Plant Growth Regul (2017) 82:79–89
DOI 10.1007/s10725-016-0240-5

ORIGINAL PAPER

The calcium-dependent protein kinase gene *VaCPK29* is involved in grapevine responses to heat and osmotic stresses

Alexandra S. Dubrovina[1] · Konstantin V. Kiselev[1,2] · Valeriya S. Khristenko[1] · Olga A. Aleynova[1]

Received: 30 March 2016 / Accepted: 3 December 2016 / Published online: 19 December 2016

Abstract Ca^{2+}-dependent protein kinases (CDPKs or CPKs) are essential primary sensors of Ca^{2+} in plants and are known to play important roles in plant abiotic and biotic stress responses. *Vitis amurensis* is a wild grapevine species with a high level of cold and disease resistance. It has previously been shown that transcription of 10 *CDPK* genes of *V. amurensis* was elevated under salt, desiccation, high mannitol, cold, and heat stress conditions. Expression of *VaCPK29* was induced under high and low temperatures, water deficit, and high mannitol stress in plant cuttings of *V. amurensis*. The present study revealed that the callus cell cultures of *V. amurensis* and soil-grown plants of *Arabidopsis thaliana* overexpressing *VaCPK29* exhibited higher tolerance to heat and high mannitol stress in comparison with the control transformed with the empty vector. Cold, salt, and drought stress tolerance of the transgenic *V. amurensis* calli and *A. thaliana* plants was comparable to that of the controls. The stress-responsive genes *AtDREB1A, AtDREB2A, AtRD29A, AtRD29B*, and *AtABF3* were up-regulated in the *VaCPK29*-overexpressing *A. thaliana* plants under heat stress. Taken together, the data indicate that the *VaCPK29* gene may act as a positive regulator in the grapevine response to heat and osmotic stresses.

Keywords Calcium-dependent protein kinase · Abiotic stress tolerance · Heat stress · Osmotic stress · *Vitis amurensis* · *Arabidopsis thaliana*

Introduction

Multiple environmental and developmental factors such as hormones, light, or environmental stresses initiate stimulus-specific elevations in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]$). The spatially- and temporally-distinct changes in $[Ca^{2+}]$, designated as "Ca^{2+} signatures", are also characterized by a specific amplitude, frequency, sub-cellular location, duration, and shape and are considered to induce proper responses to environmental or developmental signals (Reddy et al. 2011; Batistič and Kudla 2012). Perturbations in $[Ca^{2+}]$ are recognized, decoded, and transmitted by a number of Ca^{2+} sensor proteins including calmodulin (CaM), calmodulin-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), and their interacting kinases (CIPKs) (Hashimoto and Kudla 2011; Batistič and Kudla 2012). CDPKs (EC 2.7.1.37) are Ca^{2+}-regulated Ser/Thr protein kinases encoded by a large multigene family that can be found in plants, green algae, protists, and oomycetes (Valmonte et al. 2014). CDPKs are composed of a N-terminal variable domain, a kinase or catalytic domain, and a CDPK activation domain (CAD) that includes a pseudosubstrate segment and a CaM-like Ca^{2+}-binding domain (Liese and Romeis 2013). The pseudosubstrate segment is known to inhibit phosphorylation and keeps CDPKs inactive at low Ca^{2+} concentrations (Harper et al. 1994; Wernimont et al.

Electronic supplementary material The online version of this article (doi:10.1007/s10725-016-0240-5) contains supplementary material, which is available to authorized users.

✉ Alexandra S. Dubrovina
dubrovina@biosoil.ru

[1] Laboratory of Biotechnology, Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia 690022

[2] Department of Biochemistry, Microbiology and Biotechnology, Far Eastern Federal University, Vladivostok, Russia 690090

2010; Liese and Romeis 2013). Upon Ca^{2+} entry, CDPKs perceive the information encoded in the Ca^{2+} signatures and translate it into the phosporylation of specific target proteins (Hashimoto and Kudla 2011).

An increasing body of evidence has shown that CDPKs are important players in plant abiotic and biotic stress responses, immune signaling, and development (Asano et al. 2012a; Schulz et al. 2013; Boudsocq and Sheen 2013). The transcription levels and kinase activities of CDPKs are affected by various environmental stress conditions (Jaworski et al. 2011; Das and Pandey 2010; Franz et al. 2011; Dubrovina et al. 2013; Zhang et al. 2015). Various plant species have been transformed with certain *CDPK* genes from *Arabidopsis thaliana, Oryza sativa, Populus euphratica*, or *Zea mays*. Overexpression of these *CDPK* genes improved plant tolerance to various abiotic stresses (Zou et al. 2010; Asano et al. 2012b; Chen et al. 2013b; Wei et al. 2014). On the contrary, *CDPK* overexpression has also been shown to confer greater stress sensitivity (Ma and Wu 2007; Franz et al. 2011; Weckwerth et al. 2015). Thus, *CDPKs* are implicated in both positive and negative regulation of plant abiotic stress adaptation. Activated CDPKs have been shown to phosphorylate proteins (e.g., membrane channels, NADPH oxidase, or transcription factors) involved in stomatal movements, oxidative burst, and gene expression regulation (Choi et al. 2005; Kobayashi et al. 2007; Geiger et al. 2010). Nevertheless, the precise physiological functions of most plant CDPKs are still not clear.

Cultivated grapevine *Vitis vinifera* L. is an important fruit crop worldwide and represents a valuable source for wine production. Whole-genome sequencing of *V. vinifera* cv. PN40024 demonstrated the presence of 17 or 19 *CDPK* genes in its genome, depending on the study (Velasco et al. 2007; Jaillon et al. 2007; Chen et al. 2013a; Zhang et al. 2015). Several initial studies of Vitaceae *CDPKs* focused on *VvCPK7* (*ACPK1*) of *V. vinifera* and indicated that the gene is involved in fruit development and seed germination but not in abiotic stress responses (Shen et al. 2004; Yu et al. 2006, 2007). Later, Chen et al. (2013a) identified 17 *CDPK* genes in the 12x genome sequence of *V. vinifera* and analyzed the expression profiles of these *CDPKs* in various grapevine organs at different developmental stages and under certain abiotic stress conditions using the publicly available Affymetrix microarray data. Some *V. vinifera CDPKs* (*VvCPK3, 7, 11, 12, 13, 14, 17*) were found to be up-regulated in response to drought and salt stresses (Chen et al. 2013a). In a recent study, Zhang et al. (2015) identified 19 *CDPK* genes of *V. vinifera* during a genome-wide analysis of the grapevine 12x genome. The authors also analyzed the expression of the 19 homologous *CDPK* genes in the wild grape *V. pseudoreticulata* under various abiotic, biotic, and phytohormone treatments using quantitative real-time RT-PCR (qRT-PCR). The analysis revealed that a large number of *VpCDPK* genes were markedly up-regulated under the tested stress conditions, indicating the important roles *VpCPKs* play in abiotic and biotic stress resistance to *V. pseudoreticulata*. We recently identified 13 *CDPK* genes in the wild grapevine *V. amurensis* Rupr. and characterized their organ-specific and stress-induced expression patterns (Dubrovina et al. 2013). The wild grapevine *V. amurensis* possesses a remarkable abiotic and biotic stress tolerance, especially to freezing, drought, and microbial pathogens, and is currently used for wine production and as a breeding parent (Ma et al. 2010; Liu and Li 2013).

According to our data, transcription of 10 *CDPK* genes of *V. amurensis* (*VaCPK1, 2, 3, 9, 13, 16, 20, 21, 26*, and *29*) was induced under different abiotic stress treatment conditions, including water deficit, high salinity, as well as high mannitol and temperature stresses. Expression of the remaining three *CDPK* genes (*VaCPK3a, 25*, and *30*) was not responsive to these stresses (Dubrovina et al. 2013). Overexpression of the *VaCPK20* gene in callus cell cultures of *V. amurensis* and in transgenic plants of *A. thaliana* improved resistance to cold and drought stresses, while overexpression of the *VaCPK21* gene improved resistance to salt stress, demonstrating that the *VaCPK20* and *VaCPK21* genes may play a role in the positive regulation of signaling pathways involved in cold, drought, and salt stress adaptation in *V. amurensis* (Dubrovina et al. 2015, 2016a). In similar overexpression experiments, the *VaCPK3a* and *VaCPK9* genes were shown to function in growth regulation of *V. amurensis* but not in its abiotic stress adaptation (Kiselev et al. 2013; Dubrovina et al. 2016b). Nevertheless, the functions of most grape *CDPKs* in wild grape abiotic stress adaptation are largely unknown at the present time.

This study aimed to investigate the involvement of the *CPK29* gene of *V. amurensis* in the plant's adaptation to abiotic stress. Transcription of the *VaCPK29* gene was found to be elevated under desiccation, high mannitol, as well as cold and heat stresses in the plant cuttings of *V. amurensis* (Dubrovina et al. 2013), therefore *VaCPK29* has been suggested to function in stress resistance of *V. amurensis*. Transgenic callus cultures of *V. amurensis* and plants of *A. thaliana* overexpressing the *VaCPK29* gene exhibited increased resistance under heat stress and mannitol-induced osmotic stress. Thus, *VaCPK29* may play a role in the positive regulation of the signaling pathways involved in heat and osmotic stress resistance in *V. amurensis*.

Materials and methods

Plant materials and growth conditions

The V2 callus culture of wild grapevine *V. amurensis* Rupr. (Vitaceae) was established in 2002 as described previously

(Kiselev et al. 2007). The KA-0 empty vector-transformed callus cell culture was obtained in 2012 by co-cultivation of the V2 cell suspension with *Agrobacterium tumefaciens* GV3101::pMP90 strain containing pZP-RCS2-*npt*II (Tzfira et al. 2005), which contained only the kanamycin (Km) resistance gene, *npt*II, under the control of the double cauliflower mosaic virus (CaMV 35S) promoter as described previously (Kiselev et al. 2007, 2013). The *VaCPK29*-transgenic callus cell cultures of *V. amurensis* (designated KA10-I, KA10-II, KA10-III, and KA10-IV) were obtained in 2013 by transformation of the V2 cell suspension with *A. tumefaciens* strain GV3101::pMP90 containing pZP-RCS2-*VaCPK29-npt*II as described previously (Aleynova et al. 2015). The grape callus cell cultures were cultivated at 30-day subculture intervals in the dark as described (Kiselev et al. 2009; Aleynova et al. 2015). Plants (*Arabidopsis thaliana* ecotype Columbia L., stored by our lab) were grown in pots filled with commercially available rich soil in a controlled environmental chamber at 22 °C (Sanyo MLR-352, Panasonic, Japan) kept on a 16/8 h day/night cycle at a light intensity of ~120 µmol m^{-2} s^{-1}.

Isolation and sequencing of *VaCPK29*

Full-length cDNA coding sequence (1638 bp) of *VaCPK29* (*VaCPK1a*) was amplified using primers based on the predicted sequence of *VvCPK29-like* gene of *V. vinifera* (GB acc. no KC488317), subcloned, and sequenced previously (Dubrovina et al. 2013; Aleynova et al. 2015). Multiple sequence alignments were done with the BioEdit 7.0.8 program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The amino acid sequence homology analysis of VaCPK29 and other plant CDPKs was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov) by blastp algorithm (protein–protein BLAST). PROSITE (http://prosite.expasy.org/) was used to analyze domain structure of the CDPK proteins.

Overexpression of *VaCPK29* in cell cultures of *V. amurensis*

The plasmid constructions for plant cell transformations were obtained previously as described (Aleynova et al. 2015). Briefly, to generate the construction, the full-length cDNA of *VaCPK29* (GB acc. no KC488317) was amplified by PCR as described previously (Dubrovina et al. 2013; Aleynova et al. 2015). The full-length cDNA of *VaCPK29* was cloned into the pSAT1 vector under the control of the double CaMV 35S promoter (Tzfira et al. 2005). Then, the expression cassette with *VaCPK29* was cloned from pSAT1 into the pZP-RCS2-*npt*II (Tzfira et al. 2005). Plasmid DNA samples (pSAT1 and pZP-RCS2-*npt*II) were kindly provided by Professor Alexander Krichevsky (State University of New York, Stony Brook, USA). The overexpression construct of *VaCPK29* (pZP-RCS2-*VaCPK29-npt*II) or empty vector (pZP-RCS2-*npt*II) was introduced into the *A. tumefaciens* strain GV3101::pMP90 and transformed into the *V. amurensis* suspension culture V2 by co-cultivation with the bacterial cells as described (Aleynova-Shumakova et al. 2014; Aleynova et al. 2015). Transgenic callus cell cultures were selected as described (Aleynova-Shumakova et al. 2014; Aleynova et al. 2015).

Salt, cold, and heat treatments of transgenic callus cell cultures

Growth analysis and abiotic stress treatments of transgenic callus cell cultures were conducted as described (Dubrovina et al. 2015, 2016a). Briefly, salt treatment was applied by adding 50 and 100 mM of NaCl to the $W_{B/A}$ culture media. Mannitol treatment was applied by adding 200 and 300 mM of D-mannitol to the medium. Cold and heat treatments were performed by culturing the transgenic cells at 16 and 33 °C in a growth chamber (TSO-1/80 SPU, SKTB, Smolensk, Russia). The average growth rates were assessed after 30 days of cultivation under the control and stress conditions.

Overexpression of *VaCPK29* gene in *A. thaliana*

To create *A. thaliana* lines overexpressing the *VaCPK29* gene, we used the same plasmid construction as for overexpression of *VaCPK29* in cell cultures of *V. amurensis* (described above). The overexpression construct of *VaCPK29* (pZP-RCS2-*VaCPK29-npt*II) or empty vector (pZP-RCS2-*npt*II) was introduced into the *A. tumefaciens* strain GV3101::pMP90 and transformed by floral dip method into wild-type *A. thaliana* (Zhang et al. 2006). Transgenic plants were selected and confirmed by PCR as described (Dubrovina et al. 2015, 2016a) using primers S1 5′GAA TGG GGG ATG AAG CGA CT designed to the 3′ end of the *VaCPK29* protein coding region and A1 5′GAG AGA CTG GTG ATT TTT GCG designed to the CaMV 35 S terminator in the pSAT1 vector. The PCR products were verified by DNA sequencing as described (Kiselev et al. 2015). The transgenic lines used in this study were homozygous plants with single copy insertion. Four representative independent T_3 homozygous lines (L1, L2, L3, and L4) with high mRNA levels of *VaCPK29* were chosen for detailed analyses.

Drought, salt, cold, and heat tolerance analysis of transgenic *A. thaliana*

The plants were subjected to heat, freezing, drought, and salt stress treatments as described (Dubrovina et al. 2015,

2016a). Briefly, the sterilized transgenic seeds of Arabidopsis were germinated on plates and the 7-day-old seedlings were transferred to commercially available rich well-watered soil in a controlled environmental chamber at standard conditions. Then, the plants were subjected to drought by culturing without additional irrigation for 5 weeks, and then re-watered. For salt stress treatments, the transferred seedlings were cultivated without additional irrigation for 2 weeks, and then the plants were well-irrigated with 350 mM NaCl solution. No signs of drought were observed for plants before irrigation with the NaCl solution. One week after irrigation with NaCl, the pots were placed in 3 cm deep fresh water for 4 h to leach the salt from the soil. For cold tolerance assays, normally cultured *A. thaliana* plants (3-week-old) were stressed in a −10 °C freezer for 1.5 h and then cultured at 8 °C for 2 h. For heat tolerance assays, normally cultured plants (3-week-old) were stressed at 45 °C in a controlled incubator for 3 h. The survival rates were determined as the number of visibly green plants 3 days after re-watering (drought), 1 week after heat and cold stress treatments, and 1 week after salt leaching (salt stress). Two pots of plants (10 seedlings per pot) were grown for each transgenic line and each treatment in one experiment. The experiments were repeated eight times for each stress treatment type. We also assessed responses to mannitol of 1-week-old *A. thaliana* seedlings grown on the 1/2 Murashige and Skoog (MS) plates. Six-day-old VC and *35S-VaCPK29* seedlings were transferred on plates with the 1/2 MS medium supplemented with 500 mM mannitol. Survival rates were determined as the number of visibly green seedlings 6 days after the transfer.

Nucleic acid isolation and qRT-PCR

Total DNA isolation from all plants was performed as described previously (Kiselev et al. 2015). Total RNA isolation was performed using the cetyltrimethylammonium bromide-based extraction (Kiselev et al. 2012). Total RNA was isolated from the 3-week-old normally cultured and heat-stressed *A. thaliana* plants (cultivated at +22 or +45 °C for 1 h 20 min). The leaves of the control and heat-stressed plants were collected for RNA extraction after the stress treatments. Complementary DNAs were synthesized using 1.5 µg of total RNA by the MMLV RT Kit (Silex M, Moscow, Russia) as described (Kiselev et al. 2007). cDNAs of *VaCPK29, AtGAPDH, AtActin*, and the stress-responsive genes of *A. thaliana* (*ABF3, DREB1A, DREB2A, RD29A, RD29B, CSD1, CSD2, CAT1)* were amplified as described (Dubrovina et al. 2015). To evaluate the *VaCPK29* transcript levels in the transgenic *A. thaliana* plants, we used the S1 and A1 primers (mentioned above). The oligonucleotide primers, used for evaluating expression of the stress-responsive genes were designed and applied as described (Dubrovina et al. 2015). qRT-PCR data were obtained and analyzed as described previously (Dubrovina and et al. 2015). Expression was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). After the calculations, the highest expressing sample was assigned the value 1 in the relative mRNA calculation in each qPCR reaction.

Statistical analysis

All analyses of *Arabidopsis* survival rates in this work were independently repeated at least eight times with 20 replicates. The data for fresh biomass accumulation in the callus cultures were obtained from two independent experiments with ten replicates each. The data are presented as mean ± standard error (SE) and were tested by Student's *t* test. The data from qRT-PCRs (expression analysis of selected stress-responsive genes) were obtained from two independent experiments with eight replicates each. The data are presented as mean ± standard error of the mean (SE) and were subjected to a one-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) test at $P \leq 0.05$ with Tukey's Honestly Significant Difference (HSD) test at $P \leq 0.05$ using the Microsoft Office Excel 2003 program.

Results

Stress tolerance of *VaCPK29*-overexpressing callus cell cultures of *V. amurensis*

To investigate the effects of *VaCPK29* overexpression on the growth of *V. amurensis* cells cultivated in vitro, we used independent clonal callus cell cultures of *V. amurensis* transformed with the *VaCPK29* gene under the control of the double CaMV 35S promoter. The cell cultures were previously obtained from the V2 cell suspension culture of *V. amurensis* (Aleynova et al. 2015). Briefly, *A. tumefaciens* strains bearing pZP-RCS2-*npt*II or pZP-RCS2-*VaCPK29-npt*II constructs were inoculated in multiple separate flasks with cell suspensions of *V. amurensis* to establish the independently-transformed KA-0 (empty vector), KA10-I, KA10-II, KA10-III, and KA10-IV callus cell cultures. The *VaCPK29* and *npt*II genes were cloned under the control of the double CaMV 35S promoter in the constructs. We selected transgenic cell aggregates in the presence of Km, as described (Aleynova et al. 2015). The KA-0 cell culture was used as a control in all further experiments. The KA10-I, -II, -III, and -IV cell cultures represented friable, vigorously-growing, homogenous tissues that did not appear to have undergone differentiation (Fig. S1). qRT-PCR revealed that the KA10-I,-III, and -IV calli expressed the exogenous *VaCPK29* gene at a high level, while the

Table 1 Effects of cold, heat, salt and mannitol-induced osmotic stresses on the growth of 30-day-old transgenic callus cell cultures of *V. amurensis*

Cell culture	Fresh weight (g/L)	Control (heat) (%)	Heat stress, % (+33 °C, 30 days)	Control (cold), %	Cold stress, % (+16 °C, 30 days)	Control (salt), %	Salt stress, % (NaCl 50 mM, 30 days)	Salt stress, % (NaCl 100 mM, 30 days)	Control (mannitol), %	Osmotic stress, % (mannitol 200 mM, 30 days)	Osmotic stress, % (mannitol 300 mM, 30 days)
KA-0	145.9 ± 12.5	100 ± 11.3	25.9 ± 3.5**	100 ± 21.3	37.1 ± 5.0**	100 ± 21.3	77.5 ± 13.3	34.4 ± 6.2*	100 ± 3.2	46.4 ± 4.2**	23.6 ± 2.8**
KA10-I	155.3 ± 9.4	100 ± 8.1	41.0 ± 16.6**	100 ± 8.4	38.9 ± 2.8**	100 ± 8.4	63.0 ± 14.3	31.7 ± 4.7**	100 ± 14.9	104.1 ± 5.9	53.9 ± 7.9*
KA10-II	146.9 ± 13.6	100 ± 9.8	31.9 ± 10.4**	100 ± 13.7	41.4 ± 5.5**	100 ± 13.7	66.6 ± 9.0	27.7 ± 4.5**	100 ± 13.0	80.2 ± 8.2*	63.0 ± 8.9*
KA10-III	149.1 ± 6.7	100 ± 4.5	53.9 ± 12.7**	100 ± 6.3	36.8 ± 4.3**	100 ± 6.3	60.5 ± 8.5**	25.9 ± 4.7**	100 ± 6.3	73.0 ± 4.4**	31.0 ± 3.0**
KA10-IV	139.6 ± 7.9	100 ± 7.9	67.0 ± 15.8*	100 ± 11.1	40.3 ± 4.5**	100 ± 11.1	77.3 ± 12.3	34.3 ± 5.3*	100 ± 12.2	105.0 ± 10.4	59.9 ± 8.6*

*, **Significantly different from the control conditions for each stress type at $P \leq 0.05$ and 0.01, respectively, according to the Student's *t* test

KA10-II calli did not actively express it (Aleynova et al. 2015). Expression of endogenous *VaCPK29* in the KA10 callus cell cultures did not significantly differ from that in the control KA-0 culture and was at approximately the same level in all four KA10 calli.

Under standard cultivation conditions, the KA10 cell cultures accumulated biomass at approximately the same rate as the control KA-0 during the 30 d of cultivation (Table 1). To assess the effect of heat, cold, salt and osmotic stress conditions, we cultivated the transgenic calli at 16 °C (i.e., cold stress conditions) and 33 °C (i.e., heat stress conditions) for 30 days and in the presence of NaCl or mannitol for 30 days (Table 1). Under cold and salt stress conditions, the growth of the KA10 cell cultures was reduced to approximately the same level as the growth of the control KA-0 calli. Three KA10 calli showed a higher tolerance to heat stress in comparison with the tolerance of the KA-0 control calli. Under heat stress conditions, growth of the KA10-I, -III, and -IV cell cultures (active transgene expression) was reduced 1.5–2.4-fold, while the growth of KA-0 (control) and KA10-II (weak *VaCPK29* expression) was reduced 3.9- and 3.1-fold, respectively (Table 1). In response to osmotic stress generated by mannitol, the KA10-I, -II, -III, and -IV cell cultures showed an increased growth rate in comparison with that of the KA-0 control. Growth of the KA10 calli in the presence of mannitol was not inhibited or was reduced to a less degree than that of the KA-0 (Table 1). Thus, overexpression of the *VaCPK29* gene did not have a consistent and considerable effect on cold and salt stress resistance levels of the transgenic cell cultures of *V. amurensis*, while it improved growth of the KA10 calli under heat stress and high mannitol conditions. Taken together, these data indicate that a high level of *VaCPK29* expression improved the resistance of *V. amurensis* calli to heat stress and mannitol-induced osmotic stress.

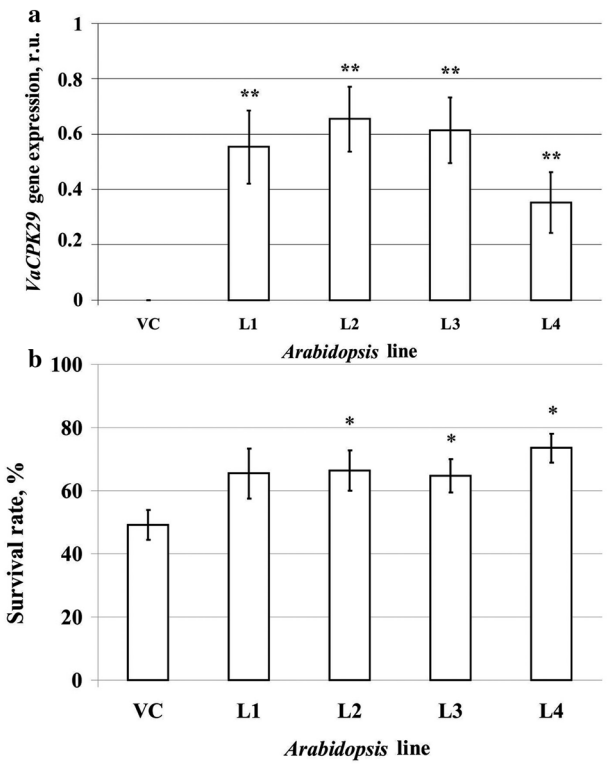

Fig. 1 Characterization of *VaCPK29*-transformed *A. thaliana* lines. **a** qRT-PCR analysis of the *VaCPK29* gene in transgenic lines. **b** Response of transgenic lines to mannitol-induced osmotic stress in vitro. Six-day-old seedlings, pre-cultured on half-strength MS medium for 1 week, were grown on semisolid half-strength MS medium supplemented with 500 mM mannitol. Survival rates were recorded as the number of visibly green seedlings present at day 6. *, **Significantly different from the vector control at $P \leq 0.05$ and 0.01, respectively, according to the Student's *t* test

Stress tolerance of *VaCPK29*-overexpressing *A. thaliana* plants

To investigate the physiological role of *VaCPK29* in abiotic stress responses, the overexpression constructs pZP-RCS2-*VaCPK29*-*npt*II and pZP-RCS2-*npt*II were transformed in Arabidopsis. Four independent fertile T_3 homozygous lines of *A. thaliana* transformed with *VaCPK29* (L1, L2, L3, and L4) and a T_3 homozygous KA-0 line of *A. thaliana* transformed with the empty vector (VC) were chosen for further experiments. Since the VC and wild type controls exhibited similar survival rates under the stress conditions tested earlier (Dubrovina et al. 2015, 2016a), the wild type control was not used in the present investigation. No consistent differences in growth and morphologies were observed between the VC, L1, L2, L3, and L4 *A. thaliana* lines. qRT-PCR demonstrated that all four transgenic *35S-VaCPK29* expressed the *VaCPK29* gene at a high level and there were no considerable differences in *VaCPK29* expression between all four lines (Fig. 1a).

The stress tolerance assays showed that the survival rates of the VC and *35S-VaCPK29* lines were affected by freezing, drought, and salinity stress treatments to approximately the same degree (Fig. 2a–c). For the heat stress treatment, the data obtained indicated that the survival rates of 35S-*VaCPK29 A.thaliana* lines were higher than that of the VC control (Fig. 2d). In all, 30–44% of the *35S-VaCPK29* L1, L2, L3, and L4 transgenic lines survived, whereas only 27% of the VC plants survived. However, the increase in heat stress tolerance was slight and was statistically significant for only two KA10 transgenic lines.

We also analyzed the survival rates of the VC and KA10 lines when the *A. thaliana* seedlings were exposed to hyperosmotic stress by transferring the seedlings to plates containing 1/2 MS medium supplemented with

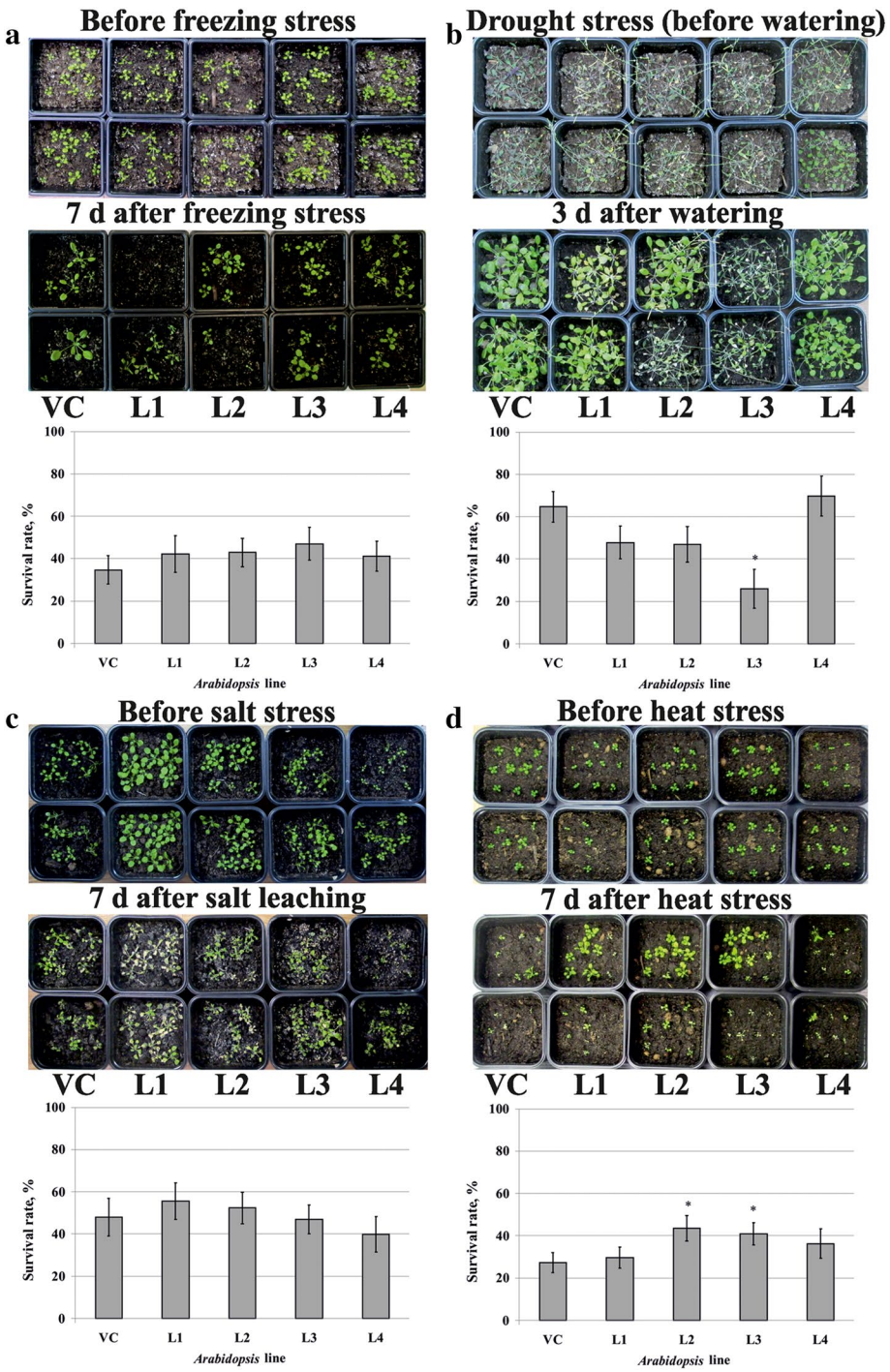

Fig. 2 Response of *VaCPK29*-transformed *A. thaliana* lines to abiotic stresses. **a** Survival rates of *A. thaliana* under cold stress. Three-week-old plants were cold stressed at −10 °C for 1.5 h and then transferred to normal conditions for recovery. **b** Survival rates of *A. thaliana* under drought stress. One-week-old plants were transplanted to soil, watered, and then cultivated for an additional 5 weeks without watering to induce drought stress, and finally were re-watered. **c** Survival rates of *A. thaliana* under salt stress. Three-week-old plants were irrigated with 350 mM NaCl solution. Free NaCl solution was removed and the plants were cultured for 1 week. Then, the pots were placed in fresh water to leach the salt from the soil. **d** Survival rates of *A. thaliana* under heat stress. Three-week-old plants were heat stressed at +45 °C for 3 h and then transferred to normal conditions for recovery. Survival rates were recorded as the number of visibly green plants after 7 days (cold, salt, and heat) or 3 days (drought). *, **Significantly different from the vector control at $P \leq 0.05$ and 0.01, respectively, according to the Student's *t* test

500 mM mannitol (Fig. 1b; Fig. S2). The analysis showed that the *35S-VaCPK29* L1, L2, L3, and L4 plants exhibit a higher tolerance for mannitol-induced osmotic stress than the VC control plants. The results indicated that the *VaCPK29* gene conferred heat and osmotic stress tolerance to the transgenic Arabidopsis at a low level and did not affect its drought, freezing, and salt stress resistance.

Expression of the stress-associated genes in *VaCPK29*-overexpressing *A. thaliana* plants

In order to investigate the role of the *VaCPK29* gene in the heat stress response pathway, the expression levels of eight stress-related genes were analyzed by qRT-PCR in the VC control plants and in the 35S-*VaCPK29* transgenic lines. We analyzed transcription levels of the stress-inducible genes (*AtABF3, AtDREB1A, AtDREB2A,*

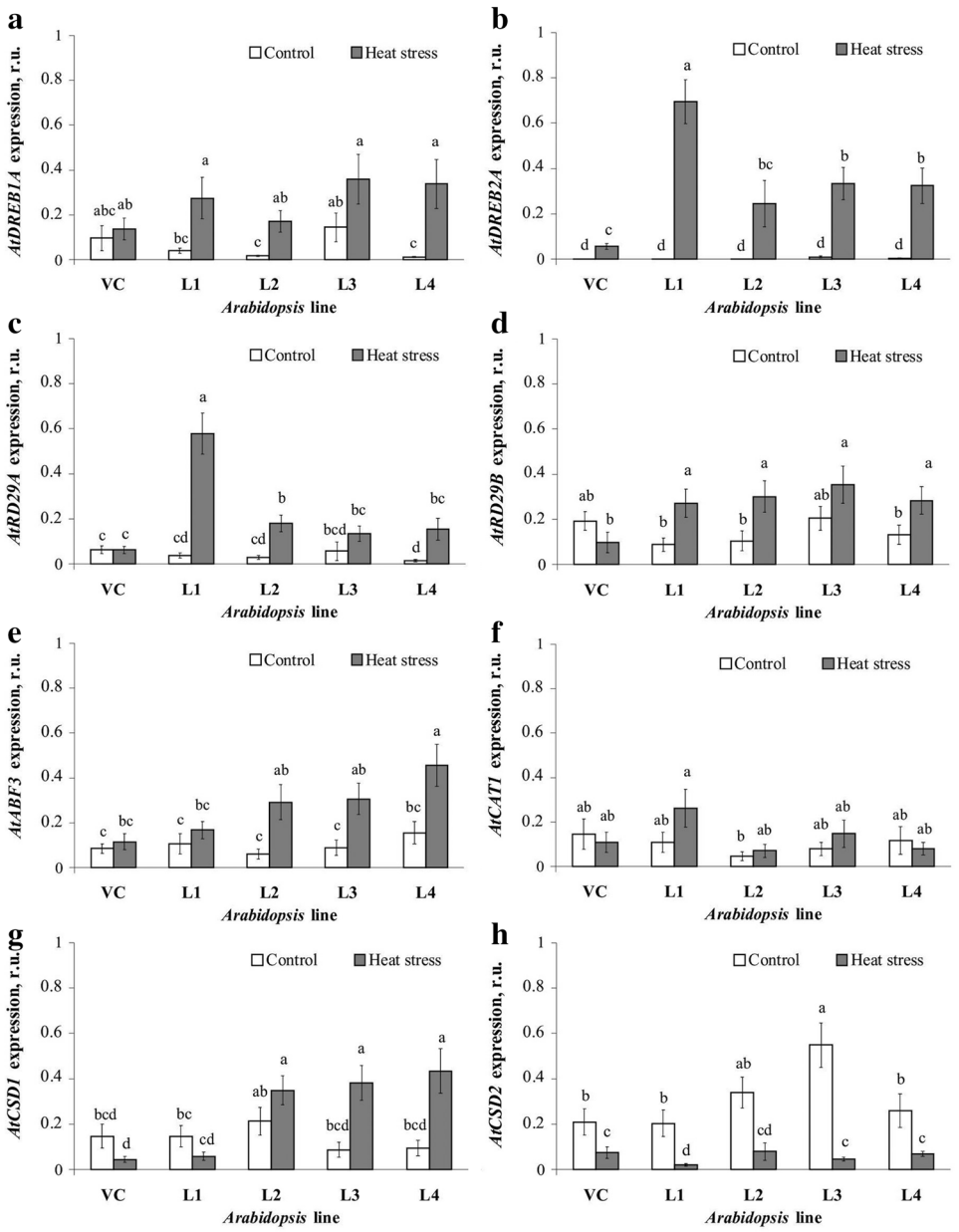

Fig. 3 Expression analysis of selected stress-responsive genes **a** *AtDREB1A*, **b** *AtDREB2A*, **c** *AtRD29A*, **d** *AtRD29B*, **e** *AtABF3*, **f** *AtCAT1*, **g** *AtCSD1*, and **h** *AtCSD2* in the 3-week-old normally cultured (+22 °C for 1 h 20 min, *white bars*) and heat-stressed (+45 °C for 1 h 20 min, *dark grey bars*) *VaCPK29*-transformed *A. thaliana* lines. Means with common letters are not significantly different at $P \leq 0.05$, according to Tukey's HSD test

AtRD29A, AtRD29B) and antioxidant genes (*AtCSD1, AtCSD2, AtCAT1*) in the 3-week-old VC and *VaCPK29*-overexpressing L1–L4 Arabidopsis plants exposed to control and heat stress conditions (Fig. 3). Under standard cultivation conditions, all lines showed the same level of expression of the stress-associated genes, with the exception of the transcription levels of *RD29A* in L4 and *CSD2* in L3 compared to that in VC and some other lines. However, under heat stress, expression levels of *AtABF3, AtDREB1A, AtDREB2A, AtRD29A*, and *AtRD29B* were up-regulated in the *VaCPK29*-overexpressing L1, L2, L3, and L4 lines but not in the VC control line. The expression of the heat-inducible gene *AtDREB2A* (Sakuma et al. 2006b; Schramm et al. 2008) displayed the most considerable up-regulation in the *VaCPK29*-overexpressing plants. Expression of *AtABF3, AtDREB1A, AtDREB2A, AtRD29A*, and *AtRD29B* in the control VC plant line was induced to a lesser extent or was not affected at all. In response to heat stress, expression of the *AtCSD1* gene significantly increased only in the L3 and L4 KA10 lines (CSD1 in L3 and L4 under heat vs. that under control conditions). Expression of the *AtCSD2* gene was down-regulated in both the VC and all four KA10 transgenic lines in response to heat stress. Thus, overexpression of *VaCPK29* enables Arabidopsis to cope with heat stress by regulating expression of the stress-inducible genes *AtDREB1A, AtDREB2A, AtRD29A, AtRD29B*, and *AtABF3*.

Table 2 Comparison of the deduced amino acid sequence of the *VaCPK29* gene (GB acc. no KC488317) and the closest homologous CDPKs of *V. vinifera, A. thaliana, O. sativa*, and *P. trichocarpa*

CDPK protein	I (%)	S (%)
VvCPK15 (VIT_18s0001g00990, GSVIVT01008749001)	98	98
AtCPK9 (GB acc. no NM_112932)	70	85
AtCPK21 (GB acc. no NM_116710)	69	82
AtCPK29 (GB acc. no NM_202421)	68	81
OsCPK1 (LOC_Os01g43410)	67	83
OsCPK15 (GB acc. no AC137608)	68	84
PtCDPK19 (POPTR_0021s00750)	73	87
PtCDPK23 (POPTR_0002s01850)	72	81
PtCDPK24 (POPTR_0005s26640)	74	83

I amino acid identities, *S* amino acid similarities

Discussion

As a result of the recent sequencing of the *V. vinifera* genome (Jaillon et al. 2007; Velasco et al. 2007) and the high stress resistance of wild-growing *V. amurensis* (Ma et al. 2010; Liu and Li 2013), the wild grape species is considered a promising experimental system to investigate plant stress adaptation mechanisms. Unraveling the mechanisms of wild grape resistance to abiotic and biotic stresses could potentially provide the knowledge to develop new efficient strategies to improve crop yield. The *VaCPK20* and *VaCPK21* genes have recently been shown to contribute to the stress resistance of *V. amurensis* by regulating its cold, drought, and salt stress responses (Dubrovina et al. 2015, 2016a). A previous study also revealed that *VaCPK29* is likely involved in abiotic stress responses in *V. amurensis* (Dubrovina et al. 2013). In this study, the effects of *VaCPK29* overexpression on the growth of the callus cell cultures of *V. amurensis* and the survival rates of *A. thaliana* plants were studied under high salinity, cold, heat, drought, and osmotic stress conditions. The stress tolerance assays showed that overexpression of *VaCPK29* improved growth of *V. amurensis* calli and survival rates of *A. thaliana* plants under heat stress and mannitol-induced osmotic stress. These results suggest that the *VaCPK29* gene is involved in the grapevine response to heat and osmotic stress conditions as a weak positive regulator, and that it likely contributes, along with other CDPKs, to the enhanced stress tolerance of *V. amurensis*.

To further understand the function of *VaCPK29* in abiotic stress responses, we analyzed whether the transcription levels of select stress-associated and antioxidant genes were altered in *VaCPK29*-overexpressing *A. thaliana* plants experiencing heat stress. The transcription levels of *ABF3, DREB1A, DREB2A, RD29A, RD29B, CSD1, CSD2*, and *CAT1* were compared in *VaCPK29*-overexpressing plants and control Arabidopsis plants transformed with empty vector. Under heat stress, expression levels of *AtABF3, AtDREB1A, AtDREB2A, AtRD29A*, and *AtRD29B* were up-regulated in the *VaCPK29*-overexpressing *A. thaliana* lines but not in the VC. The higher transcription levels of *ABF3, DREB1A, DREB2A, RD29A*, and *RD29B* suggest that the *VaCPK29* gene may act upstream to these genes in the heat stress response. The Arabidopsis abscisic acid responsive element-binding factor 3 (ABF3) is known to function in drought, cold, heat, and oxidative stress responses via regulation of stress-responsive gene transcription levels (Kim et al. 2004; Abdeen et al. 2010; Choi et al. 2013). Recent studies suggest that ABF3 could promote stomatal closure and reduce water loses under water deficit and high temperature conditions (Choi et al. 2013). The transcription factors DRE-BINDING PROTEIN 1A and 2A (DREB2A and DREB1A) were reported to regulate dehydration-responsive element (DRE)-mediated transcription of target genes under dehydration, high salinity, and cold stress conditions (Liu et al. 1998; Kasuga et al. 1999; Sakuma et al. 2006a). DREB2A had also been reported to be transcriptionally-regulated by heat shock and to play important roles in heat stress tolerance, e.g., by controlling HsfA3 transcription (Sakuma et al. 2006b; Schramm et al. 2008). Notably, in the present investigation, the most pronounced stress-responsive activation of transcription in the *VaCPK29*-overexpressing plants was observed for the *DREB2A* gene. Gene expression levels of the RESPONSIVE TO DEHYDRATION29 (RD29A and RD29B) proteins are known to be induced not only by dehydration conditions but also by salinity, cold, and/or osmotic stress (Msanne et al. 2011). *CSD1, CSD2*, and *CAT1* encode antioxidant enzymes that can detoxify reactive oxygen species (ROS) generated in plants under abiotic stress conditions (Locato et al. 2008; You and Chan 2015). It is known that ROS production increases during abiotic stress, including heat stress (Locato et al. 2008). In the present study, we found that heat stress induced the level of *CSD1* transcription in some *VaCPK29*-overexpressing *A. thaliana* lines but not in the VC line, while there were no considerable differences in *CSD2* and *CAT1* transcriptional responses in all transgenic lines. Thus, it is possible that CSD1 is responsible for ROS scavenging under heat stress in the heat-resistant *VaCPK29*-overexpressing Arabidopsis. Taken together, the results suggest that *VaCPK29* is implicated in the induction of transcription of some stress-responsive genes, thereby improving plant tolerance to heat and osmotic stress conditions.

Amino acid sequence homology analyses revealed a high degree of similarity between the deduced amino acid sequence of VaCPK29 and other CDPKs present in different plant species as shown in Table 2 and Fig. S3. VaCDPK29 shares the highest degree of similarity

to VvCPK15 of *V. vinifera* and VpCPK15 of *V. pseudoreticulata* identified by Chen et al. (2013a) and Zhang et al. (2015). qRT-PCR data presented by Zhang et al. (2015) revealed that the *CPK15* gene of wild grape *V. pseudoreticulata* was up-regulated in response to heat, salt, and cold stresses. The elevation in *VpCPK15* expression was moderate under cold and heat stress conditions and was slight under high salinity conditions. Zhang et al. (2015) did not analyze *VpCPK* transcription in response to osmotic stress. The data by Zhang et al. (2015) on the expression of *VpCPK15* in response to abiotic stresses do not contradict our hypothesis that *VaCPK29* is involved in heat stress response in grapevine. As for homologous CDPKs from Arabidopsis, it has been shown that AtCPK21 activates SLAC1 and SLAH3 channels in response to abscisic acid (ABA) and functions as a positive regulator in ABA-induced stomatal closure (Geiger et al. 2010). A study by Franz et al. (2011) reported that AtCPK21 was biochemically activated in vivo in response to hyperosmotic stress. However, overexpression of *AtCPK21* in Arabidopsis did not have a positive effect on salt and osmotic stress resistance and suggested that AtCPK21 acted as a negative regulator in abiotic stress signaling (Franz et al. 2011). According to Zuo et al. (2013), the *PtCDPK19* gene was up-regulated in response to drought stress as shown by microarray analysis. The potential biological function of AtCPK9, AtCPK29, OsCPK1, OsCPK15, and PtCDPK24 in plant stress responses remain largely unknown.

In conclusion, the characterization of the *VaCPK29* functioning suggests that the gene plays a role in regulating the signaling pathways involved in heat and osmotic stress resistance in *V. amurensis*. This finding helps to clarify details of the CDPK-mediated abiotic stress response of *V. amurensis*.

Acknowledgements This work was supported by a Grant from the Russian Science Foundation (14-14-00366).

References

Abdeen A, Schnell J, Miki B (2010) Transcriptome analysis reveals absence of unintended effects in drought-tolerant transgenic plants overexpressing the transcription factor ABF3. BMC Genom 11:69

Aleynova OA, Dubrovina AS, Manyakhin AY, Karetin YA, Kiselev KV (2015) Regulation of resveratrol production in *Vitis amurensis* cell cultures by calcium-dependent protein kinases. Appl Biochem Biotechnol 175:1460–1476

Aleynova-Shumakova OA, Dubrovina AS, Manyakhin AY, Karetin YA, Kiselev KV (2014) *VaCPK20* gene overexpression significantly increased resveratrol content and expression of stilbene synthase genes in cell cultures of *Vitis amurensis* Rupr. Appl Microbiol Biotechnol 98:5541–5549

Asano T, Hayashi N, Kikuchi S, Ohsugi R (2012a) CDPK-mediated abiotic stress signaling. Plant Signal Behav 7:817–821

Asano T, Hayashi N, Kobayashi M, Aoki N, Miyao A, Mitsuhara I, Ichikawa H, Komatsu S, Hirochika H, Kikuchi S, Ohsugi R (2012b) A rice calcium-dependent protein kinase *OsCPK12* oppositely modulates salt stress tolerance and blast disease resistance. Plant J 69:26–36

Batistič O, Kudla J (2012) Analysis of calcium signaling pathways in plants. Biochim Biophys Acta 1820:1283–1293

Boudsocq M, Sheen J (2013) CDPKs in immune and stress signaling. Trends Plant Sci 18:30–40

Chen F, Fasoli M, Tornielli GB, Dal Santo S, Pezzotti M, Zhang L, Cai B, Cheng ZM (2013a) The evolutionary history and diverse physiological roles of the grapevine calcium-dependent protein kinase gene family. PLoS ONE 8:e80818

Chen J, Xue B, Xia X, Yin W (2013b) A novel calcium-dependent protein kinase gene from *Populus euphratica*, confers both drought and cold stress tolerance. Biochem Biophys Res Commun 441:630–636

Choi HI, Park HJ, Park JH, Kim S, Im MY, Seo HH, Kim YW, Hwang I, Kim SY (2005) *Arabidopsis* calcium-dependent protein kinase AtCPK32 interacts with ABF4, a transcriptional regulator of abscisic acid responsive gene expression, and modulates its activity. Plant Physiol 139:1750–1761

Choi YS, Kim YM, Hwang OJ, Han YJ, Kim SY, Kim JI (2013) Overexpression of *Arabidopsis ABF3* gene confers enhanced tolerance to drought and heat stress in creeping bentgrass. Plant Biotechnol Rep 7:165–173

Das R, Pandey GK (2010) Expressional analysis and role of calcium regulated kinases in abiotic stress signaling. Curr Genom 11:2–13

Dubrovina AS, Kiselev KV, Khristenko VS (2013) Expression of calcium-dependent protein kinase (*CDPK*) genes under abiotic stress conditions in wild-growing grapevine *Vitis amurens*is. J Plant Physiol 170:1491–1500

Dubrovina AS, Kiselev KV, Khristenko VS, Aleynova OA (2015) *VaCPK20*, a calcium-dependent protein kinase gene of wild grapevine *Vitis amurensis* Rupr., mediates cold and drought stress tolerance. J Plant Physiol 185:1–12

Dubrovina AS, Kiselev KV, Khristenko VS, Aleynova OA (2016a) *VaCPK21*, a calcium-dependent protein kinase gene of wild grapevine *Vitis amurensis* Rupr., is involved in grape response to salt stress. Plant Cell Tissue Organ Cult 124:137–150

Dubrovina AS, Kiselev KV, Aleynova OA (2016b) Influence of overexpression of the true and false alternative transcripts of calcium-dependent protein kinase *CPK9* and *CPK3a* genes on the growth, stress tolerance, and resveratrol content in *Vitis amurensis* cell cultures. Acta Physiol Plant 38:78

Franz S, Ehlert B, Liese A, Kurth J, Cazalé AC, Romeis T (2011) Calcium-dependent protein kinase CPK21 functions in abiotic stress response in *Arabidopsis thaliana*. Mol Plant 4:83–96

Geiger D, Scherzer S, Mumm P, Marten I, Ache P, Matschi S, Liese A, Wellmann C, Al-Rasheid KA, Grill E, Romeis T, Hedrich R (2010) Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca^{2+} affinities. Proc Natl Acad Sci USA 107:8023–8028

Harper JF, Huang JF, Lloyd SJ (1994) Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. BioChemistry 33:7267–7277

Hashimoto K, Kudla J (2011) Calcium decoding mechanisms in plants. Biochimie 93:2054–2059

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–467

Jaworski K, Szmidt-Jaworska A, Kopcewicz J (2011) Two calcium dependent protein kinases are differently regulated by light

and have different activity patterns during seedling growth in *Pharbitis nil*. Plant Growth Regul 65:369–379

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 17:287–291

Kim JB, Kang JY, Kim SY (2004) Over-expression of a transcription factor regulating ABA-responsive gene expression confers multiple stress tolerance. Plant Biotechnol J 2:459–466

Kiselev KV, Dubrovina AS, Veselova MV, Bulgakov VP, Fedoreyev SA, Zhuravlev YN (2007) The *rolB* gene-induced overproduction of resveratrol in *Vitis amurensis* transformed cells. J Biotechnol 128:681–692

Kiselev KV, Dubrovina AS, Bulgakov VP (2009) Phenylalanine ammonia-lyase and stilbene synthase gene expression in *rolB* transgenic cell cultures of *Vitis amurensis*. Appl Microbiol Biotechnol 82:647–655

Kiselev KV, Shumakova OA, Manyakhin AY, Mazeika AN (2012) Influence of calcium influx induced by the calcium ionophore, A23187, on resveratrol content and the expression of *CDPK* and *STS* genes in the cell cultures of *Vitis amurensis*. Plant Growth Regul 68:371–381

Kiselev KV, Dubrovina AS, Shumakova OA, Karetin YA, Manyakhin AY (2013) Structure and expression profiling of a novel calcium-dependent protein kinase gene, *CDPK3a*, in leaves, stems, grapes, and cell cultures of wild-growing grapevine *Vitis amurensis* Rupr. Plant Cell Rep 32:431–442

Kiselev KV, Tyunin AP, Ogneva ZV, Dubrovina AS (2015) Age-associated alterations in the somatic mutation level in *Arabidopsis thaliana*. Plant Growth Regul 75:493–501

Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell 19:1065–1080

Liese A, Romeis T (2013) Biochemical regulation of *in vivo* function of plant calcium-dependent protein kinases (CDPK). Biochim Biophys Acta-Mol. Cell Res 7:1582–1589

Liu L, Li H (2013) Review: Research progress in amur grape, *Vitis amurensis* Rupr. Can J Plant Sci 93:565–575

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10:1391–1406

Locato V, Gadaleta C, De Gara L, De Pinto MC (2008) Production of reactive species and modulation of antioxidant network in response to heat shock: a critical balance for cell fate. Plant Cell Environ 31:1606–1619

Ma SY, Wu WH (2007) AtCPK23 functions in *Arabidopsis* responses to drought and salt stresses. Plant Mol Biol 65:511–518

Ma YY, Zhang YL, Shao H, Lu J (2010) Differential physio-biochemical responses to cold stress of cold-tolerant and non-tolerant grapes (*Vitis* L.) from China. J Agron Crop Sci 196:212–219

Msanne J, Lin J, Stone JM, Awada T (2011) Characterization of abiotic stress-responsive *Arabidopsis thaliana RD29A* and *RD29B* genes and evaluation of transgenes. Planta 234:97–107

Reddy AS, Ali GS, Celesnik H, Day IS (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. Plant Cell 23:2010–2032

Sakuma Y, Maruyama K, Osakabe Y, Qin F, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006a) Functional analysis of an *Arabidopsis* transcription factor, DREB2A, involved in drought-responsive gene expression. Plant Cell 18:1292–1309

Sakuma Y, Maruyama K, Qin F, Osakabe Y, Shinozaki K, Yamaguchi-Shinozaki K (2006b) Dual function of an *Arabidopsis* transcription factor DREB2A in water-stress-responsive and heat-stress-responsive gene expression. Proc Natl Acad Sci USA 103:18822–18827

Schramm F, Larkindale J, Kiehlmann E, Ganguli A, Englich G, Vierling E, von Koskull-Döring P (2008) A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of *Arabidopsis*. Plant J 53:264–274

Schulz P, Herde M, Romeis T (2013) Calcium-dependent protein kinases: hubs in plant stress signaling and development. Plant Physiol 163:523–530

Shen YY, Duan CQ, Liang XE, Zhang DP (2004) Membrane-associated protein kinase activities in the developing mesocarp of grape berry. J Plant Physiol 161:15–23

Tzfira T, Tian GW, Lacroix B, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol 57:503–516

Valmonte GR, Arthur K, Higgins CM, MacDiarmid RM (2014) Calcium-Dependent Protein Kinases in plants: evolution, expression and function. Plant Cell Physiol 55:551–569

Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A, Pruss D, Pindo M et al (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS ONE 2:e1326

Weckwerth P, Ehlert B, Romeis T (2015) ZmCPK1, a calcium-independent kinase member of the *Zea mays CDPK* gene family, functions as a negative regulator in cold stress signalling. Plant Cell Environ 38:544–558

Wei S, Hu W, Deng X, Zhang Y, Liu X, Zhao X, Luo Q, Jin Z, Li Y, Zhou S, Sun T, Wang L, Yang G, He G (2014) A rice calcium-dependent protein kinase OsCPK9 positively regulates drought stress tolerance and spikelet fertility. BMC Plant Biol 14:133

Wernimont AK, Artz JD, Finerty, Lin YH, Amani M, Allali-Hassani A, Senisterra G, Vedadi M, Tempel W, Mackenzie F et al (2010) Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. Nat Struct Mol Biol 17:596–601

You J, Chan Z (2015) ROS regulation during abiotic stress responses in crop plants. Front Plant Sci 6:1092

Yu XC, Li MJ, Gao GF, Feng HZ, Geng XQ, Peng CC, Zhu SY, Wang XJ, Shen YY, Zhang DP (2006) Abscisic acid stimulates a calcium-dependent protein kinase in grape berry. Plant Physiol 140:58–579

Yu XC, Zhu SY, Gao GF, Wang XJ, Zhao R, Zou KQ, Wang XF, Zhang XY, Wu FQ, Peng CC, Zhang DP (2007) 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 64:31–538

Zhang XR, Henriques R, Lin SS, Niu QW, Chua NH (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc 1:641–646

Zhang K, Han YT, Zhao FL, Hu Y, Gao YR, Ma YF, Zheng Y, Wang YJ, Wen YQ (2015) Genome-wide identification and expression analysis of the *CDPK* gene family in grape, *Vitis* spp. BMC Plant Biol 15:164

Zou JJ, Wei FJ, Wang C, Wu JJ, Ratnasekera D, Liu WX, Wu WH (2010) *Arabidopsis* calcium-dependent protein kinase *CPK10* functions in abscisic acid- and Ca^{2+}-mediated stomatal regulation in response to drought stress. Plant Physiol 154:1232–1243

Zuo R, Hu R, Chai G, Xu M, Qi G, Kong Y, Zhou G (2013) Genome-wide identification, classification, and expression analysis of *CDPK* and its closely related gene families in poplar (*Populus trichocarpa*). Mol Biol Rep 40:2645–2662