

Article

Genes with Cold Shock Domain from *Eutrema salsugineum* (Pall.) for Generating a Cold Stress Tolerance in Winter Rape (*Brassica napus* L.) Plants [†]

Pavel Khvatkov ^{1,2,*} , Vasiliy Taranov ¹, Alexander Pushin ^{1,3}, Galina Maletich ², Vladislav Fedorov ², Inna Chaban ¹, Alexsey Babakov ¹, Sergey Dolgov ^{1,2,3} and Mariya Chernobrovkina ¹

¹ All-Russia Research Institute of Agricultural Biotechnology, 127550 Moscow, Russia;

v.taranov1@gmail.com (V.T.); aspushin@gmail.com (A.P.); inna_chaban@rambler.ru (I.C.);

avb@iab.ac.ru (A.B.); dolgov1958@rambler.ru (S.D.); chernobrovkina@yandex.ru (M.C.)

² Nikita Botanical Gardens–National Scientific Centre, 298648 Yalta, Russia; maletich.galina@yandex.ru (G.M.); frak2006@yandex.ua (V.F.)

³ Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, 142290 Puschino, Russia

* Correspondence: khvatkov1987@gmail.com

[†] This paper is dedicated to the memory of the late Mariya Chernobrovkina.

Abstract: The increased demand in vegetable oil for food purposes and high-protein feed for livestock and poultry encourages producers to expand the production of various oil crops, while occupying rather cold agroclimatic zones. Improved cold and frost resistance of cultivated crops would significantly increase the yield and expand the range of rape cultivation in a number of cold climate regions. Nine transgenic lines of winter rape containing genes encoding proteins with a cold shock domain (CspA *nEsCSDP3*) were obtained as a result of *Agrobacterium* transformation. In total, 260 explants were involved in transformation of rape using pBI121-CSPA-plant, with a transformation efficiency of 2.3%; among 750 explants using the pBI-EsCSDP3 construction, the efficiency was 0.4%. As a result of the studies, it was shown that the expression of the new gene *Escsdp3* from the plant of *Eutrema salsugineum* was able to increase the cold and frost resistance of plants as effectively as the *cspa* gene from *E. coli*, which is classically used for this purpose. The cold resistance analysis of T₁ transgenic plants generation revealed four cold resistant winter rape lines (three lines with the *cspa-plant* gene and one line with the *Escsdp3* gene). The transfer of *Escsdp3* and *cspa-plant* genes into winter rape plants led to a significant increase in frost resistance of plants. Two winter rapeseed lines were resistant to freezing (with the *cspa-plant* gene and with the *Escsdp3* gene). Non-hardened transgenic plants remained viable after 24 h of exposure to negative temperatures up to -5°C , and plants that passed through the hardening stage survived after freezing at -16°C .

Keywords: *Brassica napus*; cold shock proteins; *Eutrema salsugineum*; freezing tolerance; *Thellungiella salsuginea*; transgenic plants; cold resistance



Citation: Khvatkov, P.; Taranov, V.; Pushin, A.; Maletich, G.; Fedorov, V.; Chaban, I.; Babakov, A.; Dolgov, S.; Chernobrovkina, M. Genes with Cold Shock Domain from *Eutrema salsugineum* (Pall.) for Generating a Cold Stress Tolerance in Winter Rape (*Brassica napus* L.) Plants. *Agronomy* **2021**, *11*, 827. <https://doi.org/10.3390/agronomy11050827>

Received: 31 March 2021

Accepted: 21 April 2021

Published: 23 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Most of the total land on Earth (about 64%) has an average minimum temperature $<0^{\circ}\text{C}$ [1]. 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 ($0\text{--}15^{\circ}\text{C}$) and freezing ($<0^{\circ}\text{C}$), negatively affects the growth and agricultural productivity of plants and limits their geographical distribution [2]. Frost tolerance is understood as the ability of plants to tolerate adverse winter conditions without damage [3,4]. Cold stress usually restricts plant growth and development. Frost tolerance of plants is determined by their ability to survive during short-term frosts or prolonged frosts and depends on hereditary characteristics.

Overwintering plants are able to increase their frost resistance when exposed to low but non-negative temperatures; this process is known as “cold acclimatization” or hardening. During this process, plants increase their tolerance to cold stress by synthesizing numerous protective substances and proteins [5].

During hardening, cellular and physiological changes occur, as well as changes in gene expression [6,7]; cold regulation (COR) genes that are involved in CBF-CORE signaling pathway are actively induced [1,8]. CBF has been found in a number of herbaceous and woody plants, all general crops, and some species of vegetables [9–19].

Freezing resistance is usually associated with the functioning of CBF/DREB transcription factors [20,21], factors that somehow modulate the membrane structure [22]. Additionally, freezing resistance has often been linked to expression of a gene of the unknown function, Eskimo1 [23], and genes of proteins with a highly conserved cold shock domain (CSD) [24]. Cold shock proteins (CSPs) are composed of a single CSD and function as RNA chaperones in bacterial proteins [25,26]. In *Escherichia coli*, CSPs are composed exclusively of CSD and function as RNA chaperones that destabilize the secondary structures of RNA. CSD proteins present in plants differ from CSPs found in prokaryotes [27]. CSP homologues, which play a major role in resistance to abiotic factors—cold, salt and drought—are found in plants [28]. A number of bacterial CSP and CSD of plants are induced under cold stress conditions [29,30]. 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 [1,31–33].

Over the past two decades, a number of genes have been identified that can increase plant resistance to chilling and freezing. For example, in study by Kim et al. [34], non-acclimated *SCOF-1* transgenic *Arabidopsis* and wild-type plants were frozen at -7°C in the dark. After being returned to normal growth temperature, almost all wild-type plants were killed, but most of the *SCOF-1* transgenic plants survived. These results demonstrate that the constitutive overexpression of the soybean transcriptional activator *SCOF-1* induced *COR* gene expression in *Arabidopsis* and enhanced the frost tolerance of non-acclimated transgenic plants. In research by Vannini et al. [35], 3-week-old wild-type plants were severely damaged by cold stress and did not survive after 24 h at freezing conditions (-6°C); *Arabidopsis* transgenic plants with *Osm4* overexpression were undamaged by cold and survived after freezing. Zhu et al. [36] demonstrated that at freezing *Arabidopsis* plants for 3 days at -4°C , the wild type is damaged by the exposure to cold stress because more than 25% of the total electrolytes are released, indicating severe tissue damage. The transgenic plant lines with over-expression of *Thp1* from *Choristoneura fumiferana* exhibited half lower electrolyte release from leaf tissue, as compared with wild-type plants. Deng et al. [37] subjected *Arabidopsis* seedlings to increasing cold stress with temperatures declining from 4°C (7 d) to -1°C (6 h) and then to -6°C (2 h). After freezing, plants were allowed 10 days for recovery. The *PeAPY2*-transgenic lines showed a significantly higher survival rate (71–83%) than wild-type plants (34%).

The property of all studied cold shock domain proteins (CSDPs) is their ability to bind nucleic acids. This ability is determined by the CSD domain structure and the presence of two conservative motifs, RNP1 and RNP2, directly interacting with RNA and DNA molecules. A characteristic feature of plant CSDPs is the presence of “zinc finger” motifs in the C-terminal amino acid sequence, separated by regions rich in glycine. CSD proteins have been shown to play an important role in developing and adapting to stresses in all the kingdoms of living organisms, where they are found in bacteria, in plants, and in animals [27].

The results obtained from the investigations of *Eutrema salsugineum* (recent name-*Thellungiella salsuginea*) revealed that EsCSDP1–4 are involved in cold acclimatization of plants and can be used in biotechnology of cultivated plants in order to increase their resistance to cold stress [38]. The *E. salsugineum* genome contains four genes encoding two “long” proteins (EsCSDP1 and EsCSDP3) with six and seven ZnFs, respectively, and two “short” proteins (EsCSDP2 and EsCSDP4) with two ZnFs [39], the functional activity of

which is associated with their RNA-chaperone activity [27], including the ability to melt the secondary structures of RNA and DNA [26,29,32]. In a study of the ability of EsCSDP1–4 proteins to specifically interact with nucleic acids, the unique ability of the cold shock domain of EsCSDP3 protein was found to effectively restore the growth of mutant strain *E. coli* (BX04 strain) cells at low temperatures. Thus, in the studies of Taranov et al. [39], of the six studied cold shock domains of *A. thaliana* and *E. salsgineum* proteins, only one, EsCSD3, showed activity in the complementation test for *E. coli* cell growth of the BX04 strain, similar to that of the native bacterial protein CspA. Freezing is a strongly stress-damaging, including to the contents of the cell nucleus. DNA structures containing alternating one and two strand sequences of nucleic acids are quite often formed as a result of destabilization of the DNA double helix, which require reparations. In an article by Zlobin et al. [40], it was shown that among the EsCSDP1–4 studied, only EsCSDP3 protein demonstrated high melting activity of partially double-stranded DNA. In all likelihood, the ability to nuclear localization of EsCSDP3 and its DNA-melting activity indicate a possible participation in the mechanisms associated with the repair of the destabilized DNA structures [40].

We assumed that the protein EsCSDP3 was able to have a significant role in the formation of resistance to cold stress. The purpose of our work was to assess the potential effectiveness of using EsCSDP3 to add additional cold and frost resistance to agricultural crops. As a plant-reference with increased cold resistance, it was decided to use transgenic rape plants expressing an efficiently increased cold resistance bacterial protein CspA. The potential for resistance to cold stress in transgenic rape was evaluated in the heterozygous T₁ generation. Winter rape was chosen as the object under study, as a crop in the life cycle of which cold stresses are critical and, at the same time, a necessary part of the vegetation season. As a result, heterozygous transgenic T₁ rapeseed lines with EsCSDP3 expression were obtained, which were not inferior in cold and frost resistance to T₁ rapeseed lines with CspA expression.

2. Materials and Methods

2.1. Gene Synthesis and Plasmid Construction

The vector constructs pBI121-CSPA-plant (Figure 1a) and pBI-EsCSDP3 (Figure 1b) were created on the basis of pBI121 vector by replacing the β -glucuronidase synthesis gene which also has the target *cspA* gene isolated from *Escherichia coli* with the optimized codon composition for the plants of the cabbage family (*Brassicaceae*) (*cspA-plant*) and the target *Escsdp3* gene, encoding the protein with the cold shock domain of *E. salsgineum* [40,41], respectively.

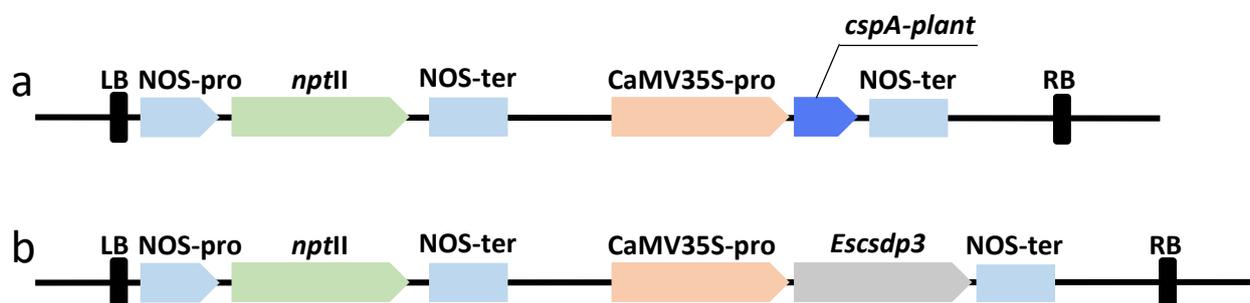


Figure 1. Schematic depiction of the expression cassettes of vectors pBI121-CSPA-plant (a) and pBI-EsCSDP3 (b) plasmids used for winter rape transformation. Target genes are under the control of the 35S promoter from the cauliflower mosaic virus (CaMV35S-pro) and the nopaline synthase terminator (NOS-ter) from *Agrobacterium tumefaciens*. The selective gene for the synthesis of neomycin phosphotransferase (*nptII*) is under the control of the regulatory elements of the nopaline synthase gene (NOS-pro).

2.2. Optimization of the Codon Usage of the *cspA* Gene

The amino acid sequence of the protein, which is 77 amino acids, was taken from the GenBank (GenBank: AIF96121.1). The encoding *CspA* protein nucleotide sequence, which is optimal for plants of *Brassicaceae* (Figure 2), was established using the JCat program [42]. Further on we amplified the full-length *cspA-plant* gene sequence, flanked by the *VspI* restriction site at the 5'-end and the *BamHI* at the 3'-end, by "PCR-based accurate synthesis (PAS) of long DNA sequences" [43].

	M S G K M T G I V K W F N A D K G F G F	
CspA	ATGTCGCGTAAAAATGACTGGTATCGTAAAAATGGTTCAACGCTGACAAAGGCTTCGGCTTC	60
CspA-plant	ATGCTGGAAAAGATGACTGGAATTGTAAAGTGGTTTAAAGCTGATAAGGGATTGGATT	60
	***** ** * ***** ** ** * ***** ** ***** ** * * * * *	
	I T P D D G S K D V F V H F S A I Q N D	
CspA	ATCACTCCTGACGATGGCTCTAAAGATGTGTTTCGTACACTTCTCTGCTATCCAGAACGAT	120
CspA-plant	ATTACTCCTGATGATGGATCTAAGGATGTTTTTGTTCATTTTCTGCTATTCAAAATGAT	120
	** ***** * ***** ***** ***** ** ** * * ***** ** * * *	
	G Y K S L D E G Q K V S F T I E S G A K	
CspA	GGTTACAAATCTCTGGACGAAGGTCAGAAAGTGTCTTCACCATCGAAAGCGGCGCTAAA	180
CspA-plant	GGATATAAGTCTTTGGATGAAGGACAAAAGGTTTCTTTTACTATTGAATCTGGAGCTAAG	180
	** ** *	
	G P A A G N V T S L *	
CspA	GGCCCCGCGAGCTGGTAACGTAACCAGCCTGTAA	213
CspA-plant	GGACCTGCTGCTGGAAAATGTTACTTCTTTGTAA	213
	** ** *	

Figure 2. Alignment of the nucleotide sequences of the native *cspA* gene and the *cspA* gene with the optimized codon composition. The 55 substitutions (marked in yellow) were made in the *cspA* gene nucleotide sequence; the percent identity of the original and modified nucleotide sequences was 74%, and the identity of the codons was 32%.

2.3. Mutant Bacteria Growth Complementation

The synthesized *cspA* gene was cloned into the pINIII expression plasmid vector [25] using the *VspI* restriction sites at the 5'-end and *BamHI* at the 3'-end, by standard procedures. The resulting genetic construct was designated as pINIII-*cspA*_(plant). The genetic construct based on the pINIII vector containing the native *cspA* gene (pINIII-*cspA*) was described previously [39]. Cells of the quadruple mutant *E. coli* BX04 ($\Delta cspA$, $\Delta cspB$, $\Delta cspE$, $\Delta cspG$) were transformed by pINIII-*cspA*_(plant), pINIII-*cspA* constructs, and original pINIII vector to test the codon-optimized *cspA-plant* gene and to restore growth ability at low temperature (+17 °C). The transformed cells were grown in a liquid medium (LB) in the presence of the antibiotics ampicillin (0.2 g L⁻¹) and kanamycin (0.05 g L⁻¹) up to an optical density of OD₅₅₀ = 0.8, then the multiplied cells were diluted to the concentrations of 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶, and after that, the cell amount of 7 μ L was applied onto the surface of solid LB medium in Petri dishes containing ampicillin (0.2 g L⁻¹), Km (0.05 g L⁻¹), and isopropyl β -D-1-thiogalactopyranoside (0.2 M). The plates were incubated at 17 °C for five days.

2.4. Creation of Genetic Constructs for Overexpression

The genetic construct harboring the *cspA-plant* gene was amplified with the DNA of the pINIII-*cspA*_(plant) plasmid using the primers 5'-ATTGGATCCACCCTTTTGGCTGTTTCCTCTC-3' with the *BamHI* restriction site (underlined) and 5'-TAAGAGCTCTTAAGCAAGCGAAGAACAATCCCT-3' with the *SacI* restriction site (underlined). The obtained PCR fragment was cloned into the pBl121 vector at the restriction sites *BamHI* and *SacI*; the nucleotide sequence of the resulting genetic construct was verified by sequencing.

Isolation of total RNA, cDNA synthesis, and PCR amplification of *Escsdp3* was performed to create a genetic construct that included the coding part of the *Escsdp3* gene. Total RNA was isolated from the leaves of the *Eutrema salsugineum* using Trizole reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. cDNA was obtained by reverse transcription using SuperScript III revertase (Invitrogen, Carlsbad, CA, USA). Amplification of the *Escsdp3* gene was performed using primers 5'-ATTGGATCCACCCTTTTGGCTGTTTCCTCTC-3' with the BamH1 restriction site (underlined) and 5'-TAAGAGCTCTTAAGCAAGCGAAGAACATTCCCT-3' with the SacI restriction site (underlined). The resulting PCR fragment was cloned into a pGEM-T vector. Its nucleotide sequence was checked by sequencing, and then recloned into the pBI121 vector for agrobacterial transformation at the BamH1 and SacI restriction sites.

Vectors for plant transformation (pBI-EsCSDP3 (Figure 1a), pBI121-CSPA-plant (Figure 1b)) were introduced into a disarmed supervirulent strain of *Agrobacterium tumefaciens* AGL0 [44] by electroporation.

2.5. Tissue Culture, Agrobacterium-Mediated Transformation, and Obtaining Generation T₀

The research was carried out using Russian winter rape of the "Severyanin" variety (® 9811769, domestic selection created at FWRC FPA). It is the first double-zero ("00") variety to be used in the Central region of Russia.

Sterile seedlings of rape were used to produce plant explants. Seeds were sterilized superficially for 35 min by 30% water solution of sodium hypochlorite, then 5-fold washed with sterile water and subsequently placed on agarized basal Murashige and Skoog medium (MS) [45] for germination and growth in vitro. Hypocotyls with a size of 7 mm were excised from 15-day-old seedlings and placed for precultivation on agarized MS medium supplemented with 1.0 mg·L⁻¹ 2,4-D (2,4-dichlorophenoxyacetic acid) [46] for further *Agrobacterium*-mediated transformation.

Bacterial strain AGL0 [44], harboring plasmid pBI121-CSPA-plant and pBI-EsCSDP3, allowing for kanamycin selection of transgenic plant tissues, respectively, was used in agro-inoculation experiments.

Experiments were conducted by co-cultivation of explants (precultured hypocotyls) jointly with *Agrobacterium tumefaciens* suspension. An overnight culture of *A. tumefaciens* was prepared in 50 mL of liquid YEB medium, supplemented with 100 mg·L⁻¹ Km and 50 mg·L⁻¹ rifampicin, and shaken in an orbital shaker (180 rpm) within 24 h at 28 °C in the dark. Then it was diluted with sterile distilled water to a final concentration at an optical density of OD₆₀₀ = 0.4–0.6. The explants were placed into a flask containing 50 mL of inoculum and kept for 30 min in an orbital shaker (100 rpm) for inoculation. After drying in air flow laminar box, the explants were transferred onto filter paper, placed on a solid surface of MS medium supplemented with 1.0 mg·L⁻¹ 2,4-D in Petri dishes, and co-cultivated in light conditions at a temperature of 21 °C for 72 h. After co-cultivation period, the explants were washed with liquid MS medium, with a lack organic components but supplemented with 300 mg·L⁻¹ Tm, and then transferred to the medium for explants cultivation and elimination of *Agrobacterium* (MS medium containing 1.0 mg·L⁻¹ 2,4-D and 150 mg·L⁻¹ tmimentine (Tm) with the addition of 100 mg·L⁻¹ kanamycin sulfate (Km)). Two weeks later, the explants were transferred to regeneration medium—MS medium supplemented with 4.0 mg·L⁻¹ N⁶-benzyladenine, mg·L⁻¹ Zeatin, 5.0 mg·L⁻¹ AgNO₃ [47], and 150 mg·L⁻¹ Tm and 100 mg·L⁻¹ Km. Every 2 weeks, explants were transferred to fresh media.

The resulting Km-resistant regenerants were rooted in a half-strength MS medium with a lack of organic components and supplemented with 0.5 mg·L⁻¹ indole-3-butyric acid and 100 mg·L⁻¹ Km.

2.6. Adaptation, Growing Plants In Vivo, and Obtaining Generation T₁

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 obtain seed generation T₁. After adaptation, the plants were transplanted into large growing containers filled with soil and grown in a greenhouse until they reached the phenological phase of readiness for overwintering (formation of the fifth–sixth layer of leaves). Then the plants were transferred to the cold compartment of the greenhouse (+8 °C) to undergo the overwintering stage for 2 months, after which the plants were returned to the compartment of the greenhouse, where the temperature was maintained at +25 °C for flowering. As the peduncles formed, insulators were put on the plants to avoid over-pollination and to obtain full-fledged T₁ generation seeds.

2.7. Analysis of Transformants

2.7.1. Polymerase Chain Reaction (PCR)

For PCR analysis, the genomic DNA was isolated from antibiotic-resistant and wild-type plants of rape (T₀ and T₁ generations) using the method of Dellaporta et al. [48]. The respective forward and reverse primer sequences for *npt II* were Npt-mf-up: 5'-TCTGATGCCGCCGTGTTCC-3' and Npt-mf-low: 5'-ATGCGCGCCTTGAGCCTG-3' (anticipated amplification fragment of 748 bp); those for *Escsdp3* were CSDP-F: 5'-AATCACTCTGGGAAGCGACG-3' and CSDP-R: 5'-GGCCAAATCCACCACAGGTA-3' (anticipated amplification fragment of 512 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 *virC* were virC1: 5'-GCACTATCTACCTACCGCTACGTCATC-3' and virC2: 5'-TTGTCGATCGGGACTGTAAATGTG-3' (anticipated amplification fragment of 548 bp); those for *Actin-1* (LOC106445773, NCBI Reference Sequence: XM_013887403.2) were Act(BN)-F: 5'-AGCGATTCTGGGAACATGG-3' and Act(BN)-R: 5'-AGACGTTCAATGTCCCTGCC-3' (anticipated amplification fragment of 557 bp).

The PCR reactions for *npt II*, *virC*, and *Actin-1* genes were carried out in a 25 µL volume containing 2.5 µL of a 10 × PCR buffer, 0.5 µL of 10 mM dNTPs, 1 µL of two 10 µM primer, 1 µL of 5 U µL⁻¹ Taq polymerase, 17 µL of mQ H₂O, and 2 µL (≈60 ng) of a DNA template. Reactions were carried out in a Mastercycler nexus gradient (Eppendorf) as follows: one cycle of 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 45 s at 60 °C, 1 min at 72 °C, and one final cycle of 5 min at 72 °C. The products were separated in 1.0% agarose gel using the electrophoresis systems [Amersham Electrophoresis Power Supply–EPS 301 (Amersham, UK) + Hoefer HE 33 Mini Horizontal Submarine Unit (Holliston, MA, USA)].

For detecting the sequences of cold shock proteins (*cspA-plant* and *Escsdp3*) gene and checking T₁ plants for possible hybrid origin (to exclude the possibility of both cold shock proteins genes' presence in a single genome), PCR was performed with the simultaneous use of four sets of primers (CSDP-F, CSDP-R, CSPA-F, and CSPA-R); the reaction conditions are described above. The products were separated in 1.2% agarose gel using the electrophoresis systems.

2.7.2. Semiquantitative RT-PCR (Reverse Transcriptase PCR) and Quantitative RT-PCR Analyzes of Transgenic Rapeseed (Real-Time qPCR)

The selection of plant material for analysis was performed twice (before and after hardening of plants), as noted in Figure 3 (point 1 and 2). RNA samples were extracted from leaves of transgenic and control T₁ generation plants using GeneJET Plant RNA Purification Mini Kit (Thermo Fisher Scientific, Waltham, MA, United States). Each sample was treated with DNase (Thermo). The cDNA was synthesized using Revert Aid Minus Reverse Transcriptase (Thermo) according to the manufacturer's protocol using oligo(dT) 18 and Random (dN) 10 primers. For each sample, 3 µg of total RNA was taken for the reverse transcription reaction in a total volume of 20 µL following 2-fold dilution. An amount of 2 µL of three nucleic acid preparations (cDNA mix for the study of gene expression, mRNA mix and total DNA for control of reaction) was used for PCR reaction. House-

keeping *Actin-7* (LOC106418315, NCBI Reference Sequence: XM_013858992.2) gene was used as internal control. RT-PCR was performed using primer sets for cold shock proteins (*cspA-plant* and *Escsdp3*) genes as described below. The respective primers sequences for *Actin-7* were Act7(BNRT)F: 5'-GATTGAGCATGGTATTGTAAGC-3' and Act7(BNRT)R: 5'-CATCTTCTCCCTGTTAGCCTTA-3' (anticipated amplification fragment of 154 bp); those for *Escsdp3* were DP3(BNRT)2F: 5'-CATTACTCCCCGATGACGGC-3' and DP3(BNRT)2R: 5'-TCGCTTCCCAGAGTGATTGC-3' (anticipated amplification fragment of 154 bp); those for *cspA-plant* were PA(BNRT)F: 5'-ATGTCTGGAAAGATGACTGG-3' and PA(BNRT)R: 5'-CCTTAGATCCATCATCAGGAG-3' (anticipated amplification fragment of 157 bp).

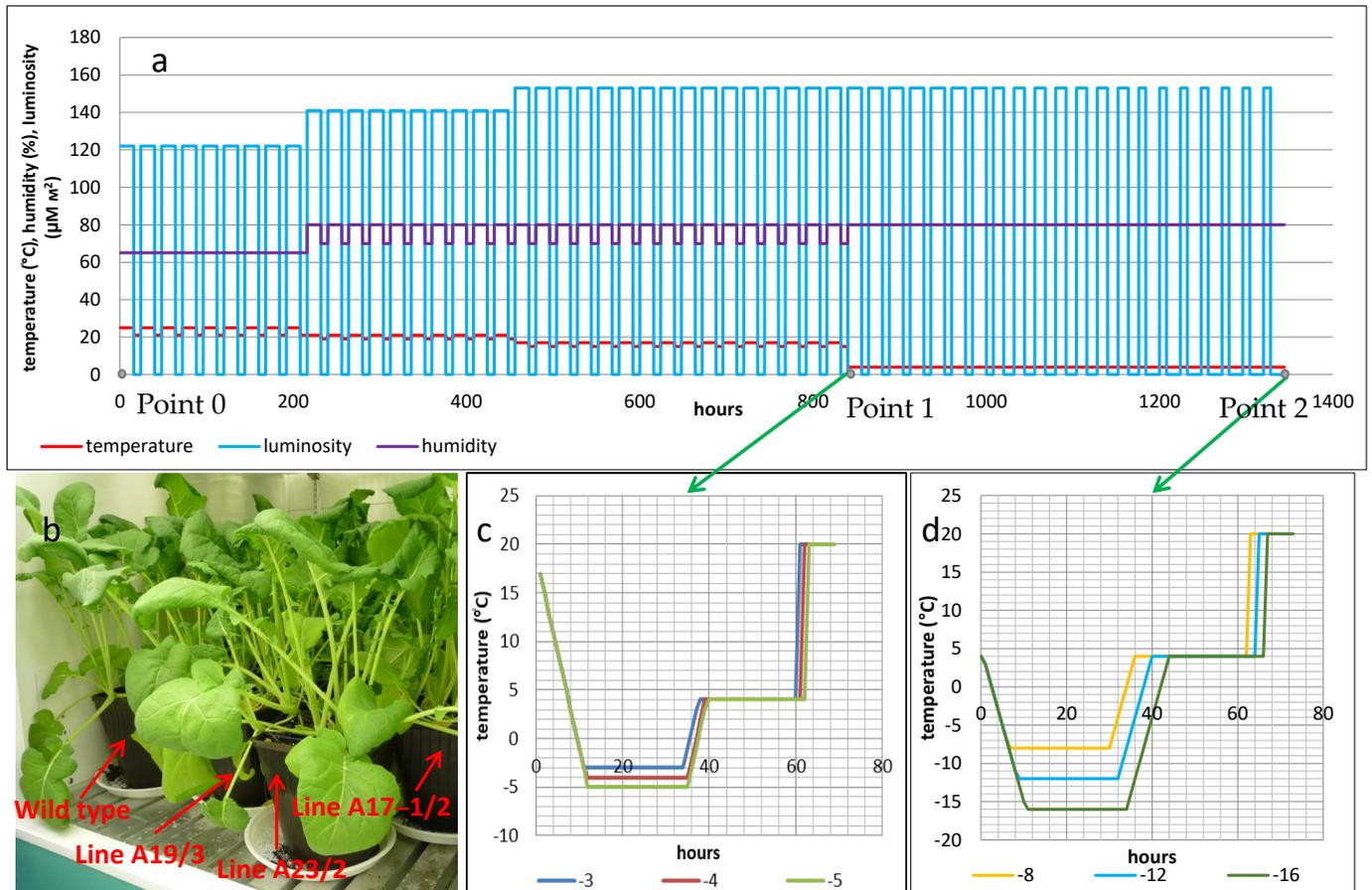


Figure 3. Cultivation of rape in climatic chamber. (a) Regimes of cultivation (point 0–point 1) and hardening (point 1–point 2). (b) The 55 day old hardened plants in climatic chamber Conviron MTR30 Reach. Programs of freezing for non-hardened (c) and hardened (d) transgenic rape lines and wild type.

The RT-PCR reactions for *Actin-7*, *cspA-plant* and *Escsdp3* genes were carried out in a 25 μL volume containing 2.5 μL of a 10 \times PCR buffer, 0.5 μL of 10 mM dNTPs, 1 μL of two 10 μM primer, 1 μL of 5 U μL^{-1} Taq polymerase, 17 μL of mQ H_2O , and 2 μL (≈ 40 ng) of a cDNA template. Reactions were carried out in a Mastercycler nexus gradient (Eppendorf) as follows: one cycle of 5 min at 94 $^{\circ}\text{C}$, followed by 35 cycles of 1 min at 94 $^{\circ}\text{C}$, 45 s at 59 $^{\circ}\text{C}$, 1 min at 72 $^{\circ}\text{C}$, and one final cycle of 5 min at 72 $^{\circ}\text{C}$. The products were separated in 1.4–1.6% agarose gel using the electrophoresis systems [Amersham Electrophoresis Power Supply–EPS 301 (Amersham, UK) + Hoefer HE 99 Horizontal Submarine Unit (Holliston, MA, USA)].

Real-Time qPCR was performed using primer sets for *cspA-plant* (PA(BNRT)F and PA(BNRT)R), *Escsdp3* (DP3(BNRT)2F and DP3(BNRT)2R) and *Actin-7* (Act7(BNRT)F and Act7(BNRT)R) gene as described above. PCR cycles were chosen so that none of analyzed samples reached a plateau at the end of the amplification; that is, they were in the ex-

ponential 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, Moscow, Russia) in a volume of 20 μ L. 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 [49] was used to normalize and calibrate the cold shock proteins (*cspA-plant* and *tscsdp3*) 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 (LightCycler[®] 96 Software 1.1 copyright F. Hoffmann-La Roche AG, Basel, Schweiz) and Microsoft Excel 2007 (copyright Microsoft Corporation, Redmond, Washington, DC, USA).

2.8. Southern Blot Analysis

T₁ generation rape genomic DNA (50 μ g) was isolated from 1 g of rapeseed plant leaves cultivated in protected ground. Isolation was carried out using a buffer containing CTAB detergent according to the procedure described in *Plant Molecular Biology Manual* [50]. DNA concentration was measured using a spectrophotometer Thermo Evolution 260 BIO (Waltham, MA, USA). An amount of 40 μ g of DNA of the selected lines and control plants were hydrolyzed overnight at 37 °C by the EcoRI enzyme (100 units) in a volume of 500 μ g. In the transferred T-DNA, the site of the selected restriction occurs once. The hydrolysis products were separated overnight by electrophoresis in 0.9% agarose gel at a voltage of 18V in the chamber Amersham MINNIE GEL UNIT (Amersham, UK). After that, the DNA was transferred to a positively charged Amersham 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 h at 80 °C. Probes for hybridization were prepared from PCR products using the AlkPhos Direct Labeling Kit (GE Healthcare, Amersham, UK). PCR products (186 bp and 512 bp) that were amplified using primers for PCR analysis were used to detect the *cspA* and *Escsdp3* genes. The integration of the *nptII* kanamycin resistance gene was confirmed using a probe consisting of 381 base pairs, which was generated using PCR and primers (5'-GCTATGACTGGGCACAACAGACAATC-3' and 5'-TCCGAGTACGTGCTCGCTCGA-3'). Pre-hybridization and hybridization were performed at a temperature from 56 °C 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 (Carestream Health, Rochester, New York, NY, USA) at room temperature. The X-ray film was scanned using an EPSON Perfection V750 PRO scanner (Suwa, Nagano, Japan).

2.9. Study of Plant Resistance to Cold and Frost

Plant Selection

After germination of T₁ generation seeds in the presence of 650 mg·L⁻¹ Km, 60 resistant plants of each line were selected for assays to determine the expression level of heterologous genes and to identify the effectiveness of introduced genes for increasing the cold and frost resistance of transgenic rapeseed lines. The seeds of the control plants were germinated in Petri dishes on filter paper moistened with distilled water.

After 10 days, the seedlings were planted into culture containers containing 2.5 L of a mixture of soil and 10% (*v/v*) agropelrite (Piter Peat), 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 (μ M m⁻²s⁻¹), humidity 80/70%, temperature 17/15 °C) until they reached the stage of 5–6 true leaves (35-day-old plants—point 1 in Figure 3a).

Some plants were selected to be tested for their frost resistance (point 1 in Figure 3a); the other part was hardened at a temperature of +4 °C in climatic chamber Conviron MTR30 Reach 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 $\mu\text{M m}^{-2}\text{s}^{-1}$ PAR intensity, humidity 80/80%, temperature 4/4 °C) (55-day-old plants—Figure 3b), and only after that were the plants subjected to cold stress (point 2 in Figure 3a).

Plants were frozen in a climatic chamber Celsius 10.0 (6 adult plants for each experimental line and control—non-transgenic plants of winter rape varieties “Severyanin”). Testing temperatures were achieved by reducing the initial cultivation temperature by 2 °C h⁻¹ (Figure 3b,c) (from +17 °C (point 1 in Figure 3a) or +4 °C (point 2 in Figure 3a) for different batches of plants). Then the plants were kept at negative temperatures for 24 h, (−3, −4, −5 °C for unhardened plants (Figure 3b) and −8, −12, −16 °C for hardened plants (Figure 3c)). After that, the temperature in the chamber was increased up to +4 °C at a rate of 2 °C h⁻¹ (Figure 3b,c). Leaf die cuts were taken from a newly formed leaf (the second leaf from the growth point along the central vascular bundle) for potentiometric analysis of the electrolytes outflow and detection of plant tissues damage by means of light microscopy. After that, the plants were kept at +4 °C for a further 24 h and then transferred to the greenhouse (16/8 h photoperiod, 21/21 °C temperature). A week later the total moisture content of plants was assessed.

2.10. Potentiometric Analysis of Electrolyte Leakage from Plant Tissue

Potentiometric analysis of electrolyte leakage from plant tissue was carried out using the method of Dexter et al. [51]. Selected die cuts were washed with distilled water and placed into falcon tubes (10 mL) with the addition of 5 mL of distilled water. The open tubes were then placed into a Bunsen flask and, by means of an IKA MVP10 basic pump, a vacuum of 15 mbar was created in the flask for 30 min, until the air bubbles completely left the solution and die-cuts, and the die-cuts dropped to the bottom of the tube, which led to the outflow of electrolytes through the damaged cell wall into distilled water. After that, the electrical conductivity of water in test tubes with cutouts (g1) was measured using the Ohaus Starter 300C device (Parsippany, NJ, USA). Next, the sample tubes were closed and autoclaved and then the conductivity of water in the tubes was re-measured, thereby determining the total electrolyte content in the sample (g2). The loss of cell electrolytes was calculated by the formula $A = \frac{g_1}{g_2} \times 100\%$ [52].

2.11. Light Microscopy and Tissue Preparation

The inspection of the selected plant material for detection of cold damage was carried out by light microscopy. The leaf fragments containing a central vascular bundle were fixed in 2.5% (w/v) glutaraldehyde dissolved in 0.1M phosphate buffer (pH 7.2), supplemented with 1.5% (w/v) sucrose. Then plant material was washed and postfixed in 1% (w/v) OsO₄, dehydrated in ethanol with increasing concentrations (30, 50, 70, 96, 100%), and subsequently in propylene oxide. Explants were stained with 0.1% methylene blue water solution and embedded in a mix of the epoxide resin Epon-812 and Araldite for light microscopy. Samples were studied and photographed with the use of an Olympus BX51 microscope (Olympus, Shinjuku, Tokyo, Japan) equipped with Color View II camera (Soft Imaging System, Munster, Germany).

2.12. Analysis of Moisture Retention in Plants

The dry matter and, accordingly, moisture content were assessed by the standard method (drying to a constant weight at 80 °C). The moisture content rate (1 unit) was taken as such in plants unexposed to cold stress (c2). The entire above-ground part of the tested plants subjected to cold stresses (including dead leaves) was dried a week after freezing (c1) to analyze the moisture content. The moisture content of the samples was determined by the formula $W = \frac{c_1}{c_2} \times 100\%$.

3. Results

3.1. Synthetic Gene and Plasmid Construction

The *cspA* gene sequence was optimized for expression in cabbage plants and was used to construct the pBI121-CSPA-plant construct. The functional activity of the synthesized gene was verified using the test for the restoration of growth ability in the fourth mutant *E. coli* (BX04: $\Delta cspA$, $\Delta cspB$, $\Delta cspE$, $\Delta cspG$) at low temperature (17 °C). Figure 4 shows that changes in the codon composition of the *cspA* gene did not affect the ability of the CspA protein to restore the growth of mutant bacteria of strain BX04 at low temperature. A positive test result indicates that the synthesized *cspA-plant* gene encodes a functionally active protein.

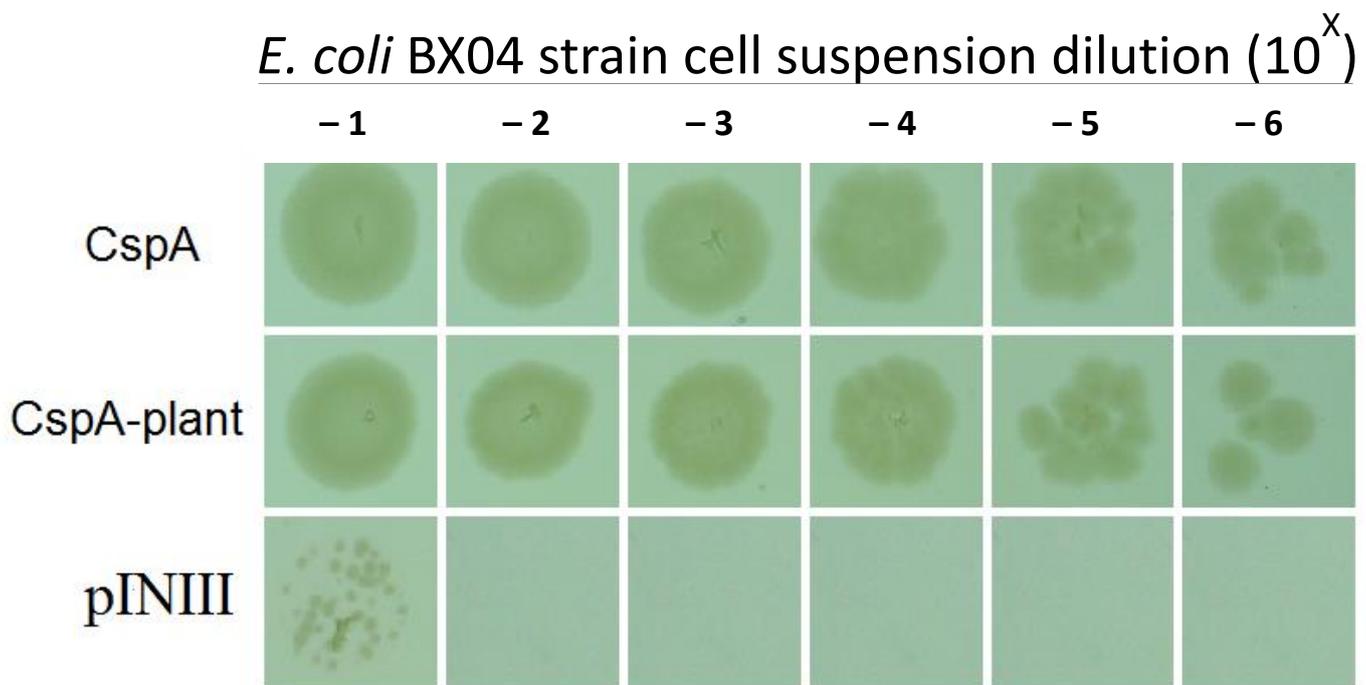


Figure 4. Plasmid expression of *cspA-plant* restores the growth capacity in the fourth mutant *E. coli* BX04 ($\Delta cspA$, $\Delta cspB$, $\Delta cspE$, $\Delta cspG$) at a low temperature (+17 °C). Mutant bacteria were transformed with an empty vector (PINIII), a vector containing the *cspA-plant* gene, and the native *cspA* gene (*E. coli*).

3.2. Agrobacterium-Mediated Transformation

As a result of the studies, nine kanamycin-resistant lines were obtained. PCR analysis revealed six transgenic rapeseed lines with the introduction of the T-DNA from pBI121-CSPA-plant (A19/3, A23/2, A17-1/2, A17-1/1, A17-2, and A10-5) construction and three lines with the insertion of the plasmid pBI-EsCSDP3 T-region (D6-1/1.2, D6/1, and D-H2). In total, 260 explants were involved in transformation of rapeseed using pBI121-CSPA-plant, and transformation efficiency was 2.3%, which is common for significant crop cultivars [53]. Seven hundred and fifty explants were involved in the transformation of rapeseed with the pBI-EsCSDP3 construction, and the efficiency was 0.4%. Apparently, already at the stage of transient synthesis, the protein CSDP3 negatively affects the regeneration potential of explants.

The obtained rapeseed lines were adapted to in vivo conditions and planted into a greenhouse to obtain seed of T₁ generation for further analysis on determination of the heterologous genes expression level and to reveal the efficiency of introduced genes on increasing of the cold resistance in transgenic rapeseed lines.

3.3. Analysis of Transformants

All transgenic lines of T₁ generation were analyzed by PCR analysis and Southern blot. The fragments of the expected size were amplified from the DNA of all analyzed transgenic lines. As it was shown by PCR, all of the nine lines contained only one type of the target cold shock gene sequence (Figure 5), which proves their non-hybrid status, despite flowering without significant spatial isolation in the greenhouse.

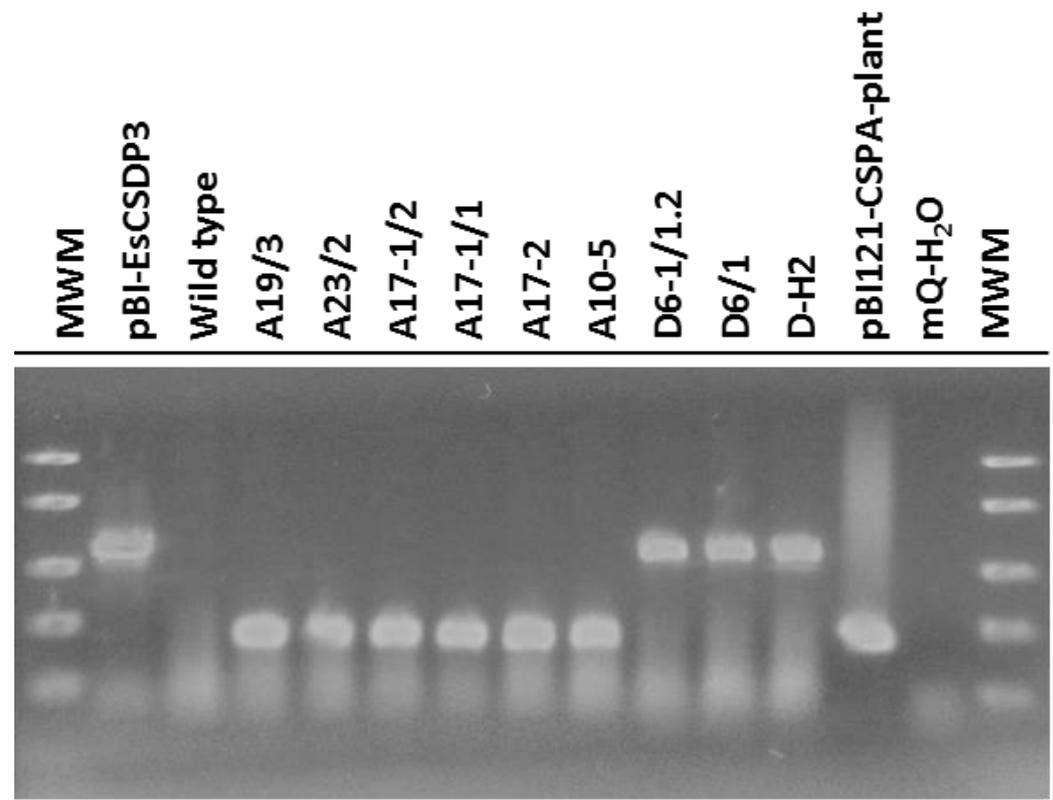


Figure 5. Multiplex-PCR analysis of *cspA-plant* and *Escsdp3* in independent Km-resistant T₁-generation of transgenic rape lines and wild type (MWM-molecular weight marker FastRuler™ Low Range DNA ladder-1500, 850, 400, 200, 50 bp).

Integration of the *cspA-plant*, *Escsdp3*, and *nptII* genes into the genome of Sevryanin rapeseed plants was confirmed by Southern blotting (Figure 6). The *cspA-plant* gene integration is shown in six lines (Figure 6a). The copy number of the gene varies among the lines. Thus, in lines A17-1/2, A17-1/1, and A10-5 there is a single copy of the *cspA-plant* gene, and in line A19/3 there are six copies, in line A23/2 there are five copies, and in line A17/2 there are four 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 (D6/1, D6-1/1.2, and D-H2), which significantly complicated the detection of the target signals on the membrane (Figure 6c, additional signals marked as red tick). Most likely it is due to the high homology of the transgene from *Arabidopsis* with the native genes of cold shock rapeseed proteins, as well as to the great diversity of these genes in the plant genome. An additional probe specific to the *nptII* gene was further used to more accurately confirm the transgenic status of the plants. It was found that there was a single copy insertion in line D6/1, two copies in line D6-1/1.2, and seven copies in line D-H2 (Figure 6b). However, the *nptII* gene was not detected in the DNA of untransformed rapeseed. Based on the profile of the bands obtained by hybridization, all lines are the result of an independent transformational event.

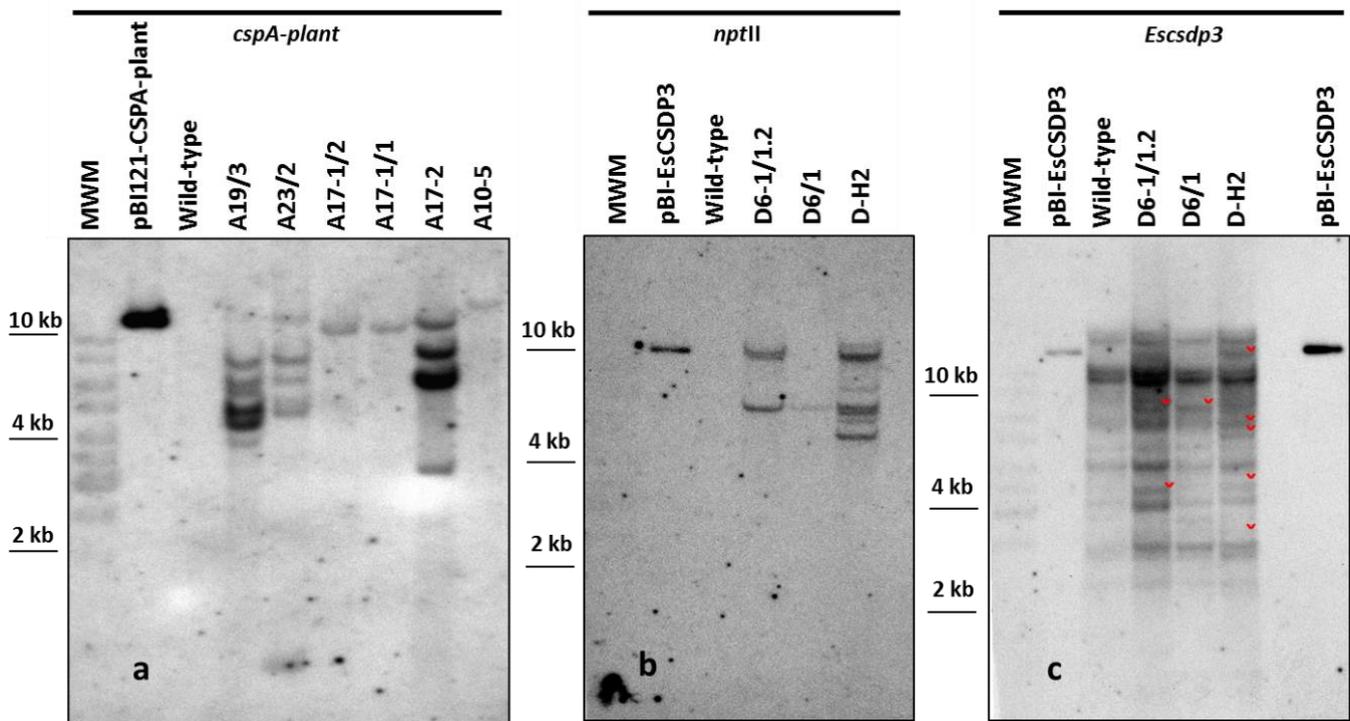


Figure 6. Southern hybridization analysis of 6 AGL0/pBI121-CSPA-plant-transformed (a) and 3 AGL0/pBI-EsCSDP3-transformed (b,c) line rape plants. MWM-molecular weight marker SM0331; Wild type—genomic DNA of non-transgenic rape plants.

Expression of target genes was shown in all transgenic lines by means of RT-PCR (Figure 7a,b). *Actin-7* was used as a reference gene. The signal of its expression on the cDNA matrix indicates the reactivity of the samples prepared for analysis. The absence of a signal on the original mRNA matrix confirms the purity of the cDNA samples that were used and indicates the reliability of the data obtained (Figure 7c). The equal size signals on the electrophoregram from the amplification products on the DNA and cDNA matrix of *Escsdp3* (Figure 7a) and *cspA-plant* (Figure 7b) genes indicates the correctness of PCR and reliably confirms the presence of target gene expression in all obtained transgenic lines.

The method of quantitative evaluation of the expression of *Escsdp3* and *cspA-plant* genes largely confirmed the results obtained with semi-quantitative RT-PCR. Expression of target genes was detected in all transgenic lines. The highest content mRNA of *Escsdp3* and *cspA-plant* genes in all lines was detected in non-hardened samples. Two lines (A19/3 and D6-1/1.2) showed a significantly higher level of target genes expression relative to the other lines (in hardened and non-hardened samples). The expression of *Escsdp3* in D6-1/1.2 line was an order of magnitude higher than in the transgenic lines D6/1 and D-H2 (Figure 8).

3.4. Study of Plant Resistance to Cold and Frost

Our studies have shown that low temperatures up to $-4\text{ }^{\circ}\text{C}$ do not adversely affect unhardened winter rape plants of the Severyanin variety. The critical temperature at which plant death begins is $-5\text{ }^{\circ}\text{C}$ (Figure 9a and Table 1). Cold damage to the leaf becomes critical and leads to rupture of the mesophyll tissue (Figure 9f, highlighted by blue arrows), and in the stomatal apertures it leads to peeling of the membrane and cell contents from the cell wall, and consequently to complete cell necrotization (Figure 9k, highlighted by blue arrows).

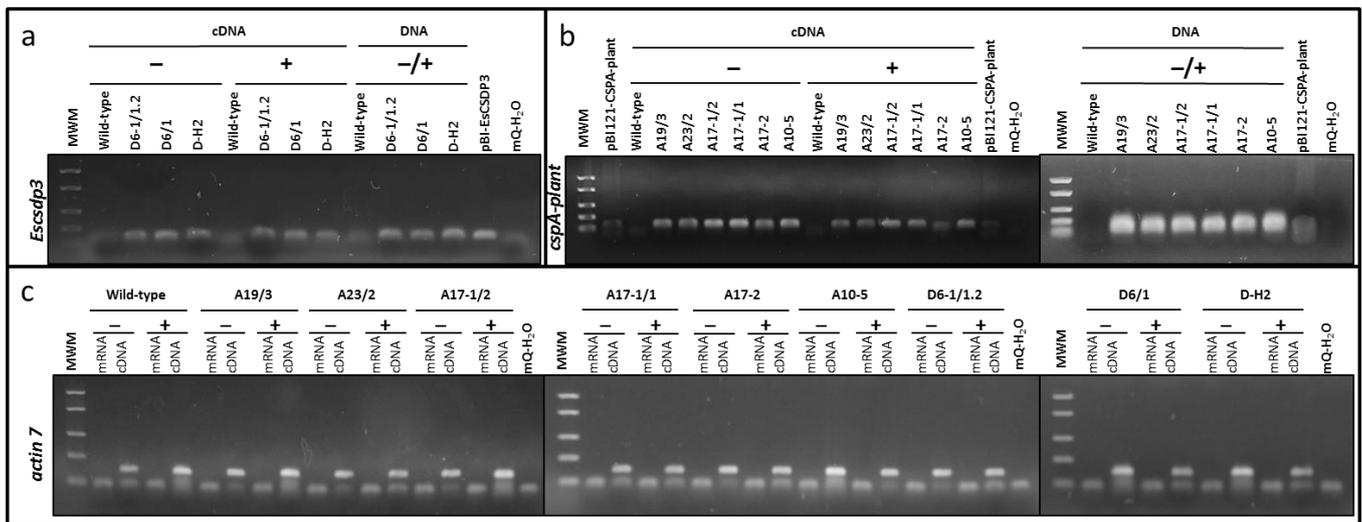


Figure 7. RT-PCR analysis of three genes (*Escsdp3* (a), *cspA-plant* (b) and housekeeping actin (*actin 7*) genes (c) in rapeseed plants. MWM-molecular weight marker (FastRuler™ Low Range DNA ladder-1500, 850, 400, 200, 50 bp); shows non-hardened (–) and hardened (+) transgenic rape lines and wild type. The PCR products from *Escsdp3* (amplification fragment of 154 bp) were separated in a 1.4% agarose gel at 100 mA (a), *cspA-plant* (amplification fragment of 157 bp) in a 1.4% agarose gel at 70 mA (b), and *actin 7* (amplification fragment of 154 bp) in a 1.6% agarose gel at 70 mA (c).

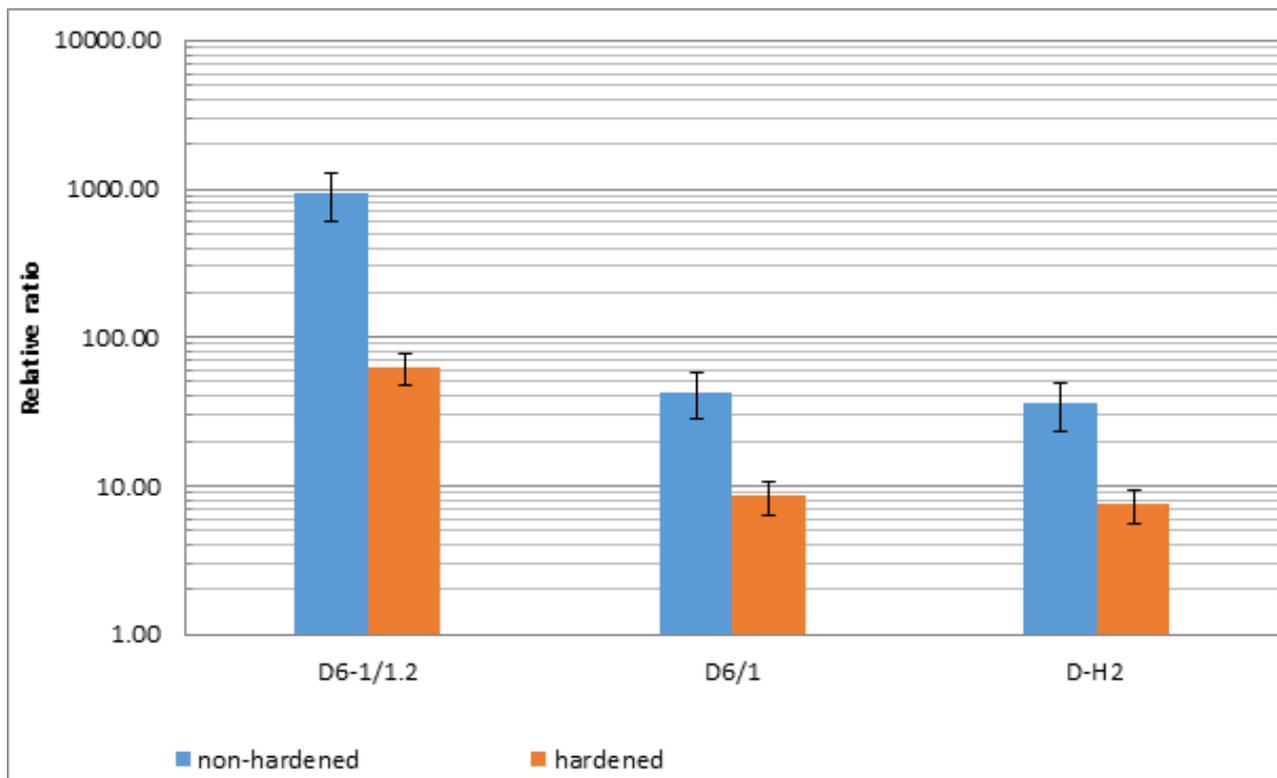


Figure 8. Relative expression levels of the *Escsdp3* gene in three transgenic rape lines. Data were normalized to the reference *actin 7* gene. Collecting plant material for real-time qPCR analyses was carried out at “Point 1” and “Point 2” according to the cultivation scheme shown in Figure 3a.

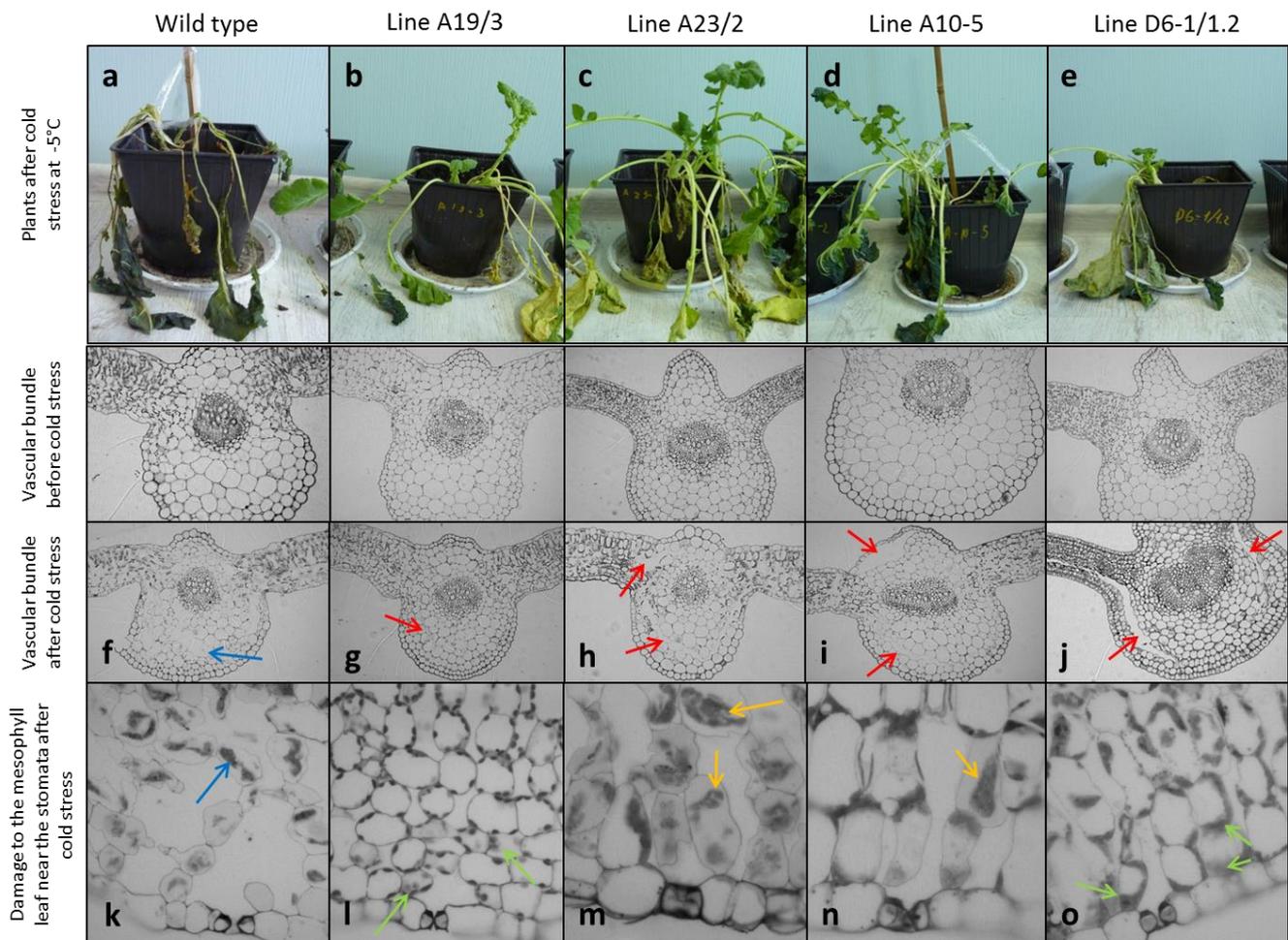


Figure 9. Light microscopy of non-hardened leaf tissue (surviving transgenic lines [A19/3 (b), A23/2 (c), A10-5 (d), and D6-1/1.2 (e)] and wild-type (a) plants before (35-day-old plants) and after freezing at -5°C according to the program shown in Figure 3c (37-day-old plants).

Table 1. Loss of electrolytes from leaves of non-hardened transgenic plants and non-transgenic plants on the first day after 24 h exposure at negative temperatures and water content in the same plants a week after exposure at negative temperatures relative to plants without freezing.

Name of Line	Loss of Electrolytes from Plant Tissues (%)			Saturation of Water Inside Plant Tissues (%)		
	-3°C at 24 h	-4°C at 24 h	-5°C at 24 h	-3°C at 24 h	-4°C at 24 h	-5°C at 24 h
Wild type	16.1 ± 1.7	23.9 ± 2.1	71.4 ± 7.4	97.3 ± 2.1	95.2 ± 2.4	42.5 ± 2.7
A19/3	10.7 ± 1.3	12.7 ± 0.8	19.0 ± 1.2	98.0 ± 2.3	96.0 ± 2.8	87.2 ± 2.4
A23/2	12.9 ± 1.6	16.1 ± 1.1	34.8 ± 2.0	100.7 ± 1.8	97.6 ± 1.7	85.1 ± 2.5
A17-1/1	21.6 ± 2.1	27.2 ± 2.9	63.7 ± 3.4	95.4 ± 2.4	57.9 ± 2.3	32.5 ± 3.3
A17-1/2	14.6 ± 1.5	27.9 ± 2.3	68.2 ± 6.5	93.6 ± 2.2	80.3 ± 2.8	54.3 ± 2.1
A17-2	14.2 ± 1.2	16.5 ± 0.7	58.3 ± 3.7	87.8 ± 3.1	81.8 ± 3.6	51.3 ± 3.8
A10-5	12.7 ± 0.9	16.7 ± 0.8	36.9 ± 2.1	100.2 ± 1.9	98.9 ± 1.7	88.5 ± 2.4
D6-1/1.2	11.0 ± 1.1	17.3 ± 1.0	38.9 ± 2.8	99.6 ± 2.2	93.9 ± 2.8	82.5 ± 2.7
D6/1	12.3 ± 1.1	16.9 ± 1.5	55.8 ± 3.3	98.0 ± 2.6	94.8 ± 2.4	40.8 ± 3.1
D-H2	13.0 ± 0.7	15.8 ± 1.2	57.1 ± 3.6	99.7 ± 2.3	96.7 ± 2.6	52.2 ± 3.6

Non-transgenic rape plant that was not subjected to freezing was used as a reference; its values were taken as 100%. Fatal results are marked in red.

As a result, the loss of electrolytes in leaf tissues of non-transgenic plants and a number of transgenic lines (A17-1/2, A17-1/1, A17/2, D6/1, and D-H2) exceeds the critical level of 46% (Table 1); thus, the plant loses its ability to retain moisture in the tissues. After a week, such plants are unable to retain even 60% of the initially moisture content (Table 1), which leads to wilting and drying of plant green mass (Figure 9a). After cold freezing at $-5\text{ }^{\circ}\text{C}$, only four lines of transgenic rapeseed plants (A19/3, A23/2, A10-5, and D6-1/1.2) remained alive (Figure 9b–e). In these lines, mesophyll tissue ruptures were local and insignificant (in Figure 9g–j, they are highlighted by red arrows). In the region of the stomatal apertures, membrane peeling was observed only in a small number of cells adjacent to the aperture (in Figure 9m–n, it is indicated by orange arrows) or had a partial character (in Figure 9l–o, it is indicated by green arrows).

Hardening has led to a significant increase in frost resistance of plants. Thus, after freezing at $-8\text{ }^{\circ}\text{C}$, all lines except the A17/2 line remained alive (Table 2); after cold processing at $-12\text{ }^{\circ}\text{C}$, lines A19/3, A 10-5, D6-1/1.2, and D6/1 survived (lines A17-1/2, A17-1/1, A17/2, A23/2, D-H2 and the wild-type control plant died). Only two transgenic lines (A19/3 and D6-1/1.2) remained alive after freezing at a temperature of $-16\text{ }^{\circ}\text{C}$. After freezing at this temperature, those lines showed the lowest outflow of electrolytes (the loss did not exceed 28.2% for the line D6-1/1.2 and did not exceed 34.7% for the line A19/3 (Table 2). A week after stress, the proportion of retained moisture in tissues was 66.7% in line D6-1/1.2 and 63.5% in line a 19/3 (Table 2). Damages at the cellular level were similar in nature to those presented in Figure 8 for plants of those lines and wild type.

Table 2. Loss of electrolytes (%) from leaves of hardened transgenic plants and non-transgenic plants on the first day after 24 h exposure at negative temperatures and water content (%) in the same plants a week after exposure at negative temperatures relative to plants without freezing.

Name of Line	Loss of Electrolytes from Plant Tissues (%)			Saturation of Water Inside Plant Tissues (%)		
	$-8\text{ }^{\circ}\text{C}$ at 24 h	$-12\text{ }^{\circ}\text{C}$ at 24 h	$-16\text{ }^{\circ}\text{C}$ at 24 h	$-8\text{ }^{\circ}\text{C}$ at 24 h	$-12\text{ }^{\circ}\text{C}$ at 24 h	$-16\text{ }^{\circ}\text{C}$ at 24 h
Wild type	11.9 ± 2.1	48.8 ± 4.3	62.7 ± 4.0	77.5 ± 3.2	56.6 ± 3.4	47.6 ± 3.8
A19/3	14.0 ± 2.4	20.6 ± 2.3	34.7 ± 2.9	95.0 ± 4.6	67.6 ± 3.4	63.5 ± 3.6
A23/2	8.0 ± 0.6	20.4 ± 2.1	48.1 ± 5.1	98.2 ± 2.2	69.8 ± 2.3	55.7 ± 2.6
A17-1/1	44.4 ± 5.6	57.9 ± 5.8	70.5 ± 6.2	66.9 ± 4.1	45.9 ± 3.6	36.8 ± 3.4
A17-1/2	11.3 ± 2.2	20.3 ± 2.0	53.3 ± 3.3	83.3 ± 3.3	56.2 ± 3.4	31.4 ± 2.8
A17-2	12.9 ± 1.2	39.4 ± 2.3	61.3 ± 4.6	75.7 ± 2.9	58.4 ± 2.7	47.1 ± 2.4
A10-5	10.2 ± 1.7	24.8 ± 2.2	47.2 ± 3.9	95.3 ± 4.6	71.9 ± 4.1	57.0 ± 3.8
D6-1/1.2	7.3 ± 0.9	13.0 ± 1.4	28.2 ± 2.0	90.4 ± 3.5	78.6 ± 3.4	66.7 ± 2.7
D6/1	6.2 ± 1.1	15.0 ± 1.5	38.4 ± 1.6	98.1 ± 2.4	59.6 ± 3.1	42.1 ± 3.6
D-H2	10.4 ± 1.5	38.4 ± 4.3	49.8 ± 3.8	98.4 ± 1.8	66.8 ± 2.4	40.6 ± 1.9

Non-transgenic hardened rape plant that was not subjected to freezing was used as a reference; its values were taken as 100%. Fatal results are marked in red.

Thus, as a result of our studies, we obtained four cold-resistant lines of winter rape—namely, three lines with the *cspA-plant* gene (A19/3, A23/2, A10-5) and the D6-1/1.2 line with the *Escsdp3* gene (Table 1). Two of them are frost-resistant (A19/3 and D6-1/1.2) (Table 2).

4. 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 [54]. Genes involved in protection or repair mechanisms could be new targets for the improvement of plant plasticity and adaptive responses to stress [55]. Finding new genes responsible for abiotic stress tolerance phenotypes is of great importance not only for a better understanding of stress responses but also for promising future crop improvement [17]. Transgenic technologies have opened up many interesting possibilities for

increasing resistance to cold stress in plants by introducing or removing a gene or genes that control a particular trait [56]. They also provide unique opportunities to improve the genetic potential of plants by creating specific crop varieties that are more resistant to cold stress. Over the past two decades, a number of genes have been identified that can increase plant resistance to cold [34–37].

In this work, we investigated the previously found *Escsdp3* gene [38–40] from the extremophyte plant *E. salsugineum* for the effectiveness of its application for imparting additional cold resistance and frost resistance to agricultural crops. Data on the level of mRNA content of the *Escsdp3* gene in plant tissues correlate with data on cold and frost resistance of the corresponding transgenic lines. Thus, the line with the highest expression of the *Escsdp3* gene (D6-1/1.2) demonstrated the highest cold and frost resistance among all tested lines with introduction of the T-DNA from pBI-TsCSDP3 (D6/1 and D-H2) construction into the rape genome. The transfer of *Escsdp3* gene into winter rape plants led to a significant increase in frost resistance of hardened plants and had a weak effect on unhardened plants. One transgenic line (D6-1/1.2) with expression of the *Escsdp3* gene remained alive after freezing 24 h at $-16\text{ }^{\circ}\text{C}$, while the wild type completely died after freezing 24 h at $-12\text{ }^{\circ}\text{C}$.

Kim et al. [57] obtained a similar result when overexpressing the *AtCSDP3* gene in *Arabidopsis*. However, according to their data, overexpression of *AtCSDP3* increased the survival of both hardened and non-hardened *Arabidopsis* plants equally by 1–2 $^{\circ}\text{C}$. The difference with our results is most likely not due to the differences in the amino acid sequences of the EsCSDP3 and AtCSDP3 proteins (91% identity) but to the differences in methods for measuring frost resistance and plant growth. In the aforementioned work, the resistance was assessed on ten-day-old seedlings grown under aseptic conditions on an artificial medium with the addition of 2% sucrose, while our experiments were carried out on six-week-old plants grown in soil and at the phenophase of readiness for hardening and overwintering.

Our results indicate the prospects of using these genes and the possibly of using the other CSD protein genes in crop biotechnology in order to increase their resistance to low-temperature stress. Our studies show how the scope for further improving the traits of traditional plants cultivars can be widened using cold shock domain proteins. For further research aimed at improving the cold resistance of plants, we are going to obtain and study transgenic plants with integration into genome *DREB1* and *Escsdp3* genes under the control of the stress-inducible *rd29A* promoter [58,59]. We assume that the use of plant inducible regulatory elements will have a positive effect on improving cold resistance in transgenic crops.

Author Contributions: The authors have made the following declarations regarding their contributions: Research planning and experiment analysis: M.C., P.K., and S.D.; gene synthesis and plasmid construction: V.T. and A.B.; *Agrobacterium*-mediated transformation: M.C. and P.K.; Southern blot analysis: A.P.; polymerase chain reaction, RT-PCR, and real-time qPCR: P.K. and G.M.; study of plant resistance to cold and frost: P.K. and V.F.; light microscopy: I.C.; contributed to the writing of the manuscript: M.C., P.K., V.T., and S.D. All authors have read and agreed to the published version of the manuscript.

Funding: Studies were carried out in accordance with the State Order (0574-2014-0024), State registration number AAAA-A17-117082950004-9, and with the State Order (0829-2016-0001), State registration number AAAA-A18-118110890010-8.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: This research was carried out using the unique scientific facilities “PHYTO-BIOGEN” of the «NBS-NSC» RAS (reg. no. 669802; Yalta, Russia) and «PHYTOTRON» reg. #2–2.9 of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS (reg. no 73597; Pushchino, Russia).

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Rihan, H.Z.; Al-Issawi, M.; Fuller, M.P. Advances in physiological and molecular aspects of plant cold tolerance. *J. Plant Interact.* **2017**, *12*, 143157. [[CrossRef](#)]
2. Leisner, C.P. Climate change impacts on food security- focus on perennial cropping systems and nutritional value. *Plant Sci.* **2020**, *293*, 1104122. [[CrossRef](#)]
3. Guo, X.Y.; Liu, D.F.; Chong, K. Cold signaling in plants: Insights into mechanisms and regulation. *J. Integr. Plant Biol.* **2018**, *60*, 745–756. [[CrossRef](#)]
4. Liu, J.; Shi, Y.; Yang, S. Insights into the regulation of CBF cold signaling in plants. *J. Integr. Plant Biol.* **2018**, *9*, 780–795. [[CrossRef](#)] [[PubMed](#)]
5. Yanglin, D.; Yiting, S.; Shuhua, Y. Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants. *New Phytol.* **2019**, *222*, 1690–1704.
6. Hannah, M.A.; Heyer, A.G.; Hinch, D.K. A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. *PLoS Genet.* **2005**, *1*, e26. [[CrossRef](#)]
7. Heidarvand, L.; Maali Amiri, R. What happens in plant molecular responses to cold stress? *Acta Physiol. Plant* **2010**, *32*, 419–431. [[CrossRef](#)]
8. Shi, Y.; Ding, Y.; Yang, S. Molecular regulation of CBF signaling in cold acclimation. *Trends Plant Sci.* **2018**, *23*, 623–637. [[CrossRef](#)]
9. Jaglo, K.R.; Kleff, S.; Amundsen, K.L.; Zhang, X.; Haake, V.; Zhang, J.Z.; Deits, T.; Thomashow, M.F. Components of the *Arabidopsis* C-repeat/dehydration responsive element binding factor cold-response pathway are conserved in *Brassica napus* and other plant species. *Plant Physiol.* **2001**, *127*, 910–917.
10. Choi, D.-W.; Rodriguez, E.M.; Close, T.J. Barley Cbf3 gene identification, expression pattern, and map location. *Plant Physiol.* **2002**, *129*, 1781–1787. [[CrossRef](#)]
11. Gao, M.-J.; Allard, G.; Byass, L.; Flanagan, A.M.; Singh, J. Regulation and characterization of four CBF transcription factors from *Brassica napus*. *Plant Mol. Biol.* **2002**, *49*, 459–471. [[CrossRef](#)] [[PubMed](#)]
12. Owens, C.L.; Thomashow, M.F.; Hancock, J.F.; Iezzoni, A.F. CBF1 orthologs in sour cherry and strawberry and the heterologous expression of CBF1 in strawberry. *J. Am. Soc. Hortic. Sci.* **2002**, *127*, 489–494. [[CrossRef](#)]
13. Dubouzet, J.G.; Sakuma, Y.; Ito, Y.; Kasuga, M.; Dubouzet, E.G.; Miura, S.; Seki, M.; Shinozaki, K.; Yamaguchi-Shinozaki, K. OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J.* **2003**, *33*, 751–763. [[CrossRef](#)] [[PubMed](#)]
14. Vágújfalvi, A.; Galiba, G.; Cattivelli, L.; Dubcovsky, J. The cold regulated transcriptional activator Cbf3 is linked to the frost-tolerance locus Fr-A2 on wheat chromosome 5A. *Mol. Genet. Genom.* **2003**, *269*, 60–67. [[CrossRef](#)]
15. Xue, G.-P. The DNA-binding activity of an AP2 transcriptional activator HvCBF2 involved in regulation of low-temperature responsive genes in barley is modulated by temperature. *Plant J.* **2003**, *33*, 373–383. [[CrossRef](#)]
16. Francia, E.; Rizza, F.; Cattivelli, L.; Stanca, A.M.; Galiba, G.; Toth, B.; Hayes, P.M.; Skinner, J.S.; Pecchioni, N. Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map. *Theor. Appl. Genet.* **2004**, *108*, 670–680. [[CrossRef](#)]
17. Sanghera, G.S.; Wani, S.H.; Hussain, W.; Singh, N.B. Engineering cold stress tolerance in crop plants. *Curr. Genom.* **2011**, *12*, 30. [[CrossRef](#)]
18. Mizoi, J.; Shinozaki, K.; Yamaguchi-Shinozaki, K. AP2/ERF family transcription factors in plant abiotic stress responses. *Biochim. Biophys. Acta* **2012**, *1819*, 86–96. [[CrossRef](#)]
19. Wisniewski, M.; Nassuth, A.; Teulier, C.; Marque, C.; Rowland, J.; Cao, P.B.; Brown, A. Genomics of cold hardiness in woody plants. *Crit. Rev. Plant Sci.* **2014**, *33*, 92–124. [[CrossRef](#)]
20. Gilmour, S.J.; Fowler, S.J.; Thomashow, M.F. *Arabidopsis* transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. *Plant Mol. Biol.* **2004**, *54*, 767–781. [[CrossRef](#)] [[PubMed](#)]
21. Vazquez-Hernandez, M.; Romero, I.; Escribano, M.I.; Merodio, C.; Sanchez-Ballesta, M.T. Deciphering the role of CBF/DREB transcription factors and dehydrins in maintaining the quality of table grapes cv. autumn royal treated with high CO₂ levels and stored at 0 °C. *Front. Plant Sci.* **2017**, *8*, 1591. [[CrossRef](#)] [[PubMed](#)]
22. Umezawa, T.; Fujita, M.; Fujita, Y.; Yamaguchi-Shinozaki, K.; Shinozaki, K. Engineering drought tolerance in plants: Discovering and tailoring genes to unlock the future. *Curr. Opin. Biotechnol.* **2006**, *17*, 113–122. [[CrossRef](#)] [[PubMed](#)]
23. Xin, Z.; Mandaokar, A.; Chen, J.; Last, R.L.; Browse, J. *Arabidopsis* ESK1 encodes a novel regulator of freezing tolerance. *Plant J.* **2007**, *49*, 786–799. [[CrossRef](#)] [[PubMed](#)]
24. Skabkin, M.A.; Skabkina, O.V.; Ovchinnikov, L.P. Multifunctional proteins with a cold shock domain in the regulation of gene expression. *Adv. Biol. Chem.* **2004**, *44*, 3–52. (In Russian)
25. Xia, B.; Ke, H.P.; Inouye, M. Acquisition of cold sensitivity by quadruple deletion of the *cspA* family and its suppression by PNPase S1 domain in *Escherichia coli*. *Mol. Microbiol.* **2001**, *40*, 179–188. [[CrossRef](#)]
26. Nakaminami, K.; Sasaki, K.; Kajita, S.; Takeda, H.; Karlson, D.; Ohgi, K.; Imai, R. Heat stable ssDNA/RNA-binding activity of a wheat cold shock domain protein. *FEBS Lett.* **2005**, *579*, 4887–4891. [[CrossRef](#)]
27. Sasaki, K.; Imai, R. Pleiotropic roles of cold shock domain proteins in plants. *Front. Plant Sci.* **2012**, *2*, 116. [[CrossRef](#)]

28. Rajasheker, G.; Kumar, A.S.; Kumari, H.P.; Kishor, K.P.B. Bacterial cold shock proteins—the molecular chaperones for multiple stress tolerance. *Adv. Biotech. Micro.* **2019**, *12*, 555837.
29. Sasaki, K.; Kim, M.H.; Imai, R. Arabidopsis cold shock domain protein 2 is a RNA chaperone that is regulated by cold and developmental signals. *Biochem. Biophys. Res. Commun.* **2007**, *364*, 633–638. [[CrossRef](#)]
30. Jung, Y.H.; Yi, J.Y.; Jung, H.J.; Lee, Y.K.; Lee, H.K.; Naicker, M.C.; Uh, J.H.; Jo, I.S.; Jung, E.J.; Im, H. Overexpression of cold shock protein A of *Psychromonas arctica* KOPRI 22215 confers cold-resistance. *Protein J.* **2010**, *29*, 136–142. [[CrossRef](#)]
31. Karlson, D.; Imai, R. Conservation of the cold shock protein of *Escherichia coli*. *Plant Physiol.* **2003**, *131*, 12–15. [[CrossRef](#)]
32. Nakaminami, K.; Karlson, D.T.; Imai, R. Functional conservation of cold shock domains in bacteria and higher plants. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 10122–10127. [[CrossRef](#)]
33. Castiglioni, P.; Warner, D.; Bensen, R.J.; Anstrom, D.C.; Harrison, J.; Stoecker, M.; Abad, M.; Kumar, G.; Salvador, S.; D'ordine, R.; et al. Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. *Plant Physiol.* **2008**, *147*, 446–455. [[CrossRef](#)] [[PubMed](#)]
34. Kim, J.C.; Lee, S.H.; Cheong, Y.H.; Yoo, C.M.; Lee, S.I.; Chun, H.J.; Yun, D.J.; Hong, J.C.; Lee, S.Y.; Lim, C.O.; et al. A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. *Plant J.* **2001**, *25*, 247–259. [[CrossRef](#)]
35. Vannini, C.; Locatelli, F.; Bracale, M.; Magnani, E.; Marsoni, M.; Osnato, M.; Mattana, M.; Baldoni, E.; Coraggio, I. Overexpression of the rice *Osm4* gene increases chilling and freezing tolerance of *Arabidopsis thaliana* plants. *Plant J.* **2004**, *37*, 115–127. [[CrossRef](#)]
36. Zhu, B.; Xiong, A.-S.; Peng, R.-H.; Xu, J.; Jin, X.-F.; Meng, X.-R.; Yao, Q.-H. Over-expression of *Thp1* from *Choristoneura fumiferana* enhances tolerance to cold in *Arabidopsis*. *Mol. Biol. Rep.* **2010**, *37*, 961. [[CrossRef](#)] [[PubMed](#)]
37. Deng, S.; Sun, J.; Zhao, R.; Ding, M.; Zhang, Y.; Sun, Y.; Wang, W.; Tan, Y.; Liu, D.; Ma, X.; et al. *Populus euphratica* APYRASE2 enhances cold tolerance by modulating vesicular trafficking and extracellular ATP in *Arabidopsis* plants. *Plant Physiol.* **2015**, *169*, 530–548. [[CrossRef](#)] [[PubMed](#)]
38. Taranov, V.V.; Berdnikova, M.V.; Babakov, A.V.; Nosov, A.V.; Galkin, A.V. Cold shock domain proteins in the extremophyte *Thellungiella salsuginea* (salt cress): Gene structure and differential response to cold. *Mol. Biol.* **2010**, *44*, 889–897. [[CrossRef](#)]
39. Taranov, V.V.; Zlobin, N.E.; Evlakov, K.I.; Shamustakimova, A.O.; Babakov, A.V. Contribution of *Eutrema salsugineum* cold shock domain structure to the interaction with RNA. *Biochemistry* **2018**, *83*, 1369–1379. [[CrossRef](#)]
40. Zlobin, N.; Evlakov, K.; Alekseev, Y.; Blagodatskikh, K.; Babakov, A.; Taranov, V. High DNA melting activity of extremophyte *Eutrema salsugineum* cold shock domain proteins EsCSDP1 and EsCSDP3. *Biochem. Biophys. Res.* **2016**, *5*, 502–508. [[PubMed](#)]
41. Zlobin, N.; Evlakov, K.; Tikhonova, O.; Babakov, A.; Taranov, V. RNA melting and RNA chaperone activities of plant cold shock domain proteins are not correlated. *RNA Biol.* **2018**, *15*, 1040–1046. [[CrossRef](#)] [[PubMed](#)]
42. Grote, A.; Hiller, K.; Scheer, M.; Munch, R.; Nortemann, B.; Hempel, D.C.; Jahn, D. JCat: A novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res.* **2005**, *33*, 526–531. [[CrossRef](#)]
43. Xiong, A.S.; Yao, Q.H.; Peng, R.H.; Duan, H.; Li, X.; Fan, H.Q.; Cheng, Z.M.; Li, Y. PCR-based accurate synthesis of long DNA sequences. *Nat. Protoc.* **2006**, *1*, 791–797. [[CrossRef](#)]
44. Lazo, G.R.; Stein, P.A.; Ludwig, R.A. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Nat. Biotechnol.* **1991**, *9*, 963–967. [[CrossRef](#)]
45. Murashige, T.; Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant* **1962**, *15*, 473–497. [[CrossRef](#)]
46. Cardoza, V.; Stewart, C.N. Canola (*Brassica napus* L.). *Agrobacterium Protoc. Methods Mol. Biol.* **2006**, *343*, 257–266.
47. Chernobrovkina, M.A.; Khvatkov, P.A.; Leonteva, A.V.; Dolgov, S.V. Study of the domestic breeding winter rape morphogenetic potential using in vitro culture. *Int. J. Appl. Fundam. Res.* **2017**, *11*, 260–264.
48. Dellaporta, S.L.; Wood, J.; Hicks, J.B. A plant DNA miniprep: Version II. *Plant Mol. Biol. Rep.* **1983**, *1*, 19–21. [[CrossRef](#)]
49. Livak, K.; Schmittgen, T. Analysis of relative gene expression data using realtime quantitative PCR and the 2^{-11C(T)}. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)]
50. Rogers, S.O.; Bendich, A.J. Extraction of Total Cellular DNA from Plants, Algae and Fungi. In *Plant Molecular Biology Manual*; Gelvin, S.B., Schilperoort, R.A., Eds.; Springer: Dordrecht, The Netherlands, 1994; Volume 1, pp. 183–190.
51. Dexter, S.T.; Tottindham, E.; Garber, L.G. Investigation of hardiness of plants by measurement of electrical conductivity. *Plant Physiol.* **1932**, *7*, 63–78. [[CrossRef](#)] [[PubMed](#)]
52. Markowski, A.; Augustyniak, G.; Janowiak, F. Sensitivity of different species of field crops to chilling temperature. III. ATP content and electrolyte leakage from seedling leaves. *Acta Physiol. Plant* **1990**, *12*, 167–173.
53. Rathore, D.S.; Doohan, F.; Mullins, E. Capability of the plant-associated bacterium, *Ensifer adhaerens* strain OV14, to genetically transform its original host *Brassica napus*. *Plant Cell Tiss. Organ Cult.* **2016**, *127*, 85–94. [[CrossRef](#)]
54. Thomashow, M.F. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Mol. Biol.* **1999**, *50*, 571–599. [[CrossRef](#)]
55. Uemura, M.; Shigematsu, S.; Minami, A.; Kawamura, Y.; Nakagawara, C.H. Responses of the plasma membrane to low temperatures. *Physiol. Plant.* **2006**, *126*, 81–89. [[CrossRef](#)]
56. Kumar, N.; Bhatt, R.P. Transgenics: An emerging approach for cold tolerance to enhance vegetables production in high altitude areas. *Indian J. Crop Sci.* **2006**, *1*, 8–12.

57. Kim, M.H.; Sasaki, K.; Imai, R. Cold shock domain protein 3 regulates freezing tolerance in *Arabidopsis thaliana*. *J. Biol. Chem.* **2009**, *284*, 23454–23460. [[CrossRef](#)] [[PubMed](#)]
58. Kasuga, M.; Miura, S.; Shinozaki, K.; Yamaguchi-Shinozaki, K. A combination of the *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiol.* **2004**, *4*, 346–350. [[CrossRef](#)] [[PubMed](#)]
59. Bihmidine, S.; Lin, J.; Stone, J.M.; Awada, T.; Specht, J.E.; Clemente, T.E. Activity of the *Arabidopsis* RD29A and RD29B promoter elements in soybean under water stress. *Planta* **2013**, *237*, 55–64. [[CrossRef](#)]