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Genes with cold shock domain for generating a frost stress tolerance in grapevine (Vitis vinifera L.) plants

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Research Article

Keywords: Vitis vinifera, cold shock proteins, cold resistance, cold tolerance, freezing tolerance, transgenic plants

Posted Date: November 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11633300/v1

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Additional Declarations:
Competing interest reported. Sergey Dolgov is one of the Associate Editors of the journal at the time of submission

Table 1 and 2 are available in the Supplementary Files section.

Genes with cold shock domain for generating a frost stress tolerance in grapevine (*Vitis vinifera* L.) plants

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Abstract

Vitis vinifera L. is sensitive to low temperatures, especially European grapevine cultivars. Winter negative temperatures and spring cold snaps have a strong negative impact on grapevine yields. Improved cold and frost resistance of cultivated crops would significantly increase the yield and expand the range of grapevine cultivation in a number of cold climate regions. 16 transgenic lines of grapevine containing genes encoding proteins with a cold shock domain (*CspA-plant* and *Escsdp3*) were obtained as a result of *Agrobacterium* transformation. As a result of our studies, we obtained three resistant to spring frosts lines of grapevine with integration of the CSD genes under the control of *35S* or *rd29A* promoters. The *rd29A* promoter allows achieving a high level of frost resistance using both bacterial (*CspA-plant*) and plant (*Escsdp3*) CSD genes.

At the same time, the highest rates of bud survival after freezing of vines in winter dormancy were noted in lines genes under the control of *35S* promoter using both bacterial and plant CSD genes. Only one line with the *Escsdp3* gene under the control of *rd29A* promoter showed a comparable survival rate, which at the same time had intermediate resistance to spring frosts compared to all transgenic lines. Thus, the use of the inducible plant regulatory elements together plant CDS gene can have a positive effect on improving frost resistance in transgenic grapevine.

Keywords: *Vitis vinifera*, cold shock proteins, cold resistance, cold tolerance, freezing tolerance, transgenic plants.

Introduction

Living organisms are constantly threatened by various abiotic stresses (Vodiasova et al. 2024). Low temperature is the most significant environmental factor limiting the productivity and geographic distribution of plants around the world (Theocharis et al. 2012). Most of the total land on Earth (about 64%) has the average minimum temperature < 0°C (Rihan et al. 2017). The need for a constant increase in food production while reducing natural resources necessitates the production of plants that can withstand adverse conditions, including the effects of low temperatures.

Such abiotic stress as cold stress, including chilling (0–15°C) and freezing (< 0°C), negatively affects the growth and agricultural productivity of plants and limits their geographical distribution (Leisner 2020). To date studies on the overexpression of cold tolerance genes are carried out mainly on transgenic *Arabidopsis* (Kim et al. 2001; Vannini et al. 2004; Zhu et al. 2010; Deng et al. 2015), but very little researchers showed on the transformation of target crops of agriculturally important cultivars with genes for resistance to cold stresses (Karlson and Imai 2003; Nakaminami et al. 2006; Castiglioni et al. 2008; Hail et al. 2017; Khvatkov et al., 2021).

Vitis vinifera L. is sensitive to low temperatures, especially European grapevine cultivars (Yu et al. 2017). Winter negative temperatures and spring cold snaps have a strong negative impact on grapevine yields. The seriousness of the problem of low cold resistance of grapevine is also evidenced by the fact that a large number of scientists in the last decade have been studying the mechanisms of response to cold stress and ways to increase the cold resistance of grapevine. Freezing resistance is usually associated with the functioning of CBF/DREB transcription factors, factors that somehow modulate the membrane structure (Gilmour et al. 1998, 2004; Uemura et al. 2006; Jaglo et al. 2001; Choi et al. 2002; Gao et al. 2002; Owens et al. 2002; Dubouzet et al. 2003; Vágújfalvi et al. 2003; Xue, 2003; Francia et al. 2004; Sanghera et al.

2011; Mizoi et al. 2012; Wisniewski et al. 2014; Vazquez-Hernandez et al. 2017). Several genes from *V. riparia* and *V. vinifera* regulated by transcription factors associated with low temperature response pathways *VrCBF1a*, *VrCBF1b*, and *VvCBF1–VvCBF4* have been cloned (Xiao et al., 2006, 2008). The transcription factor genes *VpWRKY1* and *VpWRKY2* were cloned from *V. pseudoreticulata* Baihe-35-1, and overexpression of *VpWRKY2* led to an increase in cold tolerance (Li et al. 2010). The most cold resistance wild grapevine species is *V. amurensis*. Many resistance genes have been cloned from it - *VaICE1* and *VaICE2* (Xu et al. 2014), *VaERF057* (Sun et al. 2016), *VaERD15* (Yu et al. 2017), *VaCPK20* (Dubrovina et al. 2015), *VaCPK21* (Dubrovina et al. 2016), *VaCPK29* (Dubrovina et al. 2017), *VaPUB* (Jiao et al. 2015, 2017) and *VaIPK2β* (Li et al. 2011), and overexpression of *VaPAT1* contributed to increased resistance not only to cold, but also to drought and salinity (Yuan et al. 2016). Several *V. vinifera* genes have also been annotated as involved in cold tolerance: *VvALDH11B1* and *VvALDH18B* (Zhang et al. 2012), *VvBAP1* (Hou et al. 2018), *Vv-circATS1* (Gao et al. 2019) etc.

Also, freezing resistance has often been linked to expression of genes of proteins with a highly conserved cold shock domain (CSD) (Skabkin et al. 2004). CSD proteins present in plants differ from CSPs found in prokaryotes (Sasaki and Imai 2012, Rajasheker et al. 2019). Thus, bacterial proteins of cold shock (CspA and CspB) enhanced stress adaptation in a number of plants, demonstrating improved stress resistance in maize and rice (Karlson and Imai 2003; Nakaminami et al. 2006; Castiglioni et al. 2008; Hail et al. 2017).

In our previous studies, the *Escsdp3* gene, which is involved in plant acclimatization to cold, was isolated from *Eutrema salsugineum* (Taranov et al. 2010, 2018; Zlobin et al. 2016, 2018), which showed high efficiency in increasing the resistance of transgenic rapeseed to frost treatments (Khvatkov et al., 2021). We assumed that the protein EsCSDP3 able to have a significant role in the formation of resistance to cold and frost stresses. The purpose of our research was to develop lines of grapevines with overexpression of EsCSDP3, which will make

it possible to obtain a genotype that is promising for the selection of grapevine cultivars resistant to cold stress. As a plant-reference with increased cold and frost resistance, it was decided to use transgenic grapevine plants expressing an efficiently increase cold resistance bacterial protein CspA, because it classically used for this.

Materials and methods

Gene synthesis and plasmid construction

The vector constructs pBI121-CSPA-plant (Fig. 1a), pBI121rd29A-CSPA-plant (Fig. 1b), pBI-EsCSDP3 (Fig. 1c) and pBI121rd29A-EsCSDP3 (Fig. 1d) was created on the basis of pBI121 vector. Cold-inducible *rd29a* promoter was amplified from the genomic DNA of A. thaliana Col-0 using primers 5'- ATAAGCTTGCCATAGATGCAATTCAATCAAAC - 3' and 5'- TAGGATCCAAAGATTTTTTTCTTTCCAATAGAAG - 3'. *Rd29A* promoter was cloned in pBI121 in place of *35S* promoter using HindIII and BamHI restriction enzymes. *E. coli CspA* gene with codon composition optimized (*CspA-plant*) to plants using JCAT tool (Grote et al., 2005) was amplified using primers 5'- TAGGATCCAAAAATGTCTGGAAAGATGACTGGAAT - 3' and 5'- TAGAGCTCTTACAAAGAAGTAACATTTCCAGCA - 3' from the plasmid vector with this gene (Khvatkov et al., 2021). *Escsdp3* gene was amplified from the genomic DNA *Eutrema salsugineum* (formerly *Thellungiella salsuginea*) using primers 5'- TAGGATCCAAAAATGGCGCAAGAAGATCAATCG - 3' and 5'- TAGAGCTCTTAAGCAAGCGAAGAACATTCCC - 3'. Forward primers for *CspA-plant* and *Escsdp3* contain additional AAAA sequence before start codon to boost the translation (Joshi et al., 1997; Lukaszewicz et al., 2000; Kawaguchi and Bailey-Serres, 2005; Matsuda and Dreher,

2006). *CspA-plant* and *Escsdp3* genes were cloned in pBI121(35S) and pBI121(rd29a) using BamHI and SacI restriction enzymes.

Plant material and *Agrobacterium*-mediated transformation

Inflorescences of the Podarok Magaracha local grapevine cultivar were collected from greenhouse. Inflorescences were harvested when they had reached the developmental stage showing separated flowers [developmental stage 5 (Gribaudo et al. 2004); code Baggiolini - stage H; code BBCH - stage 5(5) (Bloesch and Viret 2008)]. Surface sterilization was performed by submersion in ethyl alcohol (70% C2H5OH) for 30 sec and followed by sodium hypochlorite solution (1.5% NaOCl, 0.1% Tween 20) for 15 min and then fourfold washed with sterile water. The inflorescences were cut into fragments (5 x 5 mm) and placed 10 pieces in each Petri dish containing of PC induction media (Maletich et al., 2025).

Genetic transformation was conducted by co-cultivation of explants jointly with *Agrobacterium tumefaciens* suspension according to the protocol described by Maletich et al. (2025). The mix of somatic embryos at different developmental stages (globule, heart, torpedo and cotyledonary embryos) of the Podarok Magaracha cultivar were used as explants for transformation. Bacterial strain EHA105 (Hood et al. 1993) harboring plasmids were used in agro inoculation experiments.

In vitro plants were adapted to *in vivo* conditions. For this purpose, rooted plants were planted in a greenhouse in pots filled with soil (normalized peat + 10% agroperlite), and cultivated at 100% humidity under a protective plastic cone. After 5-7 days, the cone was removed and plants were grown to develop 8-9 new leaves.

Analysis of transformants

Polymerase chain reaction (PCR)

For PCR- analysis, the genomic DNA was isolated from antibiotic-resistant and wild type plants of grapevine using CTAB method (Murray and Thompson 1980). The respective forward and reverse primer sequences for *npt* ll were Npt-mf-up: 5'- TCTGATGCCGCCGTGTTCC-3' and Npt-mf-low: 5'- ATGCGCGCCTTGAGCCTG-3' (anticipated amplification fragment of 748 bp); those for *CspA-plant* were CSPA-F: 5'- GTCTGGAAAGATGACTGGAATTGT-3' and CSPA-R: 5'- GCAGGTCCCTTAGCTCCAGA-3' (anticipated amplification fragment of 186 bp); those for *Escsdp3* were CSDP-F: 5'- AATCACTCTGGGAAGCGACG-3' and CSDP-R: 5'- GGCCAAATCCACCACAGGTA-3' (anticipated amplification fragment of 512 bp); those for *virB* were virBf: 5'-GGCTACATCGAAGATCGTATGAATG-3‘ and virBr: 5'- GACTATAGCGATGGTTACGATGTTGAC-3' (anticipated amplification fragment of 480 bp).

The PCR reactions for *CspA-plant, Escsdp3*, *npt* ll and *virB* genes were carried out in a 25 µL volume containing 2,5 µl of a 10 x PCR buffer, 0.5 µl of 10 mM dNTPs, 1 µl of two 10 ρM primer, 1µl of 5 U μl^{-1} Taq polymerase, 17 µl of mQ H_2O and 2 µl (≈60 ng) of a DNA template. Reactions were carried out in a Mastercycler nexus gradient (Eppendorf) as the follow: one cycle of 5 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 45 sec at 60 °C, 1 min at 72 °C, and one final cycle of 5 min at 72 °C. The products were separated in 2.0 % agarose gel using the electrophoresis systems.

Southern blot analysis

Genomic DNA (50 µg) was isolated from 1 g of grapevine plant leaves cultivated in a greenhouse. Isolation was carried out using a buffer containing CTAB detergent according to the

procedure described in *Plant Molecular Biology Manual* (Rogers and Bendich 1994). DNA concentration was measured using a spectrophotometer Thermo Evolution 260 BIO. 20 μg of DNA of the selected lines and control plants were hydrolyzed overnight at 37 °C by the HindIII enzyme (60 units). The hydrolysis products were separated overnight by electrophoresis in 0.9% agarose gel at a voltage of 18V in the chamber MINNIE GEL UNIT (Amersham Biosciences, CIIIA). After that, the DNA was transferred to a positively charged Hybond N + membrane (GE Healthcare, UK) by capillary method, as described in the instructions for the membrane. DNA was fixed on the membrane by incubation for 2 hours at 80 ° C. Probes for hybridization were prepared from PCR products using the AlkPhos Direct Labeling Kit (GE Healthcare, UK). PCR products (186 bp and 512 bp) which were amplified using primers for PCR analysis were used to detect the *CspA-plant* and *Escsdp3* genes. The integration of the *npt*II kanamycin resistance gene was confirmed using a probe consisting of 381 base pairs, which was generated using PCR and primers (5'-GCTATGACTGGGCACAACAGACAATC-3' и 5'-TCCGAGTACGTGCTCGCTCGA-3'). Pre-hybridization and hybridization were performed at a temperature from 56° to 64°C, depending on the probe. DNA-probe hybridization was performed overnight. The membrane was washed in accordance with the instructions for the set (AlkPhos Direct Labeling Kit). The membrane was placed in a reagent CDP-Star (GE Healthcare, UK). For signal accumulation, the membrane in CDP-Star solution was placed overnight in a photographic cuvette with a Retina XBE blue sensitive X-ray film (Caresream Health, USA) at room temperature. The X-ray film was scanned using an EPSON Perfection V750 PRO scanner (EPSON, Japan).

Quantitative RT-PCR analyzes of transgenic grapevine (Real-Time qPCR)

199 The selection of plant material for analysis was performed twice (before and after hardening
200 of plants). RNA samples were extracted from leaves of transgenic and control plants using
201 Spectrum Plant Total RNA Kit (Sigma-Aldrich, Darmstadt, Germany). Each sample was treated
202 with DNAse (Thermo). The cDNA was synthesized using Revert Aid Minus Reverse
203 Transcriptase (Thermo) according to the manufacturer's protocol using oligo(dT) 18 and
204 Random (dN) 10 primers.
205 Housekeeping ubiquitin conjugating enzyme (*UBC*) (Gen Bank Accession: EE253706), actin
206 (*ACT*) (Gen Bank Accession: EC969944) and SAND family protein (*SAND*) (Gen Bank
207 Accession: CF405409) genes was chose as internal control as the most suitable reference genes
208 to be used in grapevine leaves, regardless of the stress (Reid et al., 2006; Borges et al., 2014).
209 Real-Time qPCR was performed using primer sets for *CspA-plant* were PA(BNRT)F: 5'-
210 ATGTCTGGAAAGATGACTGG -3' and PA(BNRT)R: 5'- CCTTAGATCCATCATCAGGAG -
211 3' (anticipated amplification fragment of 85 bp) (Khvatkov et al., 2021); those for *Escsdp3* were
212 DP3(BNRT)2F: 5'- CATTACTCCCGATGACGGC -3' and DP3(BNRT)2R: 5'-
213 TCGCTTCCCAGAGTGATTGC -3' (anticipated amplification fragment of 120 bp) (Khvatkov
214 et al., 2021); those for *UBC* were VvU(q)-F: 5'- CATAAGGGCTATCAGGAGGAC -3‘ and
215 VvU(q)-R: 5'- TGGCGGTCGGAGTTAGG -3' (anticipated amplification fragment of 161 bp)
216 (Borges et al., 2014); those for *ACT* were VvA(q)-F: 5'- CTTGCATCCCTCAGCACCTT -3‘ and
217 VvA(q)-R: 5'- TCCTGTGGACAATGGATGGA -3' (anticipated amplification fragment of 82
218 bp) (Reid et al., 2006); those for *SAND* were VvD(q)-F: 5'-
219 CAACATCCTTTACCCATTGACAGA -3‘ and VvD(q)-R: 5'-
220 GCATTTGATCCACTTGCAGATAAG -3' (anticipated amplification fragment of 76 bp) (Reid
221 et al., 2006).
222 PCR cycles were chosen so that none of analyzed samples reached a plateau at the end of the
223 amplification, that is they were in the exponential phase of amplification. The experiments were

performed on a LightCycler 96 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the qPCRmix-HS SYBR containing HS Taq polymerase and intercalating dye SYBR Green I (Evrogen, Russia) in a volume of 20 µl. The PCR reaction conditions and the melting analysis of the products were selected according to the manufacturer's recommendations. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate *CspA-plant* gene values relative to the endogenous control. Every sample was analyzed in triplicate. Data calculation and statistical analysis were performed using both the software supplied with the Roche device and Microsoft Excel.

Study of plant resistance to frost

After adaption plants to *in vivo* conditions grapevine were planted into culture containers containing 0.6 liters of a mixture of soil and 10% (v/v) agroperlite (AgroBalt-C), then they were cultured in a climatic chamber Conviron MTR30 Reach [photoperiod 16/8 h (hereinafter - day/night), illumination – 16 fluorescent lamps Sylvania PENTRON 4100K 36W which is equal to 150/0 photosynthetically active radiation (PAR) intensity (µM $m^{-2}s^{-1}$), humidity 80/70%, temperature 17/15 ° C] for until they reach the stage of 8-9 leaves.

Half of the plants [10 adult plants for each experimental line and each control – non-transgenic plants of grapevine cultivars 'Podarok Magaracha' and 'Garnacha blanca' and transgenic line of 'Podarok Magaracha' cultivars, obtained in the previous study (Maletich et al., 2025)] were selected to be hardened at a temperature of +4 °C in the same climatic chamber within 3 weeks (photoperiod 16/8 h - the first week, 12/12 h - second week, 8/16 h - third week; illumination – 16 fluorescent lamps Sylvania PENTRON 4100K 36W which is equal to 150/0 µM $m^{-2}s^{-1}$ PAR intensity, humidity 80/80%, temperature 4/4 °C) and only after that the plants were subjected to cold stress. Before cold treatments samples were taken to analyze the content

of free proline in plant tissues according to the method described by Bates et al. (1973). Determination of free proline content was carried out with a ninhydrin-based protocol with some modifications (Raldugina et al., 2021; Khaliluev et al. 2022). To simulate conditions of recurrent spring frosts plants were frozen in a climatic chamber Celsius 10.0 (5 adult plants in two repetitions for each experimental line and each control). Testing temperatures were achieved by reducing the initial cultivation temperature by 2 °C h^{-1} from +4 °C. Then the hardened plants were kept at low temperatures for 24 hours (-4; -6; -8; -10; 12; -16 °C). After that, the temperature in the chamber was increased up to +4 °C at a rate of 2 °C h^{-1} and the plants were kept at +4 °C for a further 24 hours, and then transferred to the greenhouse (25 °C temperature). Three days later, frost damage was analyzed using longitudinal sectioning of vegetative buds (Fig. 2). Only buds from woody vines were used for analysis. In total, 12 to 35 buds of each line were analyzed in each freezing. Those buds were considered alive that contained at least one intact meristem. The buds survival efficiency (SE) was calculated as the quotient of the sum of the number of buds without damage (ND) (Fig. 2a) and buds with partial damage (PD) (Fig. 2b,c) divided by the total number of buds (NB) (Fig. 2); the results are expressed as a percentage: SE = [(ND+PD)/NB] 100.

The second half of the plants (10 adult plants for each experimental line and each control – non-transgenic plants of grapevine cultivars ‘Podarok Magaracha’ and ‘Garnacha blanca’) were planted into culture containers containing 5.0 liters of a mixture of soil and 10% (v/v) agroperlite (AgroBalt-C), then they were cultured in a greenhouse for 7 months. After that, plants were transferred to induce winter dormancy at a temperature of +4 °C in a climatic chamber Conviron MTR30 Reach within 3 months (photoperiod 16/8 h - the first week, 12/12 h - second week, 8/16 h - all the rest of the time; illumination – 16 fluorescent lamps Sylvania PENTRON 4100K 36W which is equal to 150/0 μM $m^{-2}s^{-1}$ PAR intensity, humidity 80/80%, temperature 4/4 °C) and only after that the grapevines were subjected to frost stress. To simulate conditions of winter

frosts grapevines were frozen in a climatic chamber Celsius 10.0 (10 vines each, 1.5 m long, of each experimental line and each control). Testing temperatures were achieved by reducing the initial cultivation temperature by 2.5 °C h^{-1} from +4 °C with gradual hardening over 24 hours at temperatures 0 and -5 °C. Then the grapevines were kept at low testing temperatures for 12 hours (-15; -17.5; -20 °C). After that, the temperature in the chamber was increased up to +4 °C at a rate of 2.5 °C h^{-1} and the vines were kept at +4 °C for a further 24 hours, cut into three-eyed cuttings and placed in vessels with water, and then transferred to the greenhouse (25 °C temperature). Three days later, frost damage was analyzed using longitudinal sectioning of vegetative buds using SE indicator. Those buds were considered alive that contained at least one intact. In total, 13 to 38 buds of each line were analyzed in each freezing.

Results

Plasmid construction and *Agrobacterium*-mediated transformation

Two genes were used to transform grapevine plants. The first was *CspA* from *E. coli*, the major cold shock protein of this bacterium which accumulates to large amount in bacterial cells after the shift to low temperature (Goldstein et al., 1990). *CspA* acts as an RNA chaperone destabilizing secondary structures in RNA and thus facilitating transcription and translation under low temperature (Jiang et al., 1997; Bae et al., 2000; Zlobin et al., 2018). Its ectopic expression in rapeseed (Khvatkov et al., 2021) and tobacco (Nizkorodova and Iskakov, 2014) increase plant cold tolerance. The second was *Escsdp3* gene from *Eutrema salsugineum* (formerly *Thellungiella salsuginea*). *Escsdp3* belongs to the same family of cold-shock proteins as *CspA*. Ectopic expression of *Escsdp3* increase cold tolerance of rapeseed (Khvatkov et al., 2021), while it's *Arabidopsis thaliana* homologue *AtCSDP3* positively regulated freezing

299 tolerance in *Arabidopsis* (Kim et al., 2009). Both genes were cloned in pBI121 binary vector
300 under control of CaMV35S promoter, and also in the modified pBI121 variant in which
301 CaMV35S promoter was replaced by rd29A promoter. This promoter is responsive to diverse
302 abiotic stress factors (Yamaguchi-Shinozaki and Shinozaki, 1994; Bihmidine et al., 2013) and
303 was used to express stress-related transcription factors of CDF/DREB family to increase plant
304 resistance to several types of abiotic stress (Kasuga et al., 1999; Behnam et al., 2006, 2007; Wu
305 et al., 2008).

306 After agro inoculation the development of transgenic structures occurred from explants after
307 30 days of cultivation on solid medium ½ MS containing 150 mg L^{-1} and Timentin 50 mg L^{-1}
308 Kanamycin. These structures were somatic embryos with the potential to develop into single
309 plants or undifferentiated proliferating embryogenic tissue. As a result of the studies, in total 16
310 kanamycin-resistant lines were obtained.

311 The obtained grapevine lines were adapted to in vivo conditions and planted into a
312 greenhouse to further analysis on determination of the heterologous genes expression level and
313 to reveal the efficiency of introduced genes on increasing of the frost resistance in transgenic
314 grapevine lines.

315

316 **Analysis of transformants**

317

318 PCR analysis revealed 7 transgenic grapevine lines with the insertion of the plasmid
319 pBI121rd29A-EsCSDP3 T-region (PM5ADP4/2, PM5ADP10/1, PM5ADP11/1, PM5ADP12/1,
320 PM5ADP15/1, PM5ADP17/1 and PM5ADP21/1), 5 lines with the introduction of the T-DNA
321 from pBI121-EsCSDP3 (PM6SDP1/1, PM6SDP1/2, PM6SDP2/1, PM6SDP3/1 and
322 PM6SDP4/2) construction, 3 lines with the introduction of the T-DNA from pBI121rd29A-
323 CSPA-plant (PM3ACA2/1, PM4ACA2/1 and PM4ACA1/1) construction and PM1SCA11/3

lines with the insertion of the plasmid T-DNA from pBI121-CSPA-plant (Fig. 3a). Contamination by the *Agrobacterium Vir* gene was not detected in the analyzed samples. In total, 3750 explants were involved in transformation of grapevines, and transformation efficiency was 0.43%, which is common for significant crop cultivars (Ren et al. 2016, 2021; Osakabe et al. 2018; Bertini et al. 2019). No statistically significant differences in the efficiency of grapevine transformation with different vectors were found.

Integration of the *CspA-plant*, *Escsdp3* and *nptII* genes into the genome of grapevine plants has been confirmed by Southern blotting (Fig. 3b). The *CspA-plant* gene integration is shown in 4 lines. The copy number of the gene varies among the lines. So, in lines PM3ACA2/1 and PM4ACA1/1 there is a single copy of the *CspA-plant* gene, and in lines PM4ACA2/1 and PM1SCA11/3 – three copies of the gene. During the hybridization with a probe specific to the *Escsdp3 Arabidopsis* gene, a plurality of bands appeared on the membrane both in the control and in the selected lines, which significantly complicated the detection of the target signals on the membrane. Most likely it is due to the high homology of the transgene from *Arabidopsis* with the native genes of cold shock grapevine proteins, as well as to the great diversity of these genes in the plant genome. An additional probe specific to the *npt*II gene was further used to more accurately confirm the transgenic status of the plants. The *Escsdp3* gene integration is shown in 12 lines. It was found that there was a single copy insertion in 8 lines (PM6SDP2/1, PM6SDP3/1, PM5ADP4/2, PM5ADP11/1, PM5ADP12/1, PM5ADP15/1, PM5ADP17/1 and PM5ADP21/1), two copies in 3 lines (PM5ADP10/1, PM6SDP1/1 and PM6SDP4/2), and three copies in line PM6SDP1/2 (Fig. 3b). However, the *npt*II gene was not detected in the DNA of untransformed grapevine. Based on the profile of the bands obtained by hybridization, all lines are the result of an independent transformational event.

Expression of target genes was shown in 16 transgenic lines by means of RT-PCR (Fig. 3c). All housekeeping genes (*UBC*, *ACT* and *SAND*) showed similar results as reference genes

under stress conditions. When the data for each of these genes were aligned one by one, the expression profile of the target genes did not change significantly. However, when using *SAND* as a reference gene, the error bars in the expression level plot of both target genes showed the smallest deviations. We have shown that after three weeks of hardening, the *rd29a* promoter is able to provide a similar level of target genes expression in grapevine leaf tissues as the constitutive *35S* promoter (Fig. 3c).

Study of plant resistance to frost

Our studies have shown that low temperatures up to -6 °C do not adversely lethal affect hardened young grapevine plants of the Podarok Magaracha cultivar. The critical temperature at which total plant death begins is -8 °C (Table 1). From this temperature the spring frost damage to the bud becomes critical and leads to death of the meristem tissue (Fig. 2, highlighted by arrows).

Transgenic lines with *Escsdp3* gene integration under the control of *35S* promoter slightly increased their frost resistance (only 2 °C). Most of the remaining transgenic lines after freezing at -12 °C reached the SE indicator characteristic of control plants after freezing at -6 °C. As a result, after freezing at -16 °C, only 3 lines of transgenic grapevine plants (PM1SCA11/3, PM3ACA2/1 and PM5ADP12/1) alive remained (Table 1). All these transgenic grapevine lines had high levels of target genes expression (Fig. 3c, highlighted by arrows). The dramatic differences between grapevine lines were observed by a change in the contents of proline under cold treatments (Fig. 4). The content of proline in leaves of transgenic grapevine significantly increased after cold treatments, while in leaves of control grapevine the content of proline after cold treatments significantly did not differ from the control values. The most significant increase

(3 times or more) was observed in spring frost-resistant lines (PM1SCA11/3, PM3ACA2/1 and PM5ADP12/1).

Transgenic and control vines in a state of winter dormancy also increased their frost-resistance. Thus, after freezing at -15 °C all testing vines (all transgenic lines and the Podarok Magaracha cultivar) except the Garnacha blanca cultivar remained alive (Table 2), after frost processing at -17.5 °C, 9 transgenic lines showed a higher survival rate than control plants. Seven transgenic lines (PM1SCA11/3, PM4ACA2/1, PM5ADP4/2, PM5ADP10/1, PM5ADP21/1, PM6SDP1/2 and PM6SDP4/2) remained alive after freezing at a temperature of -20 °C. It is obvious that the integration of CSD genes into the grapevine genome has made it possible to significantly increase the frost resistance of vines.

Discussion

Cold is an environmental factor that limits the geographical distribution and growing season of many plant species, and it often adversely affects crop quality and productivity (Thomashow 1999). The climate of the main grapevine growing regions is characterized by extreme instability and inconstancy of air temperature in winter. Cold weather alternates with thaws, during which days with relatively high temperatures are often established. Usually, obtaining stress-resistant grapevine cultivars is limited to classical selection methods. Mostly, crossings of *V. vinifera* cultivars with native American forms of *V. vinifera* are used, or interspecific crossing of *V. vinifera* with *V. amurensis* is used as a donor of genes for resistance to cold stress. Genes involved in protection or repair mechanisms could be new targets for the improvement of plant plasticity and adaptive responses to stress for future crop improvement (Umezawa et al. 2006; Sanghera et al. 2011). Low temperatures in winter and cold snaps in spring have a strong negative impact on grapevine yields. The seriousness of the problem of low

cold and frost resistance of grapevines is also evidenced by the fact that a large number of scientists in the last decade have been studying the mechanisms of response to cold and frost stress and ways to increase the cold and frost resistance of grapevines. Over the past two decades, a number of genes have been identified that can increase plant resistance to cold (Kim et al. 2001; Vannini et al. 2004; Zhu et al. 2010; Deng et al. 2015). Several genes from *V. riparia* and *V. vinifera* regulated by transcription factors associated with low temperature response pathways *VrCBF1a*, *VrCBF1b*, and *VvCBF1–VvCBF4* have been cloned (Xiao et al., 2006, 2008). The transcription factor genes *VpWRKY1* and *VpWRKY2* were cloned from *V. pseudoreticulata* Baihe-35-1, and overexpression of *VpWRKY2* led to an increase in cold tolerance (Li et al., 2010). The most cold hardy wild grapevine species is *V. amurensis*. Many resistance genes have been cloned from it - *VaICE1* and *VaICE2* (Xu et al., 2014), *VaERF057* (Sun et al., 2016), *VaERD15* (Yu et al., 2017), *VaCPK20* (Dubrovina et al., 2015), *VaCPK21* (Dubrovina et al., 2016), *VaCPK29* (Dubrovina et al., 2017), *VaPUB* (Jiao et al., 2015, 2017) and *VaIPK2β* (Li et al. , 2011), and overexpression of *VaPAT1* contributed to increased resistance not only to cold, but also to drought and salinity (Yuan et al., 2016).

In our previous studies we used *Escsdp3* gene (Taranov et al. 2010, 2018; Zlobin et al. 2016) from the extremophyte plant *E. salsugineum* for imparting additional cold and frost resistance to rapeseed. Data on the level of *Escsdp3* gene mRNA in plant tissues correlated with data on the cold and frost resistance of the corresponding transgenic lines. One transgenic line with expression *Escsdp3* gene remained alive after freezing 24 h at -16 °C, while the wild type completely died after freezing 24 h at -12 °C. Kim et al. (2009) obtained a similar result when overexpressing the *AtCSDP3* gene in *Arabidopsis*.

In our current research aimed at improving the frost resistance of grapevines, we obtained and studded transgenic plants with integration into genome *CspA-plant* and *Escsdp3* genes under the control of the stress-inducible *rd29A* promoter (Kasuga et al. 2004; Bihmidine et al. 2013).

As a result of our studies, we obtained 3 resistant to spring frosts lines of grapevine, namely 1 line with the *CspA-plant* gene (PM1SCA11/3) under the control of *35S* promoter, 1 line with the *CspA-plant* gene (PM3ACA2/1) under the control of *rd29A* promoter and the PM5ADP12/1 line with the *Escsdp3* gene under the control of *rd29A* promoter (Tables 1). Thus, it can be assumed that the *rd29A* promoter allows achieving a high level of frost resistance using both bacterial (*CspA-plant*) and plant (*Escsdp3*) CSD genes. At the same time, the highest rates of bud survival after freezing of vines in winter dormancy were noted in lines genes under the control of *35S* promoter using both bacterial (*CspA-plant*) and plant (*Escsdp3*) CSD genes. Only one line with the *Escsdp3* gene under the control of *rd29A* promoter (PM5ADP10/1) showed a comparable survival rate, which at the same time had intermediate resistance to spring frosts compared to all transgenic lines (Tables 2). Thus, the use of the inducible plant regulatory elements together plant CDS gene can have a positive effect on improving frost resistance in transgenic grapevine.

Formatting of funding sources

This study was supported by the Russian Science Foundation Grant no. 23-76-10013.

Data availability

The data that support the findings of this study are available upon request from the corresponding author.

Ethics declarations

Conflict of interest

448

449 The authors declare that they have no conflict of interest. Sergey Dolgov is one of the Associate
450 Editors of the journal at the time of submission. This had no impact on the peer review process
451 or the final decision.

452

453 **Ethical approval**

454

455 No human participants and/or animals are involved in this work.

456

457 **Consent for publication**

458

459 All the authors agree with the publication of the article.

460

461 **Authors Contribution**

462

463 The authors have made the following declarations regarding their contributions: Research
464 planning and experiment analysis: PKh, GM, SD; Cultivation of plant material of *in vitro*
465 conditions: GM, IG, SCh; Gene synthesis and plasmid construction: NZ, AB; *Agrobacterium*-
466 mediated transformation: GM, IG, SCh, PKh; Polymerase chain reaction: PKh, GM; Southern
467 blot analysis: AP, TKh; Study of plant resistance to cold and frost: GM, IG, VK, VTs, PKh;
468 Contributed to the writing of the manuscript: PKh, YuP, SD. All authors read and approved the
469 manuscript.

470

471 **Acknowledgments**

472

This research was carried out using the unique scientific facilities "PHYTOBIOGEN" of the «NBS-NSC» RAS (reg. no. 669802; Yalta, Russia).

References

Bae W, Xia B, Inouye M, Severinov K (2000). *Escherichia coli* CspA-family RNA chaperones are transcription antiterminators. Proceedings of the National Academy of Sciences, 97(14):7784-7789

Bates LS, Waldren RA, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Behnam B, Kikuchi A, Celebi-Toprak F, Kasuga M, Yamaguchi-Shinozaki K, Watanabe KN (2007) Arabidopsis rd29A:: DREB1A enhances freezing tolerance in transgenic potato. Plant Cell Reports, 26:1275-1282

Behnam B, Kikuchi A, Celebi-Toprak F, Yamanaka S, Kasuga M, Yamaguchi-Shinozaki K, Watanabe KN (2006) The *Arabidopsis* DREB1A gene driven by the stress-inducible rd29A promoter increases salt-stress tolerance in proportion to its copy number in tetrasomic tetraploid potato (Solanum tuberosum). Plant biotechnology, 23(2):169-177

Bertini E, Tornielli GB, Pezzotti M, Zenoni S (2019) Regeneration of plants from embryogenic callus-derived protoplasts of Garganega and Sangiovese grapevine (Vitis vinifera L.) cultivars. Plant Cell Tiss Organ Cult 138:239–246. https://doi.org/10.1007/s11240-019-01619-1

Bihmidine S, Lin J, Stone JM, Awada T, Specht JE, Clemente TE (2013) Activity of the Arabidopsis RD29A and RD29B promoter elements in soybean under water stress. Planta, 237:55-64

498 Bloesch B, Viret O (2008) Stades phénologiques repères de la vigne. Revue suisse Vitic.
499 Arboric. Hortic. Vol. 40 (6): 1-4 **in French**
500 Borges AF, Fonseca C, Ferreira RB, Lourenço AM, Monteiro S (2014) Reference Gene
501 Validation for Quantitative RT-PCR during Biotic and Abiotic Stresses in Vitis
502 vinifera. PLoS ONE 9(10): e111399
503 Castiglioni P, Warner D, Bensen RJ, Anstrom DC, Harrison J, Stoecker M, Abad M, Kumar G,
504 Salvador S, D'ordine R, Navarro S, Back S, Fernandes M, Targolli J, Dasgupta S,
505 Bonin C, Luethy MH, Heard JE (2008) Bacterial RNA chaperones confer abiotic
506 stress tolerance in plants and improved grain yield in maize under water-limited
507 conditions. Plant Physiol. 147:446–455
508 Choi D-W, Rodriguez EM, Close TJ (2002) Barley Cbf3 gene identification, expression pattern,
509 and map location. Plant Physiology 129(4):1781-1787
510 Deng S, Sun J, Zhao R, Ding M, Zhang Y, Sun Y, Wang W, Tan Y, Liu D, Ma X, Hou P, Wang
511 M, Lu C, Shen X, Chen S (2015) *Populus euphratica* APYRASE2 enhances cold
512 tolerance by modulating vesicular trafficking and extracellular ATP in *Arabidopsis*
513 plants. Plant Physiology 169:530–548
514 Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K,
515 Yamaguchi-Shinozaki K (2003) OsDREB genes in rice, *Oryza sativa* L., encode
516 transcription activators that function in drought-, high-salt- and cold-responsive gene
517 expression. The Plant Journal 33(4):751-763
518 Dubrovina AS, Kiselev KV, Khristenko VS, Aleynova OA (2015) VaCPK20, a calcium-
519 dependent protein kinase gene of wild grapevine Vitis amurensis Rupr., mediates
520 cold and drought stress tolerance. J. Plant Physiol. 185:1–12. doi:
521 10.1016/j.jplph.2015.05.020

522 Dubrovina AS, Kiselev KV, Khristenko VS, Aleynova OA (2016) VaCPK21, a calcium-
523 dependent protein kinase gene of wild grapevine *Vitis amurensis* Rupr., is involved
524 in grape response to salt stress. Plant Cell Tissue Organ Cult. 124:137–150. doi:
525 10.1007/s11240-015-0882-4
526 Dubrovina AS, Kiselev KV, Khristenko VS, Aleynova OA (2017) The calcium-dependent
527 protein kinase gene VaCPK29 is involved in grapevine responses to heat and osmotic
528 stresses. Plant Growth Regul. 82:79–89. doi: 10.1007/s10725-016-0240-5
529 Francia E, Rizza F, Cattivelli L, Stanca AM, Galiba G, Toth B, Hayes PM, Skinner JS, Pecchioni
530 N (2004) Two loci on chromosome 5H determine low-temperature tolerance in a
531 'Nure' (winter) x 'Tremois' (spring) barley map. Theoretical and Applied Genetics
532 108(4):670-680
533 Gao M-J, Allard G, Byass L, Flanagan AM, Singh J (2002) Regulation and characterization of
534 four CBF transcription factors from *Brassica napus*. Plant Molecular Biology
535 49(5):459-471
536 Gao Z, Li J, Luo M, Li H, Chen Q, Wang L, Song S, Zhao L, Xu W, Zhang C, Wang S, Ma C
537 (2019) Characterization and Cloning of Grape Circular RNAs Identified the Cold
538 Resistance-Related Vv-circATS1 Plant Physiol. 180(2):966-985. doi:
539 10.1104/pp.18.01331
540 Gilmour SJ, Fowler SJ, Thomashow MF (2004) *Arabidopsis* transcriptional activators CBF1,
541 CBF2, and CBF3 have matching functional activities. Plant Molecular Biology
542 54:767-781
543 Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, Thomashow MF (1998) Low
544 temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional
545 activators as an early step in cold-induced COR gene expression. Plant J. 16:433–442

546 Goldstein, J., Pollitt, N. S., & Inouye, M. (1990). Major cold shock protein of Escherichia coli.
547 Proceedings of the National Academy of Sciences, 87(1), 283-287
548 Gribaudo I, Gambino G, Vallania R (2004) Somatic embryogenesis from grapevine anthers: the
549 optimal developmental stage for collecting explants. Am. J. Enol. Vitic. 55:427–430
550 Grote A, Hiller K, Scheer M, Münch R, Nörtemann B, Hempel DC, Jahn D (2005). JCat: a novel
551 tool to adapt codon usage of a target gene to its potential expression host. Nucleic
552 acids research, 33(suppl_2), W526-W531
553 Hail Z, Al-Issawi RM, Fuller MP (2017) Advances in physiological and molecular aspects of
554 plant cold tolerance. Journal of Plant Interactions 12(1):143-157
555 Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New Agrobacterium helper plasmids for
556 gene transfer to plants. Transgen Res2:208–218. https://doi.org/10.1007/BF01977351
557 Hou L, Zhang G, Zhao F, Zhu D, Fan X, Zhang Z, Liu X (2018) VvBAP1 Is Involved in Cold
558 Tolerance in Vitis vinifera L. Front. Plant Sci. 9:726. doi: 10.3389/fpls.2018.00726
559 Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF
560 (2001) Components of the *Arabidopsis* C-repeat/dehydrationresponsive element
561 binding factor cold-response pathway are conserved in Brassica napus and other
562 plant species. Plant Physiol. 127(3):910-917
563 Jiang W, Hou Y, Inouye M (1997) CspA, the major cold-shock protein of Escherichia coli, is an
564 RNA chaperone. Journal of Biological Chemistry, 272(1):196-202
565 Jiao L, Zhang Y, Lu J (2017) Overexpression of a stress-responsive U-box protein gene VaPUB
566 affects the accumulation of resistance related proteins in Vitis vinifera “Thompson
567 Seedless.” Plant Physiol. Biochem. 112:53–63. doi: 10.1016/j.plaphy.2016.12.019
568 Jiao L, Zhang Y, Wu J, Zhang H, Lu J (2015) A novel U-Box protein gene from “Zuoshanyi”
569 grapevine (Vitis amurensis Rupr. cv.) involved in cold responsive gene expression

570 in Arabidopsis thanliana. Plant Mol. Biol. Rep. 33:557–568. doi: 10.1007/s11105-
571 014-0783-4
572 Joshi CP, Zhou H, Huang X, Chiang VL (1997). Context sequences of translation initiation
573 codon in plants. Plant molecular biology, 35:993-1001
574 Karlson D, Imai R (2003) Conservation of the cold shock protein of Escherichia coli. Plant
575 Physiol. 131:12–15
576 Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant
577 drought, salt, and freezing tolerance by gene transfer of a single stress-inducible
578 transcription factor. Nature biotechnology, 17(3):287-291
579 Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K (2004) A combination of the
580 Arabidopsis DREB1A gene and stress inducible Rd29A promoter improved drought-
581 and low temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiol.
582 45:346–350. doi: 10.1093/pcp/pch037
583 Kawaguchi R, Bailey-Serres J (2005) mRNA sequence features that contribute to translational
584 regulation in *Arabidopsis*. Nucleic Acids Research 33(3):955-965
585 Khaliluev MR, Bogoutdinova LR, Raldugina GN, Baranova EN (2022) A simple and effective
586 bioassay method suitable to comparative *in vitro* study of tomato salt tolerance at
587 early development stages. Methods Protoc. 5:11
588 Khvatkov P, Taranov V, Pushin A, Maletich G, Fedorov V, Chaban I, Babakov A, Dolgov S,
589 Chernobrovkina M (2021) Genes with Cold Shock Domain from Eutrema
590 salsugineum (Pall.) for Generating a Cold Stress Tolerance in Winter Rape (Brassica
591 napus L.) Plants. Agronomy 11(5):827 https://doi.org/10.3390/agronomy11050827
592 Kim JC, Lee SH, Cheong YH, Yoo CM, Lee SI, Chun HJ, Yun DJ, Hong JC, Lee SY, Lim CO,
593 Cho MJ (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1,
594 enhances cold tolerance in transgenic plants. Plant J. 25:247–259

595 Kim MH, Sasaki K, Imai R (2009). Cold shock domain protein 3 regulates freezing tolerance in
596 Arabidopsis thaliana. Journal of Biological Chemistry, 284(35):23454-23460
597 Leisner CP (2020) Climate change impacts on food security- focus on perennial cropping
598 systems and nutritional value. Plant Science 293:1104122
599 Li H, Xu Y, Xiao Y, Zhu Z, Xie X, Zhao H, et al. (2010) Expression and functional analysis of
600 two genes encoding transcription factors, VpWRKY1 and VpWRKY2, isolated from
601 Chinese wild *Vitis pseudoreticulata*. Planta 232:1325–1337. doi: 10.1007/s00425-
602 010-1258
603 Li X, Hou L, Liu X, Lu J (2011). Involvement of H2O2 in regulating the expression of VvIPK2
604 in response to low temperature stress in leaves of Vitis. Acta Hortic. Sin. 38:1052–
605 1062
606 Livak K, Schmittgen T (2001) Analysis of relative gene expression data using realtime
607 quantitative PCR and the 211C(T). Methods 25:402–408
608 Lukaszewicz M, Feuermann M, Jérouville B, Stas A, Boutry M (2000). In vivo evaluation of the
609 context sequence of the translation initiation codon in plants. Plant Science,
610 154(1):89-98
611 Maletich G, Gavrilenko I, Pushin A, Chelombit S, Khmelnitskaya T, Plugatar Yu, Dolgov S,
612 Khvatkov P (2025) Somatic embryogenesis and *Agrobacterium*-mediated
613 transformation in a number of grape cultivars. Plant Cell Tiss Organ Cult 160:73
614 doi.org/10.1007/s11240-025-02997-5
615 Matsuda D, Dreher TW (2006) Close spacing of AUG initiation codons confers dicistronic
616 character on a eukaryotic mRNA. Rna, 12(7):1338-1349
617 Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K (2012) AP2/ERF family transcription factors in
618 plant abiotic stress responses. Biochim Biophys Acta. 1819:86–96

619 Murray MG and Thompson WF (1980) Rapid isolation of high molecular weight plant DNA.
620 Nucleic Acids Res 8:4321-4325
621 Nakaminami K, Karlson DT, Imai R (2006) Functional conservation of cold shock domains in
622 bacteria and higher plants. Proc. Natl. Acad. Sci. USA 103:10122–10127
623 Nizkorodova AS, Iskakov BK (2014) Transgenic tobacco plants expressing gene ECCspa tend to
624 be cold resistant. Eurasian Journal of Applied Biotechnology, (3)
625 Osakabe Y, Liang Z, Ren C, Nishitani C, Osakabe K, Wada M, Komori S, Malnoy M, Velasco
626 R, Poli M, Jung MH, Koo OJ, Viola R, Kanchiswamy CN (2018) CRISPR–Cas9-
627 mediated genome editing in apple and grapevine. Nat. Protoc. 13(12):2844–2863.
628 https://doi.org/10.1038/s41596-018-0067-9
629 Owens CL, Thomashow MF, Hancock JF, Iezzoni AF (2002) CBF1 orthologs in sour cherry and
630 strawberry and the heterologous expression of CBF1 in strawberry. Journal of the
631 American Society of Horticultural Science 127:489-494
632 Rajasheker G, Kumar AS, Kumari HP, Kishor KPB (2019) Bacterial cold shock proteins -
633 themolecular chaperones for multiple stress tolerance. Adv. Biotech. & Micro.
634 12:555837
635 Raldugina GN, Evsukov SV, Bogoutdinova LR, Gulevich AA, Baranova EN (2021) Morpho-
636 physiological testing of NaCl sensitivity of tobacco plants overexpressing choline
637 oxidase gene. Plants 10:1102
638 Reid KE, Olsson N, Schlosser J. et al. (2006) An optimized grapevine RNA isolation procedure
639 and statistical determination of reference genes for real-time RT-PCR during berry
640 development. BMC Plant Biol 6:27
641 Ren C, Liu Y, Guo Y, et al (2021) Optimizing the CRISPR/Cas9 system for genome editing in
642 grape by using grape promoters. Hortic Res. 8:52

643 Ren C, Liu X, Zhang Z, Wang Y, Duan W, Li S, Liang Z (2016) CRISPR/Cas9-mediated
644 efficient targeted mutagenesis in Chardonnay (Vitis vinifera L.). Sci Rep 6:32289.
645 https://doi.org/10.1038/srep32289
646 Rihan HZ, Al-Issawi M, Fuller MP (2017) Advances in physiological and molecular aspects of
647 plant cold tolerance. Journal of Plant Interactions 12:143– 157
648 Rogers SO and Bendlich AJ (1994) Extraction of total cellular DNA from plants, algae and
649 fungi. In: Gelvin, S.B. and Schilperoort, R.A., Eds., Plant Molecular Biology
650 Manual, 2nd Edition, Kluwer Academic Publishers, Dordrecht, 1-8
651 http://dx.doi.org/10.1007/978-94-011-0511-8_12
652 Sanghera GS, Wani SH, Hussain W, Singh NB (2011) Engineering cold stress tolerance in crop
653 plants. Curr Genomics. 12:30-43
654 Sasaki K, Imai R (2012) Pleiotropic roles of cold shock domain proteins in plants. Front Plant
655 Sci. 2:116
656 Skabkin MA, Skabkina OV, Ovchinnikov LP (2004) Multifunctional proteins with a cold shock
657 domain in the regulation of gene expression. Advances in Biological Chemistry
658 44:3–52. **in Russian**
659 Sun X, Zhao T, Gan S, Ren X, Fang L, Karungo SK, et al. (2016) Ethylene positively regulates
660 cold tolerance in grapevine by modulating the expression of ETHYLENE
661 RESPONSE FACTOR 057. Sci. Rep. 6:24066. doi: 10.1038/srep24066
662 Taranov VV, Berdnikova MV, Babakov AV, Nosov AV, Galkin AV (2010) Cold shock domain
663 proteins in the extremophyte Thellungiella salsuginea (salt cress): Gene structure and
664 differential response to cold. Molecular Biology 44:889–897.
665 Taranov VV, Zlobin NE, Evlakov KI, Shamustakimova AO, Babakov AV (2018) Contribution
666 of Eutrema salsugineum cold shock domain structure to the interaction with RNA.
667 Biochemistry (Moscow) 83:1369-1379.

668 Theocharis A, Clément C, Barka EA (2012) Physiological and molecular changes in plants
669 grown at low temperatures. Planta 235:1091–1105. https://doi.org/10.1007/s00425-
670 012-1641-y
671 Thomashow MF (1999) Plant Cold Acclimation: Freezing Tolerance Genes and Regulatory
672 Mechanisms. Annual Review of Plant Physiology & Plant Molecular Biology,
673 50:571-599.
674 https://doi.org/10.1146/annurev.arplant.50.1.571
675 Uemura M, Shigematsu S, Minami A, Kawamura Y, Nakagawara Ch. (2006) Responses of the
676 plasma membrane to low temperatures. Physiologia Plantarum 126:81–89
677 Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering
678 drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr
679 Opin Biotechnol 17:113–122
680 Vágújfalvi A, Galiba G, Cattivelli L, Dubcovsky J (2003) The cold regulated transcriptional
681 activator Cbf3 is linked to the frost-tolerance locus Fr-A2 on wheat chromosome 5A.
682 Molecular Genetics and Genomics 269(1):60-67
683 Vannini C, Locatelli F, Bracale M, Magnani E, Marsoni M, Osnato M, Mattana M, Baldoni E,
684 Coraggio I (2004) Overexpression of the rice *Osmyb4* gene increases chilling and
685 freezing tolerance of Arabidopsis thaliana plants. Plant J. 37:115–127
686 Vazquez-Hernandez M, Romero I, Escribano MI, Merodio C, Sanchez-Ballesta MT (2017)
687 Deciphering the role of CBF/DREB transcription factors and dehydrins in
688 maintaining the quality of table grapes cv. autumn royal treated with high CO2 levels
689 and stored at 0°C. Front. Plant Sci. 8:1591
690 Vodiasova E, Sinchenko A, Khvatkov P, Dolgov S (2024). Genome-Wide Identification,
691 Characterisation, and Evolution of the Transcription Factor WRKY in Grapevine

(*Vitis vinifera*): New View and Update. International Journal of Molecular Sciences, 25(11):6241. https://doi.org/10.3390/ijms25116241

Wisniewski M, Nassuth A, Teulieres C, Marque C, Rowland J, Cao PB, Brown A (2014) Genomics of cold hardiness in woody plants. Crit Rev Plant Sci. 33:92–124

Wu Y, Zhou H, Que YX, Chen RK, Zhang MQ (2008) Cloning and identification of promoter Prd29A and its application in sugarcane drought resistance. Sugar Tech, 10:36-41

Xiao H, Siddiqua M, Braybrook S, Nassuth A (2006) Three grape CBF/DREB1 genes respond to low temperature, drought and abscisic acid. Plant Cell Environ. 29:1410–1421. doi: 10.1111/j.1365-3040.2006.01524.x

Xiao H, Tattersall EA, Siddiqua MK, Cramer GR, Nassuth A (2008). CBF4 is a unique member of the CBF transcription factor family of *Vitis vinifera* and *Vitis riparia*. Plant Cell Environ. 31:1–10. doi: 10.1111/j.1365-3040.2007.01741.x

Xu W, Jiao Y, Li R, Zhang N, Xiao D, Ding X, et al. (2014). Chinese wild-growing *Vitis amurensis* ICE1 and ICE2 encode MYC-type bHLH transcription activators that regulate cold tolerance in Arabidopsis. PLoS One 9:e102303. doi: 10.1371/journal.pone.0102303

Xue G-P (2003) The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature. The Plant Journal 33(2):373-383

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. The Plant Cell, 6(2):251-264

Yu D, Zhang L, Zhao K, Niu R, Zhai H, Zhang J (2017). VaERD15, a transcription factor gene associated with cold-tolerance in Chinese wild Vitis amurensis. Front. Plant Sci. 8:297. doi: 10.3389/fpls.2017.00297

717 Yuan Y, Fang L, Karungo SK, Zhang L, Gao Y, Li S, et al. (2016) Overexpression of VaPAT1, a
718 GRAS transcription factor from Vitis amurensis, confers abiotic stress tolerance in
719 Arabidopsis. Plant Cell Rep. 35, 655–666. doi: 10.1007/s00299-015-1910-x
720 Zhang Y, Mao L, Wang H, Brocker C, Yin X, Vasiliou V, et al. (2012) Genome-wide
721 identification and analysis of grape aldehyde dehydrogenase (ALDH) gene
722 superfamily. PLoS One 7:e32153. doi: 10.1371/journal.pone.0032153
723 Zhu B, Xiong A-S, Peng R-H, Xu J, Jin X-F, Meng X-R, Yao Q-H (2010) Over-expression of
724 ThpI from *Choristoneura fumiferana* enhances tolerance to cold in *Arabidopsis*.
725 Molecular Biology Reports 37:961.
726 Zlobin N, Evlakov K, Alekseev Ya, Blagodatskikh K, Babakov A, Taranov V (2016) High DNA
727 melting activity of extremophyte Eutrema salsugineum cold shock domain proteins
728 EsCSDP1and EsCSDP3. Biochemistry and Biophysics Reports 5:502–508
729 Zlobin N, Evlakov K, Tikhonova O, Babakov A, Taranov V (2018) RNA melting and RNA
730 chaperone activities of plant cold shock domain proteins are not correlated. RNA
731 biology 15:1040-1046

Figure 1. Schematic depiction of the expression cassettes of vectors pBI121-CSPA-plant **(a)**, pBI121rd29A-CSPA-plant **(b)**, pBI121-EsCSDP3 **(c)** and pBI121rd29A-EsCSDP3 **(d)** plasmids used for grape transformation. Target genes are under the control of the *35S* promoter from the cauliflower mosaic virus (CaMV35S-pro) or stress-inducible promoter of gene transcription factor *rd29A* of plants (rd29A-pro) and the nopalinesynthase terminator (NOS-ter) from *Agrobacterium tumefaciens*. The selective gene for the synthesis of neomycinphosphotransferase (nptII) is under the control of the regulatory elements of the nopaline synthase gene (NOS-pro and NOS-ter).

Figure 2. Longitudinal sections of grapevine buds after cold treatment (bud damage is indicated by arrows), with an undamaged bud (ND) **(a)**; PD - damage to the main bud **(b)** or damage to the replacement bud **(c)**; death of bud **(d)**.

Figure 3. PCR analysis **(a)**, Southern hybridization analysis **(b)** and relative expression levels **(c)** of *CspA-plant* and *Escsdp3* in independent Km-resistant of transgenic and wild type plants. To relative expression levels data were normalized to the reference *SAND* gene. Collecting plant material for real-time qPCR analyses was carried out only after plants hardening.

Figure 4. The contents of prolin in grape leaf tissue under control conditions and after hardened at a temperature of +4 °C

Fig 1

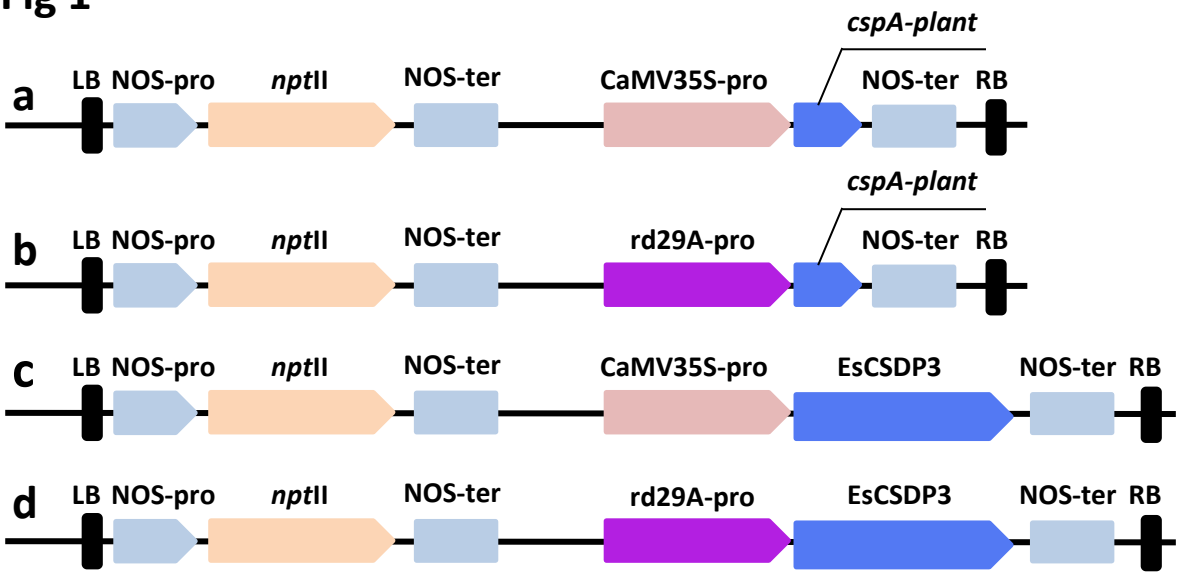

a
LB
NOS-pro
nptII
NOS-ter
CaMV35S-pro
cspA-plant
NOS-ter
RB
b
LB
NOS-pro
nptII
NOS-ter
rd29A-pro
cspA-plant
NOS-ter
RB
c
LB
NOS-pro
nptII
NOS-ter
CaMV35S-pro
EsCSDP3
NOS-ter
RB
d
LB
NOS-pro
nptII
NOS-ter
rd29A-pro
EsCSDP3
NOS-ter
RB

Fig 2

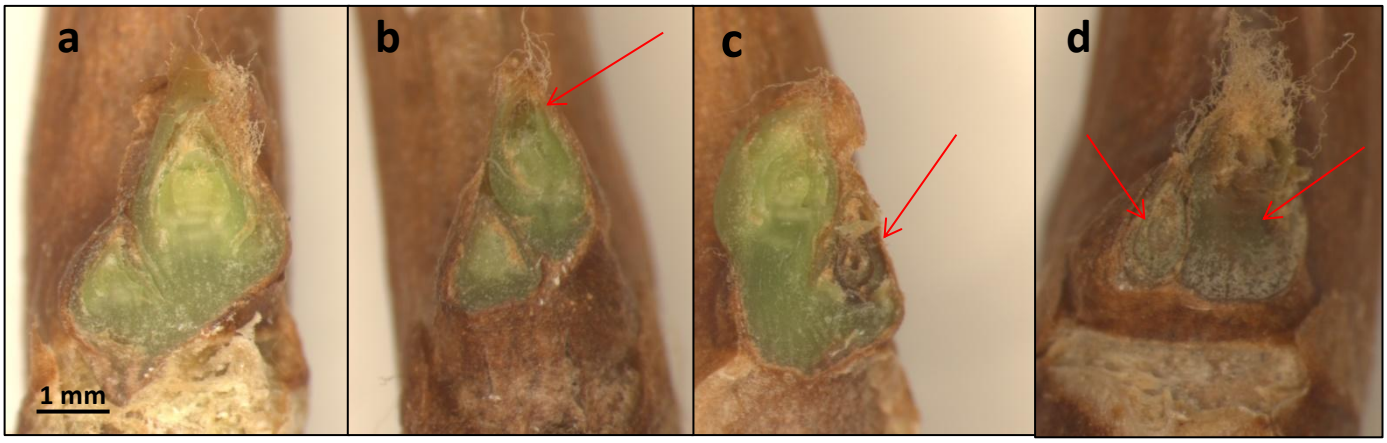


Fig 3

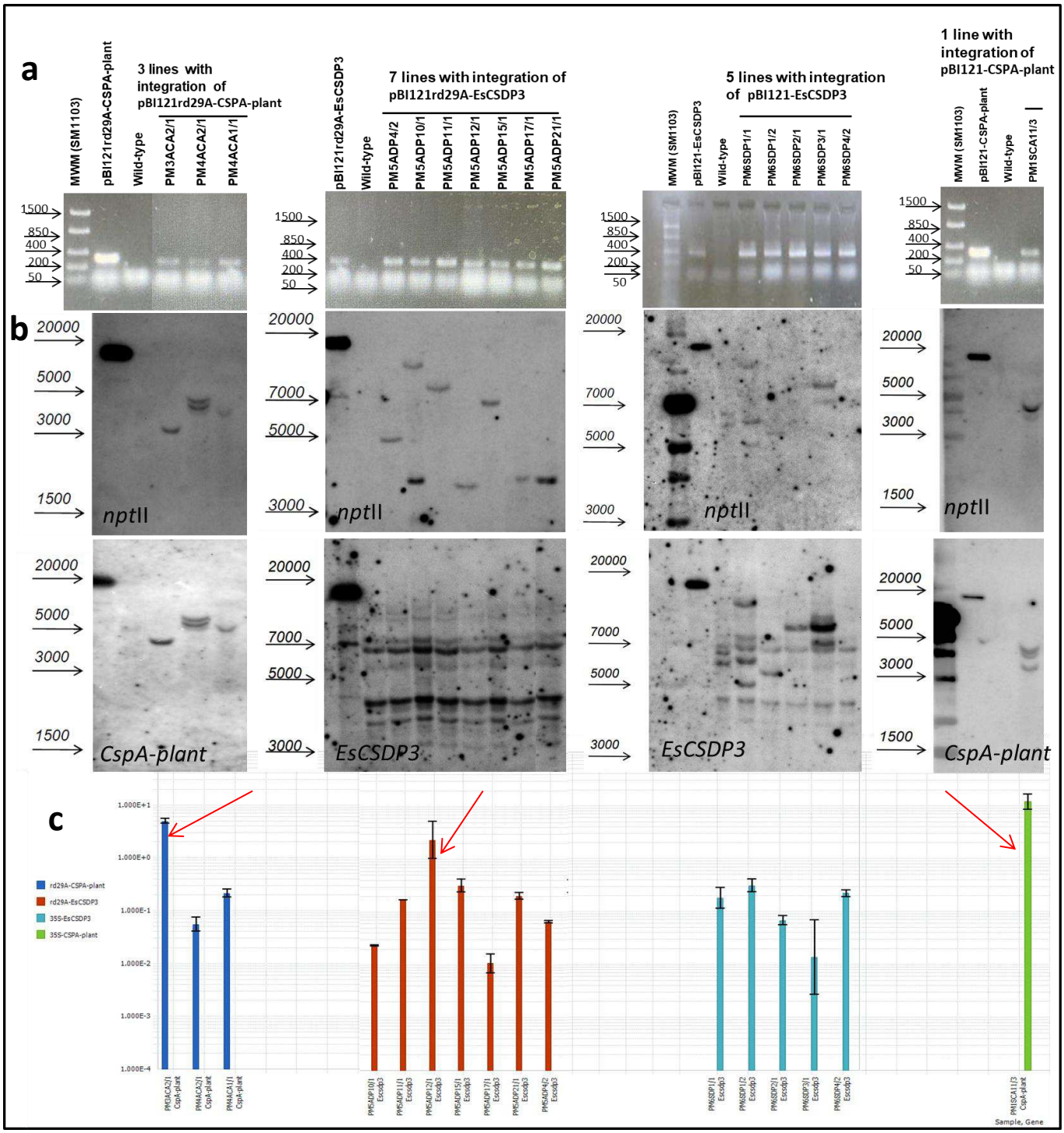
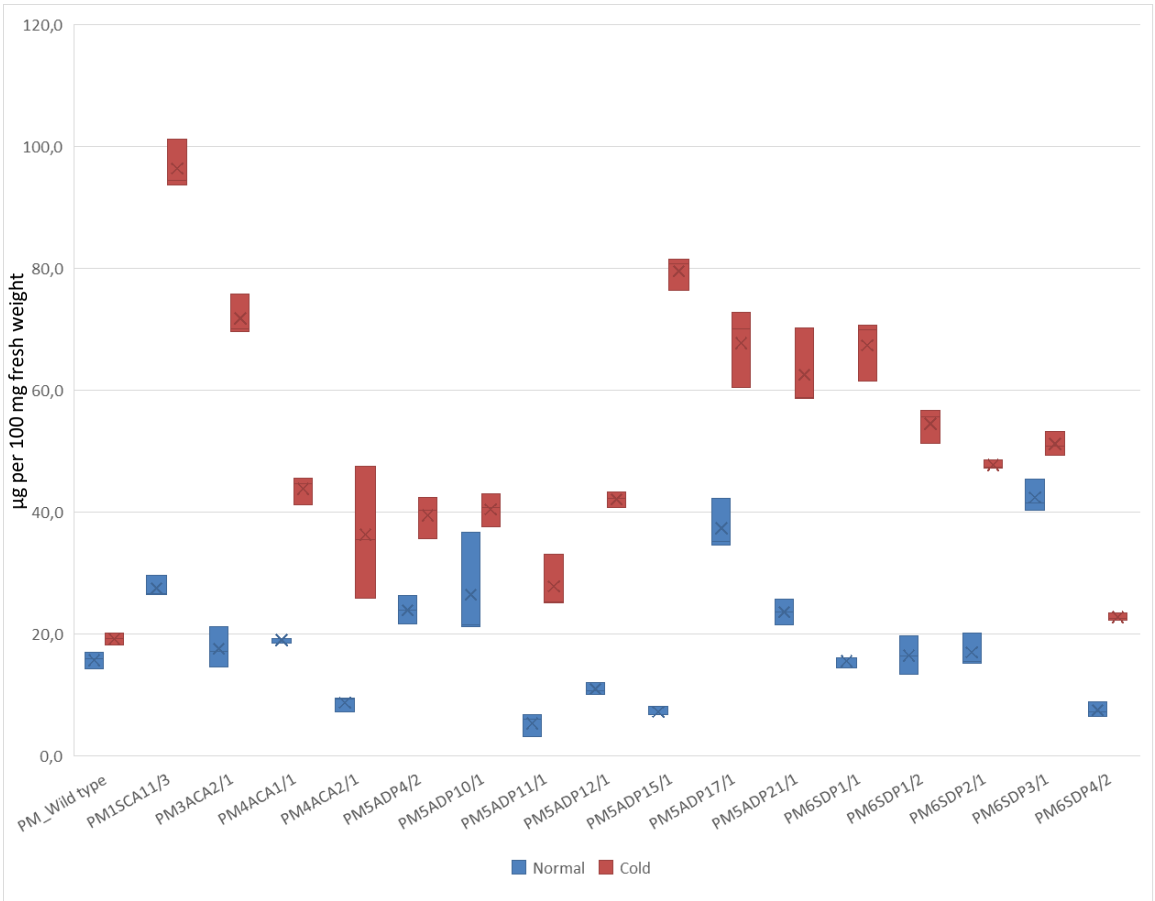

a
MWM (SM1103)
pBI121rd29A-CSPA-plant
Wild-type
3 lines with integration of pBI121rd29A-CSPA-plant
PM3ACA2/1
PM4ACA2/1
PM4ACA1/1
pBI121rd29A-EsCSDP3
7 lines with integration of pBI121rd29A-EsCSDP3
PM5ADP4/2
PM5ADP10/1
PM5ADP11/1
PM5ADP12/1
PM5ADP15/1
PM5ADP17/1
PM5ADP21/1
pBI121-EsCSDP3
5 lines with integration of pBI121-EsCSDP3
PM6SDP1/1
PM6SDP1/2
PM6SDP2/1
PM6SDP3/1
PM6SDP4/2
pBI121-CSPA-plant
1 line with integration of pBI121-CSPA-plant
PM1SCA11/3
1500
850
400
200
50
b
20000
7000
5000
3000
1500
nptII
CspA-plant
EsCSDP3
c
1.000E+1
1.000E+0
1.000E-1
1.000E-2
1.000E-3
1.000E-4
rd29A-CSPA-plant
rd29A-EsCSDP3
35S-EsCSDP3
35S-CSPA-plant
Sample, Gene

Fig 4


120,0
100,0
80,0
60,0
40,0
20,0
0,0
µg per 100 mg fresh weight
PM_Wild type
PM1SCA11/3
PM3ACA2/1
PM4ACA1/1
PM4ACA2/1
PM5ADP4/2
PM5ADP10/1
PM5ADP11/1
PM5ADP12/1
PM5ADP15/1
PM5ADP17/1
PM5ADP21/1
PM6SDP1/1
PM6SDP1/2
PM6SDP2/1
PM6SDP3/1
PM6SDP4/2
Normal
Cold

Supplementary Files

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- Tables.docx