Impact of VaCCaMK gene overexpression and its splicing isoforms on cell growth and stilbene accumulation in Vitis amurensis Rupr

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Abstract

Calcium (Ca^{2+}) functions as an essential intracellular second messenger in plants, mediating processes such as pathogen defence, stress adaptation and enzyme activation. Plants possess multiple families of calcium-binding proteins, and among these, calcium/calmodulin-dependent protein kinases (CCaMKs) remain among the least characterized. Also, their dual capacity to bind both Ca^{2+} and calmodulin makes them a subject of significant research interest. Usually, there is one *CCaMK* gene per plant genome, but when we cloned the full-length *VaCCaMK* cDNA sequence, we found several transcripts with missing exons, so it is possible that alternative splicing increases the overall diversity of *CCaMK* sequences. This study investigated the role of CCaMKs in *Vitis amurensis* Rupr. under abiotic stress conditions using grapevine cell cultures overexpressing the *VaCCaMK1* gene and its alternatives *VaCCaMK1*-s1, -s2. Results demonstrated that *VaCCaMK1*-overexpressing cultures did not exhibit increased tolerance to salt, osmotic, cold, or heat stress. Additionally, the content of secondary metabolites, as represented by stilbenes mainly produced by used grapevine cells, remained largely unchanged. However, a significant increase in both fresh and dry cell mass was observed compared with the control group: fresh biomass increased 1.1–1.7-fold, and dry biomass 1.1–1.8-fold. These findings indicate that *VaCCaMK* genes do not affect plant cell susceptibility to the tested abiotic stresses. *VaCCaMK1-s1* or *-s2* the overexpression had a similar but weaker effect on grape cells. Nevertheless, VaCCaMK appear to act as a positive regulator of cell growth and development in *V. amurensis*, suggesting their potential role in enhancing biomass accumulation.

1. Introduction

Calcium (Ca^{2+}) is a universal intracellular second messenger in plants, playing a pivotal role in transducing extracellular signals into coordinated cellular responses (McCormack et al. 2005; Kudla et al. 2010). It regulates a wide array of physiological processes, including pathogen defence, stress adaptation, enzyme activation, stomatal movement, photomorphogenesis, and growth regulation (Reddy et al. 2011; Kiselev, Dubrovina, 2025). The specificity and versatility of Ca^{2+} signalling are achieved through a complex network of calcium-binding proteins that decode the spatiotemporal patterns of Ca^{2+} concentration changes, often referred to as “calcium signatures”, into specific downstream responses (Harper and Harmon 2005).

The majority of Ca^{2+} sensor proteins contain multiple EF-hand motifs: conserved helix-loop-helix structures in which Ca^{2+} ions are coordinated within the acidic Ca^{2+}-coordinating loop (Halling et al. 2016). The major plant EF-hand-containing Ca^{2+}-binding proteins include calmodulins (CaMs), calmodulin-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs), calcineurin B-like proteins (CBLs), and calcium/calmodulin-dependent protein kinases (CCaMKs) (Hashimoto and Kudla 2011; Mohanta et al. 2019).

Among the diverse families of calcium-binding proteins in plants CCaMKs occupy a unique position due to their dual capacity to bind both Ca^{2+} ions and calmodulin. This dual regulatory mechanism allows CCaMKs to integrate calcium signals and transduce them into phosphorylation events, thereby modulating the activity of target proteins (Takezawa et al. 1996). CCaMKs are best known for their critical roles in plant–microbe interactions, particularly in establishing symbioses with nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi. In these processes, CCaMK acts as a central component of the common symbiosis signaling pathway, where it decodes Ca^{2+} oscillations and activates downstream transcriptional reprogramming (Hayashi et al. 2010).

Despite their importance in biotic interactions, the functions of CCaMKs in abiotic stress responses remain poorly understood. Abiotic stresses such as salinity, drought, osmotic imbalance, and extreme temperatures represent major constraints on plant growth and productivity worldwide (Kumar, 2020). These stressors often trigger rapid increases in cytosolic Ca^{2+} concentrations, suggesting that calcium signalling pathways, including CCaMKs, may play a role in stress perception and adaptation (Xu et al. 2022). However, direct evidence linking CCaMK activity to abiotic stress tolerance is limited, and the molecular mechanisms involved are largely unexplored (Contreras Delgado et al. 2026).

Grape *Vitis amurensis* is an excellent model for studying stress tolerance mechanisms, as it exhibits remarkable resilience to cold, pathogens, and other environmental challenges (Liu, Li, 2013; Xin et al. 2013). Grapevine cell cultures provide a controlled system to investigate molecular and physiological responses without the complexity of whole-plant architecture. The present study aimed to elucidate the role of *VaCCaMK* genes in abiotic stress responses and cell growth regulation in *V. amurensis*. Specifically, we investigated the effects of overexpressing the full-length grape *VaCCaMK* gene and its alternatively spliced variants (*VaCCaMK-s1*, *VaCCaMK-s2*) in grapevine cell cultures under salt, osmotic, cold, and heat stress conditions. We also assessed changes in stilbene production, key secondary metabolites with antioxidant and antimicrobial properties, as well as biomass accumulation.

2. Results and Discussion

2.1. Cloning and sequencing of VaCCaMK mRNA transcripts

Previously, the grapevine *Vitis vinifera* L. *VvCCaMK* sequence was determined during whole-genome sequencing in BioProject PRJNA1013121 (Jaillon et al. 2007; Shi et al. 2023). The protein-coding sequence of *VvCCaMK* consists of 1563 bp and 7 exons. Next, using primers for the beginning and end of the protein-coding sequence of the known *VvCCaMK* gene (GenBank XM_002273306) on cDNA obtained from a wild-growing *V. amurensis* grape leaf, we obtained the full-length protein-coding sequence of the *VaCCaMK* gene. The resulting *VaCCaMK* sequence was highly homologous to the *VvCCaMK* gene in terms of nucleotide composition (Identities 99.2%) and deduced amino acid sequence

(Identities 99.6%) (Fig. 1). In the available grape *V. amurensis* genome (Wang et al. 2024), we found only one copy of the *VaCCaMK* gene.

Table 1
CCaMK genes in plant species with known functions.

Species	Monocots or eudicots	Gene functions	Accession no.	Reference
Lily *Lilium longiforum*, LlCCaMK	Monocots	Preferentially expressed in developing anthers	Q43531	Patil et al. 1995
Wheat, *Triticum aestivum*, TaCCaMK	Monocots	Overexpressing *TaCCaMK* in Arabidopsis plants reduced their sensitivity to abscisic acid (ABA) treatment during seed germination and root elongation. Increased root length	HM595635	Yang et al. 2011
Maize, *Zea mays*, ZmCCaMK	Monocots	It plays a key role in the antioxidant protection of plants induced by brassinosteroids and in increasing their resistance to drought	ABD67491	Liu et al. 2020
Lotus japonicus, LjCCaMK	Eudicots	Single amino-acid replacement in a CCaMK is sufficient to turn fully differentiated root cortical cells into meristematic founder cells of root nodule primordia	CAJ76700	Tirichine et al. 2006
Medicago truncatula, MtCCaMK	Eudicots	It ensures the formation of nodules when the plant interacts with nitrogen-fixing bacteria	Q6RET7	Routray et al. 2013
Tobacco, *Nicotiana tabacum*, NtCCaMK	Eudicots	It has been shown that their expression is strictly timed to the stage of anther development, when the buds are 0.5–1.0 cm in size, which coincides with meiosis	AAD28791	Liu et al. 1998

It is important to note that while we were trying to obtain a full-length *VaCCaMK* sequence, we cloned various short *VaCCaMK* variants, even though we used a special heat-stable reverse transcriptase and high-fidelity DNA polymerase. Among these short *VaCCaMK* variants, there were two main sequences that were repeated several times in two independent transformations: *VaCCaMK-s1* and *VaCCaMK-s2*. We did not find such short variants in the *V. amurensis* genome. (Wang et al. 2024). It was only after the third transformation attempt and the analysis of more than 20 clones that we were able to obtain the standard sequence *VaCCaMK*.

We showed that the E1 and E4 exon sequences of *VaCCaMK-s1* had 8-bp short direct repeats (SDR): TGTTTCAC, resulting that the partial sequence of E1 and E4 and the complete sequence of E2 and E3

are missing from the *VaCCaMK-s1* transcript sequence and at the same time, the protein reading frame does not get messed up (Fig. 2a). This results in a 906 bp cDNA sequence that can be used to produce a shortened protein missing part of the kinase domain (Fig. 2b). The production of this *VaCCaMK-s1* transcript can be attributed to mechanisms of genomic instability and DNA recombination (Ogihara et al. 1988), but due to its high abundance during cloning, we decided to include it in our further research.

A feature of *VaCCaMK-s2* transcript was that the entire 6-th exon was missing (Fig. 2a), what is a typical example of alternative splicing (Dubrovina et al. 2013). This results in a 1503 bp cDNA sequence that can be used to produce a shortened protein missing second EF-hand, protein with two EF-hands (Fig. 2b). Also, due to its high abundance during cloning, we decided to include it in our further research.

Next, we performed a promoter analysis (2000 bp upstream of the start codon) of the *VaCCaMK* gene using PlantCARE. We identified over a hundred different *cis*-acting elements (supplementary Table S1); however, only those with well-established functions are presented in the (Fig. 3). Thus, the *VaCCaMK* gene promoter contains the highest number of light-responsive elements (6 elements). It also includes elements associated with phytohormone regulation: one for auxin, one for abscisic acid, and one for gibberellins. Additionally, elements responsive to stress hormones were detected: one for salicylic acid and one for methyl jasmonate. Elements associated with abiotic stresses were also present, albeit in smaller numbers (2 for drought and 1 for wounding).

2.2. Expression of VaCCaMK mRNA transcripts in grapevine V. amurensis leaves under abiotic stress conditions

In this section, we analyzed the expression of the *VaCCaMK* gene in grape leaves under various common abiotic stresses: water deficit, salt stress, osmotic stress, high temperatures, low temperatures, and ultraviolet C irradiation. It was shown that *VaCCaMK* expression increased in most stresses, but this increase was small and often within the error limits. Only salt stress significantly increased *VaCCaMK* expression by 1.4–1.9 times at all time points compared to untreated control cells (Fig. 4). Therefore, it was important to see how overexpression of the *VaCCaMK* gene would affect resistance to the described stresses, primarily salt stress.

2.3. VaCCaMK1 gene overexpression in the grapevine V. amurensis cell cultures

Using agrobacterial transformation we independently obtained three cell lines for each used gene construction: F1, F2, and F3 for *VaCCaMK* gene, S1-1, S1-2, and S1-3 for *VaCCaMK-s1* gene, or S2-1, S2-2, and S2-3 for *VaCCaMK-s2* gene. Next, we showed that the resulting transgenic lines actively expressed the corresponding transgenes (Fig. 5a), resulting in a significant increase in the overall expression of VaCCaMK in all transgenic cell lines, which was 3.1–8.4 times higher than in the control KA0 cell culture

(Fig. 5b). At the same time, overexpression of the *VaCCaMK* transgenes was found to reduce expression of the endogenous *VaCCaMK* gene by 1.1–2.7-fold, although this reduction did not reach statistical significance.

It was further shown that overexpression of the *VaCCaMK* gene and its alternative transcripts *VaCCaMK-s1* and *VaCCaMK-s2* increases the accumulation of fresh and dry biomass by the resulting transgenic grape cells. The accumulation of biomass by transgenic cells was greatest with overexpression of the *VaCCaMK* gene: the accumulation of fresh weight significantly increased by 1.6–1.7 times and dry mass by 2.2–2.5 times (Fig. 6). Overexpression of the *VaCCaMK-s2* transcript increased the accumulation of fresh biomass by 1.5–1.7 times and dry by 1.9–2.4 times (Fig. 6). Overexpression of the *VaCCaMK-s1* transcript also activated an increase in fresh and dry weight, but this increase was to a lesser extent (1.0-1.5 and 1.8–2.4 times) and was not significant for all three cell lines (Fig. 6).

It is well established that plant calcium sensors play key roles in stress resistance. We therefore investigated the effect of *VaCCaMK* transcript overexpression on the tolerance of transgenic grape cells to temperature extremes (high and low), salt stress, and osmotic stress (Fig. 7). The data show that the applied conditions significantly reduced fresh biomass accumulation in the control KA0 cell culture (Fig. 7), confirming the efficacy of the selected temperatures and substance concentrations. However, no significant differences in growth were observed between *VaCCaMK*-transgenic cells and KA0 control cells under these stress conditions.

All cell lines overexpressing the *VaCCaMK* gene and its short variants exhibited reduced growth at 16°C compared to the control; however, these differences relative to KA0 cell growth were not statistically significant (Fig. 7). Additionally, several *VaCCaMK*-expressing cell lines showed marginally enhanced growth at elevated temperatures (33°C), though the observed increase fell within the range of measurement error (Fig. 7a,b).

We also analyzed the content of valuable secondary metabolites capable of being synthesized by grape cells – specifically, stilbenes. In grape cell cultures, stilbenes occur in eight forms: resveratrol diglucoside, trans-piceid, cis-piceid, trans-piceatannol, trans-resveratrol, cis-resveratrol, ε-viniferin, and δ-viniferin (Aleynova et al. 2023). Following transformation with the *VaCCaMK* gene and its short variants, the stilbene content was in the range of 0.26–0.50 mg/g dry weight (DW), whereas the stilbene content in the control cell culture KA0 was 0.38 ± 0.05 mg/g DW (supplementary Table S2). Thus, transformation with the *VaCCaMK* gene did not lead to significant changes in stilbene accumulation.

3. Conclusions

In this study, we successfully cloned and sequenced the full-length *VaCCaMK* gene from *V. amurensis*, revealing a high degree of homology (99.6% at the amino acid level) with the *VvCCaMK* gene of *V. vinifera*. During cloning, two short transcript variants, *VaCCaMK-s1* and *VaCCaMK-s2*, were also identified. *VaCCaMK-s1* arises due to genomic instability and DNA recombination, featuring 8-bp short direct

repeats and a truncated cDNA sequence (906 bp). *VaCCaMK-s2* is a product of alternative splicing, lacking the 6-th exon and resulting in a 1503 bp cDNA sequence.

Typically, plant genomes contain a single *CCaMK* gene, implying strong evolutionary conservation of this kinase function (Wang et al. 2015). However, recent findings suggest that post-transcriptional regulation, particularly alternative splicing, may expand the functional diversity of CCaMK proteins. During cloning of the full-length *VaCCaMK* cDNA from grape *V. amurens*is, a wild grapevine species notable for its high cold and disease resistance (Liu, Li, 2013), we identified multiple transcript variants with missing exons. This observation raises the possibility that alternative splicing generates functionally distinct isoforms of CCaMK proteins, potentially enabling fine-tuned regulation of calcium signaling under different conditions. This is an interesting example, and it is possible that other low-copy plant genes undergo similar modifications; however, this requires further research.

Transgenic cell lines overexpressing *VaCCaMK* and its variants exhibited increased biomass accumulation, most notably with the full-length gene (fresh weight: 1.6–1.7 times; dry mass: 2.2–2.5 times). However, no significant improvement in tolerance to temperature extremes, salt, or osmotic stress and no increase in the secondary metabolites was observed in transgenic cells compared to controls. Previous studies have demonstrated that overexpression of the *TaCCaMK* gene enhances wheat root growth (Yang et al. 2011). Similarly, CCaMK genes are frequently involved in nodule formation and development during bacterial-plant interactions (Tirichine et al. 2006; Routray et al. 2013). Therefore, existing publications also indicate the growth-promoting properties of these genes in other plant species, which is consistent with our findings.

Overall, our findings characterize *VaCCaMK* and its splice variants in *V. amurensis* and suggest their role in biomass regulation rather than in abiotic stress tolerance or stilbene production, this is also important for plant biotechnology in the regulation of the biosynthetic properties of plant cells.

4. Materials and Methods

4.1. Plant material and drought, salt, cold and heat treatments of V. amurensis leaves

Wild-type 10-12-year-old plants of gape *V. amurensis* were sampled from a non-protected natural population near Vladivostok (Akademgorodok, Russia). For *VaCCaMK* expression analysis, the *V. amurensis* vines were collected in September 2025 and divided into cuttings (excised young stems, approximately 8 cm long, with one healthy leaf). The cuttings were then placed in cups filled with filtered sterile water. Salt treatment was performed by adding 50 and 100 mM of NaCl to the water in cups. Cold and heat treatments were performed by 16°C and 33°C in a growth chamber (TSO-1/80, Smolenskoe SKTB SPU, Smolensk, Russia). Mannitol treatment (osmotic stress) was applied by adding 0.2 and 0.3 M of D-mannitol (Serva, New York, USA) to the water in cups (Aleynova et al. 2024).

4.2. Overexpression of VaCCaMK transgenes in cell cultures of V. amurensis

The *VaCCaMK* transgene was used in three forms, including *VaCCaMK* (full-length), short *VaCCaMK-s1* and *VaCCaMK-s2*. To generate the construction for plant cell transformation, the sequences of the *VaCCaMK* gene transcript were amplified from cDNA of grapevine *V. amurensis* leaves by PCR using the primers presented in the supplementary Table S3.

We used a heat-stable RNAscribe RT (Biolabmix, Novosibirsk, Russia) to obtain cDNA and then in PCR we used a high-fidelity Tersus polymerase (Evrogen, Moscow, Russia) to obtain *VaCCaMK* 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 VaCCaMK PCR products were subcloned into a pJET1.2 using the CloneJET PCR Cloning 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 Bgl II restriction site and the reverse primer containing a Sal I restriction site (supplementary Table S3). The *VaCCaMK* transcripts were cloned into the pSAT1 vector (Tzfira et al., 2015) by the Bgl II and Sal I sites. Then, the expression cassette from pSAT1 with the *VaCCaMK* genes was cloned into the pZP-RCS2-nptII vector (Tzfira et al., 2015) using the PalAI (AscI) sites. All transgenes in the used vectors were under the control of the double cauliflower mosaic virus promoter.

The *VaCCaMK* overexpression constructs of (pZP-RCS2-VaCCaMK-nptII, pZP-RCS2- VaCCaMK-s1-nptII, or pZP-RCS2-VaCCaMK-s2-nptII) or empty vector (pZP-RCS2-nptII) were introduced into the *Agrobacterium tumefaciens* strain (GV3101::pMP90), which was used for the transformation of the suspension V7 culture of *V. amurensis*. 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 *VaCCaMK* transgenic cell lines and KA0 cell line were obtained again: three lines for each genetic construction used, except for the one vector KA0 cell line, which overexpressed only the *nptII* gene and used as control. Grapevine cell lines were grown in the dark at 24–25°C for 35 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.

4.3. 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 RNAscribe RT kit (Biolabmix, Novosibirsk, Russia) with oligo(dT)15 at 55°C for 50 min as described in the manufacturer's protocol.

The mRNA transcript levels of the transgenes were determined by the $2^{-\Delta\Delta CT}$ method (Livak, Schmittgen, 2001) with two internal controls, including *VaGAPDH* (XM_002263109) and *VaActin1* (DQ517935) for grape *V. amurensis* as described (Aleynova et al., 2022). Primers designed for qPCRs are shown in the supplementary Table S3.

4.4. Analysis of promoter cis-acting elements

The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html, accessed on 15 Apryl 2026) was used to predict cis-regulatory elements in the 2000 bp promoter regions upstream of the transcription start sites of the *VaCCaMK* gene. The results were visualized using gggenes (version 0.6.0) (Wilkins, 2025) and tidyverse (version 2.0.0) (Wickham et al. 2019) R packages.

4.5. HPLC and mass spectrometry stilbene analysis

Stilbenes levels were analyzed by HPLC with diode array detection as described (Dubrovina et al., 2010). 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.

4.5. Statistical analysis

Three independent experiments with ten technical replicates in each experiment were performed for biomass accumulation and three independent experiments with three technical replicates in each experiment for the stilbene analysis in the callus cell lines. For the analysis of the *VaCCaMK* total, transgene, and endogenous 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). 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).

Declarations

Author Contributions:

KVK and ASD performed research design, interpretation and paper preparation. AAB, AAA, EVT, and OAA performed experiments with agrobacterial transformation, experiments on the cell lines, RNA isolations, RT-qPCRs, and data analysis. NNN carried out the bioinformatics and statistical analyses. ARS conducted the HPLS analysis.

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Figures

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                                                  K - Kinase domain
VvCcamk   MG-QETRRLLDEYEVSDVLGRGGFSVVRRGTRKSSSGE----NPVAIKTLKRCGQTNLP-GLPRNRGSEKRVASMAFPTWKQVSISDALLTNEILVMRKI
VaCCaMK   MG-QETRRLLDEYEVSDVLGRGGFSVVRRGTRKSSSGE----NPVAIKTLKRCGQTNLP-GLPRNRGSEKRVASMAFPTWKQVSISDALLTNEILVMRKI
MtCCamk   MG-YGTRKLSDEYEVSEILGRGGFSVVRKGTKKSSIEEEKSQSQVAIKTLRRLGASNNPSGLPRKKDIGE-KSTIGFPTMRQVSVSDTLLTNEILVMRRI
TaCCaMK   MSTTESRRLSDDYEVADVLGRGGFSIVRRGVSKSDEGK----TQVAIKTLRRLGPAMTGMGMQQ--GSKDAPSSGGLPLWKQVSISDALLTNEILVMRRI
          *.   :*:* *:***:::*******:**:*. **.  :    . ******:* * :    *: :  .  .  :: .:*  :***:**:**********:*
                                                  K - Kinase domain
VvCcamk   VEHVSPHPNVINLHDVYEDPSGVHLVLELCSGGELFDRIVAQARYSEAGAAAVVKQLAEGLKALHQANIIHRDLKPENCLFLDKSEDATLKIMDFGLSSV
VaCCaMK_  VEHVSPHPNVINLHDVYEDPSGVHLVLELCSGGELFDRIVAQARYSEAGAAAVVKQLAEGLKALHQANIIHRDLKPENCLFLDKSEDATLKIMDFGLSSV
MtCCamk_  VENVSPHPNVIDLYDVYEDTNGVHLVLELCSGGELFDRIVAQDKYSETEAATVVHQIASGLEAVHRANIVHRDLKPENCLFLDVRKDSPLKIMDFGLSSV
TaCCaMK   VENVAPHPNVISLHDVYEDAHGVHLVLELCSGGELFDRIIGRERYSEFDAAAVVRQIAGGLKALHKANIIHRDLKPENCLFSDRKEDSTLKIMDFGLSSV
          **:*:******.*:*****. ******************:.: :***  **:**:*:* **:*:*:***:********** *  :*:.**********
                                                  K - Kinase domain
VvCcamk   EEFTDPVVGLFGSIDYVSPEVLSQGKISSASDMWSLGVILYILLSGYPPFIAQSNRQKQQMIIAGDFSFYEKTWKNISSSAKQLISSLLTVDPERRPTAH
VaCCaMK   EEFTDPVVGLFGSIDYVSPEVLSQGKISSASDMWSLGVILYILLSGYPPFIAQSNRQKQQMIIAGDFSFYEKTWKNISSSAKQLISSLLTVDPERRPTAQ
MtCCamk   EEFTDPVVGLFGSIDYVSPEALSQGKITTKSDMWSLGVILYILLSGYPPFIAQNNRQKQQMIMNGNFSFYEKTWKGISQPAKNLISSLLTVDPSKRPSAL
TaCCaMK   EDFSDPVVALFGSVDYVSPEALSRQEVSTASDMWSVGVILYILLSGCPPFHAATNQEKQQRILQGEFSFQEHTWKTITSSAKDLISSLLSVEPYKRPTAS
          *:*:****.****:******.**: :::: *****:********** *** * .*::*** *: *:*** *:*** *:..**:******:*:* :**:*
                                          CaM
VvCcamk   QLLQHPWVMGDSAKQDQMDAEIVSRLQSFNARRKFRAAAIASVWSSTVFLRTKKLKTLVGSHDLTQEELENLRIHFKEICLKGDNATLSEFEQVLKAMNM
VaCCaMK   QLLQHPWVMGDSAKQDQMDAEIVSRLQSFNARRKFRAAAIASVWSSTVFLRTKKLKTLVGSHDLTQEELENLRIHFKEICLKGDNATLSEFEQVLKAMNM
MtCCamk   ELLSDPWVKGEKAKDVQMDPEIVSRLQSFNARRKLRAAAIASVWSSTIFLRTKKLKSLVGSYDLKEEEIENLRMHFKKICADRDNATLSEFEEVLKAMNM
TaCCaMK   DLLMHPWVIGDCAKQDRIDAEVVSKLQRFNARRKLRAAAIASVLSSKVALRTKRLRSLLGTHDLSSEELDNLRQHFARICADGENATLTEFEQVLKAMKM
          :** .*** *: **: ::*.*:**:** ******:******** **.: ****:*::*:*:::**..**::*** ** .** . :****:***:*****:*
                          EF1                                     EF2                                        EF3
VvCcamk   SSLIPLAGRIFDLFDNNRDGTVDMREILCGFSSLRNSQGDDALRLCFQMYDTDRSGCITKEEVASMLRALPDDCLPADITEPGKLDEIFDLMDANSDGKV
VaCCaMK   SSLIPLAGRIFDLFDNNRDGAVDMREILCGFSSLRNSQGDDALRLCFQMYDTDRSGCITKEEVASMLRALPDDCLPADITEPGKLDEIFDLMDANSDGKV
MtCCamk   LSLIPFASRIFDLFDNNRDGTVDMREILCGFSSLKNSKGEDALRLCFQMYDTDRSGCISKEEVASMLRALPYDCLPTDITEPGKLDEIFDLMDANNDGKV
TaCCaMK   DSLIPLAPRVFDLFDNNRDGTVDMREILCGLSSLRNSRGDNALRLCFQMYDADRSGCISKEELASMLRALPEECLPGDIAEPGKLDEMFDTMDANGDGEV
           ****:* *:**********:*********:***:**:*::*********:******:***:******** :*** **:*******:** ****.**:*
VvCcamk   TFEEFKTAMQRDSSLQDVVLSSLRPL--
VaCCaMK   TFEEFKTAMQRDSSLQDVVLSSLRPL--
MtCCamk   TFDEFKAAMQRDSSLQDVVLSSIRP---
TaCCaMK   TFDEFKAAMQKGSALQDEVLSSLRPGQQ
          **:***:***:.*:*** ****:**
```

Figure 1

Multiple sequence alignment of the conserved domains of several plant CCaMKs. K – kinase domain (red font), CaM – calmodulin binding domain (green font), EF – three EF-hands or the visinin-like domain (blue font). The DDBJ/GenBank/EMBL accession numbers of the above four CCaMKs were listed in Table 1.

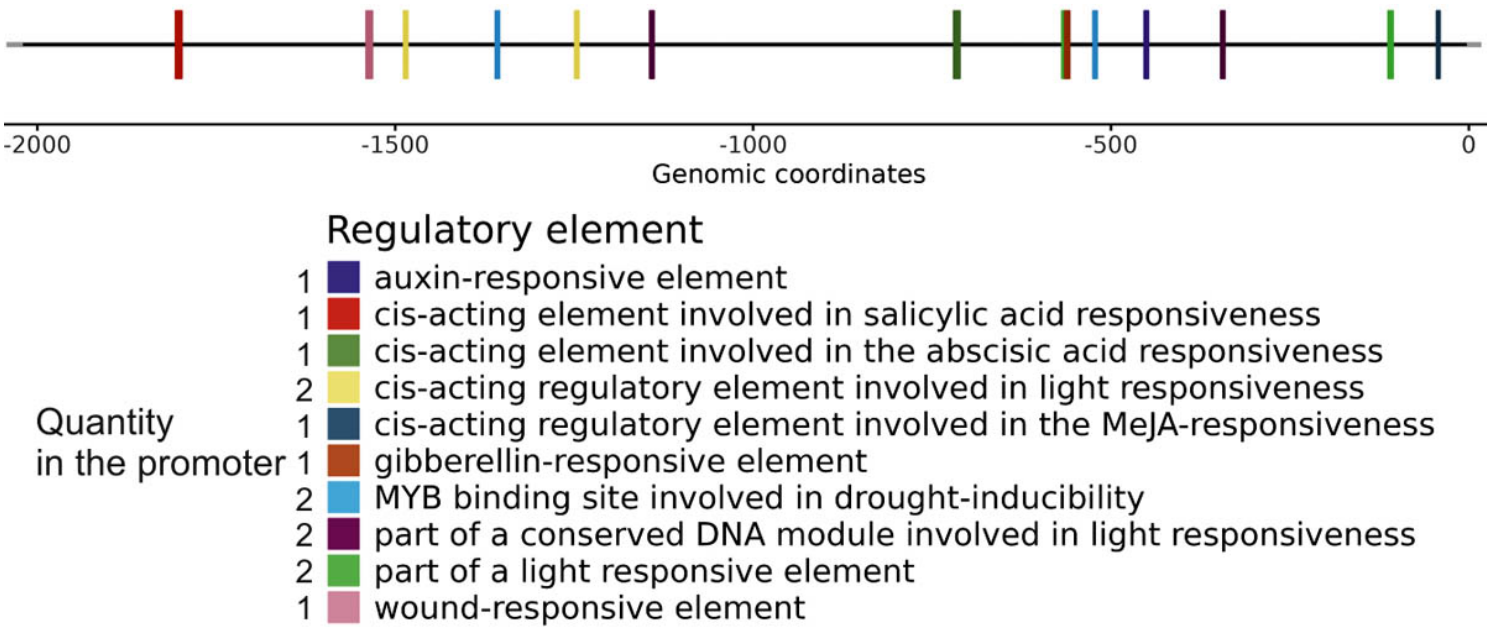

Figure 2

a) The structure representation of *VaCCaMK* 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 F and R – primes used for *VaCCaMK* expression levels in the leaves of grapevine *V. amurensis* cuttings. b) CCaMK functional domains: N – N-terminal domain, K – kinase domain (red font), CaM – calmodulin binding domain (green font), EF – three EF-hands or the visinin-like domain (blue font), C - C-terminal domain.

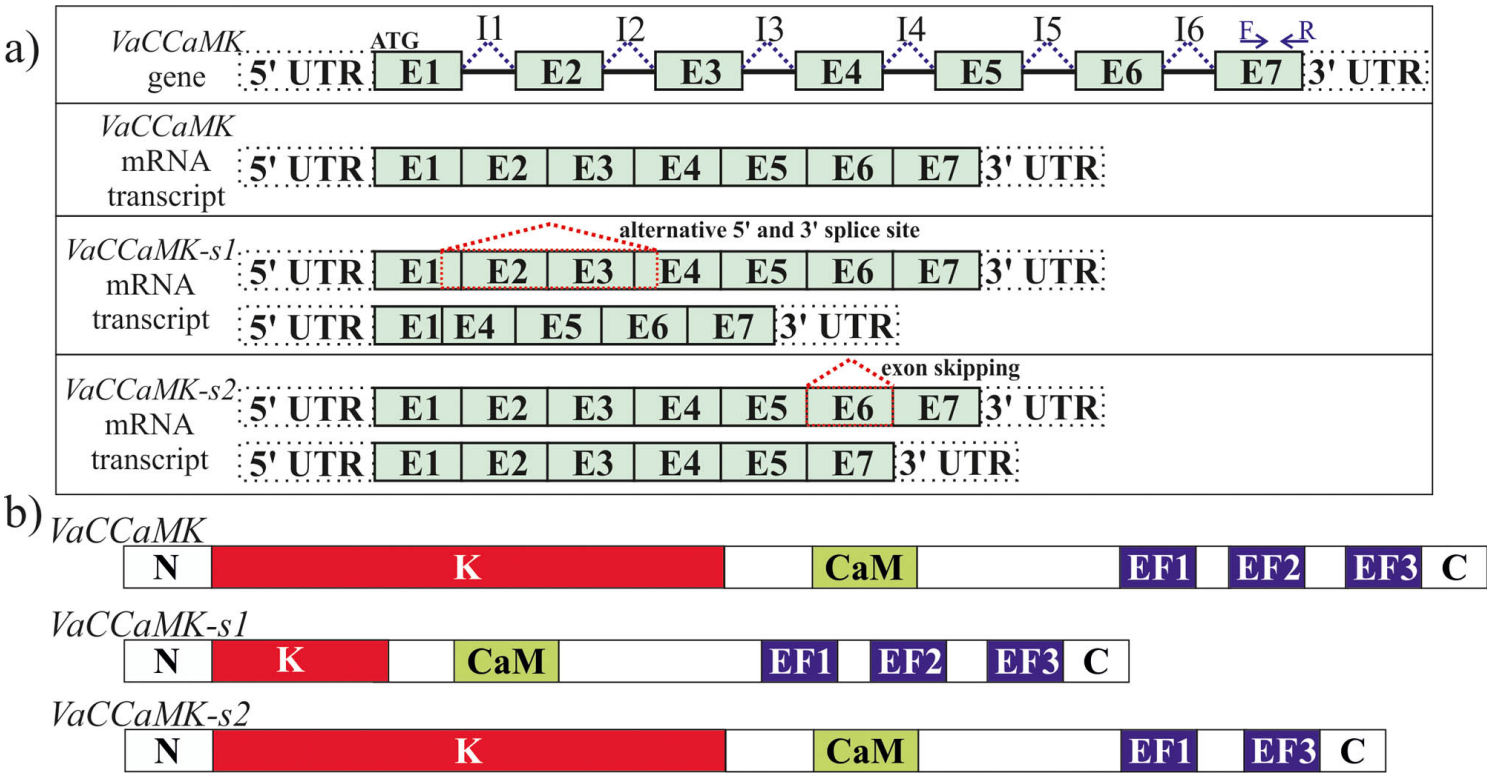

Figure 3

Analysis of *cis*-acting elements in the promotor region of the grape *VaCCaMK* gene. *Cis*-regulatory element analysis of *VaCCaMK* gene. Sequences 2000 bp upstream of the start codon were extracted and cis-acting elements were predicted by PlantCARE and visualized using gggenes (version 0.6.0) (Wilkins, 2025) and tidyverse (version 2.0.0) (Wickham et al. 2019) R packages.

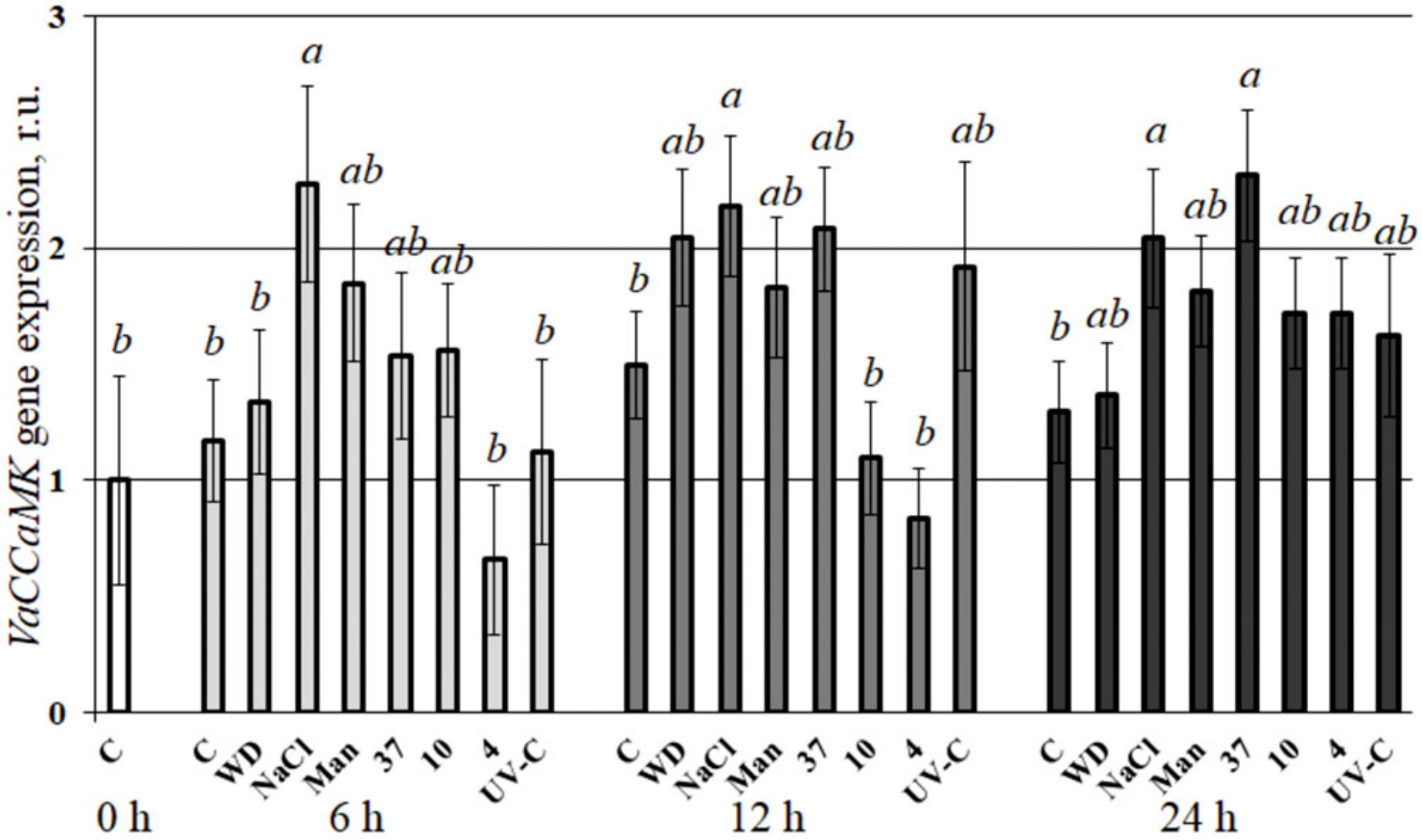

Figure 4

VaCCaMK expression in grape *Vitis amurensis* leaves under different stress conditions. The *VaCCaMK* expression levels were determined by quantitative RT-PCR and analyzed before stress conditions (0 h) and after 6 h, 12 h, and 24 h of treatments. C – non-stress conditions (filtered water, 25^{o}C); WD – water-deficit stress (cuttings constantly laid on a paper towel, 25^{o}C); NaCl – salt stress (constantly 0.4 M NaCl, 25^{o}C); Man – mannitol (constantly 0.4 M mannitol, 25^{o}C); 37 – heat stress (constantly filtered water, 37^{o}C); 10 or 4 – cold stress (constantly filtered water, 10^{o}C or and 4^{o}C), UV-C – ultraviolet C irradiation (for 20 min at a wavelength of 254 nm, 230 μW cm^{-2}, 15 cm from the lamp VL-215.MC, provided by Vilber Lourmat company (Marne-la-Vallée, France). The data are presented as mean ± standard error; r.u. – related units. Means followed by the same letter were not different using Student's t test, where $p < 0.05$ was considered to be statistically significant.

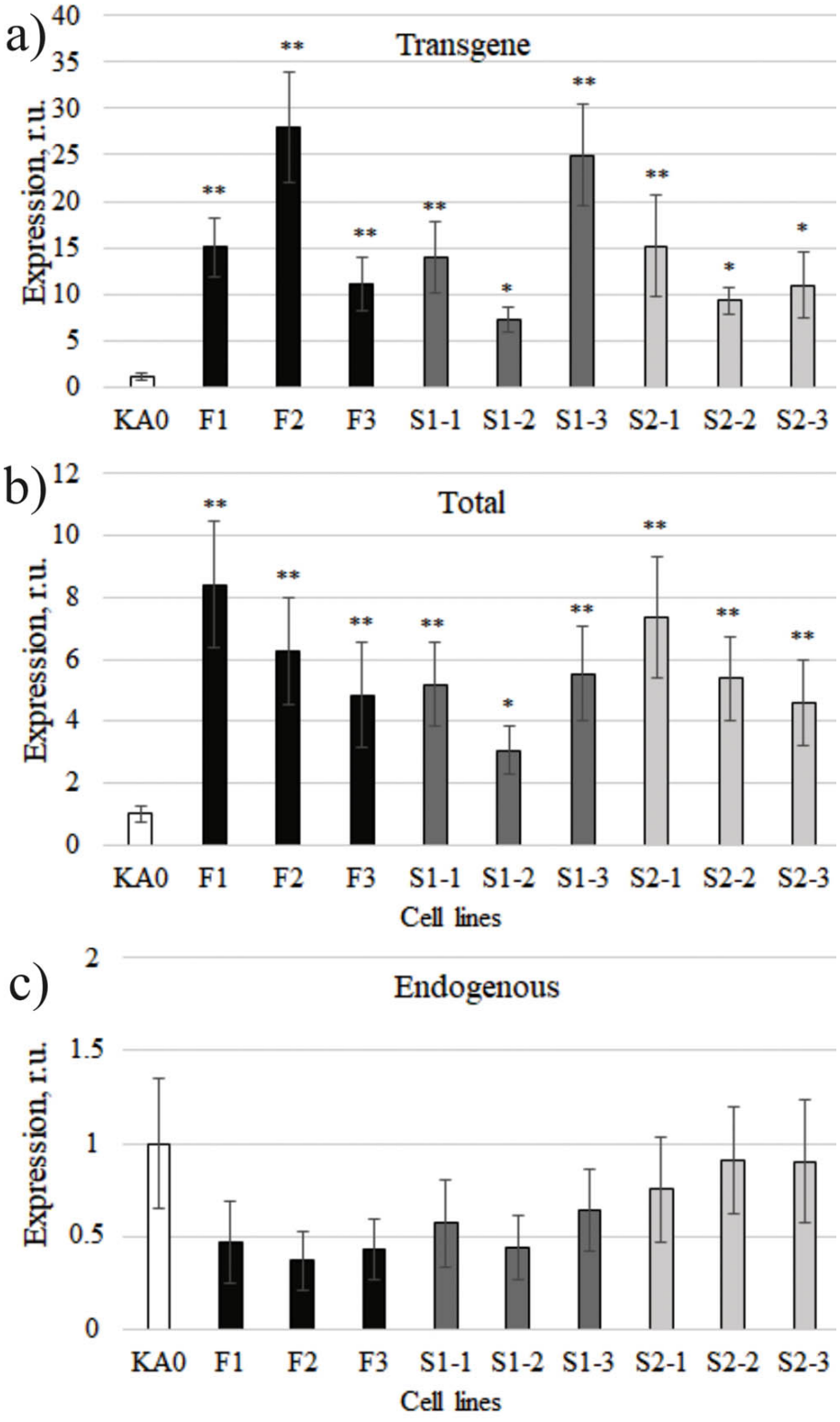

Figure 5

Quantification the transgene (a), total (b), and endogenous (c) *VaCCaMK*expression in the transgenic cell cultures of *V. amurensis* performed by quantitative real-time RT-PCR. RNA was extracted from the vector control (KA0), *VaCCaMK*-transformed (F1, F2, F3), *VaCCaMK-s1*-transformed (s1-1, s1-2, s1-3), and *VaCCaMK-s2*-transformed (s2-1, s2-2, s2-3) cell lines of *V. amurensis*. The data are presented as mean ±

standard error; r.u. – related units. *p < 0.05; **p < 0.01 versus values of fluorescence in the KA0 cell culture transformed with the empty vector (Student's *t* test).

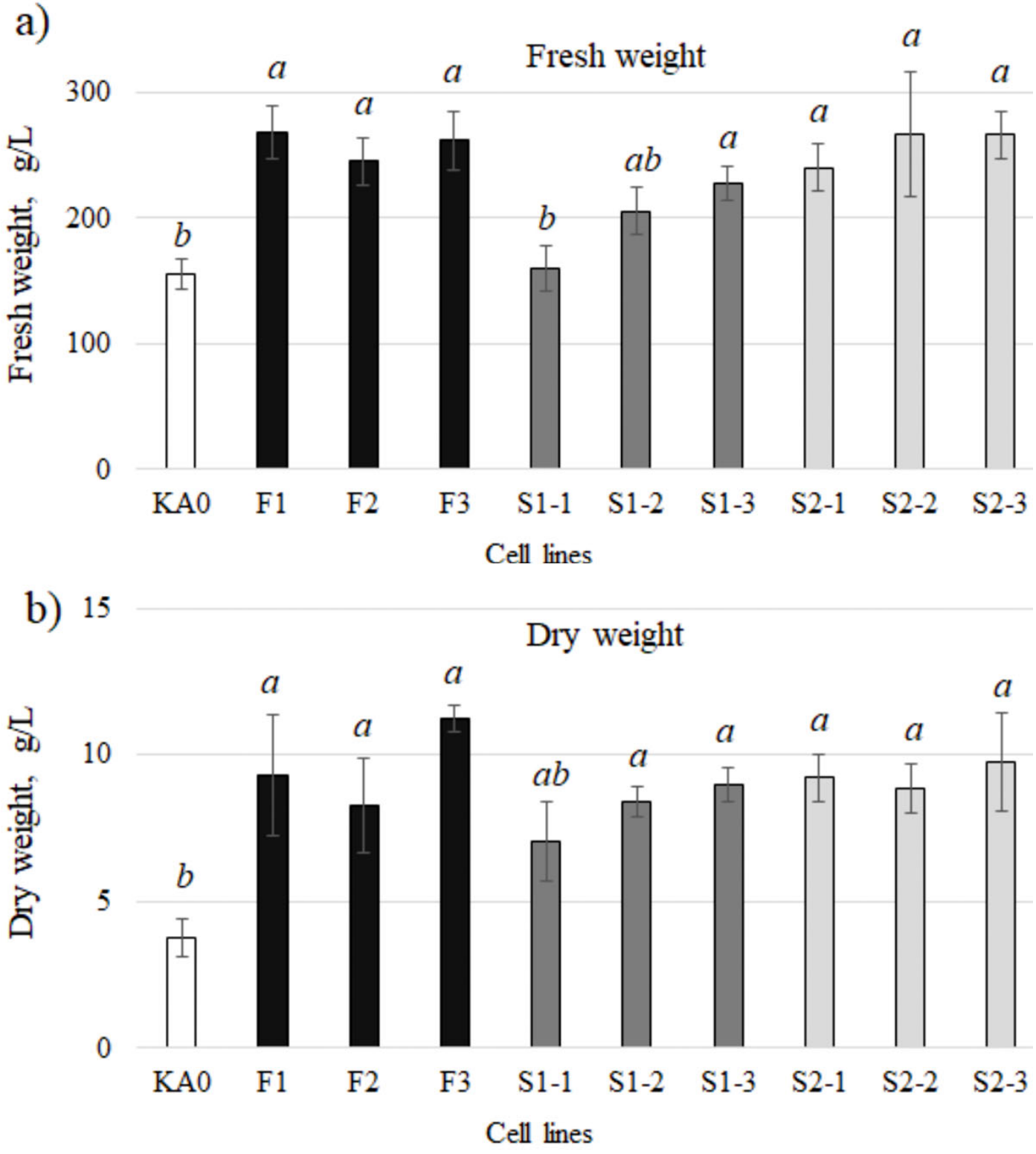

Figure 6

Fresh and dry biomass accumulation in the cell lines KA0 (vector control), F1, F2, F3 (overexpressed *VaCCaMK* gene), S1-1, S1-2, S1-3 (overexpressed *VaCCaMK-s1*transcript), S2-1, S2-2, S2-3

(overexpressed *VaCCaMK-s2* transcript) cell lines. The callus tissue samples were harvested from the 35 days old cultures. The data are presented as mean ± standard error; r.u. – related units. Means followed by the same letter were not different using Student's *t* test. $p < 0.05$ was considered to be statistically significant.

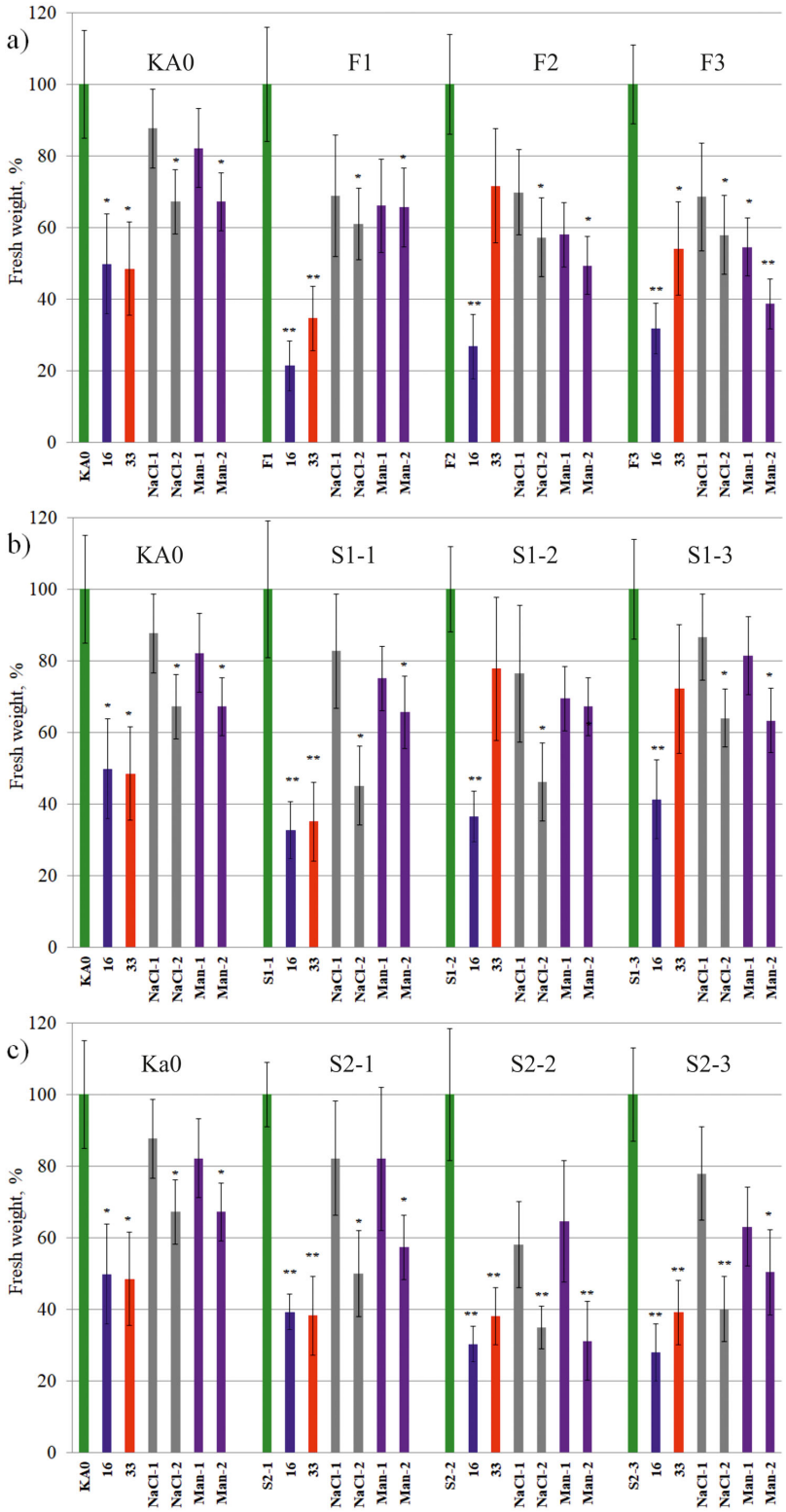

Figure 7

Effects of cold (16, 16^{o}C), heat (35, 35^{o}C), salt (NaCl, 1: 50 mM and 2: 100 mM), and mannitol (Man, 1: 200 and 2: 300 mM) induced osmotic stresses on the growth of 35-day-old transgenic callus cell cultures of *Vitis amurensis*. KA0 (vector control), F1, F2, F3 (a, overexpressed *VaCCaMK* gene), S1-1, S1-2, S1-3 (b, overexpressed *VaCCaMK-s1* transcript), S2-1, S2-2, S2-3 (c, overexpressed *VaCCaMK-s2* transcript) cell lines. The data are presented as mean ± standard error; for 100% - growth in the control conditions for each cell line. Statistical significance was determined by Student's t-test: *p < 0.05 and **p < 0.01 versus growth values of the respective cell line cultured under stress-free control conditions (100%, marked in green).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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