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Low Temperatures Stimulate Alternative Splicing of the *CPK26* Gene in *Vitis amurensis* Grapes

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Abstract—Alternative splicing (AS) is a non-canonical gene splicing process that allows a single gene to synthesise multiple protein isoforms and enhance a variety of protein functions. In this study, the involvement of AS in the generation of plant resistance to abiotic stresses was investigated using the *VaCPK26* calcium-dependent protein kinase (CPK) gene, which is responsible for the resistance of *Vitis amurensis* Rupr. grapes to soil salinity and drought. The level of *VaCPK26* transcription in grape leaves was studied under the influence of different environmental factors. Under low temperature exposure, in addition to the full-length *VaCPK26* transcript, a short-spliced *VaCPK26s1* transcript was obtained that lacked the 2nd exon out of the 7 exons that make up the full-length *VaCPK26*. Recombinant *VaCPK26* increased the resistance of grape cells to salt stress and drought, and overexpression of the spliced *VaCPK26s1* transcript in *V. amurensis* grape cell cultures had no effect on resistance to the stresses tested. These results show that AS can lead to the loss of properties of spliced transcripts characteristic of the original full-length form, which is important for complete understanding of the biological functions of CPK and alternative splicing.

Keywords: abiotic stress, grapes, calcium, protein kinase, cold, exon

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INTRODUCTION

In plants, like in other eukaryotes, premessenger RNA (pre-mRNA) consists of noncoding segments (introns) and coding segements (exons). During RNA processing, introns are removed, while exons are spliced together with the formation of mature messenger RNA (mRNA). Alternative splicing (AS) is a complex process when coding and noncoding gene regions are rearranged differently by spliceosomes in different splice site regions [1]. This results in the formation of numerous mRNA transcripts generated from the same pre-mRNA molecule.

Recent studies have shown that AS is much more widespread in plants than in animal cells as it was supposed previously. Whole-genome mapping of the *Arabidopsis thaliana* transcriptome has shown that up to 61% of intron-containing genes undergo AS [2, 3]. AS plays the key role in plant response to environmental stresses and various developmental signals [4–6]. AS influences the expression of many genes involved in signaling pathways, including transcription factors, ubiquitin ligases, protein kinases, and the genes related to plant defense against pathogens and abiotic stresses.

Protein kinases are the most important regulators of signal transduction in plants; however, relatively little is known about how AS modulates their activity and functions. It has been reported only on several cases of AS in plant protein kinases [7–9].

Mitogen-activated protein kinase (MAPK) cascades are signaling pathways that play a key role in regulation of cell responses to various stimuli, including stress, growth factors and hormones. These cascades consist of a number of protein kinases, and each of them phosphorylates and activates the next one, enhancing the signal. The *OsMAPK5* gene in rice produces two differentially spliced transcripts, which results in the production of isoforms with different protein sequences [10]. The *ZmMIK* gene in maize, which encodes GCK-like *ZmMAP4K*, undergoes AS to generate at least four mature mRNA transcripts. These isoforms have different kinase activities [11].

The AtAUR2 kinase in *A. thaliana* has a splicing variant, AtAUR2S, which lacks part of the kinase domain. AtAUR2S loses the ability to phosphorylate H3 histone but retains the function of histone binding [7]. As a result of AS of the *OsBWMK1* gene, isoforms with contrasting subcellular localization are produced in rice. These isoforms play different roles in hormonal signaling pathways [8]. The *AtMPK13* gene in *A. thaliana* synthesizes three variants of spliced transcripts. The full-length *AtMPK13* is completely spliced, while *AtMPK13*-I4 and *AtMPK13*-I5 retain

the fourth and fifth introns, respectively. *AtMPK13*-I4 and *AtMPK13*-I5 exhibit neither kinase activity nor the interaction with upstream MAPKKs, which suggests that splicing regulates functions [9].

Another important kinase involved in signal transduction in plants is calcium-dependent protein kinase (CPK). CPKs are the family of Ca^{2+}-regulated Ser/Tre-protein kinases playing an importnat role in the regulation of different cellular processes in plants, green algae and protozoans [12, 13]. CPKs are characterized by conserved structural organization (classified as EC 2.7.1.37) with the N-terminal variable domain, the catalytic kinase domain, the autoinhibitory domain, and the calmodulin-like Ca^{2+}-binding domain with EF hand motifs mediating Ca^{2+} binding [14, 15]. At low Ca^{2+} concentrations, the autoinhibitory domain inhibits phosphorylation, maintaining CPK in an inactive state [16]. Under the conditions of Ca^{2+} influx, the calmodulin-like Ca^{2+}-binding domain binds Ca^{2+} and induces conformational changes that relieve autoinhibition and activate the kinase domain.

Individual CPK isoforms perform various functions and participate in numerous signaling pathways, including immune responses, stress responses and developmental processes. They regulate different cellular processes such as stem elongation [17], stomatal closure in response to drought [18], and generation of reactive oxygen species (ROS) under stress conditions [19, 20]. CPKs are encoded by multigene families with different number of genes in different plant species. Whole-genome sequencing in the rice *Oryza sativa*, *A. thaliana* and *Populus trichocarpa* has revealed 31, 34 and 30 *CPK* genes, respectively [21–23]. Different CPK isoforms demonstrate different expression patterns, subcellular localization and sensitivity to Ca^{2+} levels, which contributes to the diversity of their functional roles [15].

Previously, AS has been shown for five CPK genes. For example, the *CPK* gene of hepatica has two almost identical exons that are alternatively spliced in two different mRNAs, which suggests the absence of independent splicing of the intron between them [24]. AS of the *OsCPK2* gene in rice results in the production of isoforms with different kinase activities and stress responses [25].

Previously, several atypical variants of the *VaCPK3a* and *VaCPK9* transcripts containing minor deletions or insertions in the kinase domain have been described in the wild grape *Vitis amurensis* Rupr. [26]. Modified *VaCPK3a* transcripts were expressed in *Escherichia coli* to analyze protein kinase activity *in vitro*. The findings demonstrate that the synthesis of short variants of the *VaCPK3aSF2* and *VaCPK3aSF3* transcripts may result in modified protein kinases with reduced phosphorylation activity [27]. Later, the expression of the *VaCPK21* gene, which increases the resistance of grape and *A. thaliana* plant cells to salinity, was studied in detail in the grape *V. amurensis* [28]. High temperature stress resulted in the appearance of short (the loss of some exons) and long (intron retention) *VaCPK21* transcripts with different properties compared to the canonical *VaCPK21*. For example, the overexpression of short *VaCPK21* transcripts did not make them resistant to abiotic stresses; in case of overexpression of the long *VaCPK21L* form, grape cells were characterized both by the higher resistance to salinity and by the new property of resistance to mannitol-induced osmotic stress [29].

In the new research, AS has been studied in another grape CPK, *VaCPK26*, which provides resistance to osmotic stress and soil salinity [30].

MATERIALS AND METHODS

Plant material and plant cell cultures. Wild grape *V. amurensis* plants used for experiments were collected in summer, at 13:00–14:00, in sunny weather in the vicinity of Vladivostok. Then young vines were divided into cuttings of 15–20 cm in length, with one whole leaf for experiments. In the control, grape cuttings in a 10-mL glass with sterile filtered water were placed for 12 h into a climatic chamber (MLR-352, Panasonic, Japan) with a 16/8-h light/dark photoperiod, at a light intensity of 70 μmol m^{-2} s^{-1}, 22°C. For salt stress, grape cuttings were placed for 12 h into a saline solution (350 mM NaCl). For temperature stress, in contrast to the control conditions, the settings in the climatic chamber were changed: to 5 and 37°C for the lower and higher temperatures, respectively. For water deficiency, a grape cutting was left in a chamber (70 μmol m^{-2} s^{-1}, 22°C) on a filter paper for 12 h without watering.

The V7 callus cultures were obtained in 2017 from young stems of wild *V. amurensis* plants in the vicinity of Vladivostok as described [31]. V7 cells were grown for 30–32 days in the dark on a modified Murashige and Skoog medium $W_{B/A}$ [31] with the addition of 0.5 mg/L 6-benzylaminopurine, 2 mg/L α-naphthaleneacetic acid, and 8 g/L agar [31]. The data on fresh biomass were obtained from three independent experiments and are the mean of 10 technical replicates for each independent experiment presented as the mean ± standard error.

In grape V7 cell cultures, osmotic and salt stresses were induced with mannitol (0.2 and 0.3 M) and sodium chloride (NaCl, 0.05 and 0.1 M), respectively; low and high temperature stresses were induced by the cultivation of grape cells at 16 and 33°C, respectively. The selected temperature mode was milder than the mode for grape cuttings described above, because the cells in the culture were more sensitive and all of them turned black and died when the temperature decreased below 16°C or increased above 33°C.

Nucleic acid extraction and quantitative PCR. Extraction by the cetyltrimethylammonium bromide

Table 1. The sequence of primers used for the amplification of *VaCDPK26* and *VaCDPK26s1* sequences

Name	Primer sequence, 5'–3'
VaCPK26-nachS	ATGGGCAACACATGCCGGGGA
VaCPK26-konA	CTAAAAAGCTCCTGGTGCATCTC
VaCPK26-realS	TGCTATGATGCAAAAGGGCAATG
VaCPK26-realA	CTAAAAAGCTCCTGGTGCATCTC
VaCPK26s1S	CAAGGATTATTGTAGGGGTTGTC
VaCPK26s1A	ATTCCCTGTTGAGTTTCTGGTTTAAA
VaCPK26-SacI	GCTCGAGCTCATGGGCAACACATGCCGGGGA
VaCPK26-KpnI	TCGAGGTACCCTAAAAAGCTCCTGGTGCATCTC
VaGAPDH-realS	CACTGAAGATGATGTTGTTTCC
VaGAPDH-realA	GCTATTCCAGCCTTGGCAT
VaActin1-realS	GTATTGTGCTGGATTCTGGTGAT
VaActin1-realA	AGCAAGGTCAAGACGAAGGATAG

(**CTAB**) method was used for total RNA extraction as described [31]. cDNA was obtained using an RT-PCR kit MMLV with oligo(dT)$_{15}$ (RT-PCR, Evrogen, Russia) as described [31].

The levels of transgenic mRNA transcripts were determined by real-time PCR (**PCR RT**). The results of PCR RT were calculated by the $2^{-\Delta\Delta CT}$ method [32] using two internal controls, including *VaGAPDH* (GenBank XM_002263109) and *VaActin1* (GenBank DQ517935), as described [30]. The primers for PCR RT, VaGAPDH-realS/A and VaActin1-realS/A, are given in Table 1.

The reactions of PCR RT were performed in a volume of 20 μL with a real-time PCR kit (Evrogen) as described [30]; the kit contained 1× Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each oligonucleotide primer, 1× SybrGreen I Dye for real-type PCR, 1 μL of cDNA, and 1 U Taq DNA polymerase (Evrogen). The analysis was carried out with a DTprime 4M1 thermocycler (DNA Technology, Russia) programmed for the initial stage of denaturing for 2 min at 95°C, followed by 50 cycles: 10 s at 95°C and 25 s at 62°C.

Overexpression of the *VaCDPK26* and *VaCDPK26s1* transcripts in grape cells. For creating a construct for plant cell transformation, the whole-length sequences of the *VaCDPK26* (KC488323) gene and the *VaCDPK26s1* transcript were amplified by PCR with the VaCPK26-nachS and VaCPK26-konA primers selected for the 5' and 3' ends of coding sequences presented in Table 1. cDNA used for amplification of transcript *VaCDPK26* and *VaCDPK26s1* was obtained from a *V. amurensis* grape leaf after low temperature exposure (12 h at 5°C).

The resultant PCR products, *VaCDPK26* and *VaCDPK26s1*, were subcloned in pJET1.2 with a CloneJET PCR Cloning kit (ThermoFisher Scientific, United States) and sequenced with an ABI 3130 genetic analyzer (Applied Biosystems, United States) according to the manufacturer's instructions. Then PCR was performed with a forward primer containing the *Sac* I restriction site and a reverse primer containing the *Kpn* I restriction site (Table 1).

VaCDPK26 and *VaCDPK26s1* were cloned in the pSAT1 vector [33] at the *Sac* I and *Kpn* I sites. Then the expression cassette from pSAT1 with the *VaCDPK26* or *VaCDPK26*s1 genes was cloned in the pZP-RCS2-*nptII* vector [33] using *PalA*I (*Asc*I) sites. The pZP-RCS2-*nptII* construct also contained the neomycin phosphotransferase (*nptII*) gene. All transgenes in the vectors used were under the control of the promoter of the cauliflower mosaic virus (CaMV 35S). The constructs for the overexpression of *VaCDPK26* (pZP-RCS2-*VaCDPK26-nptII* or pZP-RCS2-*VaCDPK26*s1-*nptII*) or empty vector (pZP-RCS2-*nptII*) were transferred into the strain *Agrobacterium tumefaciens* (GV3101::pMP90), which was used for the transformation of V7 *V. amurensis* suspension culture [30].

Multiple sequence alignment was performed with ClustalX2 [34].

Statistical processing of the results. The *VaCDPK26* gene expression was analyzed in two independent experiments with ten technical replicates (five PCR RT reactions normalized for one internal control gene and five PCR RT reactions normalized for the other gene in each independent experiment). The mass of callus tissue was analyzed in three independent experiments with ten technical replicates in each experiment. The data are presented as the mean ± standard error (SE) and assessed by the Student's *t*-test or one-

Table 2. The number of the *Vitis amurensis VaCPK26* gene transcripts cloned from RNA extracted from the leaves of the grape *V. amurensi* cuttings obtained at low (5°C) and high (37°C) temperatures, water deficit (WD), and in the control (Control)

Active factor	Standard*	Short**
Control	31	0
NaCl	36	0
5 ^{0}C	36	5
37 ^{0}C	42	0
WD	33	0
Total number of clones	178 (97.3%)	5 (2.7%)

* Standard, the sequence completely corresponds to the *VaCPK26* gene sequence (GenBank, KC488323).
** Short, the sequences lacking some regions of the protein-coding sequence.

way analysis of variance (ANOVA), followed by the Tukey HSD multiple comparison test performed in Excel with the XLSTAT software, where the value of $p < 0.05$ was statistically significant.

RESULTS AND DISCUSSION

Diversity and expression of spliced variants of the *VaCPK26* gene under the conditions of abiotic stresses. Using the primers at the beginning and the end of coding sequence of the *VaCPK26* gene, 183 sequences of the *VaCPK26* gene transcripts from cDNA of grape leaves exposed to different stresses (salinity, low and high temperatures, water deficit) were cloned and sequenced (Table 2). The sequences of altogether 31 to 42 cloned transcripts of the *VaCPK26* gene were obtained and analyzed. It is noteworthy that the complete sequence submitted to GenBank, *VaCPK26* (3d, KC488323), was noted in Table 2 as "standard". If any regions of the protein-coding sequence were absent, such sequences were referred to as "short" (Table 2).

All of the cloned sequences of the *VaCPK26* gene from cDNA obtained under normal conditions (31 sequences), under the exposure to NaCl (36 sequences), high temperature (42 sequences) and water deficit (33 sequences) had a standard structure: 1686 nucleotides, 561 amino acids (Table 2). Only among 41 cloned sequences from cDNA obtained from DNA of the leaves exposed to low temperatures there were 5 short *VaCPK26s1* transcripts (12.2% of all sequences). All of the *VaCPK26s1* transcripts were characterized by the absence of 144 nucleotides (48 amino acids); eventually, the full-length sequence of *VaCPK26s1* included 1542 nucleotides (513 amino acids), and the reading frame was not shifted (Fig. 1). The *VaCPK26* gene consists of 7 exons. The second exon was completely missing in the *VaCPK26s1* sequence, i.e., there was exon skipping, one of the most widespread types of alternative splicing [5]. 48 amino acids were missing in the region of the kinase domain (with hijacked subdomains VIII and IX) (Fig. 1); therefore, translation from the *VaCPK26s1* matrix can yield a protein that lacks part of the kinase domain (Fig. 2).

Then, the real-time quantitative PCR (PCR RT) and specific primers was used to analyze the accumulation of transcripts of all known forms of the *VaCDPK26* gene (Fig. 3a) and the accumulation of individual transcripts of the alternative variant of *VaCDPK26s1*

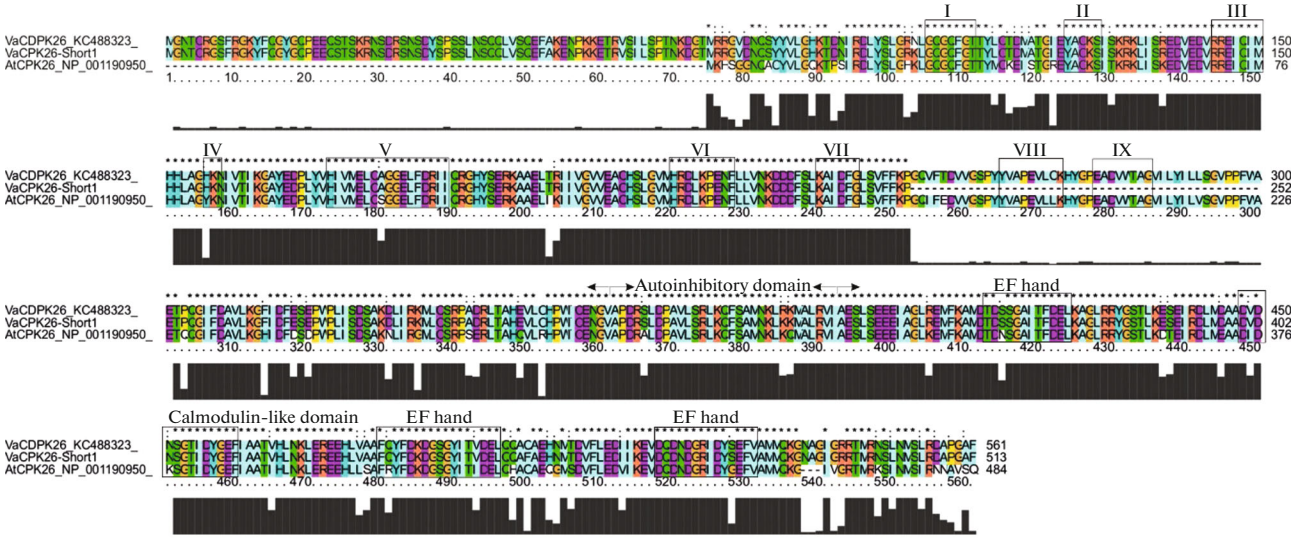

Fig. 1. Domain analysis of amino acid sequences of VaCPK26 (KC488323), VaCPK26s1 and AtCPK26 from *Arabidopsis thaliana* (NP_001190950). Roman numerals indicate subcatalytic domains of the kinase domain.

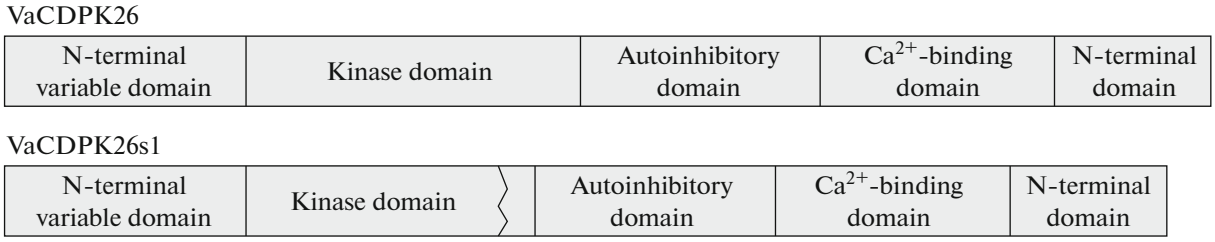

Fig. 2. Domain analysis of the obtained amino acid sequences of VaCPK26 and VaCPK26s1.

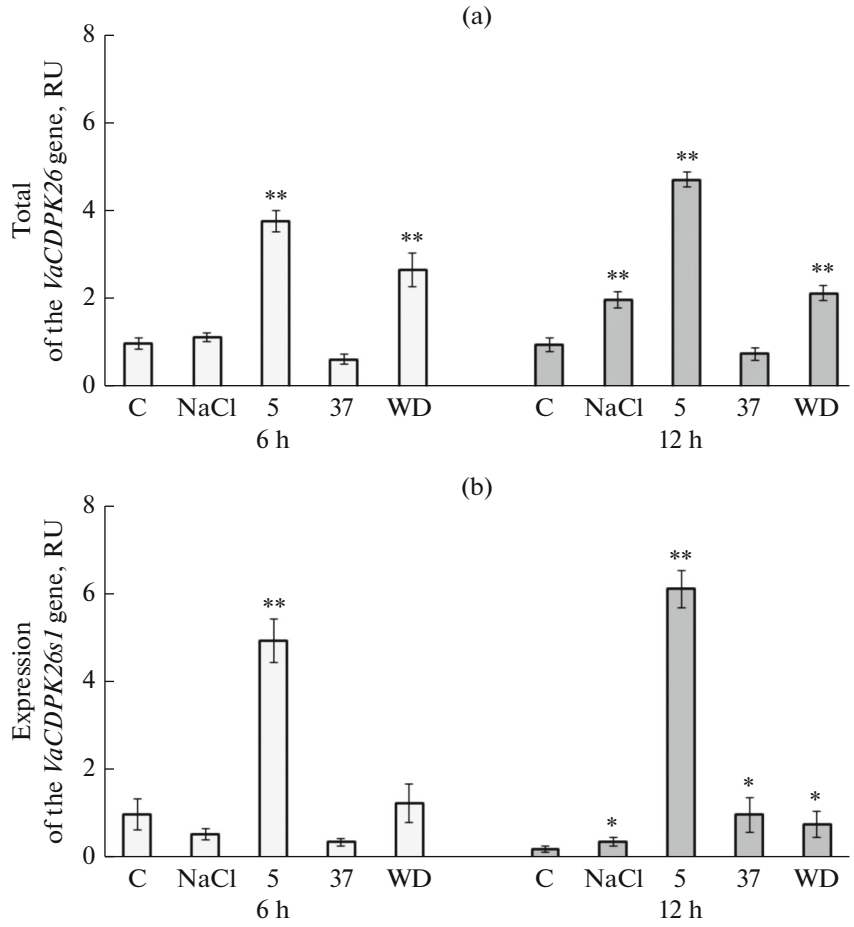

Fig. 3. Quantitative assessment of the total level of transcripts of the *VaCDPK26* gene (a) and short transcript *VaCDPK26s1* (b) in the leaves of *Vitis amurensis* grape cuttings after the exposure to salt stress (NaCl), cold (5) and high temperature stress (37), water deficit (WD), and in the control (C). * $p < 0.05$, ** $p < 0.01$ (paired Student's *t*-test) compared to the values of the level of transcripts in the control (C). RU, relative units.

(Fig. 3b). It was shown that the overall expression of the *VaCDPK26* gene reliably increased 3.8–4.7 times at a low temperature, 2.1–2.7 times under water deficit, and 1.2–2.0 times under salt stress (Fig. 3a).

It is worth noting that specific primers for the *VaCDPK26s1* transcript made it possible to show that the maximum number of *VaCDPK26s1* transcripts was typical of cDNA samples obtained after 6- and 12-h

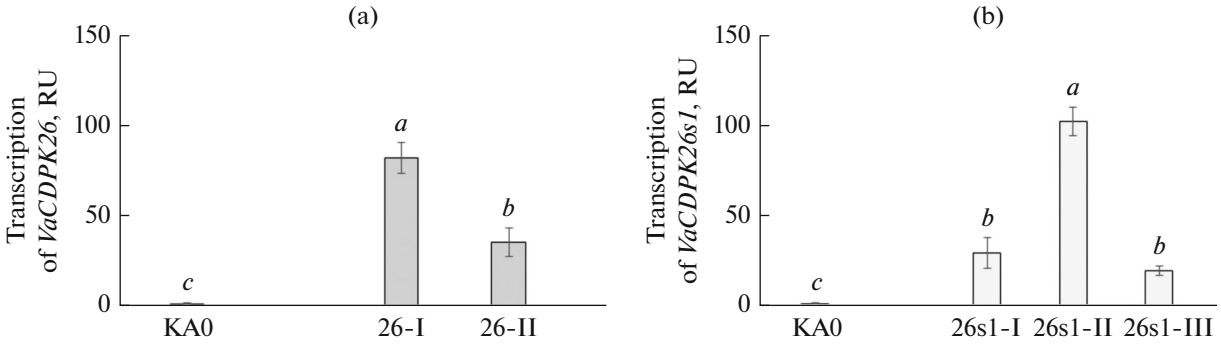

Fig. 4. Quantification of the levels of total expression of the *VaCDPK26* (a) and *VaCDPK26s1* (b) transcripts in transgenic lines of the grape *Vitis amurensis* KA0, 26-I, 26-II, 26s1-I, 26s1-II and 26s1-III. Statistically significant value was considered to be $p < 0.05$. RU, relative units.

low temperature stress (Fig. 3b). These results confirmed the data obtained by the cloning of full-length PCR products (Table 2).

Overexpression of spliced variants of the *VaCPK26* gene in cell cultures of the grape *V. amurensis.* After the procedure of agrobacterial transformation and selection of transgenic cells of the grape *V. amurensis,* the transcription of the *VaCPK26* and *VaCPK26s1* transgenes in the resultant lines was studied (Fig. 4). A total of two grape cell lines with the full-length form of *VaCDPK26,* 26-I and 26-II, and three lines with the spliced form of *VaCPK26s1*, 26s1-I, -II, -III were obtained. The KA-0 cell line obtained as a result of transformation by an empty vector carrying only the *nptII* gene in its sequence was used as a control. Transgenes were actively expressed in all independently obtained cell cultures of the grape *V. amurensis*; the highest expression of the *VaCDPK26* transgene was observed in line 26-I; in line 26-II, it was 2.3 times lower (Fig. 4a). The highest expression of the *VaCPK26s1* transgene was observed in the culture of the 26s1-II cell line; the expression of *VaCPK26s1* in lines 26s1-I and 26s1-III was reliably lower: 3.5–5.4 times (Fig. 4b).

Then, after proving the active transcription of the *VaCPK26* transgene in the resultant cell lines, cell resistance to the following abiotic stresses was analyzed: water deficit, salt stress, low and high temperatures (Fig. 5). Osmotic stress was induced by mannitol (0.2 and 0.3 M) (Fig. 5a); salt stress was induced by sodium chloride (NaCl, 0.05 and 0.1 M) (Fig. 5b); low and high temperature stresses were induced by the cultivation of grape cells at 16 and 33°C, respectively (Fig. 5c).

It was shown that transformation by the full-length transcript of the *VaCPK26* gene increased the resistance to osmotic stress (Figs. 5a, 5b) and salinity of the nutrient medium (Fig. 5b), as had been confirmed by previous results [30].

The most interesting thing was to study the resistance of grape cells overexpressing the spliced variant of the *VaCPK26* gene: *VaCDPK26s1*. It was shown that the growth of all transgenic lines overexpressing the *VaCDPK26s1* transcript was significantly worse under the conditions of osmotic, salt, high and low temperature stresses, compared to the cells overexpressing the full-length *VaCPK26* and the control KA0 cells (Fig. 5); however, these differences in growth were within the margin of error.

Thus, overexpression of the modified *VaCDPK26s1* transcript did not provide resistance in case of osmotic and salt stresses like the full-length *VaCDPK26* transcript. This may indicate the absence or weakness of kinase activity of the spliced *VaCDPK26s1* form, which does not transduce further stress resistance signal to plant cells. In addition, *VaCDPK26s1*-transgenic grape cells were not resistance to low temperatures, in spite of activation of the level of *VaCDPK26s1* transcription under low temperature stress. Probably, the resultant form of the *VaCDPK26s1* transcript is involved in other signaling networks necessary for plants, but this assumption requires further research.

* * *

Alternative splicing (AS) is a process that allows one gene to generate numerous diverse mRNA transcripts. By changing the structure and sequence of coded proteins, AS can modulate their activity, subcellular localization and interaction between new proteins and other components of signaling pathways. The analyzed literature data and the findings of the present work by the example of the *VaCDPK26* gene suggest that AS most often leads to the loss of properties typical of the initial form, which appears as a result of canonical splicing. However, there are examples when AS leads to the appearance of new properties not characteristic of canonical transcripts (e.g., *VaCPK21L*). Thus, AS has a significant effect on the function of

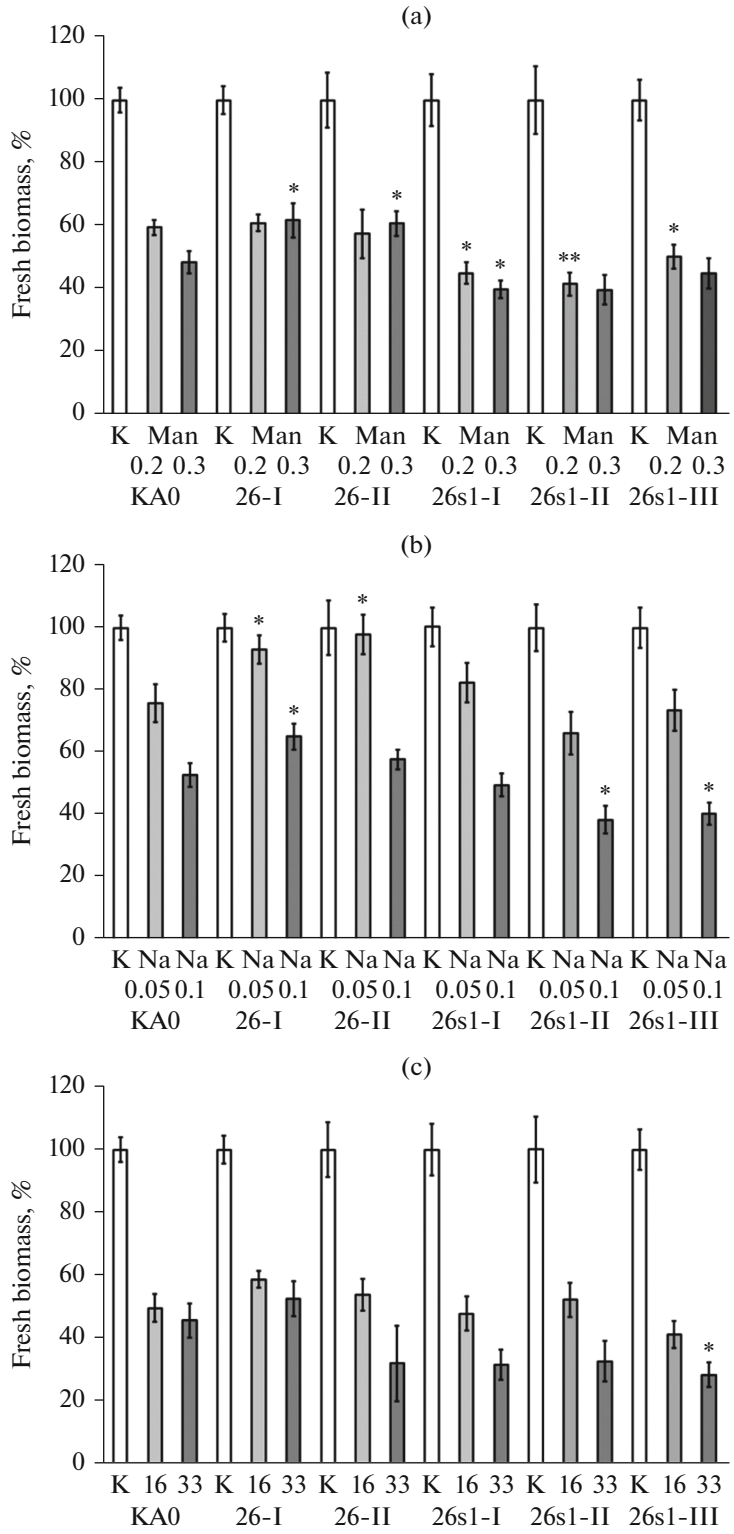

Fig. 5. Accumulation of fresh biomass within 30 days in the KA0, 26-I, 26-II, 26s1-I, 26s1-II and 26s1-III cell lines exposed to osmotic (a), salt (B), cold (c) and heat (d) stresses: KA0, the cells overexpressing only the selective marker *nptII* gene; 26-I and 26-II, the cells overexpressing the full-length *VaCPK26* gene and the *nptII* gene; 26s1-I, 26s1-II and 26s1-III, the cells overexpressing the spliced *VaCDPK26s1* transcript and the *nptII* gene. Statistical significance was calculated by Student's *t*-test. **, $p < 0.01$ and *, $p < 0.05$ for *VaCDPK26* or *VaCDPK26s1* transgenic cells compared to the values of biomass accumulation in the control culture of KA0 cells.

many plant genes, and the features of this effect need further studies.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not contain any studies involving human and animal subjects.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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