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Overexpression of *CmDUF239-1* Enhances Cold Tolerance in Melon Seedlings by Reinforcing Antioxidant Defense and Activating the ICE-CBF-COR Pathway

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Abstract

Low-temperature stress is a major factor that limits the productivity and geographical distribution of melon (*Cucumis melo* L.). This study elucidates that *CmDUF239-1* is a positive regulator of cold stress, and its underlying mechanisms are investigated using root-specific overexpression lines. Seedlings overexpressing *CmDUF239-1* exhibited increased biomass and reduced relative electrical conductivity under cold stress. *CmDUF239-1* overexpression promoted the accumulation of soluble sugar and proline, which was accompanied by enhanced activity of the proline biosynthetic enzyme Δ^1-pyrroline-5-carboxylate synthase (P5CS) and suppressed activity of the proline-degrading enzyme proline dehydrogenase (PDH). Molecular analysis revealed that *CmDUF239-1* overexpression upregulated antioxidant enzyme-related genes, sugar metabolism related genes, and proline-related genes. Furthermore, it activated key genes in the ICE-CBF-COR signaling pathway, including *CmCBF1*, *CmCBF2*, and the downstream effector gene *CmCOR413-2*. In conclusion, the *CmDUF239-1* gene enhances melon cold tolerance by modulating antioxidant defense, enhancing osmolyte (sugar and proline) metabolism and activating a core signaling pathway. This study not only characterizes a novel function for a DUF family gene but also provides a promising candidate gene for the genetic improvement of cold resilience in melon and other related crops.

Keywords: *CmDUF239-1*; cold stress; *Cucumis melo* L.; DUF gene; antioxidant enzyme activity; ICE-CBF-COR pathway; gene expression

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1. Introduction

Cold stress, encompassing chilling (0–15 °C) and freezing (<0 °C) conditions, is a major environmental factor limiting crop growth, productivity, and geographical distribution [1]. At the physiological level, low temperatures impair the root system's ability to absorb water, leading to a water imbalance and cellular dehydration [2]. At the molecular level, cold stress disrupts cellular homeostasis by altering the fluidity of the cell membrane, destabilizing protein and RNA secondary structures and affecting enzymatic activities [3,4]. This disruption triggers oxidative stress through the excessive accumulation of reactive oxygen species (ROS), thereby impairing photosynthesis, respiration, and metabolic processes,

ultimately exerting a toxic effect on the plant's life activities [5]. Cold stress in plants can lead to leaf wilting, growth inhibition, and, in severe cases, plant death.

To mitigate damage caused by low temperatures, plants have evolved a series of cold tolerance mechanisms, including the osmotic protection system, the redox system and specific regulatory signaling pathways [6]. Osmotic adjustment is achieved by accumulating organic osmolytes, such as soluble sugars, proline, and quaternary ammonium compounds, which buffer the internal water deficit [7,8]. To reduce oxidative damage, plants utilize a sophisticated ROS scavenging system composed of enzymes like Catalase (CAT), Superoxide Dismutase (SOD), Peroxidase (POD), whose activities are often transiently increased upon cold exposure in various species [9–14]. The ICE1-CBF-COR module acts as a central signaling pathway that rapidly responds to cold stress [15,16]. Upon cold stimulus, ICE1 (Inducer of CBF expression 1) binds to the promoter regions of CBF (C-Repeat Binding Factor) genes and induces the expression of CBFs, which in turn activate downstream COR (Cold-Responsive) genes to enhance plant cold tolerance [6]. The CBF gene family is functionally conserved across diverse plant species; for instance, in both *Arabidopsis* and tomato, *CBF1*, *CBF2*, and *CBF3* all positively regulate the cold tolerance [15,16]. In melon, silencing of the *CmCBF* genes via VIGS was shown to significantly reduce seedling cold tolerance [17]. In addition, a *DUF793* family protein-BYPASS1-LIKE, in *Arabidopsis,* has been shown to participate in freezing tolerance through the CBF-dependent pathway, indicating that this previously uncharacterized protein family may play a role in modulating this core signaling cascade [18].

The Domain of Unknown Function (DUF) constitutes a large superfamily of proteins widely distributed in plants, many of which remain functionally uncharacterized [19]. Accumulating evidence suggests that DUF proteins are pivotal not only in plant development but also in response to abiotic stresses, including drought, salinity, and temperature extremes [20,21]. Several DUF-containing proteins have been identified as positive regulators of plant cold tolerance. In rice, the expression of *OsDUF568* and *OsSRDP (DUF740)* is induced by low temperatures, thereby enhancing cold tolerance [22,23]. Similarly, *DUF506* family members in *Arabidopsis* and rice are implicated in cold-induced signal transduction via the regulation of intracellular Ca^{2+} concentrations [24]. In cotton, overexpression of *AmDUF1517* has been shown to enhance cold stress tolerance by maintaining ROS homeostasis and alleviating cell membrane damage [25,26]. In melon, *CmDUF239-1* has previously been shown to be involved in salt and heat stress response, implying a potential role in a broader stress-response network [27,28].

Melon (*Cucumis melo* L.) is an important economic crop cultivated extensively worldwide. However, as a typical thermophilic crop with an optimal growth temperature between 25 and 35 °C, its productivity is highly sensitive to low-temperature stress, particularly during the seedling stage [29]. Although several cold-responsive genes in melon, including CmGLP and CmPIP2;3, have been reported to enhance tolerance to low temperatures, the underlying molecular mechanisms that regulate cold tolerance in this species remain largely unresolved [30,31]. Previously, research has demonstrated that under heat stress, *CmDUF239-1* enhances thermotolerance by upregulating antioxidant defense, proline metabolism, and heat shock protein (HSP) accumulation in melon seedlings [28]. Given the partial overlap between molecular responses to cold and heat stress [3], we hypothesized that *CmDUF239-1* may also contribute significantly to the cold stress response. To test this hypothesis, the present study was conducted to investigate the role of *CmDUF239-1* in melon seedlings under low-temperature conditions and to uncover its associated molecular mechanisms.

2. Materials and Methods

2.1. Plant Material, Growth Conditions, and Generation of Transgenic Melon Lines

Seeds of melon cultivar-*Cucumis melo* L. cv. 'Xizhou Honey No. 17' was used in this experiment. Initially, seeds were sterilized in sterile water at 55 °C for 15 min, followed by soaking at 25 °C for 6 h. Germination was carried out in the dark at 28 °C on Petri dishes (10 cm) lined with three layers of moist filter paper. After germination, seedlings were transplanted into 50-cell trays and cultivated in an LED growth chamber (ZRX-460, WEGA, Weifang, China) under a 12 h/12 h photoperiod, a light intensity of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a day/night temperature of 30 °C/20 °C.

An overexpression construct for *CmDUF239-1 (MELO3C022991)* was generated following the protocol described by Peng et al. and introduced into *Escherichia coli* DH5α. Subsequently, both the resulting plasmid and the empty pKSE403 vector were transferred into *Agrobacterium tumefaciens* strain K599 (Weidi, Shanghai, China) [32]. Melon cotyledons were infected with the K599 strain, and seedlings were transplanted four days later. Every five days, transgenic roots exhibiting positive red fluorescence were retained and the expression level of *CmDUF239-1* was verified by qRT-PCR. Seedlings confirmed as positive at the one-leaf, one-heart stage were transferred to hydroponic cups containing 400 mL of half-strength Hoagland solution. The nutrient solution consisted of 4 mM $CaCl_2$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $Ca(H_2PO_4)_2 \cdot H_2O$, 60 mM KNO_3, and 74.93 mg/L of a solid trace element, with pH 6.5 adjusted by 1 M KOH.

2.2. Cold Stress Treatment and Sampling

Melon seedlings were subjected to two treatments: normal conditions (control, CK) with day/night temperatures of 30 °C/20 °C, and low-temperature stress (cold stress, CS) with day/night temperatures of 15 °C/6 °C, both under a 12 h photoperiod. Each treatment included three biological replicates, with six seedlings per replicate [33].

After 24 h of cold treatment, a subset of melon roots was harvested, immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent transcriptome sequencing. At the same time, another subset of melon roots was sampled to determine the content of malondialdehyde (MDA), sucrose, glucose, fructose, and proline (PRO). The activities of SOD, POD, CAT, neutral invertase (NI), sucrose phosphate synthase (SPS), Δ^1-pyrroline-5-carboxylate synthetase (P5CS), and proline dehydrogenase (PDH) were also assayed. On day 5 of the cold treatment, plants were randomly sampled to photograph and record phenotypes. The fresh weight, dry weight, and relative electrical conductivity of the above-ground parts and below-ground parts were measured. Distinct sets of plants were used for biomass determination, physiological assays, and transcriptome sequencing to ensure independent measurements.

2.3. Measurement of Phenotypic Indicators

After 5 days of cold stress, melon shoots and roots were harvested. Their fresh weight (FW) was measured by an electronic analytical balance [34]. To determine dry weight (DW), the sample is placed in an oven and dried.

2.4. Measurement of Physiological Indicators

On day 5 of the low temperature treatment, the relative electrical conductivity (REC) of seedling leaves and roots was determined. For REC measurement, 0.1 g of melon leaves or roots was weighed, rinsed three times with distilled water, and blotted dry with filter paper. The sample was then placed in a 15 mL centrifuge tube containing 5 mL of distilled water and subjected to vacuum treatment at 0.8 MPa for 20 min. The conductivity of the resulting leachate (R_1) was subsequently measured using a DDSJ-308F conductivity meter

(DDSJ-308F, Leici, Shanghai, China). Subsequently, the samples were boiled for 30 min, cooled to room temperature, mixed completely, and the final leachate conductivity (R2) was measured. The REC was calculated using the following formula: Relative Electrical Conductivity (%) = (R1/R2) × 100% [35].

On day 1 of the low temperature treatment, the contents of malondialdehyde (MDA), sucrose, glucose, fructose, proline (PRO), and the activities of SOD, POD, CAT, neutral invertase (NI), sucrose phosphate synthase (SPS), P5CS, and pyruvate dehydrogenase (PDH) were measured in the roots. Testing was performed using kits supplied by Aidi Biological Co., Ltd. (Yangzhou, China, http://www.adsbio.cn) and the product catalog numbers are as follows: MDA (ADS-W-YH002) [36], sucrose, glucose, and fructose (ADS-F-DF018) [37], PRO (ADS-W-AJS004) [38], SOD (ADS-W-KY011-48) [39], POD (ADS-W-YH002) [40], CAT (ADS-W-KY002-48) [41], NI (ADS-F-ZT008) [42], SPS (ADS-F-ZT006), P5CS (ADS-F-AJS011) [38], and PDH (ADS-F-AJS014) [43]. Three biological replicates were used for each measurement, with samples randomly collected from different plants to ensure representativeness.

2.5. Transcriptome Analysis

The melon seedling roots were subjected to a transcriptomic analysis one day after being exposed to cold stress. The RNA sequencing was carried out by Beijing Qingke Biotechnology Co., Ltd. (Beijing, China). Differential expression analysis between treatment groups was performed using the DESeq2 software (version 1.26) [44]. The criteria for identifying differentially ex-pressed genes (DEGs) were a Log2(Fold Change value) > 1.0 and a p-value < 0.05. [45]. Transcriptome data have been uploaded to NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1357663, accessed on 6 November 2025).

2.6. Analysis of Quantitative Real-Time PCR

Root tissues from melon seedlings exposed to one day of cold stress were collected for RNA extraction. Both RNA isolation and qRT-PCR were conducted following the protocols described by Bai et al. [46].

The genes selected for analysis, including those related to SOD, POD, CAT, sugar metabolism (ZT, GT), proline metabolism (PRO), and the CBF pathway, were identified through the Cucurbit Genomics Database using annotation for melon (*Cucumis melo* L.) [47]. *CmActin* (*MELO3C025848*) was used for the internal reference gene and primer sequences used for qRT-PCR are listed in Table S1.

2.7. Data Statistical Analysis and Graphical Presentation

The effect of *CmDUF239-1* overexpression was evaluated using a Student's t-test. All other datasets were analyzed by two-way analysis of variance (ANOVA) to assess treatment effects, with a significance threshold of $p < 0.05$. When significant differences were observed, multiple comparisons were conducted using Duncan's new multiple range test (DMRT). All statistical analyses and graphical visualizations were performed in RStudio (version 4.0.3).

3. Results

3.1. Effects of CmDUF239-1 Overexpression on the Phenotype and Membrane Stability of Melon Seedlings Under Cold Stress

To investigate the role of *CmDUF239-1* in regulating melon growth and cold tolerance, the phenotypic and physiological responses of a *CmDUF239-1* root-overexpressing transgenic line (OEDUF239-1) and empty vector controls (EV) were evaluated under normal (CK) and cold stress (CS) conditions. After 5 days of cold stress, the OEDUF239-1 seedlings displayed a growth advantage over the EV seedlings under both control and cold stress

conditions (Figure 1a). Under control conditions, OEDUF239-1 plants showed significantly higher shoot and root fresh weight (FW) and dry weight (DW) than EV plants. Concurrently, leaf relative electrical conductivity (REC) was significantly lower in OEDUF239-1 plants, although root REC showed a slight increase (Figure 1c–h), suggesting that *CmDUF239-1* overexpression promotes plant growth and partially enhances membrane stability even in a non-stress environment. Under cold stress, OEDUF239-1 plants maintained showing significantly higher FW and DW in both shoots and roots, and significantly lower REC in both leaves and roots compared to EV controls (Figure 1c–h), indicating that root-specific *CmDUF239-1* overexpression effectively alleviates cold stress damage in melon. Under cold stress, compared to EV, the fresh weight of shoots and roots in OEDUF239-1 increased significantly, with dry weight of shoots and roots rising significantly by 48.9% and 68.5%, respectively. Additionally, the relative electrical conductivity of both leaves and roots showed a significant decrease. Collectively, these findings indicate that root-specific overexpression of *CmDUF239-1* enhances plant growth and contributes to cold tolerance in melon seedlings by maintaining biomass accumulation and improving cell membrane stability under low-temperature conditions.

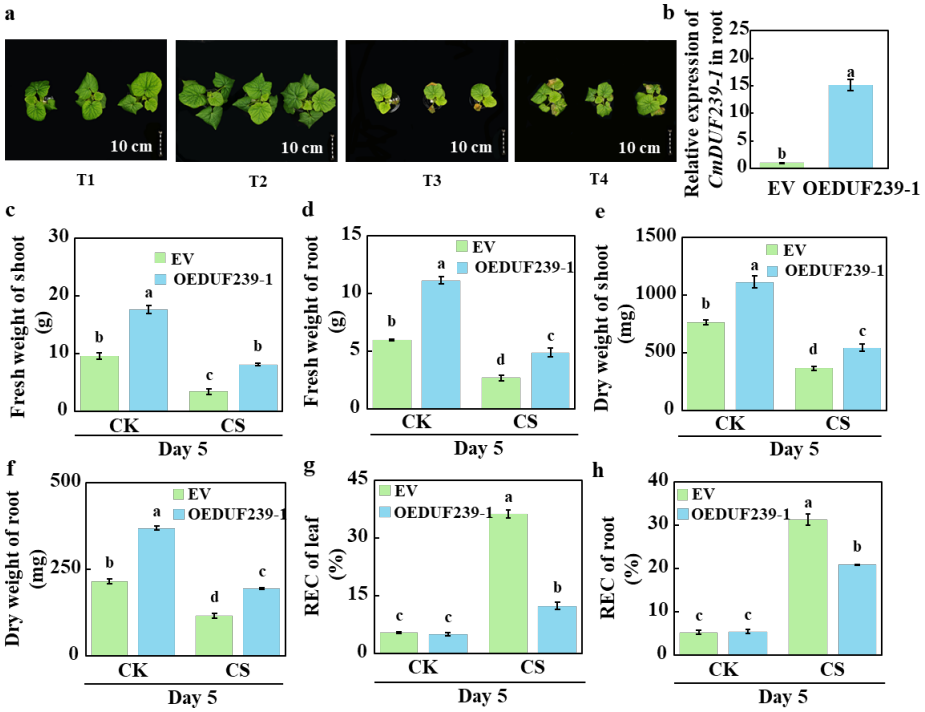

Figure 1. Effects of overexpressing *CmDUF239-1* on melon seedling growth and relative electrical conductivity. (**a**) Phenotype of seedlings after 5 days of low-temperature treatment. (**b**) Relative expression level of the *CmDUF239-1* gene in OEDUF239-1 roots. (**c**) fresh weight of shoots, (**d**) fresh weight of roots, (**e**) dry weight of shoots, (**f**) dry weight of roots, (**g**) leaf relative electrical conductivity (REC) of leaves (**g**) and roots (**h**) after 5 days of low-temperature treatment. Data are presented as "mean ± standard error" (n = 3). Different lowercase letters show significant differences among various treatments ($p < 0.05$). EV: empty vector transformed into melon seedlings; OEDUF239-1: root-specific overexpressing *CmDUF239-1* lines; CK: control condition; CS: cold stress; T1: EV lines under control conditions; T2: OEDUF239-1 lines under control conditions; T3: EV lines under cold stress treatment, and T4: OEDUF239-1 lines under cold stress treatment.

3.2. Differential Gene Analysis

To explore the molecular mechanisms underlying the enhanced cold tolerance, transcriptome sequencing was performed on the roots of melon seedlings after one day of cold stress. Principal Component Analysis (PCA) showed that all three replicates for each

treatment group were clustered within the 95% confidence ellipse, suggesting good sample repeatability (Figure 2a). Differential expression analysis revealed that, after one day of cold stress, 2784 genes were up-regulated and 2680 genes were down-regulated in the roots of EV plants relative to the control. In OEDUF239-1 plants, 3187 genes were up-regulated, and 2800 genes were down-regulated under the same conditions (Figure 2b). Gene Ontology (GO) enrichment analysis of the 5614 differentially expressed genes from the OEDUF239-1 plants revealed significant enrichment in molecular function categories related to oxidoreductase activity and peroxidase activity (Figure 2c). These results suggest that *CmDUF239-1* may confer cold tolerance by regulating the activity of antioxidant enzymes in the roots.

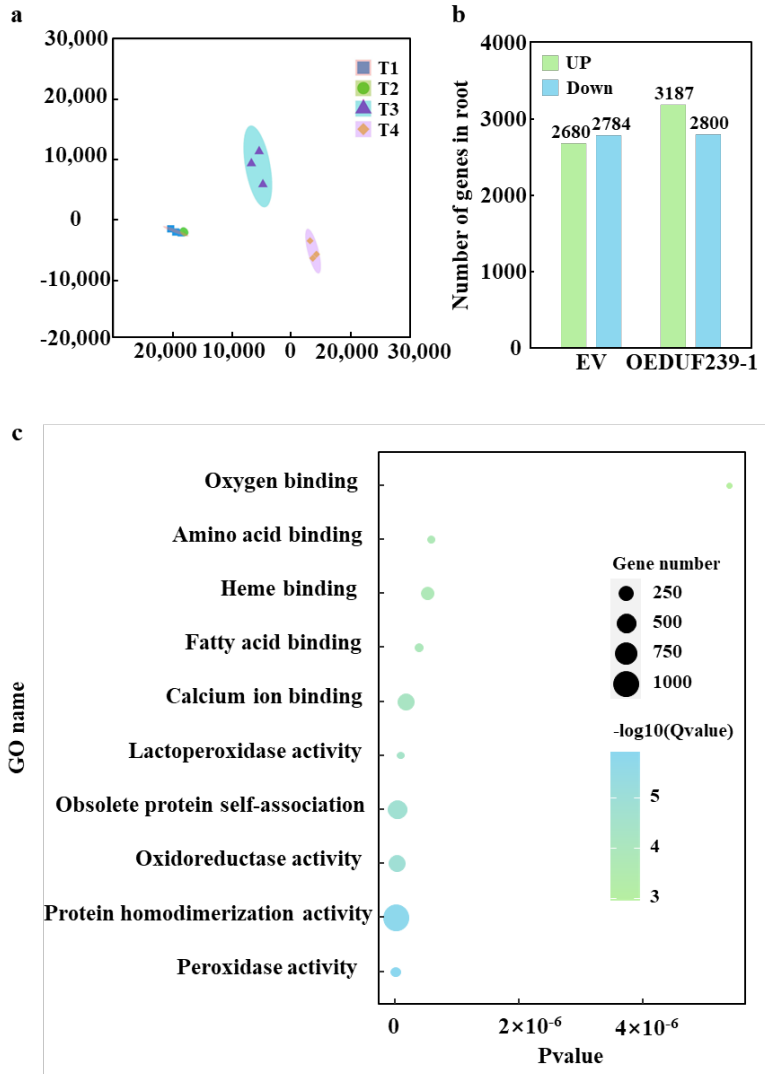

Figure 2. Analysis of transcriptome data. (**a**) Principal Component Analysis of the transcriptome data. (**b**) Analysis of the number of DEGs. (**c**) Analysis of Gene Ontology (GO) enrichment.

3.3. The Effect of Overexpressing CmDUF239-1 on Antioxidant Enzyme Activity in Roots

To elucidate the effect of overexpression of *CmDUF239-1* on the antioxidant system of melon seedlings under cold stress, the content of MDA and the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were measured. Under control temperature conditions, no significant differences were detected in these parameters between OEDUF239-1 and EV plants after one day of treatment, indicating that overexpression of *CmDUF239-1* does not disrupt basal antioxidant homeostasis (Figure 3a–d). However, after 24 h of cold stress, the activities of SOD, POD, and CAT in OEDUF239-1 plants were significantly higher than those in the EV plants, while the accumulation of

MDA was significantly lower (Figure 3a–d). This result indicates a stronger reactive oxygen species (ROS) scavenging capacity and consequently less severe membrane damage in the transgenic lines. Relative to their respective non-stressed controls, cold treatment led to increases in SOD, POD, and CAT activities in EV plants by 37.3%, 108.1%, and 17.8%, respectively, accompanied by a 1.6-fold rise in MDA content. In contrast, the OEDUF239-1 plants mounted a much more robust antioxidant response, with SOD, POD, and CAT activities increasing substantially by 109.2%, 318.6%, and 18.2%, respectively. Consequently, the increase in MDA content was limited to only 65.6%. Collectively, these results confirm that root overexpressing *CmDUF239-1* enhances the cold tolerance of melon seedlings by coordinately boosting antioxidant enzyme activities and suppressing membrane lipid peroxidation. To assess the regulatory role of *CmDUF239-1* on antioxidant enzyme-related genes at the transcriptional level, the expression level was analyzed in melon roots after one day of cold stress. Transcriptome data indicated that among the SOD genes, *CmCSD1* and *CmMSD1* were the most highly expressed. Although their expression was only modestly induced or even suppressed by cold stress in the EV line, these genes were markedly upregulated in OEDUF239-1 plants (Figure 3e). For the POD and CAT gene families, *CmPOD1*, *CmPA2*, *CmCAT2-1*, and *CmCAT2-2* were identified as the predominantly expressed and cold-inducible members in both genotypes (Figure 3f,g).

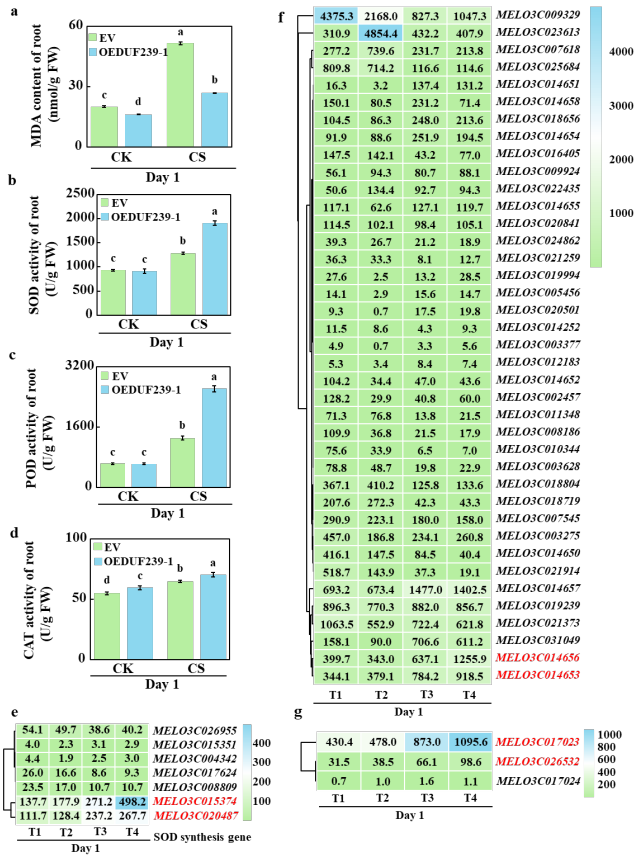

Figure 3. Effect of *CmDUF239-1* overexpression on MDA content, antioxidant enzyme activity and related genes in roots after 1 day of cold stress. (**a**) MDA content in the roots. Enzyme activity of SOD (**b**), POD (**c**), and CAT (**d**). Heatmap analysis of the expression of genes encoding SOD (**e**), POD (**f**), and CAT (**g**) after cold stress treatment. Data are presented as "mean ± standard error" (n = 3). Different lowercase letters show significant differences among various treatments ($p < 0.05$). *MELO3C015374*(*CmCSD1*), *MELO3C020487*(*CmMSD1*), *MELO3C014656*(*CmPOD1*), *MELO3C014653*(*CmPA2*), *MELO3C017023*(*CmCAT2-2*), *MELO3C026532*(*CmCAT2-1*).

3.4. CmDUF239-1 Overexpression Upregulates the Expression of Antioxidant Enzyme-Related Genes Under Cold Stress

qRT-PCR was conducted to validate the transcriptomic results. The analysis confirmed that, although antioxidant-related genes were induced in both genotypes following one day of cold stress, the level of induction was generally greater in OEDUF239-1 plants (Figure 4a–f). Compared to their respective non-stressed controls, the expression of *CmCSD1* and *CmMSD1* in EV plants increased by 51.0% and 93.3%, respectively. In contrast, the transcript level of *CmCSD1* in OEDUF239-1 plants increased by a substantial 196.8% (*CmMSD1* showed a 79.2% increase). A similar pattern was observed for the POD gene *CmPOD1*, with its expression increasing by 187.9% in EV plants and by a more significant 256.2% in OEDUF239-1 plants. For the CAT genes, *CmCAT2-1* and *CmCAT2-2* transcripts increased by 31.0% and 165.4% in the EV line, while in the OEDUF239-1 line, they increased by 47.8% and 156.1%, respectively. These findings demonstrate that overexpression of *CmDUF239-1* more effectively induces the transcription of key antioxidant enzyme genes under cold stress, thereby enhancing the plant's ROS scavenging capacity.

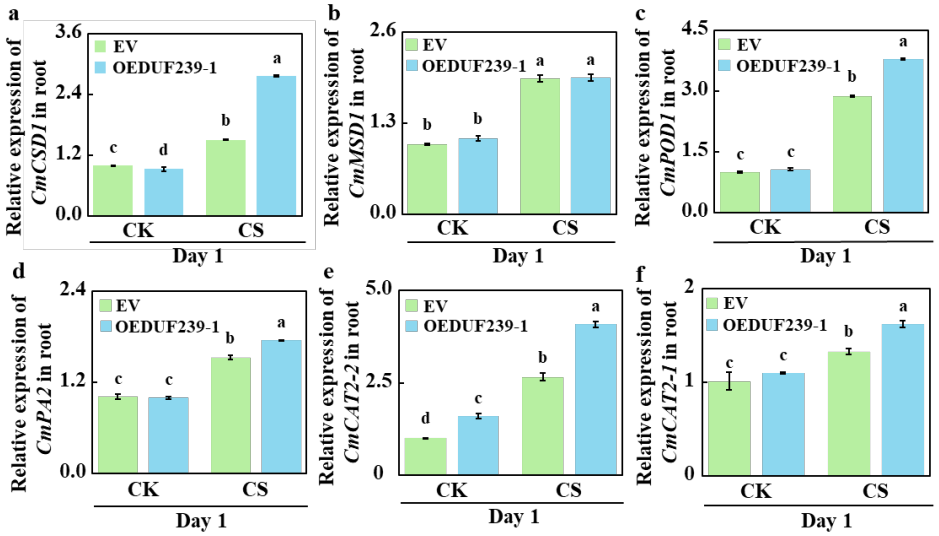

Figure 4. The impacts of overexpressing *CmDUF239-1* on the expression of genes associated with antioxidant enzymes in root tissue following 1 day of cold stress. Relative expression levels of *CmCSD1* (**a**), *CmMSD1* (**b**), *CmPOD1* (**c**), *CmPA2* (**d**), *CmCAT2-2* (**e**), and *CmCAT2-1* (**f**) in melon seedling roots after 1 day of cold stress treatment. Data are presented as "mean ± standard error" (n = 3). Different lowercase letters show significant differences among various treatments ($p < 0.05$).

3.5. CmDUF239-1 Overexpression Regulates Soluble Sugar Content, Related Enzyme Activities and the Expression of Sugar Metabolism-Related Genes

To assess the impact of *CmDUF239-1* overexpression on sugar metabolism in melon seedlings, the contents of glucose, fructose, and sucrose, as well as the activities of neutral invertase (NI) and sucrose phosphate synthase (SPS), were measured under both control and cold stress conditions. Under control conditions, the OEDUF239-1 seedlings already exhibited significantly higher levels of glucose and fructose, along with greater NI and SPS activities, compared to the empty vector (EV) controls. The sucrose content was also elevated slightly, indicating that *CmDUF239-1* has a potential impact on basal sugar metabolism (Figure 5a–e).

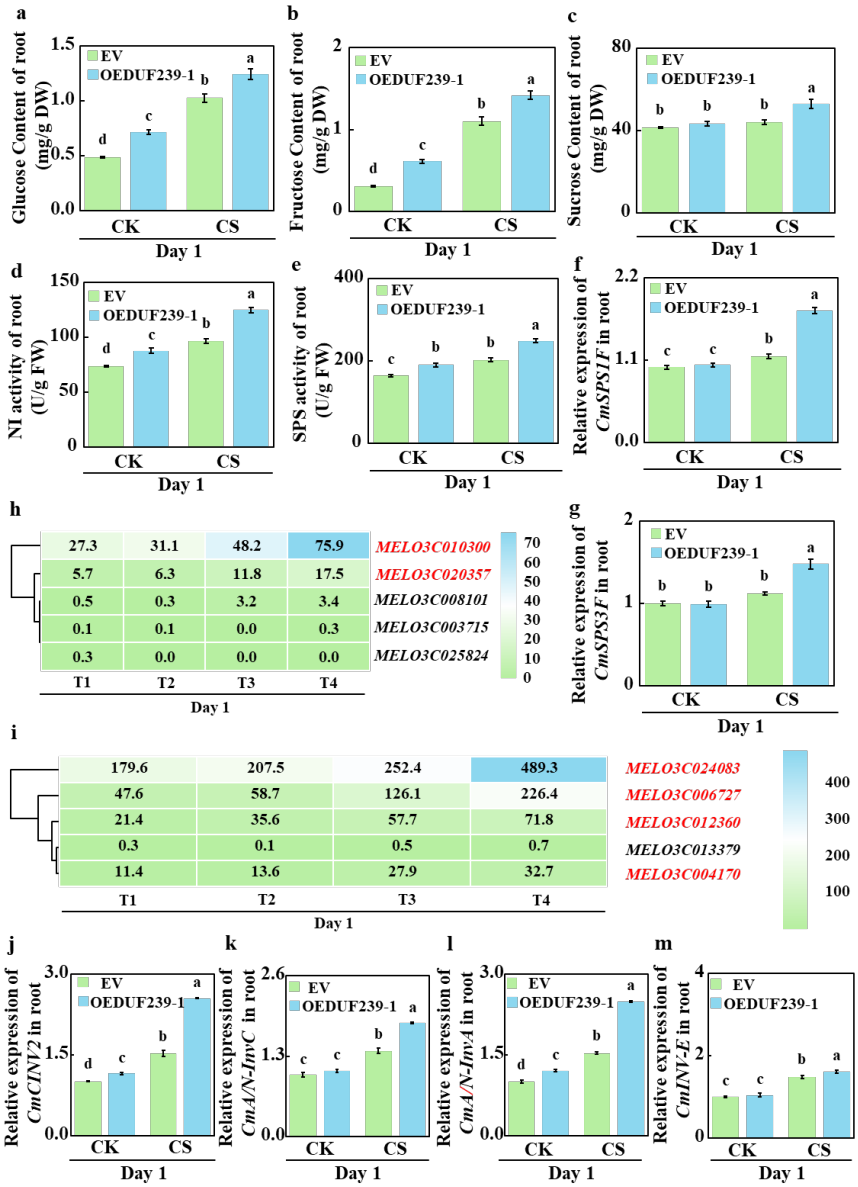

Figure 5. Impacts of overexpressing *CmDUF239-1* on sugar content, enzyme activities, and expression of sugar metabolism-related genes in roots after 1 day of cold stress. (**a**) glucose content, (**b**) fructose content, (**c**) sucrose content, (**d**) neutral invertase (NI) activity, (**e**) sucrose phosphate synthase (SPS) activity. Heat map analysis of the expressions of synthesis genes for sucrose (**h**) and fructose (**i**). Relative expression levels of sucrose-related genes including *CmSPS1F* (**f**), *CmSPS3F* (**g**), *CmCINV2* (**j**), *CmA/N-InvC* (**k**), *CmA/N-InvA* (**l**), and *CmINV-E* (**m**) in melon seedling roots after 1 day of cold stress treatment. Data are presented as "mean ± standard error" (n = 3). Different lowercase letters show significant differences among different treatments ($p < 0.05$). *MELO3C010300*(*CmSPS1F*), *MELO3C020357*(*CmSPS3F*), *MELO3C024083*(*CmCINV2*), *MELO3C006727*(*CmA/N-InvC*), *MELO3C012360*(*CmA/N-InvA*), *MELO3C004170*(*CmINV-E*).

In comparison to their respective controls, cold treatment induced an increase in soluble sugar content and related enzyme activities in both genotypes. In EV plants, the contents of glucose, fructose, and sucrose increased by 112.4%, 253.1%, and 6.1%, respectively, while NI and SPS activities increased by 31.3% and 22.9% (Figure 5a–e). After one day of cold treatment, the OEDUF239-1 seedlings showed significantly higher contents of glucose, fructose, and sucrose, and greater NI and SPS activities than the EV seedlings. Specifically, under cold stress, the contents of glucose, fructose, and sucrose in OEDUF239-1 plants were 21.1%, 28.2%, and 20.4% higher, respectively, while NI and SPS activities were

29.4% and 22.6% higher than those in EV plants (Figure 5a–e). These results, consistent with the phenotypic data (Figure 1a–h), suggest that *CmDUF239-1* positively regulates the cold tolerance of melon seedlings by enhancing the activities of sugar synthesis and conversion enzymes.

To investigate the regulatory mechanism of *CmDUF239-1* on sugar metabolism-related genes, qPCR analysis was performed on root samples after one day of cold stress. Among the sucrose-related genes, *CmSPS1F* and *CmSPS3F* were the most highly expressed, while among the invertase genes, *CmCINV2*, *CmA/N-InvA*, *CmA/N-InvC*, and *CmINV-E* showed the highest transcript abundance (Figure 5h,i). Although cold stress elicited only a modest induction of these genes in EV plants, their expression was markedly upregulated in OEDUF239-1 plants. Compared to their respective non-stressed controls, the transcript levels of *CmSPS1F*, *CmSPS3F*, *CmCINV2*, *CmA/N-InvA*, *CmA/N-InvC*, and *CmINV-E* in EV plants increased by 14.4%, 11.9%, 52.1%, 53.0%, 38.2%, and 47.4%, respectively. In contrast, the expression of these genes in the OEDUF239-1 plants was enhanced more substantially, with increases of 70.3%, 50.1%, 1.22-fold, 1.07-fold, 73.3%, and 55.2%, respectively (Figure 5f,g,j–m). These findings indicate that the overexpression of *CmDUF239-1* significantly activates the expression of key genes involved in sugar metabolism under cold stress, thereby likely enhancing the capacity for carbon assimilation and osmotic adjustment in melon seedlings.

3.6. CmDUF239-1 Differentially Regulates Proline Metabolism at the Physiological and Transcriptional Levels

To investigate the impact of *CmDUF239-1* overexpression on the osmotic adjustment of melon seedlings in response to cold stress, we measured the root proline content and the activities of its key synthesis enzyme (P5CS) and degradation enzyme (PDH). Under control conditions, no significant differences in proline levels, P5CS activity, or PDH activity were observed between the OEDUF239-1 and empty vector (EV) lines (Figure 6a–c). However, after one day of cold stress treatment, the OEDUF239-1 line exhibited significantly higher proline content and P5CS activity compared to the EV line, but PDH activity was significantly lower in the OEDUF239-1 plants (Figure 6a–c). When compared to their respective control groups, cold treatment led to a 24.4% increase in proline content and a 44.8% increase in P5CS activity in EV plants, while PDH activity decreased by 50.9%. In contrast, the OEDUF239-1 plants mounted a stronger response, with proline content increasing by 103.5%, P5CS activity rising by 80.8%, and PDH activity decreasing by a substantial 74.0% (Figure 6a–c). These results are consistent with the phenotypic observations, indicating that *CmDUF239-1* enhances proline accumulation under cold stress by activating P5CS and inhibiting PDH, thereby positively regulating the cold tolerance of melon seedlings.

To determine if this regulation occurred at the transcriptional level, we analyzed the expression of proline metabolism-related genes. Transcriptome data identified *CmP5CS1* (a key synthesis gene) and *CmERD5* (a drought-responsive gene) as the most highly expressed members of their respective families (Figure 6d). Under control conditions, no significant difference in the expression of these two genes was observed between EV and the OEDUF239-1 plants. After cold treatment, the expression of both genes was unexpectedly down-regulated in both genotypes. In EV plants, *CmP5CS1* and *CmERD5* transcript levels decreased by 6.3% and 38.4%, respectively. Notably, this transcriptional repression was significantly amplified in the OEDUF239-1 plants, where the expression of *CmP5CS1* and *CmERD5* was reduced by 24.4% and 69.3%, respectively (Figure 6e,f). These results indicate that *CmDUF239-1* overexpression intensified the suppression of proline biosynthesis and drought-responsive gene expression under cold stress, suggesting a potential negative impact on melon cold tolerance through the downregulation of proline accumulation.

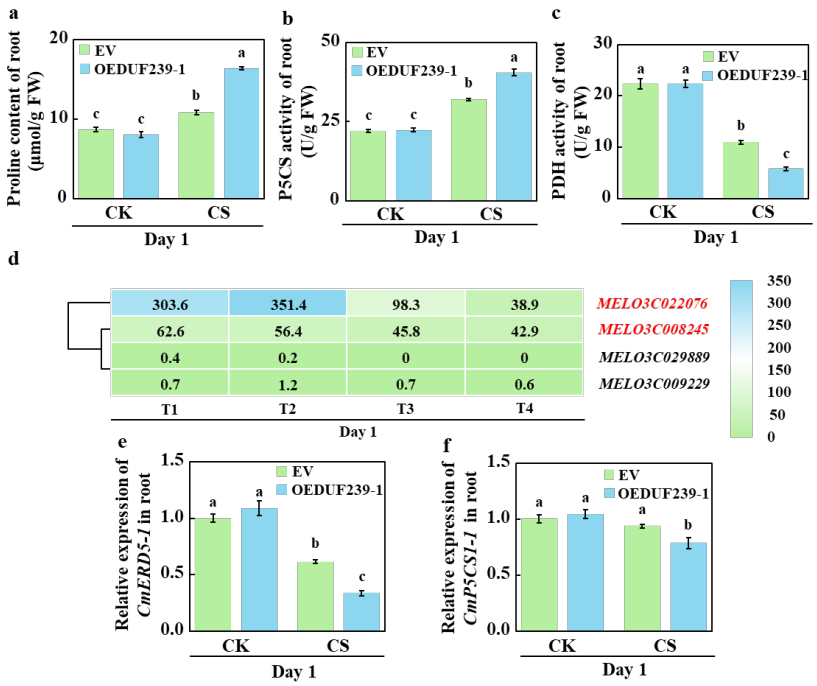

Figure 6. Impacts of overexpressing *CmDUF239-1* on proline content, synthase and dehydrogenase activity, and related gene expression in roots after 1 day of cold stress. (**a**) Proline content in the root. Activity of P5CS (**b**) and PDH (**c**). (**d**) Heat map analysis of gene expression involved in proline synthesis and degradation. Relative expressions of *CmERD5-1* (**e**) and *CmP5CS1-1* (**f**) in the roots of melon seedlings after 1 day of cold stress. Data are presented as "mean ± standard error" (n = 3). Different lowercase letters show significant differences among various treatments ($p < 0.05$). EV: empty vector transformed into melon seedlings; OEDUF239-1: root-specific overexpressing *CmDUF239-1* lines; CK: control condition; CS: cold stress; T1: EV lines under control conditions; T2: OEDUF239-1 lines under control conditions; T3: EV lines under cold stress treatment, and T4: OEDUF239-1 lines under cold stress treatment. *MELO3C022076*(*CmERD5-1*), *MELO3C008245*(*CmP5CS1-1*).

3.7. Overexpression of CmDUF239-1 Amplifies the Transcriptional Response of the ICE-CBF-COR Pathway

To elucidate the effect of *CmDUF239-1* overexpression on the transcriptional regulation of the core cold-response pathway in melon seedlings, the expression levels of key genes within the ICE-CBF-COR cascade were measured after 1 day of cold stress. The results showed that key genes, including *CmCBF1*, *CmCBF2*, *CmICE2*, and the downstream target *CmCOR413-2*, were highly responsive to the cold treatment (Figure 7a). Under normal temperature conditions, the expression of these four genes showed no significant difference between the EV and OEDUF239-1 lines. This indicates that overexpression of *CmDUF239-1* does not alter the basal transcriptional state of the cold signaling network under non-stress conditions. Upon cold treatment, four genes exhibited an induced upregulation level in EV plants, with *CmICE2* showing the highest induction level. It is worth noting that in OEDUF239-1 plants, the cold-induced upregulation of these genes was further enhanced, particularly for *CmCOR413-2*, which displayed the most significant increase (Figure 7a). qPCR quantification results identified that the expression levels of *CmCBF1*, *CmCBF2*, *CmICE2*, and *CmCOR413-2* in EV plants were upregulated by 51.4%, 53.5%, 72.7%, and 53.5%, respectively, compared to their controls. Moreover, in OEDUF239-1 plants, these genes showed even greater induction, with expression increasing by 73.1% for *CmCBF1*, 100.7% for *CmCBF2*, 70.6% for *CmICE2*, and a remarkable 150.1% for *CmCOR413-2* (Figure 7b–e). These findings demonstrate that *CmDUF239-1* overexpression significantly enhances melon seedling cold tolerance by synergistically amplifying the transcriptional response of the cold-induced ICE-CBF-COR pathway.

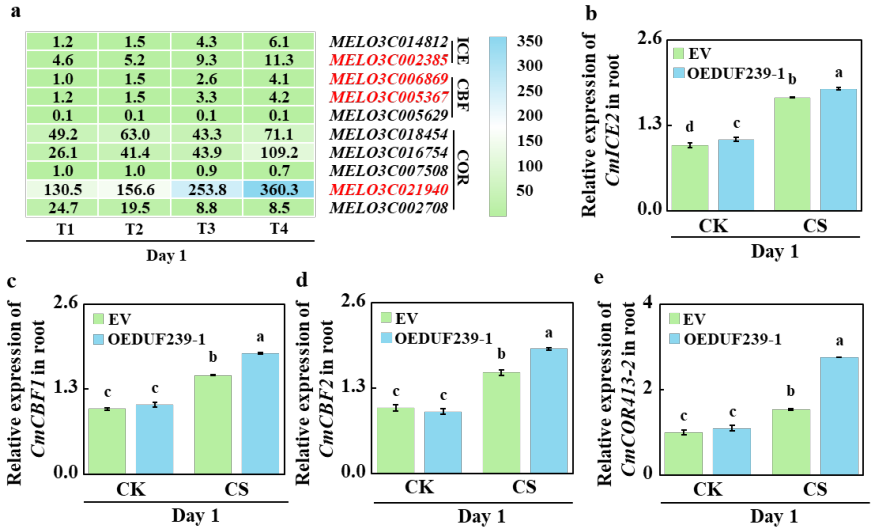

Figure 7. Impacts of overexpressing *CmDUF239-1* on the transcriptional level of ICE-CBF-COR pathway-related genes in roots after 1 day of cold stress. (**a**) Heat map analysis of the genes involved in the ICE-CBF-COR pathway. Relative expression levels of *CmICE2* (**b**), *CmCBF1* (**c**), *CmCBF2* (**d**), and *CmCOR413-2* (**e**) in melon seedling roots after 1 day of cold stress. Data are presented as "mean ± standard error" (n = 3). Different lowercase letters show significant differences among different treatments ($p < 0.05$).

4. Discussion

4.1. Positive Regulation of Cold Tolerance in Melon by CmDUF239-1

Among the various abiotic stresses that constrain plant growth and development, low temperature is a primary factor that significantly affects crop yield and quality. To cope with these adverse conditions, plants activate complex regulatory networks that integrate antioxidant defenses, osmotic regulation, and specific signaling pathways [48]. DUF proteins play a pivotal role within this framework, modulating the expression of stress-responsive genes and proteins to enhance resilience across various species [49]. For instance, overexpression of *OsSGL1* (a member of the rice DUF1645 protein family), significantly enhanced plant drought-tolerance in both *Arabidopsis* and rice, as evidenced by increased root biomass, improved recovery capacity, reduced leaf curling and wilting, and a marked decrease in malondialdehyde (MDA) content [50]. In cucumber, *CsDUF966* acts as a positive regulator of crop tolerance, and which overexpression of *CsDUF966* promoted plant height under salt and drought stresses [51].

In this study, we provide evidence that *CmDUF239-1* is also a key positive regulator of cold tolerance in melon. Our results demonstrate that overexpressing *CmDUF239-1* significantly enhanced the growth of melon seedlings under cold stress. Compared to the controls, the OEDUF239-1 lines exhibited significantly greater fresh and dry mass in both shoots and roots after 5 days of cold exposure (Figure 1c–f). OEDUF239-1 seedlings displayed significantly lower relative electrolyte leakage (REC) in both leaves and roots under cold stress, indicating enhanced cell membrane integrity and reduced chilling injury (Figure 1g,h). These performances are consistent with the protective effects previously observed for this gene under heat stress [28]. These findings extend the functional scope of *CmDUF239-1* beyond heat and salt tolerance, suggesting that it acts as a broad-spectrum regulator capable of enhancing tolerance to extreme temperatures.

4.2. CmDUF239-1 Enhances Cold Tolerance in Melon Seedlings by Increasing Antioxidant Enzyme Activity

Upon the occurrence of cold stress, the endomembrane system of plant cells is disrupted, electron transfer and redox dynamic balance are broken, and a large amount of ROS is accumulated [52]. Therefore, the ability to rapidly activate the antioxidant defense system is crucial for stress tolerance. DUF proteins have been reported to enhance cellular antioxidant defenses by regulating the activities of enzymes. For example, *AmDUF1517* in cotton enhances ROS scavenging capacity by increasing the activities of antioxidant enzymes (SOD, POD, CAT, and GST) and decreasing MDA content [25]. A previous study confirmed that in melon, *CmDUF239-1* could significantly enhance heat tolerance by increasing the activities of SOD, POD, CAT, APX, and GR, and upregulating the expression of their corresponding genes [28].

In this study, transcriptome data analysis of melon seedling roots after 1 day of low-temperature treatment revealed that molecular functions of DEGs were significantly enriched in categories related to oxidoreductase activity and peroxidase activity (Figure 2c). This suggests that *CmDUF239-1* likely confers cold stress resistance via the antioxidant system. At the physiological level, OEDUF239-1 seedlings accumulated significantly less MDA content, a marker of lipid peroxidation, while the activities of primary ROS-scavenging enzymes (SOD, POD, and CAT) were significantly higher than in the EV controls (Figure 3a–d). Concurrently, *CmDUF239-1* could more effectively activate the expression of antioxidant enzyme genes under cold stress at the transcriptional level, where key antioxidant genes such as *CmCSD1*, *CmMSD1*, and *CmPOD1* showed a more potent and rapid upregulation (Figure 4). This mechanism is consistent with findings from this gene in heat tolerance studies, indicating that this antioxidant-mediated pathway is a core and conserved function of *CmDUF239-1*. This findings position *CmDUF239-1* as a fundamental regulator of the ROS scavenging system, protecting melon seedlings from a wide range of extreme temperature stresses.

The empty vector (EV) control lines exhibited slightly elevated basal activities of CAT and POD compared to the Wild Type (Figure 3). This suggests a possible minor "priming" effect, which may be a non-specific physiological response to the *Agrobacterium* mediated transformation or T-DNA insertion. However, the OEDUF239-1 lines displayed a significantly more robust increase in these enzyme activities compared to the EV, confirming that the observed enhancement in antioxidant capacity can be primarily and specifically attributed to the function of *CmDUF239-1*. Furthermore, a key consideration in developing stress-tolerant crops is the potential "metabolic burden" associated with constitutive overexpression, especially for an uncertain stress. This study was designed using root-specific overexpression to avoid placing an unnecessary metabolic load on photosynthetic tissues under non-stress conditions. Therefore, these findings not only validate the specific role of *CmDUF239-1* but also present an agronomically viable approach that simultaneously enhances stress tolerance while maintaining optimal growth potential.

4.3. CmDUF239-1 Modulates Sugar Metabolism and Proline Regulation to Maintain Cellular Homeostasis Under Cold Stress

In addition to antioxidant defense, the accumulation of osmotic regulators is a fundamental strategy for plants to maintain cellular turgor and protect macromolecular structures under stress [53], with proline and soluble sugars being two of the most critical osmoprotectants [54]. DUF proteins have been reported to maintain water balance by promoting the accumulation of osmotic regulatory substances. For example, in soybean, overexpression of *GmDUF4228-70* was found to significantly increase proline content, thereby enhancing its drought tolerance [55]. In rice, lines overexpressing *OsDUF846.2* exhibited more severe

damage, with decreased proline and soluble sugar contents under salt and heat stress [56]. Furthermore, previous research has also confirmed the key role of *CmDUF239-1* in proline metabolism. Overexpressing *CmDUF239-1* leads to significant proline accumulation by concurrently upregulating the key synthesis gene *CmP5CS1* and repressing the key degradation gene *CmPDH*. This established a clear dual-regulatory mechanism for proline regulation by *CmDUF239-1* under high temperatures [28].

This study reveals a more complex and multifaceted regulatory strategy by *CmDUF239-1*, which involves both conserved physiological phenotype and distinct molecular pathways. First, consistent with the heat stress findings, OEDUF239-1 seedlings accumulated significantly more proline under cold stress (Figure 6a). This accumulation was also achieved by enhancing the activity of the synthesis enzyme P5CS and suppressing the activity of the degradation enzyme PDH (Figure 6b,c). However, in a major departure from the heat stress mechanism, the transcript level of the *CmP5CS1* gene was found that significantly repressed in OEDUF239-1 lines under cold, not upregulated instead (Figure 6e,f). The observed discrepancy between elevated enzyme activity and relatively low gene transcription levels suggests that *CmDUF239-1* may operate via a distinct regulatory mechanism under cold stress. One possible explanation is that, although de novo transcription of CmP5CS1 is suppressed under cold stress, the existing *CmP5CS1* mRNA pool may be stabilized, potentially through the action of RNA-binding proteins. This stabilization could prolong mRNA half-life and ensure continued translation into functional P5CS enzyme. It is crucial to emphasize that this specific molecular hypothesis is not directly tested in our study and requires further experimental verification, for instance, through mRNA decay assays. Second, a novel finding of this study is the profound effect of *CmDUF239-1* on soluble sugar metabolism, a mechanism not highlighted in the previous heat stress report. Under cold stress, OEDUF239-1 plants accumulated significantly higher levels of glucose, fructose, and sucrose (Figure 5a–c). This was directly correlated with increased activities of sucrose synthesis (SPS) and invertase (NI) enzymes (Figure 5d,e), and was strongly supported at the transcriptional level by the significant upregulation of sugar metabolism genes, including *CmSPS1F*, *CmSPS3F*, and *CmCINV2* (Figure 5f–m). Therefore, *CmDUF239-1* maintains cellular homeostasis under cold via a dual osmotic strategy: enhancing proline accumulation (likely through post-transcriptional control) and simultaneously promoting soluble sugar accumulation (through transcriptional upregulation of metabolic genes).

4.4. CmDUF239-1 Activates the Central ICE-CBF-COR Cold Response Pathway

The ICE-CBF-COR module is widely recognized as the central signaling cascade and master regulator of cold acclimation in diverse plant species. Upon cold perception, the upstream transcription factor ICE1 activates the expression of CBF genes [57]. These CBF transcription factors then bind to the promoters of COR genes, which promote osmolyte accumulation and antioxidant enzyme activation that lead to enhanced tolerance [18,58]. In melon, *CmCBF4* and *CmABF1* directly activate the expression of *CmADC* (Arginine decarboxylase), a key enzyme in the biosynthesis of cold-protective polyamines [19]. In cucumber, overexpression of the *CsCBF* genes enhances the proteins that directly bind to the promoters of their target genes *CsCOR15A* and *CsKIN1* to activate their expression and thereby enhance plant cold tolerance [59]. Notably, in *Arabidopsis*, the *DUF793* family protein BYPASS1-LIKE has been reported to participate in freezing tolerance through the CBF-dependent pathway [18], providing a direct precedent for crosstalk between DUF proteins and the core cold signaling machinery.

In this study, transcriptional analysis provides convincing evidence for *CmDUF239-1* integrating with this central cold signaling pathway (Figure 7). Under cold stress, the OEDUF239-1 seedlings exhibited significantly higher induction levels of key pathway

components compared to the empty EV plants. Specifically, the transcript levels of *CmCBF1*, *CmCBF2*, *CmICE2*, and the downstream effector gene *CmCOR413-2* were all more strongly upregulated in the OEDUF239-1 lines, aligning with findings from previous studies in cucumber and melon. The enhancement was particularly pronounced for *CmCBF2* (100.7% increase in OE and 53.5% in EV) and *CmCOR413-2* (150.1% increase in OE and 53.5% in EV). The ICE-CBF-COR pathway serves a conserved molecular chaperone role in plants, mediating responses to cold stress. This finding is particularly significant, as it strongly indicates that *CmDUF239-1* acts as an upstream positive regulator or enhancer of the ICE-CBF-COR pathway. This activation provides a unified molecular basis for explaining how *CmDUF239-1* can systemically and simultaneously enhance both the antioxidant defense system and the osmotic adjustment pathways.

5. Conclusions

To sum up, our study provides novel insights into the role of the *CmDUF239-1* gene in enhancing cold tolerance in melon seedlings. Our findings support the hypothesis that *CmDUF239-1* enhances cold tolerance by upregulating key genes associated with antioxidant defense, sugar metabolism, proline metabolism, and the ICE-CBF-COR signaling pathway. Our combined findings position *CmDUF239-1* as a high-level, broad-spectrum regulator of thermotolerance. It appears to engage conserved downstream defense mechanisms (antioxidant and osmotic systems) while differentially activating stress-specific master pathways—the HSP pathway for heat tolerance and the CBF pathway for cold tolerance. This discovery not only provides novel insights into the complex regulatory network of plant temperature responses but also offers a valuable candidate gene for the genetic improvement of climate-resilient melon cultivars with enhanced tolerance to diverse temperature extremes.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy15122725/s1, Table S1. Primers of q-RT PCR.

Author Contributions: Conceptualization, Y.P. and Z.T.; methodology, J.Z.; software, Y.L. (Yang Li); validation, Y.L. (Yanjun Liu), X.W. and Y.L. (Yang Li); formal analysis, Y.L. (Yang Li) and X.W. investigation, Y.L. (Yang Li), J.Z. and Y.L. (Yanjun Liu); resources, Y.P. and Z.T.; data curation, Y.L. (Yang Li); writing—original draft preparation, Y.P., J.Z. and Z.T.; writing—review and editing, Y.P.; visualization, Y.P.; supervision, Y.P.; project administration, Y.P. and Z.T.; funding acquisition, Y.P. and Z.T. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

MDA	malondialdehyde
PRO	proline
SOD	superoxide dismutase
POD	peroxidase
CAT	catalase

NI	neutral invertase
SPS	sucrose phosphate synthase
P5CS	Δ^1-pyrroline-5-carboxylate synthetase
PDH	proline dehydrogenase
GO	Gene Ontology

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