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ORIGINAL ARTICLE

Alternative splicing diversified abiotic stress response of *VaCPK21* gene of wild-growing grapevine *Vitis amurensis*

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Abstract

Alternative splicing (AS) is a non-canonical gene splicing process that allows a single gene to produce multiple protein isoforms and increase the diversity of protein functions. This study investigates the involvement of AS in plant tolerance to abiotic stresses using the calcium-dependent protein kinase (*VaCPK21*) gene involved in the tolerance to salinity in grapevine *Vitis amurensis* Rupr. In this study, we investigated expression of *VaCPK21* under the effect of various environmental stresses, and, in addition to the full-length *VaCPK21*, we obtained two short spliced transcripts and one longer transcript. While recombinant *VaCPK21* enhanced salt stress resistance, overexpression of the short *VaCPK21s1* and *VaCPK21s2* transcripts in grapevine cell cultures and Arabidopsis did not confer tolerance to the tested stresses. Overexpression of the longer *VaCPK21L1* did not confer tolerance to salt stress but conferred a novel tolerance to osmotic stress induced by mannitol. Overexpression of all *VaCPK21* transcripts did not affect the biosynthesis of stilbenes, valuable phenolic secondary metabolites. Thus, the *VaCPK21s1* and *VaCPK21s2* isoforms appear to lose the stress tolerance properties of the full-length *VaCPK21*, while the *VaCPK21L1* isoform confers novel tolerance to osmotic stress. These findings show that AS can result in the loss of properties characteristic of the original canonical form or the appearance of new properties, which is important for full understanding of *CPK* and alternative splicing biological functions.

Key message

Alternative splicing can result in the loss of properties characteristic of the original canonical form of the grape calcium-dependent protein kinase (VaCPK21) or the appearance of new properties.

Keywords *Arabidopsis* · Calcium · Calcium-dependent protein kinase · Overexpression · Salinity · Salt stress · Stilbenes · Stress tolerance

Introduction

In plants, as in other eukaryotes, pre-messenger RNAs (pre-mRNAs) consist of non-coding segments (introns) and coding segments (exons). During RNA processing, introns are removed, and exons are spliced together to form mature messenger RNAs (mRNAs). Alternative splicing (AS) is a complex process in which coding and non-coding regions of a gene are rearranged in different ways by spliceosomes at different splice sites (Tognacca et al. 2023). This results in multiple mRNA transcripts generated from the same pre-mRNA molecule.

Recent studies have shown that AS is far more pervasive and crucial in plants than previously believed. It significantly contributes to the extensive complexity of plant transcriptomes and proteomes (Reddy 2007; Syed et al. 2012). Genome-wide mapping of the *Arabidopsis thaliana* transcriptome revealed that up to 61% of intron-containing genes undergo AS (Filichkin et al. 2010; Marquez et al. 2012). AS plays a central role in plant responsiveness to environmental stresses and various developmental signals (Mastrangelo et al. 2012; Carvalho et al. 2013; Dubrovina et al. 2013a). It affects the expression of many genes involved in signalling

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pathways, including transcription factors, ubiquitin ligases, serine/arginine (SR) proteins, and defence-related genes.

One area of interest is the regulation of plant protein kinases by AS. Protein kinases are crucial regulators of signal transduction in plants. However, relatively little is known about how AS modulate their activities and functions. Only a few cases of AS in plant Ser/Thr protein kinases have been reported (Xiong and Yan 2003; Castells et al. 2006; Kurihara et al. 2007; Koo et al. 2007; Lin et al. 2010). Mitogen-activated protein kinase (MAPK) cascades are ubiquitous signalling pathways that play a critical role in mediating cellular responses to various stimuli, including stress, growth factors, and hormones. These cascades are composed of a series of protein kinases, with each kinase phosphorylating and activating the next, amplifying the signal. AS, a process that allows a single gene to generate multiple distinct mRNA transcripts, is a major contributor to the regulation of MAPK signalling. By altering the structure and sequence of the encoded proteins, AS can modulate their activity, subcellular localisation, and interactions with other components of the pathway. The *OsMAPK5* gene in rice produces two differentially spliced transcripts, resulting in isoforms with distinct protein sequences (Xiong and Yan 2003). MIK in maize, a gene encoding a GCK-like MAP4K, undergoes AS to produce at least four mature mRNA transcripts. These isoforms have different kinase activities and respond differently to activation by the maize atypical receptor kinase (MARK) (Castells et al. 2006).

The Aurora kinase *AtAUR2* in *A. thaliana* has a splicing variant, *AtAUR2S*, which lacks part of its kinase domain. While *AtAUR2S* loses its ability to phosphorylate histone H3, it retains its histone binding capacity (Kurihara et al. 2007). AS of *OsBWMK1* in rice generates isoforms with contrasting subcellular localisations. These isoforms play distinct roles in hormone signalling pathways (Koo et al. 2007). The *MPK13* gene in *A. thaliana* produces three splice variants. The full-length *MPK13* is fully spliced, while *MPK13 I4* and *I5* retain the fourth and fifth introns, respectively. *I4* and *I5* lack both kinase activity and interactions with upstream MAPKKs, suggesting that splicing regulates their functionality (Lin et al. 2010).

Another important kinase in plant signaling are calcium-dependent protein kinase (CDPKs or CPKs). CPKs are a family of Ca^{2+}-regulated Ser/Tre protein kinases that play a prominent role in the regulation of various cellular processes in plants, green algae, and protozoa (Hrabak 2000; Harper et al. 2004; Dekomah et al. 2022). CPKs share a conserved structural organization (classified under EC 2.7.1.37) with an N-terminal variable domain, a kinase catalytic domain, an autoinhibitory domain, and a calmodulin-like Ca^{2+}-binding domain with EF-hand motifs that mediate Ca^{2+} binding (Roberts and Harmon 1992; Klimecka and Muszyska 2007). At low Ca^{2+} concentrations, the autoinhibitory domain acts as a pseudosubstrate inhibitor, preventing phosphorylation and maintaining the CPK in an inactive state (Harper et al. 1994). Upon Ca^{2+} influx, the calmodulin-like Ca^{2+}-binding domain binds Ca^{2+} and inducing conformational changes that release the autoinhibition and activate the kinase. Individual CPK isoforms have diverse functions and are involved in numerous signalling networks, including immune responses, stress responses, and developmental processes. They regulate various cellular events, such as: stem elongation in plants (Kohler and Blatt 2002), stomata closure in response to drought conditions (Kobayashi et al. 2007), reactive oxygen species (ROS) production under stress conditions (Matschi et al. 2013; Wang and Song 2013). CPKs are encoded by multigene families with varying numbers of genes in different plant species. Genome-wide sequencing analyses in rice *Oryza sativa*, *A. thaliana*, and *Populus trichocarpa* revealed 31, 34, and 30 *CPK* genes, respectively (Cheng et al. 2002; Asano et al. 2005; Ray et al. 2007). Different CPK isoforms exhibit distinct expression patterns, subcellular localizations, and sensitivities to Ca^{2+} levels, contributing to their diverse functional roles (Klimecka and Muszyska 2007). While CPKs have been identified primarily in plants, homologous proteins have also been found in green algae and protozoa.

To date, AS has been observed in four CPK genes. Nishiyama et al. (1999) found that the liverwort CPK gene has two nearly identical exons that are alternatively spliced into two different mRNAs, suggesting no independent splicing of the intron between them. AS of CPK2 in rice results in isoforms with different kinase activities and responses to stress conditions (Kawasaki et al. 1999). Previously, several atypical *VaCPK3a* and *VaCPK9* transcript variants containing small deletions or insertions in the kinase domain were identified and described in wild grapevine *Vitis amurensis* Rupr. cell cultures (Dubrovina et al. 2009; 2013b). The modified *CPK3a* transcripts were expressed in *Escherichia coli* and assayed for protein kinase activity in vitro. The data showed that synthesis of the short *VaCPK3aSF2* and *VaCPK3aSF3* transcript variants could lead to the production of modified protein kinases with altered phosphorylation activities.

V. amurensis VaCPK21 is a member of the CPK family, which plays a crucial role in various physiological and developmental processes in plants. In particular, studies have shown that *VaCPK21* overexpression enhances grape cells and *A. thaliana* plants tolerance to salinity (Dubrovina et al. 2016). Under salt stress conditions some stress-related genes demonstrated strong upregulation in the *VaCPK21*-transgenic but not in the control Arabidopsis plants. Among these genes were the genes of dehydrins like *AtCOR15* or *AtCOR47* (Szlachtowska and Rurek 2023), antioxidant genes like *AtCAT1* or *AtCSD1* (Nghia et al. 2020), vacuolar Na^+/H^+ antiporter gene *AtNHX1* (Pabuayon et al. 2021), Ser/Thr protein kinase AtKIN1 and transcription factor AtRD26

(Fujita et al. 2004; Yamaguchi-Shinozaki and Shinozaki (2004). The *VaCPK21* gene is also subject to AS, which is investigated in detail in this paper.

Results and discussion

Cloning, sequencing, and expression of *VaCPK21* mRNA transcripts under abiotic stress conditions

Using specific primers designed to amplify the coding sequence of the *VaCPK21* gene (Supplementary Table S1), we performed a comprehensive cloning and sequencing analysis of *VaCPK21* mRNA transcripts derived from grape leaves exposed to different stressors. These stressors included salinity, extreme temperatures (both low and high), and water deficiency. From each experimental treatment, we obtained and sequenced a substantial number of *VaCPK21* gene sequences (Table 1). Also, in reverse transcription we use high temperature (65 °C) heat-stable reverse transcriptase (ThermoScript) in order to get rid of false transcripts as a result of reverse transcriptase (Dubrovina et al. 2014).

The number of sequenced transcripts was significant, e.g. 11 (after water deficit), 41 (after elevated temperature), 48 (after salinity), and 56 (in control). However, only two transcripts could be sequenced after low temperature treatment (Table 1). This low yield may be due to the previously reported observation that low temperature significantly reduces *VaCPK21* gene expression compared to other stressors (Dubrovina et al. 2013b). Furthermore, the PCR product obtained from samples after cold stress showed the weakest signal. It is important to note that the complete sequence of the *VaCPK21* gene (*VaCPK1d*, KC488318) deposited in the Gene Bank was marked by us as 'Normal' in Table 1. If there were no parts of the protein coding sequence in the sequence, such sequences were called "Short", and if there were inserts, they were called "Long" (Table 1).

For the *VaCPK21* gene under control growth conditions, out of 56 cloned sequences, only 1 *VaCPK21s2* sequence (1.8%) contained only 393 nucleotides out of 1638 nucleotides characteristic of the full-length normal sequence (Supplementary Figs. S1, S2, S4). This sequence was generated due to the loss of the 2nd, 3rd, 4th, 5th, and 6th exons and partially of the 1st and 7th exons (Fig. 1). It is known that in most cases plant introns, as introns of other eukaryotes, start with the canonical splice sites GT at the 5' end (donor splice site) and an AG at the 3' end (acceptor splice site) (Reddy 2007). We showed that *VaCPK21s2* variant did not include any of these canonical donor–acceptor sites but possessed short direct repeated sequences (SDR) instead (Figs. 2, 3, and S4). The correct

Table 1 Number of *Vitis amurensis VaCPK21* gene transcripts cloned from RNA isolated from leaves of grapevine *V. amurensis* cuttings 12 h after treatment by salt stress (NaCl, 350 mM), cold (5, 5 °C), heat (37, 37 °C), water deficit (WD, a leaf on filter paper without watering), and control conditions (16/8 h light/dark photoperiod: light intensity ~ 70 µmol m^{-2} s^{-1} at 22 °C)

VaCPK21 gene (KC488318)	Normal	Short	Long
Control	55	1 (s2)	0
NaCl	48	0	0
5	2	0	0
37	38	2 (s1)	1
WD	11	0	0
Total number of clones	154 (97.5%)	3 (1.9%)	1 (0.6%)

Normal—the sequence is completely identical to the sequence deposited in the Genbank, KC488318 for *VaCPK21*; Short—sequences lacking sequences of the protein coding part of the *VaCPK21* gene; Long—sequences containing nucleotide insertions uncharacteristic for the protein coding sequence of the *VaCPK21* gene; s1—*VaCPK21s1*; s2—*VaCPK21s2*

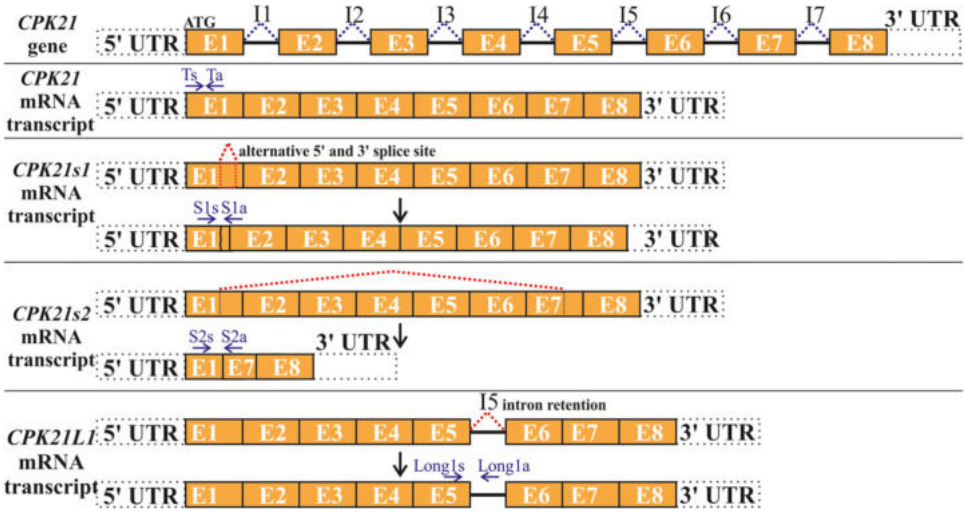

Fig. 1 The structure representation of *VaCPK21* mRNA splice variants in grapevine *Vitis amurensis* (not to scale). Exons (E) and introns (I) are shown using boxes and lines, respectively, with white dashed boxes representing untranslated regions (UTRs). In blue font arrows and Ts and Ta-primes used for *VaCPK21* total expression levels in the leaves of grapevine *V. amurensis* cuttings; S1a and S1a—for *VaCPK21s1*; S2a and S2a—for *VaCPK21s2*; Long1s and Long1a—for *VaCPK21L1*

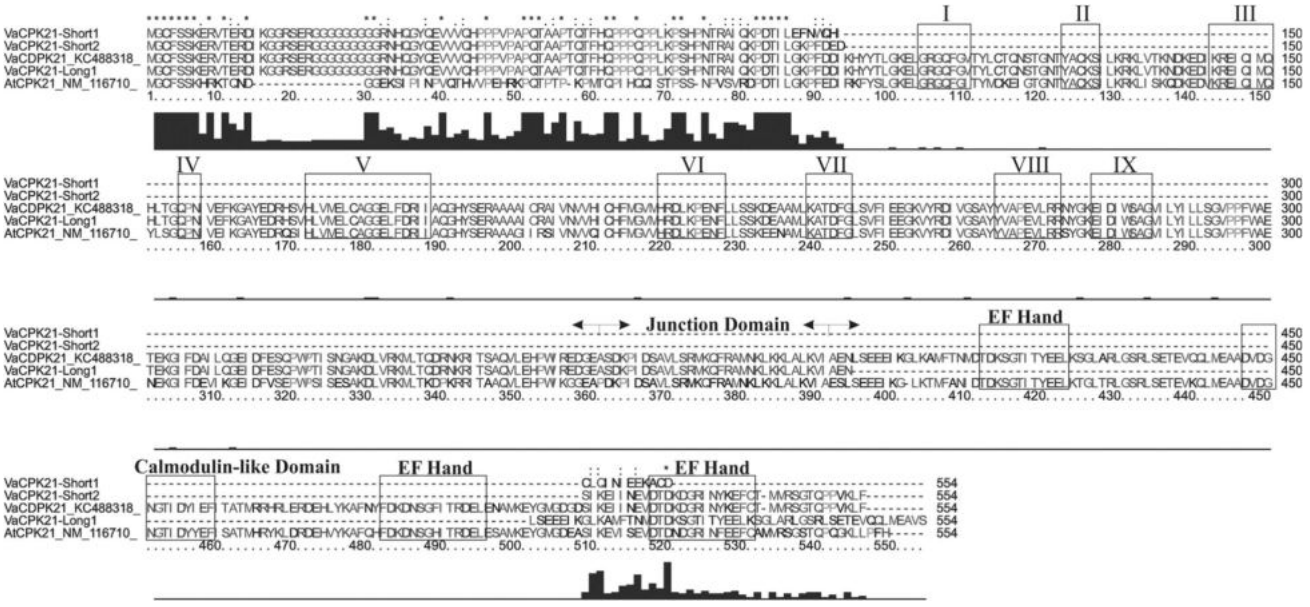

Fig. 2 Domain analysis of the deduced amino acid sequences of VaCPK21 (KC488318), VaCPK21s1, VaCPK21s2, VaCPK21L1, and AtCPK21 (NM_116710). The kinase domain consisting of I-IX catalytic domains, autoinhibitory or junction domain, and Ca^{2+}-binding domain with EF hands are indicated in the figure as described (Dubrovina et al. 2014). The alternatively spliced *Vitis amurensis VaCPK21* transcript variants identified in this work (*VaCPK21s1*, *VaCPK21s1*, *VaCPK21L1*) were predicted by gene sequence analysis. Multiple sequence alignment was performed using ClustalX2 software (Larkin et al. 2007)

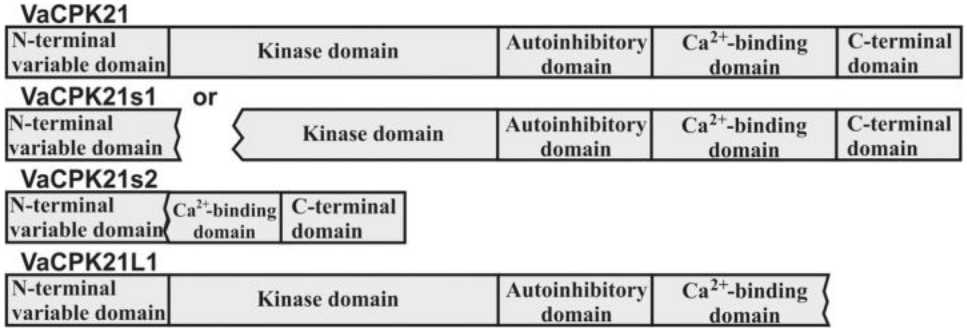

Fig. 3 Domain analysis of the deduced amino acid sequences of VaCPK21 (KC488318), VaCPK21s1, VaCPK21s2, and VaCPK21L1. The N-terminal, kinase, autoinhibitory, and Ca^{2+}-binding domains are shown in the figure

reading frame of *VaCPK21s2* sequence was not lost, and in the translated protein there are 131 amino acids. These amino acids contain only an N-terminal variable domain located on one side of the Ca^{2+} domain and a C-terminal domain (Figs. 2 and 3). It is possible that *VaCPK21s2* is an example of alternative splicing (exon skipping) or a methodological artifact, but we decided to take this sequence into expression analysis as an example of a *CPK* sequence without kinase activity.

After stressful effects, only elevated temperature (37 °C) increased the number of *VaCPK21* short sequences to 4.9% and led to the appearance of a single long sequence *VaCPK21L1* (2.4% of all sequenced sequences). Other effects (such as low temperatures, water deficiency) did not affect the number of short and long sequences of the *VaCPK21* gene (Table 1).

The *VaCPK21-long1* (*VaCPK21L1*) sequence was characterized by the insertion of 103 nucleotides (Supplementary Figs. S1, S2, S3, and Fig. 1, retention of the 5th intron), which led to a failure of the reading frame, which eventually led to the appearance of numerous stop codons after the 1341st nucleotide (containing 447 amino acids), suggesting that the protein loses part of its activity (Figs. 2 and 3). Moreover, the short *VaCPK21s1* sequences found in the 37 °C samples had a different structure compared to *VaCPK21s2*: 89 nucleotides were missing from their sequence, which led to shifted reading frame and predicted transcription site starting from the 315th nucleotide (somewhere from the 105th amino acid). The reading frame was shifted and numerous stop codes were formed, which means that it can be assumed that a possible protein would lose its functional features—the deduced

VaCPK21s1 sequences contained only the N-terminal variable domain (Figs. 2 and 3). However, it is also possible to obtain a protein deletion of 89 nucleotides where alternative start codons are present, in this case one can expect to obtain a shortened protein without the N-terminal variable domain and lacking only the initial part of the kinase domain (Fig. 3). The *VaCPK21s1* transcript was generated via canonical a GT at the 5' donor splice site and an AG at the 3' acceptor splice site inside the first exon (Fig. 1). Next, the expression of different variants of the *VaCPK21* gene was analysed by real-time PCR (qPCR) using the primers presented in Supplementary Table S1. The primers were selected for general sequences (total expression analysis, Fig. 4a) and specific sequences of the studied transcripts (short and long sequences, Fig. 4b–d).

It was shown that the total expression of the *VaCPK21* gene (primers designed for all known *VaCPK21* transcripts) significantly increased with salt stress and water deficiency (Fig. 4a), which largely confirms the results obtained previously (Dubrovina et al. 2013b). Furthermore, we showed that the presence of the *VaCPK21L1* transcript was higher in the samples compared with control leaves after high temperature treatment (37 °C), which confirms the data obtained in the Table 1, but this increase was not statistically significant (Fig. 4b). Perhaps, because there are very few of these *VaCPK21L1* transcripts in the analyzed probes. At the same time, the qPCR data also confirmed that the number of *VaCPK21s1* transcripts was significantly higher in samples treated at high temperature (37 °C, Fig. 4c), confirming the data obtained by cloning the PCR products (Table 1). The presence of *VaCPK21s2* transcripts also did not differ significantly in the analyzed samples (Fig. 4d), which is also possible due to the low presence of these transcripts, which does not allow high fluorescence values in qPCR. Thus, using 2 approaches (gene cloning and qPCR), a strong positive correlation was shown between an increase in the number of *VaCPK21s1* transcripts during high temperature treatment ($r = 0.73–0.85$), and a slight correlation ($r = 0.66–0.75$) in *VaCPK21L1* transcripts during heat. Correlations between the number of *VaCPK21s2* transcripts and used stress conditions was low ($r = 0.10–0.24$) therefore, the appearance of the *VaCPK21s2* transcript was accidental.

In the total, we did not show the presence of VaCPK21s1, VaCPK21s2, or VaCPK21L1 proteins in our cells, but similar short or truncated proteins of poplar PtRD26 protein that senescence-associated NAC family transcription factor were shown in the paper of Wang and co-authors (Wang et al. 2021). This indicates that our proteins are capable of existing in different forms, making it intriguing to explore the effects of overexpressing these variants in plant cells and the plants themselves.

Overexpression of spliced VaCPK21 gene variants in grapevine cell cultures

Using agrobacterial transformation, we obtained 10 cell lines overexpressing different new transcript variants of the *VaCPK21* gene. Next, using the qPCR with specific pairs of primers (supplementary Table S1), we investigated the expression of transgenes in the obtained lines. It was shown that in all cell culture lines the transferred *VaCPK21* sequences were expressed at a high level in all cell culture lines compared to the control vector cell line VC (Fig. 5a). Moreover, the full-length *VaCPK21* transcript was highly expressed in cell line F21 full-length *VaCPK21* transcript (Fig. 5a). The expression of the *VaCPK21s1* transgene was highest in cell line 21s1-1, in lines the 21s1-2 and 21s1-3 the expression of *VaCPK21s1* transgene was 1.6–1.9 times lower than in the 21s1-1 line (Fig. 5b). The expression of the *VaCPK21s2* transgene was highest in cell lines 21s2-1 and 21s2-2, in line 21s2-3 it was 1.5–1.8 times lower than in lines 21s2-1 and 21s2-2 (Fig. 5c). The expression of the *VaCPK21L1* transgene was highest in cell line 21L1-1, in lines 21sL1-2 and 21L1-3 it was 2.8–3.3 times lower than in line 21L1-1 (Fig. 5d).

Next, the tolerance of the obtained transgenic cell lines to salt, low and high temperatures, and osmotic stress was investigated (Fig. 6). It is important to note that the F21 cell line, which actively expresses the full-length *VaCPK21* gene, was also obtained. First, F21 cell line did not have tolerance to other stresses (low and high temperatures, osmotic stress) compared to the stability of the control line VC (Fig. 6b, c). The data obtained on the stability of the cell line expressing the full-length *VaCPK21* gene are generally consistent with previously published data (Dubrovina et al. 2016). In short variants of the *VaCPK21* gene (s1, s2), systemic tolerance to salinity disappeared, only individual lines were stable at some doses, and the level of tolerance was lower than in lines overexpressing the full-length *VaCPK21* gene (Fig. 6a). The resulting s1-, s2-cell lines were not resistant to the other stresses used: temperature and osmotic stress (Fig. 6b, c). It is interesting to note that the transformation by the *VaCPK21L1* transcript, which contains part of the intron, maintained the salinity tolerance of most of the obtained lines (Fig. 6a), moreover, the obtained lines showed tolerance to osmotic stress: 2 out of 3 lines to high mannitol concentration (0.3 M, Fig. 6c).

It has been shown that in the culture of *V. amurensis* grape cells, the main secondary metabolites are stilbenes—resveratrol and its derivatives (Dubrovina and Kiselev 2017). Stilbenes are phenolic compounds with pronounced protective properties and health-promoting activities (Dubrovina and Kiselev 2017). In order to understand how new alternative forms of the *VaCPK21* gene can affect the secondary metabolism of plants, we investigated the content of stilbenes in

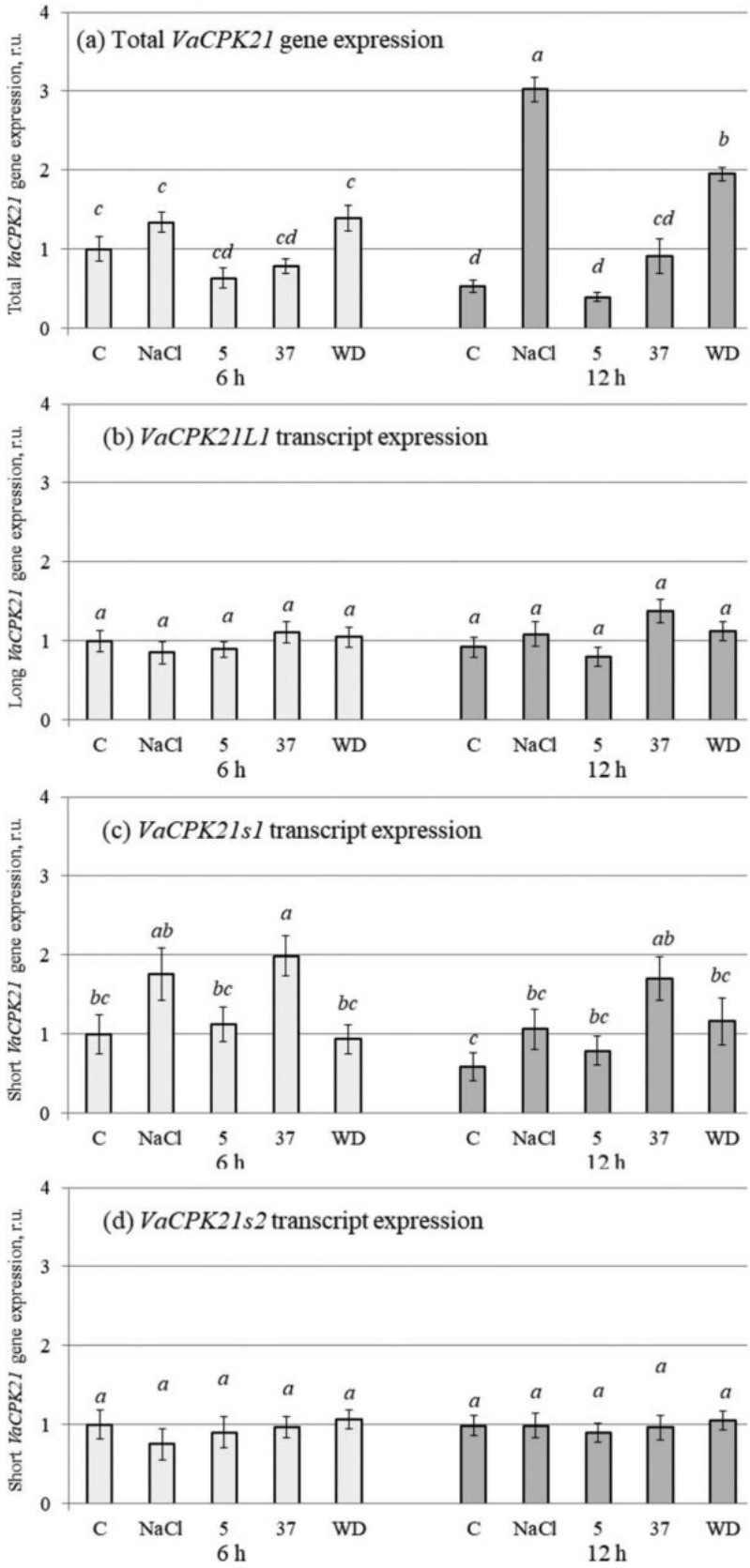

Fig. 4 Quantification of *VaCPK21* total (**a**), *VaCPK21L1* (**b**), and *VaCPK21s1* (**c**) expression levels in the leaves of grapevine *Vitis amurensis* cuttings treated by standing in a glass of filtered water 6 h and 12 h after treatment by salt stress (NaCl, 350 mM of NaCl), cold (5, 5 °C), heat (37, 37 °C), water deficit (WD, a leaf on filter paper without watering), and control conditions (C, 16/8 h light/dark photoperiod: light intensity ~ 70 µmol m^{-2} s^{-1} at 22 °C). The qPCR data were obtained from three independent experiments and are averages of 10 technical replicates for each independent experiment (five qPCR reactions normalized to *VaActin1* and five qPCR reactions normalized to *VaGAPDH* expression) and presented as mean ± standard error. Means followed by the same letter were not different using Student's *t* test. $p < 0.05$ was considered statistically significant

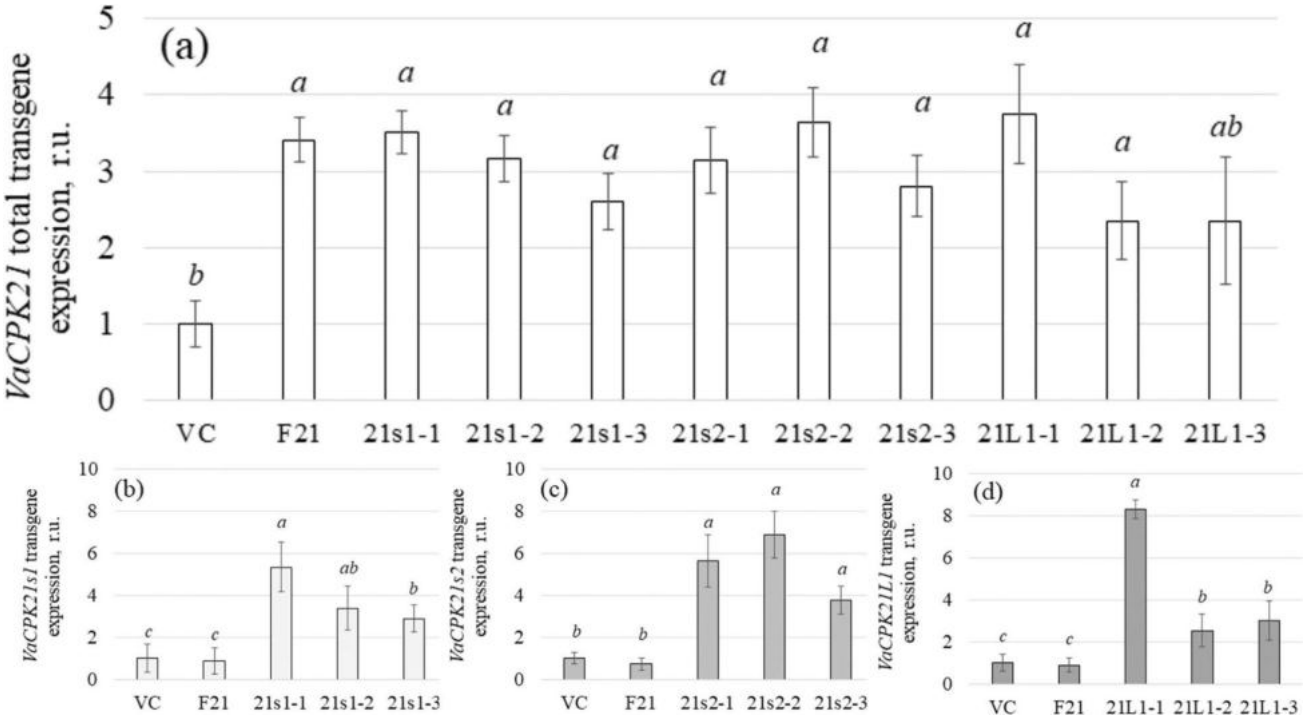

Fig. 5 Quantification of *VaCPK21* transgene in grapevine control vector cell line VC and cell lines expressing full-length 2×35S-*VaCPK21* transgene (F21), short 2×35S-*VaCPK21s1*, 2×35S-*VaCPK21s2* (21s1, 21s2), and long 2×35S-*VaCPK21L1* (21L) transgenes. **a** total *VaCPK21* expression, primers designed for all known *VaCPK21* transcripts; **b** *VaCPK21s1* expression, primers designed for *VaCPK21s1* transcripts; **c** *VaCPK21s2* expression, primers designed for *VaCPK21s2* transcripts; **d** *VaCPK21L1* expression, primers designed for *VaCPK21L1* transcripts. qPCR data were obtained from two independent experiments and are averages of 8 technical replicates for each independent experiment (five qPCR reactions normalized to *VaActin1* and five qPCR reactions normalized to *VaGAPDH* expression) and presented as mean ± standard error. Means followed by the same letter were not different using Student's *t* test. $p < 0.05$ was considered statistically significant

the obtained transgenic grape cells. In the used grapevine *V. amurensis* cell cultures we determined 8 individual stilbenes: *t*-resveratrol diglucoside, *t*-piceid (t-resveratrol glucoside), *cis*-piceid, *t*-piceatannol, *t*-resveratrol, *cis*-resveratrol, *ε*-viniferin, and *δ*-viniferin, as described previously (Aleynova et al. 2023). Overexpression of the full-length *VaCPK21* gene was shown to have no significant effect on stilbene content (Fig. S5), confirming previously obtained results (Aleynova et al. 2015). Overexpression of the short and long variants of the *VaCPK21* transcript also had no effect on stilbene biosynthesis, suggesting that the *VaCPK21* gene and its alternative variants are not involved in the regulation of phenolic secondary metabolite biosynthesis.

Overexpression of spliced VaCPK21 gene variants in Arabidopsis thaliana plants

Next, we overexpressed the *VaCPK21* genes in *A. thaliana* plants. Homozygous plants with a single transgene insert were obtained. It is important to note that despite numerous attempts, it was not possible to obtain transgenic plants overexpressing the *VaCPK21L1* sequence. 5 independent attempts were made, but at the T1 plants, grown on the selective antibiotic kanamycin (Km), did not produce seeds, they were sterile. We cannot explain the reason for sterility. We can only say that we independently obtained three transgenic *Arabidopsis* plant lines expressing the *VaCPK21L1* transcript, but none produced seeds. So, sterility is not the result of accidental mutagenesis due to the insertion of transfer DNA with *VaCPK21L1* transcript. Thus, these data suggest that this is a consequence of the expression of the *VaCPK21L1* transcript.

In all the obtained *VaCPK21*s-transgenic *A. thaliana* plant lines, the expression of the transgene was high, indicating the successful production of transgenic *Arabidopsis* plants (Fig. 7). The expression of the *VaCPK21s1* transgene was highest in the plant line 21s1-1, while in the lines 21s1-2 and 21s1-3 the expression of *VaCPK21s1* was 2.1–4.2 times lower (Fig. 7a). The expression of the *VaCPK21s2* transgene was highest in the plant line 21s2-1, and 2-3 times lower in the 21s2-2 and 21s2-3 lines (Fig. 7b). Next, the tolerance of *A. thaliana* transgenic plants to abiotic stresses was investigated: salinity, cold, heat, and drought. Seeds from previously obtained transgenic plants overexpressing the

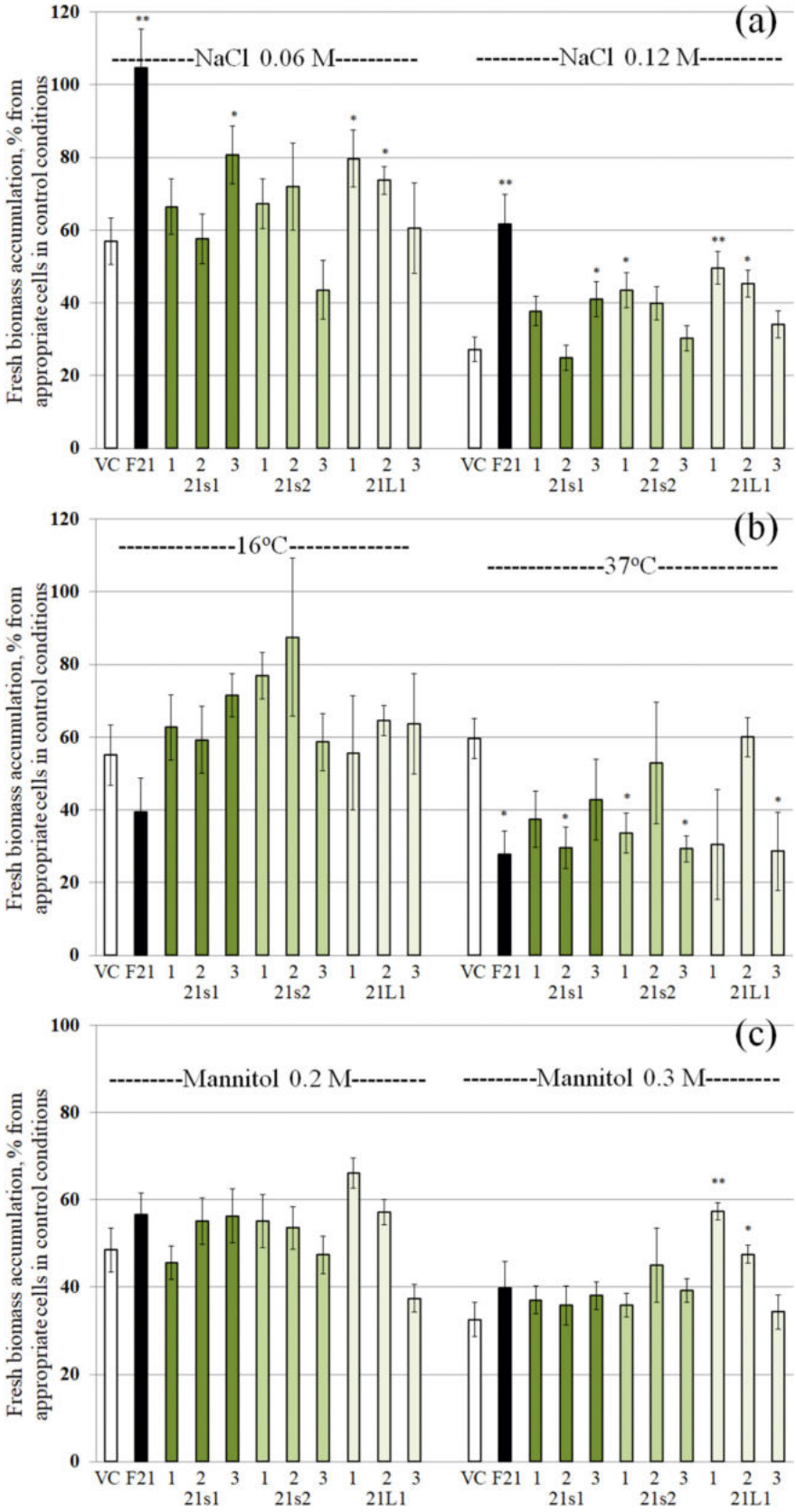

Fig. 6 Fresh biomass accumulation for 30 d in the VC, F21, 21s1, 21s2, and 21L1 cell lines and the influence of salt (NaCl), cold (16°C), heat (37°C), and osmotic stresses (Mannitol) on the growth of the transgenic cell lines overexpressing only the *nptII* selective marker (VC) or the *nptII* gene with full-length *VaCPK21* transgene (F21), short *VaCPK21* transcripts (21s1, 21s2), and long *VaCPK21* transcripts (21L1). Fresh biomass data were obtained from three independent experiments and are averages of 10 technical replicates (10 test tubes) for each independent experiment and are presented as mean ± standard error. Statistical significance was calculated by Student's *t* test. **—$p < 0.01$ and *—$p < 0.05$ in *VaCPK21*-transgenic cells versus values of biomass accumulation in the empty vector-transformed VC cell culture in the appropriate conditions: *VaCPK21*-transgenic cells under stress versus VC under same stress conditions

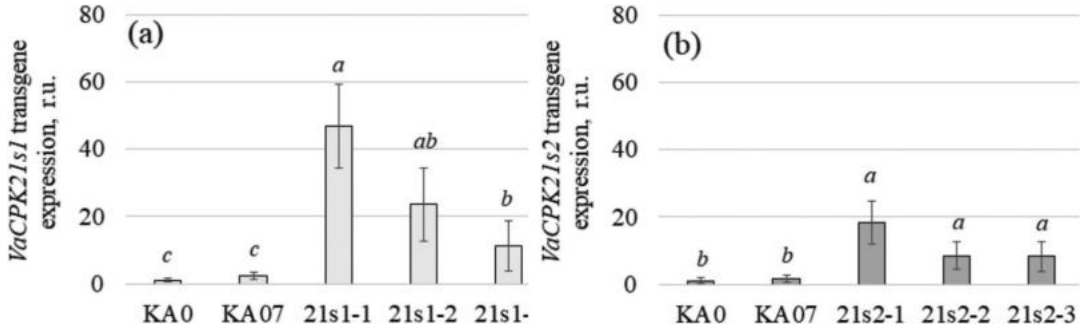

Fig. 7 Quantification of the 2×35S-*VaCPK21s1* (**a**) and 2×35S-*VaCPK21s2* (**b**) transgene in *Arabidopsis thaliana* plants in the control vector KA0 plants, plants overexpressing the full-length *VaCPK21* transgene (KA07), short (21s1, 21s2), and long (21L) *VaCPK21* transgenes. qPCR data were obtained from two independent experiments and are averages of 10 technical replicates for each independent experiment (five qPCR reactions normalized to *AtActin* and five qPCR reactions normalized to *AtGAPDH* expression) and presented as mean ± standard error. Means followed by the same letter were not different using Student's *t* test. $p < 0.05$ was considered statistically significant

full-length *VaCPK21* gene (KA07 line) were added to the newly obtained transgenic lines of *A. thaliana* plants. It was previously shown that these KA07 plants were resistant to salt stress (Dubrovina et al. 2016), which was confirmed in our new experiments (Figs. 8a, S6). These KA07 plants were not resistant to other stresses.

In the obtained transgenic *A. thaliana* plants overexpressing short transcript variants of the *VaCPK21* gene (21s1 and 21s2), tolerance to soil salinity was observed in one line, only one plant line 21 s-2 was resistant in plants overexpressing the *VaCPK21s1* transgene (Figs. 8a; S6). However, if just one out of the three lines shows tolerance, it is likely due to the insertion of T-DNA, which indicates a non-specific outcome of the transformation method applied. Consequently, we posit that the tolerance observed in the CPK21s1-2 line arises from the T-DNA insertion rather than from an increased expression of the *VaCPK21s1* transcript. Thus, transformation by short *VaCPK21s* transcripts does not lead to salinity tolerance. To other stresses, plants overexpressing short variants of the *VaCPK21* gene were not resistant as like *A. thaliana* plants overexpressed the full-length *VaCPK21* transgene (Fig. 8b–d).

Conclusions

Thus, our results indicate that the *VaCPK21* gene undergoes alternative splicing normally (*CPK21s2*) and under stress conditions (*CPK21s1*, *L1*). The strongest effect, leading to the appearance of alternative sequences, was heat treatment. Perhaps this is the plant cell's response to unfavourable conditions, or simply that at such a temperature, polymerase errors can occur in the plant cells themselves. We have not cloned such transcripts under other stresses, although qPCR data suggest that, for example, the form of *CPK21s1* increases slightly under water shortage and salt stress.

Previous and current studies have shown that *VaCPK21* overexpression enhances plant tolerance to salinity (Dubrovina et al. 2016). This protective effect is probably due to the involvement of calcium-dependent protein kinase VaCPK21 in the regulation of ion homeostasis, osmotic adjustment, and antioxidant defense systems by phosphorylation of the specific regulatory (e.g. same Myb transcription factors) or functional proteins (e.g. NADPH oxidases, peroxidases). Interestingly, overexpression of the alternative *VaCPK21S1* and *VaCPK21S2* variants in grape cells and *A. thaliana* plants did not, in most cases, lead to the salinity tolerance typical of the full-length *VaCPK21* transcript. In this case, AS leads to the loss of an important function that is characteristic of VaCPK21 probably the loss of a part of the VaCPK21 protein changes the spectrum of protein partners, reduces the number of important for tolerance to salinity proteins that can bind to VaCPK21S. At the same time, grape cells overexpressing the *VaCPK21L1* form retained their tolerance to salinity, but at the same time a new property appeared—greater tolerance to osmotic stress, compared with control vector VC cells and F21 cells overexpressing the full-length *VaCPK21* transcript. Probably, in case of VaCPK21L1, the insertion leads to the loss of the C-terminal domain and a change in the spectrum of partner proteins, was appear new targets of VaCPK21L1 for phosphorylation. In addition, we were not able to obtain *A. thaliana* plants overexpressing the *VaCPK21L1* transcript, because the plants did not form seeds, which also indicates the appearance of some new regulatory functions in this transcript as a result of alternative splicing. Further study of the partner proteins of VaCPK21 proteins will help answer a number of interesting questions that are important for understanding the function of calcium-dependent protein kinase in plants.

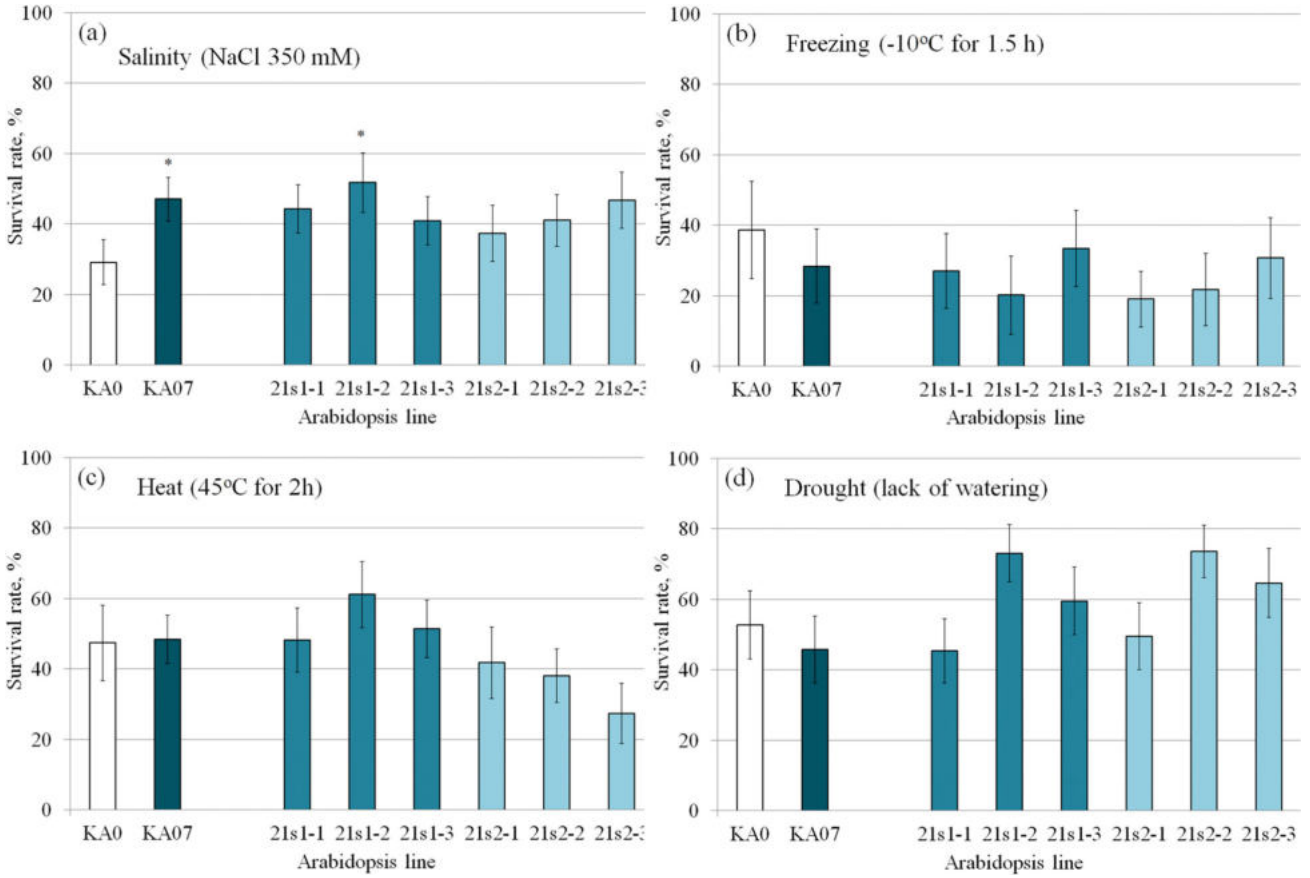

Fig. 8 Responses to salinity (**a**), freezing (**b**), heat (**c**), and drought (**d**) of vector control (KA0), 2×35S-*VaCPK21* (KA07), 2×35S-*VaCPK21s1* (21s1-1, 21s1-2, 21s1-3), and 2×35S-*VaCPK21s2* (21s2-1, 21s2-2, 21s2-3) *Arabidopsis thaliana* plants. **a**—three-week-old KA0 and 2×35S-*VaCPK21* (lines KA07, 21s1, 21s2) plants were soaked in saline solution (NaCl 350 mM) for 2 h and then transferred to normal conditions without watering, after half of the plants turned yellow, all the plants were soaked in a filtered water; **b** plants were cold stressed at − 10 °C for 1.5 h and then transferred to normal conditions; **c** plants were heat-stressed at 45 °C for 2 h and then transferred to normal conditions; **d** after planting in moist soil, the plants were not watered for 5 weeks until the withering of half of the plants wilted due to drought, then the plants were soaked in a filtered water and transferred to normal conditions for recovery. Survival rates were determined as the number of visibly green plants after the applied stress conditions. Values are the mean ± SE. Twenty plants of each line were used in each of ten experiments. Means followed by the same letter were not different using Student's *t* test. $p < 0.05$ was considered to be statistically significant

The variation in transcript numbers suggests differential regulation of *VaCPK21* expression under control and stress conditions, potentially reflecting its involvement in specific stress response pathways. This information may help in the development of genetically modified grapevine varieties or other plants with improved stress tolerance, which is important for the efficient production of agricultural products.

Materials and methods

Plant material and drought, salt, cold and heat treatments of transgenic callus cell lines and transgenic Arabidopsis plants

Wild-type 8–10-year-old plants of gape *V. amurensis* were sampled from a non-protected natural population near Vladivostok (Akademgorodok, Russia). For *VaCPK21* expression analysis, the *V. amurensis* vines were collected in September 2020 and divided into cuttings (excised young stems, approximately 8 cm long, with one healthy leaf). All transgenic cell lines were grown in the dark at 24–25 °C for 32 days on Murashige and Skoog modified medium (Dubrovina et al. 2010) supplemented with 0.5 mg/L BAP, 2 mg/L NAA, and 8 g/L agar in the dark. Inoculum biomass was 0.15–0.17 g (each callus was weighed on an electronic balance).

Salt treatment was performed by adding 50 and 100 mM of NaCl to the culture media. Cold and heat treatments were performed by culturing the transgenic cells at 16 °C and 33 °C in a growth chamber (TSO-1/80, Smolenskoe SKTB SPU, Smolensk, Russia) on the culture media. Mannitol treatment was applied by adding 0.2 and 0.3 M of D-mannitol to the medium (BioChemica, Darmstadt, Germany). Average growth rates were assessed after 32 days of cultivation under control conditions, heat, cold, and salt stresses.

Plants of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia-0 (stored in our laboratory) were grown five plants in one pot (8 × 8 cm) filled with commercially available rich soil (Universalniy, Fasko, Russia) in an environmental control chamber (JIUPO Fujian Jiupo Biotechnology Co., Fuzhou City, China) kept on a 16/8 h d/night cycle at 22 °C and a light intensity of approximately 200 µmol m^{-2} s^{-1}.

The *A. thaliana* plants were subjected to drought stress treatments by culturing without additional irrigation until lethal effects were observed for all genotypes (approximately 5 weeks without additional irrigation) and then re-watered. For salt stress treatments, the 7-day-old seedlings, transferred to the well-watered soil, were grown without additional irrigation for 2 weeks, and plants were then well-irrigated with NaCl solution (350 mM) applied to the bottom of the pots. When the soil was completely saturated with salt water, the free NaCl solution was removed and the plants were grown as normal. 1 week after irrigation with NaCl, the pots were placed in 3 cm of fresh water for 4 h to leach the salt from the soil. For freezing tolerance assays, normally cultured *Arabidopsis* plants (3-week-old) were stressed in a − 10 °C freezer for 2 h and then cultured at 8 °C for 2 h to facilitate recovery, and then cultured under normal growth conditions for 3 days. For heat tolerance assays, normally cultured *Arabidopsis* plants (3-week-old) were stressed at 45 °C in a controlled incubator (TSO-1/80, Smolenskoe SKTB SPU, Smolensk, Russia) for 4 h without light, and then cultured under normal growing conditions for 1 additional week. Survival rates in all experiments were determined as the number of visibly green plants 3 days (drought, freezing) or 1 week (salinity, heat) after stress conditions.

Overexpression of *VaCPK21* transgenes in Arabidopsis plants and cell cultures of *V. amurensis*

The *VaCPK21* transgene was used in four forms, including *VaCPK21* (full-length), short *VaCPK21s1* and *VaCPK21s2*, and long *VaCPK21L1* transcripts (suppl. Figs. S2, S3). To generate the construction for plant cell transformation, the sequences of the *VaCPK21* gene transcript were amplified from cDNA of grapevine *V. amurensis* leaves by PCR using the primers presented in the supplementary Table S1. We used a heat-stable RT ThermoScriptTM (Invitrogen, Life Technologies, USA) to obtain cDNA and then in PCR we used a high-fidelity Pfu polymerase ("Silex M", Moscow, Russia) to obtain VaCPK21 PCR products for cloning to plasmids. We have previously shown that this completely eliminates mutations or deletions (Kiselev et al. 2011; Dubrovina et al. 2014).

The obtained *VaCPK21* PCR products were subcloned into a pJET1.2 using the CloneJET PCR Cloninig Kit (ThermoFisher Scientific, Waltham, MA, USA) and sequenced using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manuphacturer's instructions. Next, we performed PCR with the forward primer containing a *Sal* I restriction site and the reverse primer containing a *Bam*H I restriction site (supplementary Table S1). The *VaCPK21* transcripts were cloned into the pSAT1 vector (Tzfira et al. 2005) by the *Sal* I and *Bam*H I sites. Then, the expression cassette from pSAT1 with the *VaCPK21* genes was cloned into the pZP-RCS2-*npt*II vector (Tzfira et al. 2005) using the *Pal*AI (*Asc*I) sites. All transgenes in the used vectors were under the control of the double cauliflower mosaic virus (2 × 35S) promoter.

The *VaCPK21* overexpression constructs of (pZP-RCS2-*VaCPK21-nptII,* pZP-RCS2-*VaCPK21s1-nptII,* pZP-RCS2-*VaCPK21s2-nptII,* or pZP-RCS2-*VaCPK21L1-nptII*) or empty vector (pZP-RCS2-*nptII*) were introduced into the *Agrobacterium tumefaciens* strain (GV3101::pMP90), which was used for the floral-dip transformation of *A. thaliana* (Zhang et al. 2006) or for the transformation of the suspension V7 culture of *V. amurensis* (Aleynova-Shumakova et al. 2014; Kiselev et al. 2021). The V7 callus cultures were established in 2017 from young stems of the wild-growing mature V. amurensis vines near Vladivostok as described in (Tyunin et al. 2019) and are maintained in our laboratory.

All *VaCPK21* transgenic cell lines were obtained again: three lines for each genetic construction used, except for the control vector VC cell line, which overexpressed only the *nptII* gene and the F21 cell line, overexpressed the full-length *VaCPK21* and the *nptII* genes. We obtained one VC and one F21 cell line. We used previously obtained control vector KA0 (overexpressing only the *nptII* gene) and KA07 (overexpressing the full-length *VaCPK21* and *nptII*

genes) *A. thaliana* plants (Dubrovina et al. 2016). In new paper, we have independently obtained three transgenic plant lines for *VaCPK21s1* (21s1-1, 21s1-2, 21s1-3) and three for *VaCPK21s2* transcripts (21s2-1, 21s2-2, 21s2-3).

Fertile T_3 homozygous lines of *A. thaliana* transformed with the pZP-RCS2-*VaCPK21-nptII* (KA07), pZP-RCS2-*VaCPK21s1-nptII* (21s1), or pZP-RCS2-*VaCPK21s2-nptII* (21s2) were selected for detailed analyses. The transgenic lines used in this study were homozygous plants with a single copy insertion (Gadaleta et al. 2011).

HPLC and mass spectrometry stilbene analysis

Stilbenes levels were analyzed by HPLC with diode array detection as described (Dubrovina et al. 2010; Kiselev et al. 2017). The extracts were separated on Shim-pack GIST C18 column the on HPLC LC-20AD XR analytical system (Shimadzu, Japan), equipped with an SPD-M20A photodiode array detector. The mobile phase consisted of a gradient elution of 0.1% aqueous formic acid (A) and acetonitrile (B). 1 µL of the sample extract was injected with a constant column temperature maintained at 40 ℃.

Nucleic acid purification and quantitative real-time PCR (qPCR)

Cetyltrimethylammonium bromide (CTAB)-based extraction was used for total RNA isolation as described (Kiselev et al. 2013). cDNAs for qPCR were prepared using the MMLV Reverse transcription PCR Kit with oligo(dT)15 (qPCR, Evrogen, Moscow, Russia) at 37 °C as described (Kiselev et al. 2013). cDNAs for cloning different *VaCPK21* transcripts were synthesized at 65 °C using 1.5 µg of RNA for 1 h and heat-stable reverse transcriptase (ThermoScript, Invitrogen, Life Technologies, Waltham, Massachusetts, USA). We use heat-stable reverse transcriptase in order to get rid of false transcripts as a result of reverse transcriptase (Dubrovina et al. 2014).

The mRNA transcript levels of the transgenes were determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) with two internal controls, including *AtGAPDH* (NM_111283.4) and *AtEF* (XM_002864638) for *A. thaliana* and *VaGAPDH* (XM_002263109), *VaActin1* (DQ517935) for grape *V. amurensis* as described (Aleynova et al. 2022). Primers designed for qPCRs are shown in the supplementary Table S1.

Statistical analysis

Three independent experiments with ten technical replicates in each experiment were performed for callus cell lines and three independent experiments with three technical replicates in each experiment for the stilbene analysis. For drought, salt, cold and heat treatments of transgenic *Arabidopsis* plants, twenty plants of each line were used in each of ten experiments. Data are presented as mean ± standard error (SE) and were evaluated by Student's t test performed in Microsoft Excel Standard 2019 (Microsoft Office, Microsoft, Redmond, Washington, USA). For the analysis of the *VaCPK21* transgene expression, we performed three independent experiments with ten technical replicates (five qPCR reactions normalized to one internal control gene and five qPCR reactions—to the second internal gene in each independent experiment).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11240-024-02931-1.

Author contributions ASD and KVK performed planning experiments, data interpretation, and writing a paper. ZVO and OAA performed experiments with cell cultures, transgenic plants, RNA isolations, qPCRs, and data analysis. ARS performed stilbene analysis.

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Data availability The data presented in this study are available within the article and Supplementary Materials.

Declarations

Conflict of interest The authors declare no conflict of interest. KVK is one of the Associate Editor of the journal at the time of submission. This had no impact on the peer review process and the final decision.

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