an *iQ5* thermal cycler (*Bio-Rad Laboratories*, Hercules, USA) supplied with optical system software *v.2.0*. Normalized expression was compared to actin, and scaling options were set at highest. The data on *CDPK* expression from real-time PCR were obtained from five independent experiments.

Biomass accumulation, *CDPK* gene expression and clone composition were analyzed in three independent experiments. Statistical analysis was done using the *Statistica 8.0* program. The results are represented as mean ± SE and using a paired Student's *t*-test differences were considered significant at $P < 0.05$.

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

Gene	Primers, 5' - 3'	*TaqMan* probe, 5' - 3'
PgCDPK1a	5'-CGATTTTGGTCTGTCTGTCTTCA	5'-CGTGATATAGTTGGTAGTGCTTACTATGTTGCTC
PgCDPK1b	5'-CCATAACTACGCCGCAACACTTC	
PgCDPK1c		
PgCDPK2a	5'-TTCTGAAATTGTTGGGAGTCCAT	5'-ATTTCTGGTCCATAGTTTCGCTTGAGCACCT
PgCDPK2b	5'-GAATGACTCCAGCACTCCATATA	
PgCDPK2c	5'-TGTGATGAAGGGTCAGTATAGT	5'-TCAATTGTTTTCAAGGCTGCTGTTTCCTTCTT
	5'-AACCCCCAAAGAATGGCAAG	
PgCDPK2d	5'-CATTATAGCGAGAGAGCGGC	5'-TGACCACTTCCGCCACCGTCCTCGCAA
	5'-CAAGAAATTCTCGGGCTTCAAGT	
PgCDPK3a	5'-CATTCAGAGGGGACATTACAGTG	5'-ACCTACAATAATCTTTGTCAATTCAGCAGCCTT
	5'-ACCAACAAGAAATTCTCAGGCTT	
PgCDPK3c	5'-TGTGATGAAGGGTCAGTATAG T	5'-TCCACAACCCCAACAATAGTCTTGAGAAGCT
	5'-AACCCCCAAAGAATGGCAAG	
P. ginseng actin 1	5'-GTATGTTGCTATTCAAGCCGATC	5'-ACCTGTTGTACGACCACTAGCATACAGGGA
	5'-ACCATCACCAGAATCCAGCACA	

Results

Cell cultures of *P. ginseng*: Phenotypic features of the ginseng cell cultures were described previously (Kiselev *et al.* 2008). In this article, we wanted to focus on the most important differences. Transforming the 1c cell culture with an *A. tumefaciens* strain containing a binary vector, GV3101/pPCV002, without other inserts (a GV cell culture) gave a phenotype similar to the 1c culture (Kiselev *et al.* 2008). We used this GV cell culture as a control. Several *rolC* transgenic (2c2, 2cR2, 2c3, CII, CIII) and one *rolB* transgenic (GB) cell cultures were also obtained as a result of *A. tumefaciens* transformation. Expression of *rolC* gene led to the appearance of multiple morphological effects: roots, somatic embryos and shoots (Kiselev *et al.* 2008). In this study, we used two *rolC*

transformed callus cell cultures with different levels of *rolC* gene expression: high (2cR2) and low (2c2) (Fig. 1, Table 2). We did not succeed in generating several *rolB* transformed cell cultures because even low *rolB* gene expression led to cell death. We generated only one *rolB* transgenic cell culture, GB, with a medium to low level of *rolB* expression based on numerous weak *rolB* transcripts (Fig. 1, Table 2).

Table 2. Quantification of the *rolB, rolC* and *CDPK* transcripts by microchip technology (relative fluorescence units) in different cultures. Means ± SE, $n = 3$.

Gene	GV	GV NaCl	2c2	2cR2	GB
rolB					
rolC	0	0	1.71±0.29	10.2±1.28	1.45±0.28
CDPK	0.99±0.13	1.27±0.23	0.93±0.18	0.77±0.14	1.31±0.15

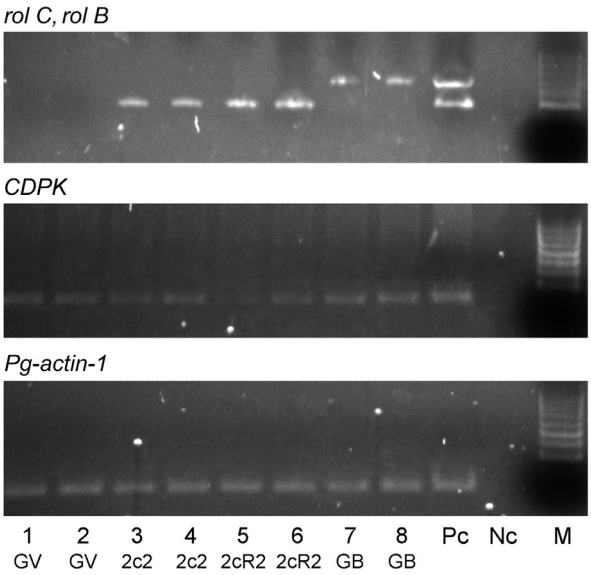

Fig. 1. Expression of *CDPK*, *rolC* and *rolB* in ginseng cell culture at 35 d of cultivation. Electrophoretic separation of *P. ginseng* RT-PCR products of *CDPK*, *rolC*, *rolB* and *Pg-actin-1*. *Lanes* 1, 3, 5, and 7 - GV, 2c2, 2cR2, and GB cultures, 0.5 mm^3 of the templates diluted with H_2O 1:4. *Lanes* 2, 4, 6, and 8 - the cultures presented in the same order, 0.5 mm^3 of undiluted templates; Pc - positive control (plasmids comprising cDNAs for *CDPK, rolC*, *rolB* and *PgActin1*); Nc - negative control (PCR mixture without plant cDNA), M - synthetic marker.

Increased salt tolerance of *rolB* and *rolC* transformed cells: Ginseng cell cultures were grown on medium containing 0 to 200 mM NaCl. All transgenic cell cultures were more tolerant to salt stress than the control GV culture (Table 3). The concentration of NaCl that decreased callus growth on 50 % (IC_{50}) was 30, 45, 60, and 85 mM for the GV, 2c2, GB, and 2cR2 callus lines, respectively. The difference between the GV and GB and 2cR2 cultures was significant. In *rolC* transgenic cells, salt tolerance depended on the level of *rolC* gene expression: it was the highest in the 2cR2 cell culture, which also had the highest *rolC* gene expression (Table 3). The GB cell culture had an intermediate tolerance level: more than that of the 2c2 but less than that of the 2cR2 cell culture (Table 3). The *rolC* and *rolB*-cultures could grow in the presence of NaCl concentrations as high as 200 mM, whereas growth of the control culture was completely inhibited at the same concentrations.

Table 3. Effect of NaCl (0 - 200 mM) on biomass production in control (CV) and the *rolC* and *rolB* transgenic cell cultures (2c2, 2cR2, GB). The biomass accumulation under control conditions was set to 100 %. Means ± SE, $n = 3$. *, ** denotes the statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively.

NaCl [mM]	GV	2c2	2cR2	GB
0	100±8	100±5	100±3	100±15
20	71±6	78±6	98±8	99±13
60	22±4	42±4*	61±5**	52± 8*
200	0	3±1**	6±1**	6± 1**

Inhibition of the CDPK proteins: Salt tolerance is known to be controlled by CDPK kinase activities (Xiong *et al.* 2002). We cultivated cells on medium supplemented with NaCl and NaCl with CDPK inhibitor. The addition of 60 mM NaCl significantly inhibited growth of the GV control cells. Biomass growth of the transgenic 2c2 and 2cR2 cell cultures was inhibited to a lesser degree (Table 4).

We used CDPK inhibitor W7 at concentrations 5 and 30 μM that inhibited cell growth within 30 % of the control. The addition of a CDPK inhibitor to the medium led to a considerable reduction of salt tolerance in the transgenic cell cultures and led to cell death in the control culture (Table 4).

Total *CDPK* gene expression in the ginseng cell cultures: Total *CDPK* gene expression in the control and transgenic ginseng cell cultures and in the control culture under salt stress (60 mM NaCl) was measured using semiquantitative RT-PCR with degenerate primers to the conservative sequences of the *CDPK* serine/threonine kinase domain. Visual electrophoresis results corresponded with the results of quantitative PCR analysis using an *Agilent 2100* bioanalyzer (Fig. 1, Table 2). Total expression of the *CDPK* gene family appeared approximately equivalent among all of the investigated cell cultures. We noticed the following tendencies: *1*) salt stress activated total expression 1.1 - 1.2 times, *2*) *rolC* gene inhibited total *CDPK* gene expression 1.2 - 1.3 times, *3*) *rolB* gene increased total *CDPK* gene expression

Table 4. Fresh mass accumulation in different cell cultures as affected by 60 mM NaCl and CDPK protein inhibitor (5 or 30 μM W7). Means ± SE, $n = 3$. *, ** denotes the statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively.

Culture	Control	5 μM W7	30 μM W7	NaCl	NaCl + 5 μM W7	NaCl + 30 μM W7
GV	1.83±0.08	1.75±0.19	1.51±0.11	0.27±0.04**	0.04±0.02**	0.01±0.01**
2c2	1.91±0.16	1.93±0.15	1.68±0.17	0.92±0.18*	0.55±0.07**	0.43±0.04**
2cR2	0.82±0.10	0.77±0.19	0.65±0.11	0.52±0.11	0.20±0.09**	0.15±0.09**
GB	0.73±0.10	0.71±0.15	0.66±0.08	0.51±0.06	0.35±0.08*	0.30±0.05**

Table 5. *PgCDPK* expression in the control GV, NaCl-treated GV, 2c2, 2cR2 and GB cell cultures. Means ± SE, $n = 3$. *, ** denotes the statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively.

	GV	GV + NaCl	2c2	2cR2	GB
PgCDPK1a	0.41±0.02	0.37±0.02	0.31±0.07	0.34±0.05	0.20±0.07
PgCDPK1b	0.13±0.03	0.05±0.01*	0.07±0.03	0.11±0.04	0.17±0.04
PgCDPK1c	0.03±0.02	0.09±0.01**	0.04±0.01	0.15±0.09*	0.11±0.02*
PgCDPK1d	0.03±0.01	0.01±0.01	0.09±0.07	0.07±0.05	0.02±0.02
PgCDPK1as	0	0	0	0	0.04±0.04
PgCDPK2a	0.01±0.01	0.02±0.02	0.01±0.01	0.02±0.01	0.11±0.02**
PgCDPK2b	0.06±0.04	0.11±0.06	0.05±0.03	0.07±0.01	0.09±0.04
PgCDPK2c	0.01±0.01	0.15±0.02**	0.01±0.01	0.01±0.01	0.10±0.03**
PgCDPK2d	0.01±0.01	0.02±0.02	0.01±0.01	0.06±0.01**	0.07±0.06
PgCDPK2ds	0	0	0	0.01±0.01	0.01±0.01
PgCDPK3a	0.25±0.04	0.09±0.05*	0.16±0.02	0.10±0.03 *	0.20±0.07
PgCDPK3b	0.04±0.04	0.12±0.05	0.01±0.01	0.01±0.01	0.08±0.05
PgCDPK3c	0	0.14±0.05**	0	0	0
PgCDPK3L	0	0	0	0	0.02±0.02
PgCDPK4a	0.03±0.01	0.11±0.02**	0.01±0.01	0.01±0.01	0.12±0.02**
PgCDPK4as	0	0.02±0.02	0	0	0

1.1 - 1.2 times. Previously, we determined that total *CDPK* expression did not change considerably, as a rule (Kiselev *et al.* 2008). We showed substantial inhibition of total *CDPK* expression only in the embryogenic 2c3 ginseng cell culture (Kiselev *et al.* 2008). Significant diversity has been found in the composition of the expressed *CDPK* genes. Therefore, the amplicons of *CDPK* genes were further cloned and analyzed by sequence analysis.

***CDPK* gene expression in NaCl-treated cell cultures:** PCR products of the *CDPK* genes were cloned and sequenced in NaCl-treated *P. ginseng* cell cultures. The fragments were shown to code for a portion of the kinase domain of several CDPKs. All kinase domain fragments were highly similar to known plant CDPKs. The ginseng CDPK amino acid sequences were aligned using the *BioEdit* program and formed four subfamilies, similar to *A. thaliana* CDPKs (Cheng *et al.* 2002). The gene subfamilies were accordingly designated *PgCDPK1*, *PgCDPK2*, *PgCDPK3* and *PgCDPK4* (Kiselev *et al.* 2008). Each subfamily was represented by several groups of genes. In total, 13 genes were identified as follows. The *PgCDPK* nucleotide sequences of the amplified fragments were used to deduce the amino acid sequences. A difference in amino acid sequence in more than one residue suggested a new group. The genes of one group could differ in nucleotide sequence, but their regions flanked by the degenerate primers coded for identical amino acid sequences. Sequenced fragments of *CDPK* genes were deposited to GenBank (GenBank accession numbers indicated in parentheses): *PgCDPK1a* (DQ421785), *PgCDPK1b* (EF546429), *PgCDPK1c* (EF621917), *PgCDPK1d* (EU378948), *PgCDPK2a* (DQ422856), *PgCDPK2b* (EF541125), *PgCDPK2c* (EF600555), *PgCDPK2d* (EU073200), *PgCDPK3a* (DQ422857), *PgCDPK3a* (DQ981494), *PgCDPK3c* (EU939532), *PgCDPK3L* (EU672431), *PgCDPK4a* (EU043518).

The amino acid sequence similarity of CDPKs was no less than 85 % within a subfamily and no more than 70 % between subfamilies. Among all known CDPKs, the highest sequence similarity of the kinase domain fragment was observed with NtCDPK1 for PgCDPK1a (87 %), with AtCPK10 for PgCDPK2a (84 %), with OsCPK7 for PgCDPK3a (94 %) and with AtCDPK17 for PgCDPK4a (94 %).

The greatest difference in *CDPK* expression between

Table 6. Comparison of *PgCDPK* expression in control and 60 mM NaCl-treated GV cell culture of *P. ginseng* using real-time PCR and PCR with degenerate primers, by frequency analysis of RT-PCR products. Means ± SE, $n = 3$. *, ** denotes the statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively.

	Real-time PCR		PCR with degenerate primers	
	GV	GV + NaCl	GV	GV + NaCl
PgCDPK1a, PgCDPK1b, PgCDPK1c	0.93±0.08	0.87±0.06	0.56±0.07	0.50±0.03
PgCDPK2a, PgCDPK2b	0.61±0.25	0.62±0.19	0.07±0.05	0.12±0.08
PgCDPK2c	0.13±0.07	0.81±0.19*	0.01±0.01	0.15±0.01**
PgCDPK2d	0.69±0.21	0.70±0.09	0.01±0.01	0.02±0.02
PgCDPK3a	0.84±0.15	0.58±0.09*	0.26±0.04	0.09±0.04*
PgCDPK3c	0.03±0.02	0.92±0.21*	0	0.14±0.05*

Table 7. Comparison of *PgCDPK* expression in control GV and *rol* transgenic *P. ginseng* cell cultures using real-time PCR and PCR with degenerate primers, by frequency analysis of RT-PCR products. Means ± SE, $n = 3$. *, ** denotes the statistically significant differences at $P < 0.05$ and $P < 0.01$, respectively.

	Real-time PCR				PCR with degenerate primers			
	GV	2c2	2cR2	GB	GV	2c2	2cR2	GB
PgCDPK1a, 1b,1c	0.69±0.18	0.66±0.11	0.53±0.12	0.54±0.19	0.56±0.07	0.42±0.11	0.60±0.18	0.48±0.13
PgCDPK2a, 2b	0.34±0.15	0.35±0.14	0.70±0.10	0.52±0.05	0.07±0.05	0.06±0.04	0.09±0.02	0.20±0.06
PgCDPK2c	0.12±0.05	0.12±0.07	0.13±0.08	0.74±0.09*	0.01±0.01	0.01±0.01	0.01±0.01	0.10±0.03*
PgCDPK2d	0.01±0.01	0.01±0.01	0.23±0.07*	0.03±0.01	0.01±0.01	0.01±0.01	0.06±0.01**	0.07±0.05
PgCDPK3a	0.53±0.09	0.51±0.12	0.20±0.02*	0.45±0.04	0.26±0.04	0.16±0.02	0.13±0.03*	0.20±0.07
PgCDPK3c	0.01±0.01	0.01±0.01	0.02±0.01	0.02±0.01	0	0	0	0

control cells and NaCl-treated cells was the significant increase of the *PgCDPK3c* expression. *PgCDPK3c* expression increased from 0 to 0.17 r.u. (Table 5). Also, *PgCDPK2c* expression was significantly increased (6 to 15 times) in NaCl-treated cells compared to the control cells. The expression of *PgCDPK1c* and *PgCDPK4a* increased 2 - 4 times, while the expression of *PgCDPK1b* and *PgCDPK3a* significantly decreased (2 - 3 times) (Table 5). It is important to note that the NaCl-treated cells expressed *PgCDPK4as*, the serine/threonine kinase domain of which lacked part of catalytic subdomain VII, while the reading frame was not shifted.

In order to confirm the above data on the frequency analysis of RT-PCR products, *CDPK* expression levels were estimated by real-time PCR. The results were consistent with the results of frequency analysis of RT-PCR products (Table 6), suggesting that *PgCDPK2c* and *PgCDPK3c* expression in NaCl-treated cultures was considerably increased, while *PgCDPK3a* expression was considerably decreased compared to the control (Table 6). Real-time PCR showed that traces of *PgCDPK3c* transcripts were present in the control, while the proportion of *PgCDPK3c* transcripts increased dramatically in the NaCl-treated cells. The amplitude of changes varied slightly (Table 6), which could be explained by measurement errors.

***CDPK* gene expression in NaCl tolerant *rolC* and *rolB* transgenic *P. ginseng* cell cultures:** It is important to note that in 2cR2 and GB ginseng cell cultures, some *CDPK* transcripts were found to have missing sequences, encoding the VI and VII subdomains of the serine/threonine kinase domain or in the region between domains. However, these amino acid excisions did not result in frameshifts. Amino acid sequences of these *CDPK* genes were identical to sequences described earlier. They were consistent in groups of "short" transcripts of the ginseng *CDPK* genes: *PgCDPK1as*, *PgCDPK2ds*, and *PgCDPK4as*.

One of the transcripts, *PgCDPK3L*, was "chimeric" because the 5' portion of the transcript was highly homologous to *PgCDPK3b,* and 3' portion was very similar to *PgCDPK3a*. These 5' and 3' portions were connected by an additional L amino acid, therefore, the transcript was called *PgCDPK3L*. It is important to note that similar "short" and "chimeric" transcripts were found in cDNA probes of *rolB* transformed *Vitis amurensis* cell cultures (Dubrovina *et al.* 2009).

PgCDPK2ds was found as two different transcripts in different ginseng cell cultures: *PgCDPK2ds1* in GB cell culture, with the elimination of the VI and VII catalytic subdomains, and *PgCDPK2ds2* in 2cR2 cell culture, with the elimination of five amino acids between the VII and

VIII catalytic subdomains.

Data on the qualitative composition of clones of the *CDPK* genes became the basis for generating data on *CDPK* gene expression in the ginseng cell cultures (Table 5). In GV cell culture, *PgCDPK1a*, *PgCDPK1b* and *PgCDPK3a* genes were typically more highly expressed than other genes.

Expression of *rolC* gene led to a considerable reduction in *PgCDPK3a* expression, which was high in the control cell culture (Table 5). This reduction in *PgCDPK3a* expression depended on the level of the *rolC* gene expression. *PgCDPK1c* and *PgCDPK2d* expression were substantially increased in the *rolC* transformed cell cultures, and "short" *PgCDPK2ds* transcripts also appeared.

Expression of *rolB* gene in ginseng cell cultures led to a significant increase in *PgCDPK1c, PgCDPK2a, PgCDPK2c,* and *PgCDPK4a* expression. "Chimeric" transcripts of *PgCDPK3L* and "short" transcripts of *PgCDPK1as* and *PgCDPK2ds* also appeared (Table 5).

In order to confirm the above data on the frequency analysis of RT-PCR products, *CDPK* expression levels were estimated by real-time PCR. The results were consistent with the results of frequency analysis of RT-PCR products, suggesting that *PgCDPK2d* expression in *rolC* transgenic cell cultures was significantly increased, while *PgCDPK3a* expression was decreased compared to the control (Table 7). Expression of *PgCDPK2c* in the *rolB* transgenic cell culture was significantly increased (Table 7). Real-time PCR showed that traces of *PgCDPK3c* transcripts were presented in the control and *rol* transgenic cell cultures (Tables 6 and 7).

Discussion

Salt tolerance is known to be caused by the activity of CDPK (Xiong *et al.* 2002). Adding a CDPK inhibitor led to a considerable reduction in salt tolerance in the ginseng transgenic cell cultures and cell death in the control culture. We suggest that salt tolerance in the control and *rol* transgenic cell cultures of *P. ginseng* primarily depended on CDPK activity. Presumably, the salt tolerance in the *rol* transgenic cell cultures is caused by the increase of *CDPK* gene expression. For *P. ginseng*, these genes are *PgCDPK1c, PgCDPK2c, PgCDPK3c,* and *PgCDPK4a*. Along with increased expression of some *CDPK* genes, we found reduced *PgCDPK1b* and *PgCDPK3a* expression. Obviously, more work should be done to confirm this hypothesis and identify the functions of every ginseng *CDPK* gene. These findings are of great importance given that *P. ginseng* is one of the most valuable Chinese traditional medicinal herbs (Yue *et al.* 2007). *P. ginseng* was not studied as well as *O. sativa* or *A. thaliana*. It was impossible to use modern molecular approaches (*e.g.* DNA microarray and real-time PCR) at the outset of these studies. Therefore, we developed a new approach of *CDPK* gene expression analysis for *P. ginseng* involving frequency analysis of RT-PCR products obtained using degenerate primers. These data correlated with real-time PCR data, which could be used after we had identified the nucleotide sequences of ginseng *CDPKs*. We were able to identify unknown earlier ginseng *CDPK* gene that was actively expressed in response to salt stress (*PgCDPK3c*).

We attempted to compare our results with the expression of known *CDPK* genes from other plants. Many studies have identified ESTs from NaCl-treated plant cells such as *Glycine soja* (Ji *et al.* 2006). Among 2 003 ESTs we did not find any close homologues to *PgCDPK* genes, therefore, we could not use ESTs in our studies. This lack of *CDPK* homologues could be due to the fact that *CDPKs* are regulatory genes, thus, frequency of *CDPK* transcript occurrence is lower than that of other genes, and there should be more transcripts in an EST analysis to analyze *CDPK* expression.

Many studies have looked at the expression of one or more *CDPK* genes, but only one study looked at the expression of 31 *CDPK* genes in *O. sativa* cells under salt stress using an *Affymetrix 57K GeneChip*® and real-time PCR (Ray *et al.* 2007). In this study, CDPK close in amino acid sequences were shown to be regulated differently by salt stress. For example, the expression of *OsCPK15* was increased, while the expression of *OsCPK1* was decreased (91 % aa identity between OsCPK15 and OsCPK1). Similarly, some of the *PgCDPK* genes, close in amino acid sequences, were shown to be regulated differently by salt stress (*PgCDPK3a*, *PgCDPK3b*, *PgCDPK3c*).

The genes of the *PgCDPK3a* group were similar to *VCPK1* (*ACPK1*), a CDPK of *V. labrusca* × *V. vinifera*, which enhanced biomass growth in *V. labrusca* × *V. vinifera* cell culture (Yu *et al.* 2007). Similar results were obtained in our experiments: a decrease in biomass growth correlated with a decrease in *PgCDPK3a* expression in the transgenic ginseng cultures. In addition, amino acid sequences of *PgCDPK3a* transcripts were similar to *O. sativa OsCDPK7* (*OsCDPK13*) (94%) or to *Phalaenopsis amabilis PaCDPK1*. Overexpression of *OsCDPK7* (*OsCDPK13*) increased salt tolerance of rice plants (Saijo *et al.* 2000). *PaCDPK1* transcription was enhanced in response to low temperature, wounding, and pathogen infection (Tsai *et al.* 2007). There are several ginseng *CDPKs* similar to the *OsCDPK7* (*e.g. PgCDPK3c* - 80 %, *PgCDPK3L* - 86 %). In the present studies, we found a significant increase in *PgCDPK3c* expression in NaCl-treated ginseng cells. *PgCDPK3c* in ginseng cells appears to be the most functionally similar gene to *OsCDPK7*. Large difference in amino acid sequences between PgCDPK3c and OsCDPK7 may be

explained by the large differences between monocot (*O. sativa*) and dicot (*P. ginseng*) plants. *P. ginseng* is more closely related to the dicot species *V. labrusca* × *V. vinifera* than to the monocot species *O. sativa* or *P. amabilis*.

The amino acid sequences of PgCDPK1a and PgCDPK1b are most similar to NtCDPK1, which is highly expressed in rapidly proliferating tissues and cell cultures (Lee *et al.* 2003). NtCDPK1 interacts with the 26S proteasome and catalyzes the degradation of many enzymes and regulatory proteins, promoting the cell transition to mitosis (Lee *et al.* 2003). It is possible that some protein factors that initiate morphogenesis degraded in the GV culture. Consequently, GV cells did not give origin to somatic embryos. According to this scenario, *rolC* expression decreases the expression of *PgCDPK1a* and *PgCDPK1b*, which inhibits 26S proteasomes and increases the concentration of protein factors that trigger somatic embryogenesis in 2c3 cell culture (Kiselev *et al.* 2008). Also, the embryogenic 2c3 culture actively expresses the *PgCDPK2* subfamily (*PgCDPK2b* and *PgCDPK2d*), whose protein products are most similar to AtCPK30. AtCPK30 acts as a Ca^{2+} sensor and is involved in hormonal signal transduction and root development, and elevated *AtCPK30* expression correlates with morphogenetic processes in cell and organ cultures (Yuan *et al.* 2007). However, the differences in *CDPK* gene expression might be caused by the development of somatic embryogenesis in cell culture. This problem may be solved in the further experiments by performing gain- or loss-of-function of several genes, *e.g. PgCDPK1a*, *PgCDPK1b, PgCDPK2b* and *PgCDPK2d*.

Our current study identified "short" and "chimeric" transcripts in *rol* transformed ginseng cell cultures that were not known previously. Also, salt stress led to the presence of "short" *CDPK* transcripts in the control cell culture, suggesting that it was not an effect of transformation with *rol* genes. These oncogenes could potentially activate a natural process that has been already presented in the plant cells. Previously, we detected "short" *CDPK* transcripts in *rolC* transformed embryogenic 2c3 cell culture of ginseng (Kiselev *et al.* 2008). Moreover, it has been recently shown that enhanced phytoalexine accumulation in *rolB* transgenic cell cultures of *V. amurensis* correlates with the appearance of "short" and "chimeric" transcripts of *CDPK* kinase domains, similar to that of *P. ginseng* (Dubrovina *et al.* 2009). It is interesting to note that the number and localization of the excisions in *CDPK* kinase domains were individual for the described "short" transcripts of *CDPKs* (present investigation, Dubrovina *et al.* 2009). Generally, there is a lack of literary data concerning alternative splicing or RNA editing of *CDPKs*. Nishiyama *et al.* (1999) reported one case of alternative splicing of a *CDPK* gene, where sequences corresponding to EF hands were modified. "Short" or "chimeric" transcripts of serine/treonine kinase domains similar to that of *P. ginseng* and *V. amurensis* were not previously reported for *CDPKs* from other plant species and their physiological significance is not known. However, several examples of alternative splicing in kinase domains of other serine/treonine protein kinases are known. For example, it has been shown that "short" *MAPK*, *AtAUR2* (Aurora kinase) and *PKR* [interferon-induced, double-stranded RNA (dsRNA)-dependent protein kinase] transcripts were generated by excisions of a 312-bp sequence corresponding to the III-VI kinase subdomains of MAPK (Xiong and Yang 2003), 96-bp sequence corresponding to the VI kinase subdomain of AtAUR2 (Kurihara *et al.* 2007), and 159-bp sequence corresponding to the kinase subdomains III and IV of PKR (Park *et al.* 2006), leading to the production of serine/treonine kinase proteins lacking kinase activity. The data concerning "short" and "chimeric" *PgCDPK* transcripts presented in the current work, the results of Dubrovina *et al.* (2009) and the cited above modifications of the serine/treonine kinase domains together suggest that the "short" and "chimeric" *P. ginseng* and *V. amurensis* transcripts of *CDPK* kinase domains were most likely subjected to alternative splicing. The unusual (short and long) *P. ginseng CDPK* transcripts described in our study might be pseudokinases (Boudeau *et al.* 2006). Further study is needed to confirm this hypothesis.

We showed that salt stress imposed on the control culture changed the expression of six *PgCDPK* genes (*PgCDPK1b*, *PgCDPK1c*, *PgCDPK2c*, *PgCDPK3a*, *PgCDPK3c*, and *PgCDPK4a*). The data suggest that protein products of these *CDPK* genes play a key role in salt tolerance. However, we showed that salt tolerant *rol* transformed cell cultures of *P. ginseng* expressed different patterns of *CDPK* genes. Expression of only a few of the six genes was significantly changed in *rolC* and *rolB* transformed ginseng cultures. Based on the changes in *CDPK* gene expression in NaCl-treated ginseng cells and *CDPK* expression patterns in *rol* transgenic ginseng cell cultures, we suggest *PgCDPK* genes that could promote NaCl-tolerance in *rolC* and *rolB* transgenic ginseng cell cultures. Salt tolerance in the *rolC* cell cultures could be caused by increased *PgCDPK1c* expression or decreased *PgCDPK3a* expression. Expression of the *rolB* gene could cause salt tolerance by the increase in expression of *PgCDPK1c, PgCDPK2c* or *PgCDPK4a*. The appearance of the "short" *CDPK* transcripts might also play a role in salt tolerance caused by *rolB* or *rolC* transformations. In NaCl-treated ginseng cells, the expression of the "short" *CDPK* transcripts from the *PgCDPK4* subfamily was increased, while, in the *rol* transgenic ginseng cells, the expression of the "short" *CDPK* transcripts from the *PgCDPK1* and *PgCDPK2* subfamilies was increased. The reasons for this difference await further investigation.

References

Asano, T., Tanaka, N., Yang, G., Hayashi, N., Komatsu, S.: Genome-wide identification of the rice calcium-dependent protein kinase and its closely related kinase gene families: comprehensive analysis of the *CDPKs* gene family in rice. - Plant Cell Physiol. **46**: 356-366, 2005.

Boudeau, J., Miranda-Saavedra, D., Barton, G.J., Alessi, D.R.: Emerging roles of pseudokinases. - Trends Cell Biol. **16**: 443-452, 2006.

Bulgakov, V.P., Veselova, M.V., Tchernoded, G.K., Kiselev, K.V., Fedoreyev, S.A., Zhuravlev, Y.N.: Inhibitory effect of the *Agrobacterium rhizogenes rolC* gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures. - Planta **221**: 471-478, 2005.

Bulgakov, V.P., Kiselev, K.V., Yakovlev, K.V., Zhuravlev, Y.N., Gontcharov, A.A., Odintsova, N.A.: Agrobacterium-mediated transformation of sea urchin embryos. - Biotechnol. J. **1**: 454-461, 2006.

Cheng, S.H., Willmann, M.R., Chen, H.C., Sheen, J.: Calcium signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. - Plant Physiol. **129**: 469-485, 2002.

Dubrovina, A.S., Kiselev, K.V., Veselova, M.V., Isaeva, G.A., Fedoreyev, S.A., Zhuravlev, Y.N.: Enhanced resveratrol accumulation in *rolB* transgenic cultures of *Vitis amurensis* correlates with unusual changes in *CDPK* gene expression. - J. Plant Physiol. **166**: 1194-1206, 2009.

Filippini, F., Rossi, R., Marin, O., Trovato, M., Costantino, P., Downey, P.M., Lo Schiavo, F., Terzi, M.: A plant oncogene as a phosphatase. - Nature **379**: 499-500, 1996.

Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C.: An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. - Methods **25**: 386-401, 2001.

Hwang, I., Sze, H., Harper, J.F.: A calcium-dependent protein kinase can inhibit a calmodulin-stimulated Ca^{2+} pump (ACA2) located in the endoplasmic reticulum of *Arabidopsis*. - Proc. nat. Acad. Sci. USA **97**: 6224-6229, 2000.

Ji, W., Li, Y., Li, J., Dai, C.H., Wang, X., Bai, X., Cai, H., Yang, L., Zhu Y.M.: Generation and analysis of expressed sequence tags from NaCl-treated Glycine soja. - BMC Plant Biol. **6**: 1-7, 2006.

Kiselev, K.V., Dubrovina, A.S., Bulgakov, V.P.: Phenylalanine ammonia-lyase and stilbene synthase gene expression in *rolB* transgenic cell cultures of *Vitis amurensis*. - Appl. Microbiol. Biotechnol. **82**: 647-655, 2009.

Kiselev, K.V., Dubrovina, A.S., Veselova, M.V., Bulgakov, V.P., Fedoreyev, S.A., Zhuravlev, Y.N.: The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. - J. Biotechnol. **128**: 681-692, 2007.

Kiselev, K.V., Gorpenchenko, T.Y., Tchernoded, G.K., Dubrovina, A.S., Grishchenko, O.V., Bulgakov, V.P., Zhuravlev, Y.N.: Calcium-dependent mechanism of somatic embryogenesis in *Panax ginseng* cell cultures expressing the *rolC* oncogene. - Mol. Biol. **42**: 243-252, 2008.

Kiselev, K.V., Grishchenko, O.V.: Enhanced salt tolerance in cells of *Panax ginseng*, transformed with *rolC* and *rolB* oncogenes from *Agrobacterium rhizogenes*. - In: Nosov A.M. (ed): The Proceedings of the IX International Conference "The Biology of Plant Cell *in Vitro* and Biotechnology". P. 175. FBK-Press, Zvenigorod 2008.

Kiselev, K.V., Kusaykin, M.I., Dubrovina, A.S., Bezverbny, D.A., Zvyagintseva, T.N., Bulgakov, V.P.: The *rolC* gene induces expression of a pathogenesis-related beta-1,3-glucanase in transformed ginseng cells. - Phytochemistry **67**: 2225-2231, 2006.

Kurihara, D., Kawabe, A., Matsunaga, S., Nakagawa, K., Fujimoto, S., Uchiyama, S., Fukui, K.: Characterization of a splicing variant of plant Aurora kinase. - Plant Cell Physiol. **48**: 369-374, 2007.

Lee, S.S., Cho, H.S., Yoon, G.M., Ahn, J.W., Kim, H.H., Pai, H.S.: Interaction of *NtCDPK1* calcium-dependent protein kinase with NtRpn3 regulatory subunit of the 26S proteasome in *Nicotiana tabacum*. - Plant J. **33**: 825-884, 2003.

López-Carrión, A.I., Castellano, R., Rosales, M.A., Ruiz, J.M., Romero, L.: Role of nitric oxide under saline stress: implications on proline metabolism. - Biol. Plant. **52**: 587-591, 2008.

Martin, M.L., Busconi, L.: A rice membrane-bound calcium-dependent protein kinase is activated in response to low temperature. - Plant Physiol. **125**: 1442-1449, 2001.

Melgar, J.C., Syvertsen, J.P., Martínez, V. García-Sánchez, F.: Leaf gas exchange, water relations, nutrient content and growth in citrus and olive seedlings under salinity. - Biol. Plant. **52**: 385-390, 2008.

Moriuchi, H., Okamoto, C., Nishihama, R., Yamashita, I., Machida, Y., Tanaka, N.: Nuclear localization and interaction of RolB with plant 14-3-3 proteins correlates with induction of adventitious roots by the oncogene *rolB*. - Plant J. **38**: 260-275, 2004.

Nishiyama, R., Mizuno, H., Okada, S., Yamaguchi, T., Takenaka, M., Fukuzawa, H., Ohyama, K.: Two mRNA species encoding calcium-dependent protein kinases are differentially expressed in sexual organs of *Marchantia polymorpha* through alternative splicing. - Plant Cell Physiol. **40**: 205-212, 1999.

Palazón, J., Cusidó, R.M., Gonzalo, J., Bonfill, M., Morales, S., Piñol, M.T.: Relation between the amount the *rolC* gene product and indole alkaloid accumulation in *Catharantus roseus* transformed root cultures. - J. Plant Physiol. **153**: 712-718, 1998.

Park, S.H., Choi, J., Kang, J.I., Choi, S.Y., Hwang, S.B., Kim, J.P., Ahn, B.Y.: Attenuated expression of interferon-induced protein kinase PKR in a simian cell devoid of type I interferons. - Mol. Cells **21**: 21-28, 2006.

Radyukina, N.L., Ivanov, Y.V., Kartashov, A.V., Shevyakova, N.I., Rakitin, V.Y., Khryanin, V.N., Kuznetsov, V.V.: Inducible and constitutive mechanisms of salt stress resistance in *Geum urbanum* L. - Russ. J. Plant Physiol. **54**: 612-618, 2007.

Ray, S., Agarwal, P., Arora, R., Kapoor, S., Tyagi, A.K.: Expression analysis of calcium-dependent protein kinase gene family during reproductive development and abiotic stress conditions in rice (*Oryza sativa* L. ssp. indica). - Mol. Genet. Genomics **278**: 493-505, 2007.

Saijo, Y., Hata, S., Kyozuka, J., Shimamoto, K., Izui, K.: Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. - Plant J. **23**: 319-327, 2000.

Spena, A., Schmulling, T., Koncz, C., Schell, J.S.: Independent

and synergistic activity of *rolA, B* and *C* loci in stimulating abnormal growth in plants. - EMBO J. **6**: 3891-3899, 1987.

Su, G.X., Bai, X.: Contribution of putrescine degradation to proline accumulation in soybean leaves under salinity. - Biol. Plant. **52**: 796-799, 2008.

Tähtiharju, S., Sangwan, V., Monroy, A.F., Dhindsa, R.S., Borg, M.: The induction of *kin* genes in cold-acclimating *Arabidopsis thaliana*. Evidence of a role for calcium. - Planta **203**: 442-447, 1997.

Tiwari, R.K., Trivedi, M., Guang, Z.C., Guo, G.Q., Zheng, G.C.: *Agrobacterium rhizogenes* mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots. - Biol. Plant. **52**: 26-35, 2008.

Tsai, T.M., Chen, Y.R., Kao, T.W., Tsay, W.S., Wu, C.P., Huang, D.D., Chen, W.H., Chang, C.C., Huang, H.J.: PaCDPK1, a gene encoding calcium-dependent protein kinase from orchid, *Phalaenopsis amabilis*, is induced by cold, wounding, and pathogen challenge. - Plant Cell Rep. **26**: 899-1908, 2007.

Urao, T., Katagiri, T., Mizoguchi, T., Yamaguchi-Shinozaki, K., Hayashida, N., Shinozaki, K.: Two genes that encode Ca^{2+}-dependent protein kinases are induced by drought and high salt stresses in *Arabidopsis thaliana*. - Mol. Gen. Genet. **224**: 331-340, 1994.

Veena, V., Taylor., C.G.: *Agrobacterium rhizogenes*: recent developments and promising applications. - *In Vitro* cell. dev. Plant Biol. **43**: 383-403, 2007.

Xiong, L., Schumaker, K.S., Zhu, J.K.: Cell signaling during cold, drought, and salt stress. - Plant Cell **14**: 165-183, 2002.

Xiong, L.Z., Yang, Y.N.: Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. - Plant Cell **15**: 745-759, 2003.

Yu, X.C., Zhu, S.Y., Gao, G.F., Wang, X.J., Zhao, R., Zou, K.Q., Wang, X.F., Zhang, X.Y., Wu, F.Q., Peng, C.C., Zhang, D.P.: Expression of a grape calcium-dependent protein kinase *ACPK1* in *Arabidopsis thaliana* promotes plant growth and confers abscisic acid-hypersensitivity in germination, postgermination growth, and stomatal movement. - Plant mol. Biol. **64**: 531-538, 2007.

Yuan, X., Deng, K.Q., Zhao, X.Y., Wu, X.J., Qin, Y.Z., Tang, D.Y., Liu, X.M.: A calcium-dependent protein kinase is involved in plant hormone signal transduction in *Arabidopsis*. - J. Plant Physiol. mol. Biol. **33**: 227-234, 2007.

Yue, P.Y., Mak, N.K., Cheng, Y.K., Leung, K.W., Ng, T.B., Fan, D.T., Yeung, H.W., Wong, R.N.: Pharmacogenomics and the Yin/Yang actions of ginseng: anti-tumor, angiomodulating and steroid-like activities of ginsenosides. - Chin. Med. **6**: 1-21, 2007.