



## Article

# Hormone Profiles and Antioxidant Activity of Cultivated and Wild Tomato Seedlings under Low-Temperature Stress

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**Abstract:** Low temperature is a major limiting factor for the growth and reproduction of some plant species, such as tomato. So far, few studies have been conducted on the effects of low temperature, and the mechanisms of plants' response to this type of stress is not fully clear. In the current study, the effects of low, nonfreezing temperature (10 °C for three days) on the hormone content, antioxidant activity, and expression patterns of cold-related genes in the leaves of cold-tolerant species (*Solanum habrochaites* Accession 'LA1777') and cold-susceptible species (*Solanum lycopersicum* cultivar 'Moneymaker') were investigated. Low temperature increased the abscisic acid (ABA) content in both tomato species, while the content of zeatin-type cytokinins (ZT) increased in the cold-tolerant species. However, the content of indole-3-acetic acid (IAA) and gibberellic acid (GA) reduced in response to low temperature in susceptible species. Accordingly, cytokinin (CK) is identified as an important hormone associated with low-temperature stress in tomato. In addition, our results indicate that the *C-repeat/DRE binding factor 1 (CBF1)* gene is less induced in response to low temperature in tomato, although transcription of the *inducer of CBF expression 1 (ICE1)* gene was upregulated under low temperature in both tomato species. It seems that *ICE1* may modulate *cold-regulated (COR)* genes in a CBF-independent way. In addition, in response to low temperature, the malondialdehyde (MDA) level and membrane stability index (MSI) increased in the susceptible species, indicating that low temperature induces oxidative stress. Additionally, we found that glutathione peroxidase is highly involved in reactive oxygen species (ROS) scavenging induced by low temperature, and antioxidants are more induced in tolerant species. Overall, our results suggest that sub-optimal temperatures promote oxidative stress in tomato and CK is introduced as a factor related to the response to low temperature that requires deeper attention in future breeding programs of tomato.

**Keywords:** ABA; ROS; auxin; cytokinins; *CBF1* gene; phytohormones; cold-related genes



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## 1. Introduction

Abiotic stresses, such as low temperature, as limiting factors affect plant production and plant distribution [1–3]. Temperatures less than 12 °C can cause necrosis, and long-term exposure to temperatures below 12 °C inhibits photosynthesis and disrupts membrane integrity [4]. Low-temperature damage is more pronounced in tropical or subtropical plants. During low, nonfreezing temperatures, changes occur in the structure and metabolism of plant cells, which are more visible in tropical plants species. The change in membrane conformation that results in a decrease in membrane fluidity is one of the first structural changes related to low-temperature stress [5]. Furthermore, the alteration of membrane conformation can cause oxidative stress by rapidly inducing reactive oxygen species (ROS), and then, the concentration of cryoprotective compounds increases in the cytoplasmic matrix [2,6,7]. However, plants use various mechanisms, including morphological and structural modifications, as well as biochemical mechanisms to modulate cellular homeostasis to reduce low, nonfreezing temperature-induced damage [2,8,9].

Approximately 10% of the genes in *Arabidopsis* are involved in the response to cold stress and are known as *cold-regulated* (*COR*) genes [10,11]. The promoter of *COR* genes contains a CRT/DRE motif as a cis-regulatory element that is bound by transcription factors such as C-REPEAT BINDING FACTOR (CBFs) proteins under cold and drought stress as well as high-salt stress [10,12,13]. In addition, *CBF* genes are activated by other transcription factors of upstream elements, such as INDUCER OF CBF EXPRESSION (ICE) proteins [14]. However, it was reported that CBF-regulon in chilling-sensitive plants such as tomato is different from other model plants such as *Arabidopsis*. It seems that the CBF-like proteins are more induced by chilling stress in tomato [15].

Phytohormones and reactive oxygen species (ROS) play basic roles in regulating plant responses to abiotic stresses, such as low-temperature stress [16,17]. Phytohormones, including abscisic acid (ABA), jasmonic acid (JA), cytokinins (CKs), auxins (AUXs), gibberellins (GAs), salicylic acid (SA), ethylene (ET), brassinosteroids (BRs), nitric oxide (NO), and strigolactones (SLs), are chemical messengers involved in multiple cellular processes, and they regulate plant growth and development and cell signaling [18–20]. However, phytohormones are involved in all aspects of plant life, but each phytohormone has major and minor roles in the plant life cycle under different environmental conditions, and there are antagonistic and synergistic interactions between them [19,21].

Among the phytohormones, ABA, as a signaling agent, can increase tolerance to drought and cold stress by decreasing water loss and activating downstream signaling [22]. Furthermore, calcium ( $\text{Ca}^{2+}$ ) signaling, which is involved in the front line of stress signaling, is induced by ABA signaling [23,24]. In addition, previous studies have shown that JA, SA, and ET are also involved in the response to low-temperature stress [25–28]. Under low temperature, the JA levels rapidly increase, and *Arabidopsis* mutant lines for JA biosynthesis are more susceptible to low temperature [29]. Additionally, SA is involved in low-temperature stress, and endogenous SA levels increase in *Arabidopsis* shoots under low temperature [6]. In addition, the exogenous application of SA can increase cold tolerance by inducing *COR* genes and can reduce chilling damage in plants by modulating cellular hydrogen peroxide [27]. Based on previous studies, ET negatively affects cold tolerance, and exogenous application of 1-aminocyclopropane-1-carboxylic acid (ACC), an ET precursor, reduces the cold tolerance of *Arabidopsis* seedlings [28]. Moreover, *Arabidopsis* mutant lines for ET signaling genes *ein3-1*, *ein2-5*, *ein4-1*, and *etr1-1* show improved cold tolerance [28]. Other phytohormones, such as AUXs, GAs, CKs, and BRs, which have key roles in regulating signaling and growth, are also induced by low-temperature stress [6]. Besides, it seems that root gravitropism is influenced by cold stress decreasing indole-3-acetic acid (IAA) concentrations, whereas cold stress affects the auxin transport system more than it affects auxin signaling [30]. In addition, the GA content decreases in response to low-temperature stress in wheat plants [6]. CBFs that are induced specifically under cold stress can upregulate GA2 oxidase (*GA2ox*), a GA-catabolizing gene [31]. Overexpression of *CBF1* increases the level of DELLA proteins by decreasing the GA content [32]. There is also evidence of the positive effect of BRs on increasing cold tolerance. For instance, exogenous BR application can induce the expression of genes related to antioxidant enzymes and osmoprotectants [6].

Cultivars of *Solanum lycopersicum* are susceptible to cold stress, while the wild tomatoes such as *S. habrochaites* LA1777 are tolerant species. Previous studies revealed that various mechanisms are associated with cold tolerance in wild tomato species, including fast stomata closure, maintaining water potential and shoot turgor, antioxidants, and hormone content [1,33]. In addition, Chen et al. [34] found that the gene expression patterns for wild tomato species and cultivated species are dissimilar, and 21% and 23% of genes are significantly induced by cold stress in *S. lycopersicum* and *S. habrochaites*, respectively. Besides, Lu et al. [35] found that diethyl aminoethyl hexanoate (DA-6), as a synthetic elicitor, could increase the tomato tolerance to low night temperature by affecting the synthesis of endogenous cytokinin and maintaining the photochemical activity and chloroplast structure. Additionally, it was stated that a PHYTOCHROME-INTERACTING

TRANSCRIPTION FACTOR in tomato, SIPIF4, can regulate the biosynthesis and signaling of phytohormones, including gibberellin, abscisic acid, and jasmonate, in response to low temperature [36]. Moreover, it was reported that the content of H<sub>2</sub>O<sub>2</sub>, melatonin, and zeatin riboside are significantly increased in response to cold stress [37]. Due to the limiting effects of low temperature in tomato cultivation, the response mechanisms of tomato species under long-term, low-temperature stress are important to understand in different wild *Solanum* germplasm resources to be exploited for breeding new tomato varieties. In the present research, the response of the cultivated tomato *S. lycopersicum* (varietal genotype “Moneymaker”) and the wild related species *S. habrochaites* (accession “LA1777”) to low-temperature stress was investigated on the basis of phytohormone profiles, the expression of genes involved in both low-temperature tolerance and hormone biosynthesis, and antioxidant activity in order to expand our understanding and to develop strategies for improving tomato tolerance to low, nonfreezing temperatures. We investigated auxin, cytokinin, and gibberellin as well as abscisic acid profiling as plant hormones associated with the adaptation of tomato species in response to long-term low temperatures (10 °C for 3 days). Our findings clearly exhibit which hormone is more involved in the response to long-term, low-temperature stress with the specific tomato materials utilized here, and also shed light on the relationship between phytohormone profiles and key genes involved in the cold stress (*ICE1* and *CBF1*). Overall, our results offer additional insights that, if combined with the existing knowledge in this field, could find utility in future programs of tomato for further improving crop yields at low, nonfreezing temperatures.

## 2. Materials and Methods

### 2.1. Plant Material and Temperature Treatments

Seeds of two tomato species, *Solanum habrochaites* Accession ‘LA1777’ (cold-tolerant) and *Solanum lycopersicum* cultivar Moneymaker (cold-susceptible), that were supplied by the Tomato Genetics Research Center (University of California, Davis, CA, USA), were sown in plates (15 cm × 10 cm × 15 cm dimensions), containing 60% vermicompost and 40% perlite. Plates were grown at 25 ± 2 °C under a 14 h photoperiod (82.5 μmol m<sup>-2</sup> s<sup>-1</sup> of PPFD (photosynthetic photon flux density)). Forty-day-old seedlings were separated into two groups: one group was transferred to the growth chamber under a 14 h photoperiod (82.5 μmol m<sup>-2</sup> s<sup>-1</sup> of PPFD) and 10 ± 1 °C (as low-temperature stress), and the other group was maintained at 25 ± 2 °C as a control condition. After three days, the fresh shoots (young and old leaves) of forty-three-day-old seedlings (under the normal and low-temperature conditions) were harvested, immediately frozen in liquid nitrogen, and then stored at −80 °C for measurements. However, some fresh leaves were used to analyze the malondialdehyde (MDA) content and membrane stability index (MSI). All treatments of the experiment were repeated as part of four biological replicates. One replication consisted of three seedlings.

### 2.2. Hormone Analysis

In the present study, to find the relationships between phytohormones profile and long-term, low-temperature stress, three hormones involved in regulating growth and development, auxin, cytokinin, and gibberellin, with the related stress hormone, abscisic acid (ABA), were studied. The leaves of the seedlings in the treatments were ground into a powder in liquid nitrogen for phytohormone measurements. The free forms of abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellin (GA<sub>3</sub>), and zeatin (ZT) contents were analyzed using High Performance Liquid Chromatography (HPLC) (Unicam Crystal 200, Loughborough, UK) with a reverse-phase column (4.6 mm × 250 mm Diamonsic C18, 5 μm). Standards of the studied phytohormones were obtained from Sigma–Aldrich (Steinheim, Germany) with catalog numbers: A4903, 45533, 36575, and Z0164 for ABA, IAA, GA<sub>3</sub>, and ZT, respectively. The ABA content was measured based on the method described by Li et al. [38]. IAA, GA<sub>3</sub>, and ZT were measured according to the method described by Tang et al. [39].

### 2.3. RNA Extraction and Real-Time PCR Analysis

Whole shoots in each treatment were homogenized in liquid nitrogen. Total RNA was extracted using RNXTM-Plus (Sinaclon, Iran) according to the manufacturer's protocols. The quality and quantity of RNA samples were investigated by agarose gel electrophoresis and a Nano Photometer (Implen N50). Reverse transcription was carried out using 1 µg of total RNA treated with RNase-free DNase I (Thermo Scientific) and reverse transcriptase (Promega, Madison, MI, USA). The volume of each cDNA pool was adjusted to give the same exponential phase PCR signal strength according to the expression level in tomato. In this study, genes involved in hormone signaling (*CKX* and *DELLA-like* genes) and antioxidant biosynthesis (*CAT1* and *SOD* genes) and two genes encoding cold-related transcription factors (*ICE1* and *CBF1* genes) were investigated. The specific primers of selected genes with *EF-1-α* (Solyc06g005060), as an internal control gene, were designed by the online Primer3 Plus tool (Table 1). Real-time PCR was performed using RealQ Plus 2× Master Mix Green High ROXTM (Ampliqon) on an Applied Biosystems StepOne instrument. Each 10 µL aliquot for real-time PCR consisted of 5 µL of 2× master mix, 0.5 µL of each primer, and diluted cDNA (1/10). The real-time PCR conditions were based on the method described by Heidari et al. [40]. The relative expression of the selected genes was calculated using the 2−ΔΔCt method [41].

**Table 1.** List of primers of the studied genes.

Gene Name	Gene ID	Primer (5'-3')	Product Size (bp)
<i>CKX6</i>	Solyc04g016430	F: TTCCATTAGGGGACAAGCCA R: ACCACCAACGGTAAGGTACA	229
<i>DELLA-like</i>	Solyc10g086380	F: ATGGCCAGCACTTTTACAGG R: AATTCCTGTGAGCCGAAGAG	70
<i>CAT1</i>	Solyc12g094620	F: CCTCTAAGTATCGCCCATCAAG R: GGTCCAACAGTCAAGGAAGAA	100
<i>SOD</i>	Solyc11g066390	F: AGGGCAACTCTAATGTTGAGG R: CCAGGAGCAAGTCCAGTTATAC	94
<i>ICE1</i>	Solyc03g118310	F: ATGGAGGAACTGGTTCTTGG R: TCCACACCTCCATCATCAAC	139
<i>CBF1</i>	Solyc03g026280	F: CCTGCTTCCTCCAACCTCTAAA R: CTCATCCACGAAGTCACTACTC	135
<i>EF-1-α</i>	Solyc06g005060	F: GGAACCTTGAGAAGGAGCCTAAG R: CAACACCAACAGCAACAGTCT	158

### 2.4. Lipid Peroxidation Analysis and the Membrane Stability Index (MSI)

The content of malondialdehyde (MDA), the final product of lipid peroxidation, was determined according to the methods of Campos et al. [42]. We used the 1% TCA (*w/v*) and 0.5% TBA for extraction, and then MDA content was measured at 532 nm. The membrane stability index (MSI) of the tomato leaves was estimated by measuring the electrical conductivity (EC) based on the method described by Sairam et al. [43]. In summary, 0.1 g of tomato leaf was transferred to 10 mL double-distilled water and then samples were placed in a water bath at 40 °C for 10 min, and the first EC (EC1) was measured. Subsequently, the same samples were put in a water bath at 90 °C for 10 min and then EC2 was measured.

### 2.5. Catalase and Glutathione Peroxidase Activities

Leaf tissue (250 mg) was ground to a powder in liquid nitrogen and then homogenized in 2.5 mL of 0.1 M phosphate buffer (pH 7.5), after which the mixture was briefly vortexed. The samples were then centrifuged at 18,000× *g* for 15 min at 4 °C. The supernatant of the samples was removed to distinguish the enzyme activities. Catalase (CAT; EC 1.11.1.6) activity was defined as described by Aebi [44], and glutathione peroxidase (GPX; EC 1.11.1.9) activity was determined as described by Elia et al. [45].

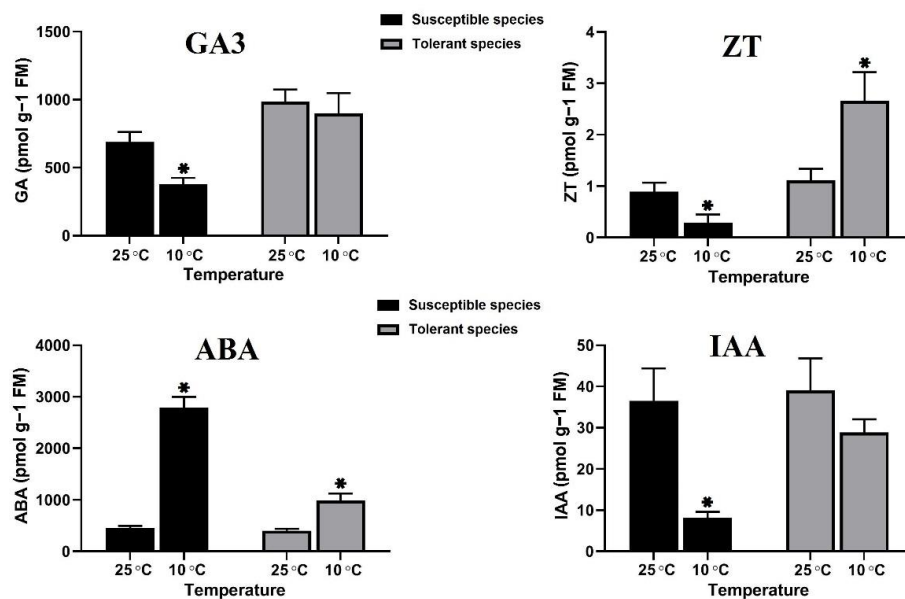
## 2.6. Statistical Analyses

All experimental treatments included four independent biological replicates. Student's *t*-test was used to calculate the significant difference ( $p$ -value < 0.05) between the low-temperature and normal-temperature treatment results using Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Besides, correlation was calculated between hormones in susceptible and tolerant species using the Pearson method. All graphs were created based on the mean of each treatment and the standard deviation (SD).

## 3. Results

### 3.1. Effects of Low Temperature on Endogenous Hormones

In the current study, the concentration of three hormones, including auxin, cytokinin, and gibberellin, which are most involved in regulating growth and development of plant cells [6], were studied along with the well-known stress hormone, abscisic acid [46,47], to provide new insights into the role of these hormones in responding to long-term, low-temperature stress. We found that the content of endogenous hormones, including ZT, ABA, IAA, and GA<sub>3</sub>, especially in the susceptible species, are influenced by low, nonfreezing temperatures to regulate downstream pathways related to stress responses (Figure 1). The GA<sub>3</sub> content was influenced by decreasing temperature, and sharply decreased by 1.83 times in the susceptible species. In addition, tomato species showed a significant differential response to low temperatures based on the ZT concentration. The ZT type of cytokinin decreased by 3.11 times with decreasing temperature in the susceptible species, while it increased by 2.39 times in the tolerant species in response to low temperature. Furthermore, the ABA content was significantly higher in both tomato species under low-temperature stress than normal temperature. However, the ABA concentration was greater in the susceptible species than in the tolerant species. In addition, the content of indole-acetic acid (IAA), an auxin, significantly decreased by 4.45 times in the susceptible species in response to low-temperature stress. Additionally, a strong negative correlation based on correlation analysis (Table 2) was observed between the ABA and IAA profiles in the tomato species, while a positive correlation was found between ABA and ZT in tolerant species (Table 2). The observed positive correlation between ABA and ZT indicates the regulatory potential of cytokinin in response to low-temperature stress.



**Figure 1.** Hormone content profiles, including abscisic acid (ABA), indole-3-acetic acid (IAA), gibberellin (GA<sub>3</sub>), and zeatin (ZT) contents, in tomato leaves in response to temperature change (10 °C for three days). An asterisk above a bar shows a significant difference ( $p$ -value < 0.05) between the low-temperature and normal-temperature treatments ( $n = 4$ ) (according to Student's *t*-test).

**Table 2.** Correlation between hormones in susceptible species (below the diagonal) and tolerant species (above the diagonal) under normal and low-temperature conditions.

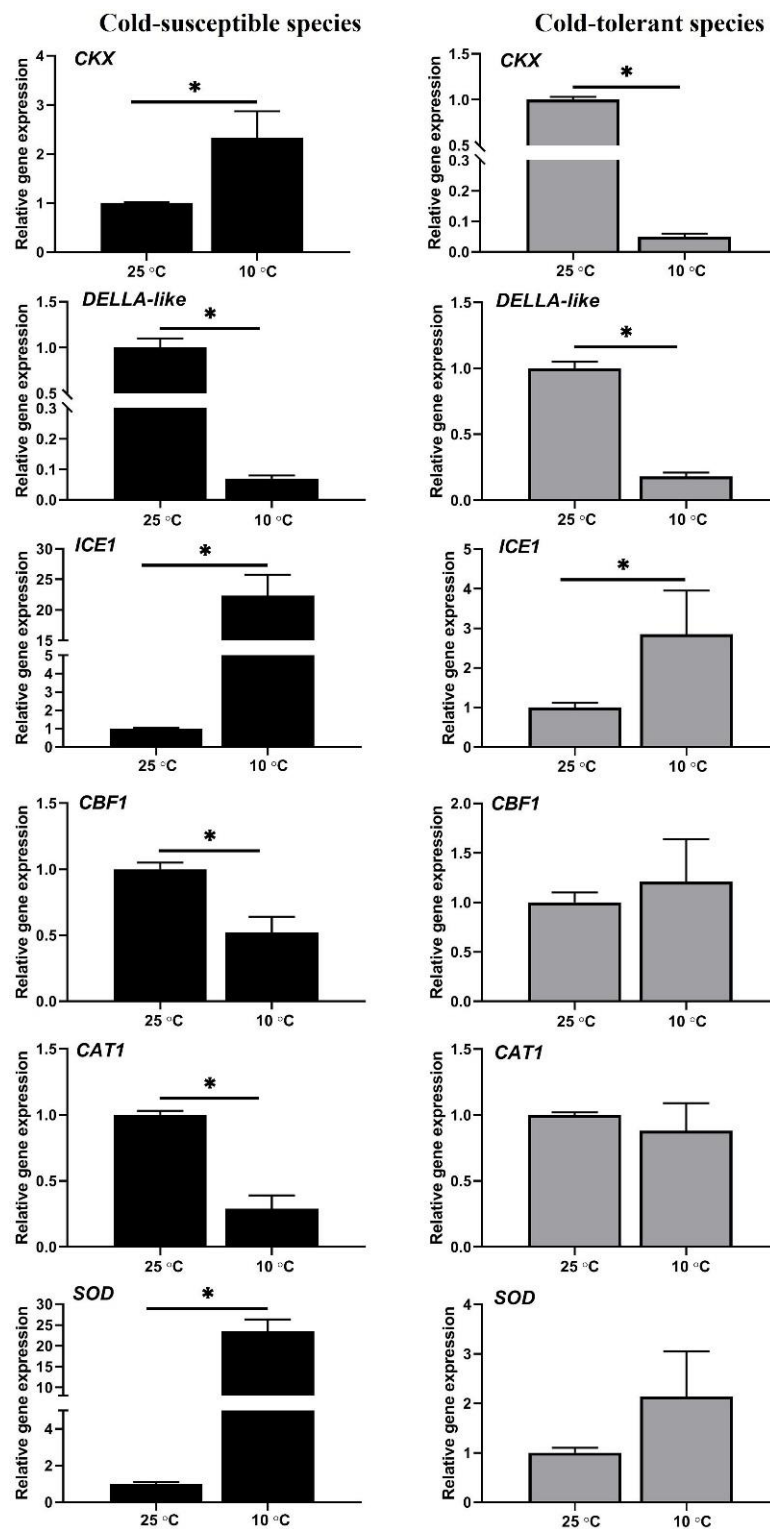
	GA <sub>3</sub>	ZT	ABA	IAA
GA <sub>3</sub>		−0.45	−0.62	0.74
ZT	0.85		0.91	−0.83
ABA	−0.79	−0.73		−0.77
IAA	0.87	0.75	−0.98	

### 3.2. Effects of Low Temperature on Gene Expression

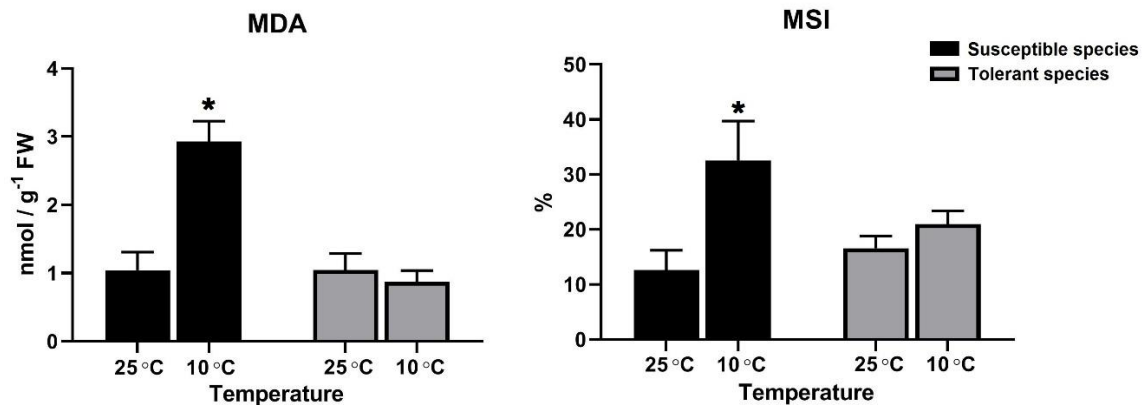
The expression patterns of the CKX (as a cytokinin oxidase/dehydrogenase gene involved in cytokinin signaling), *DELLA-like* (as a GA signaling suppressor), *ICE1* and *CBF1* (as the cold-related transcription factors), *CAT1* (as a catalase gene involved in catalase biosynthesis), and *SOD* gene (involved in superoxide dismutase biosynthesis) are shown in Figure 2. The results revealed that low-temperature stress could affect the expression of the studied genes in the susceptible species. *CKX* and *DELLA* are also involved in response to abiotic stress [48,49], and the study of their expression patterns along with cold response genes, including *ICE1* and *CBF1*, can provide valuable information. In the current study, the *CKX* gene showed differential expression in response to low temperature in the tomato species, where it was significantly upregulated in the susceptible species and downregulated in the tolerant species. In this study, the expression pattern of the *DELLA-like* gene, which encodes an inhibitor of GA biosynthesis, was evaluated. According to the gene expression results, low temperature as a repressor decreased the expression of *DELLA-like* under low-temperature stress. Under low temperature, expression of the *ICE1* gene was upregulated in both genotypes, while *ICE1* expression was sharply upregulated in the susceptible species. In addition, the expression level of the *CBF1* gene slightly decreased in response to low temperature in the susceptible species; however, it was not induced by low temperature in the tolerant species. In this study, the expression pattern of two genes involved in antioxidant biosynthesis was investigated. As shown in Figure 2, the expression of the *CAT1* gene was downregulated in the susceptible species under low temperature, whereas it was not induced by low temperature in the tolerant genotype. Under low temperature, *SOD* expression was significantly upregulated in the susceptible species. The expression pattern results showed that *ICE1* and *SOD* genes were more highly expressed in response to low-temperature stress than to normal temperature in tomato.

### 3.3. Effects of Low Temperature on Lipid Peroxidation and the Membrane Stability Index

Adverse environmental conditions can damage the cell membrane of plant cells by inducing oxidative stress. To investigate the effects of low temperature on cell membrane stability, the MDA content and MSI were measured. The MDA concentration in the cold-susceptible species significantly increased under low temperature and was nearly three times greater than that under normal temperature (Figure 3). Additionally, the membrane stability index (MSI) was influenced by low-temperature stress, and the MSI (%) significantly increased by 2.55 times in the susceptible species in response to low-temperature stress. Our results revealed that low temperature causes oxidative stress that increases lipid peroxidation, the MDA content, and cell membrane stability.



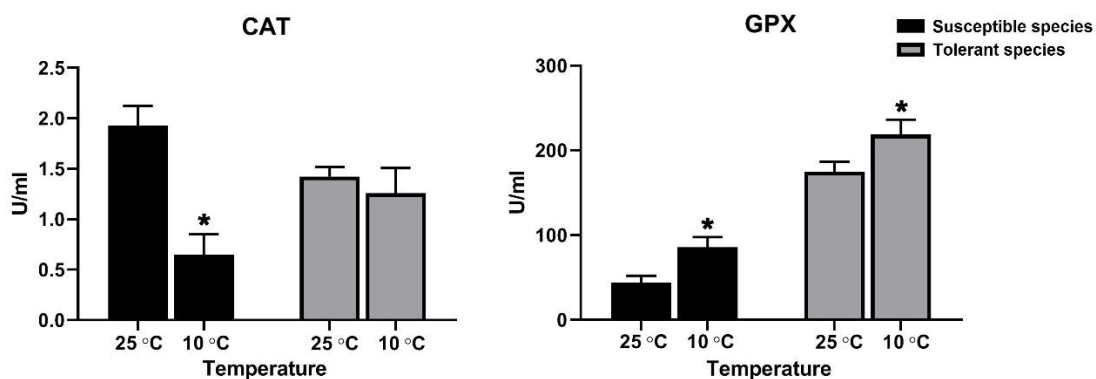
**Figure 2.** Expression patterns of the cytokinin oxidase/dehydrogenase (CKX), *DELLA-like* (as a GA signaling suppressor), INDUCER OF CBF EXPRESSION 1 (ICE1), C-REPEAT BINDING FACTOR 1 (CBF1), CAT1 (as a catalase biosynthesis gene), and superoxide dismutase (SOD) genes under low temperature (10 °C for three days). An asterisk above a bar shows a significant difference ( $p$ -value < 0.05) between the low-temperature and normal-temperature treatments ( $n = 4$ ) (according to Student's  $t$ -test).



**Figure 3.** Effects of low temperature (10 °C for three days) on malondialdehyde (MDA) content (nmol/g<sup>-1</sup> FW) and membrane stability index (MSI %). An asterisk above a bar shows a significant difference ( $p$ -value < 0.05) between the low-temperature and normal-temperature treatments ( $n = 4$ ) (according to Student's  $t$ -test).

### 3.4. Effects of Low Temperature on Antioxidant Activities

In the present study, the activities of catalase (CAT) and glutathione peroxidase (GPX) were evaluated in tomato species under low temperature. The CAT activity in the cold-susceptible species significantly decreased under low-temperature stress, while a non-significant change was observed in the CAT activity of the tolerant species compared to the control treatment. The decrease in CAT activity under low temperature in the susceptible species was nearly three times that under normal temperature (Figure 4). In addition, the levels of GPX activity increased in both tomato species in response to low-temperature stress. The increase in GPX activity in the susceptible species was 92.55%, while the increase in tolerant species was 24.32% compared with that under normal temperature.



**Figure 4.** Catalase (CAT) and glutathione peroxidase (GPX) activity in different tomato species under low temperature (10 °C for three days). An asterisk above a bar shows a significant difference ( $p$ -value < 0.05) between the low-temperature and normal-temperature treatments ( $n = 4$ ) (according to Student's  $t$ -test).

## 4. Discussion

Low temperature is among the negative factors that reduce the yield and growth of greenhouse and low-tolerance crops such as tomato [50]. The results of this study determined that low temperature could induce oxidative stress. The content of antioxidants and phytohormones, including GA, IAA, ABA, and CK, were modified as responses of cultivated tomato species to decreasing temperature. In addition, our findings showed that changes in the content of cytokinin, auxin, and gibberellin could be among the key factors affecting the tolerance to low-temperature stress in tomato species. In the following sections, the important results are further interpreted.



#### 4.1. CKs Are Responsive to Low-Temperature Stress

Cytokinin (CK) hormones can regulate various biological processes in plants as well as responses to abiotic stresses. The negative and positive effects of CKs have been observed under stress conditions [51]. In this study, low temperature reduced the zeatin (ZT) concentration in the leaves of seedlings of the cold-susceptible tomato species, and the expression of the *CKX* gene was upregulated in response to low-temperature stress. However, different responses were observed in the cold-tolerant species based on the CK content and *CKX* gene expression. The evidence from previous studies also shows that the CK concentration increases in response to drought and water stress [52,53], whereas numerous investigations have reported that CK levels decrease in plant species under adverse conditions [54–56]. Furthermore, exogenous application of CKs could enhance the freezing tolerance of *Arabidopsis* in a CBF1-independent manner [57]. The cytokinin oxidase/dehydrogenase (*CKX*) enzyme can regulate endogenous CK levels, and the constitutive overexpression of *CKX* genes reduces the CK content and improves drought and heat stress tolerance [58–60]. Plants use different mechanisms to adapt to adverse environmental conditions, one of which is regulation of cytokinin metabolism [61]. Additionally, several crosstalk between CKs and other phytohormones such as ABA have been observed to increase plant tolerance to adverse conditions [61]. Besides, we found a positive correlation between ABA and ZT content in tolerant species. It seems that the change in cytokinin content is probably associated with the tolerance to low-temperature stress in tomato species, that in future tomato genetics programs, focusing on the biosynthesis and signaling pathway of cytokinin hormone, may be useful.

#### 4.2. ABA Regulates Cold Responses in Tomato Seedlings

The signaling mechanisms of ABA in model plant species such as *Arabidopsis thaliana* under adverse environmental stresses have been well-described [62]. Cold-related genes are induced by both ABA-independent and ABA-dependent pathways [22]. In addition, the exogenous application of ABA can enhance cold resistance by reducing cell membrane injury and increasing the content of proline and soluble sugars [63,64]. In this study, ABA concentration was more increased in cold-susceptible cultivars under long-term, low-temperature stress. More ABA concentration may reduce the damage of dehydration by closing stomata and keeping the cellular water [22,23]. It seems the cold-susceptible cultivars respond more to the low temperature, and ABA concentration is increased for controlling the negative effects of low temperature. Additionally, previous studies have revealed that ABA regulates cold responses through a CBF-independent pathway [65,66]. However, there is also evidence of ABA effects on the expression of *CBF* genes under cold stress. For instance, open stomata 1 (*OST1*) is a serine/threonine protein kinase induced by ABA in response to cold stress that interacts with *CBF* genes [67]. Besides, *OST1* may control *CBF* expression levels via affecting phosphorylation of *ICE1*, which increases the stability and transcriptional activity of *ICE1* [68]. It seems that ABA can regulate the expression of *COR* genes either by affecting *CBF* transcription or through a CBF-independent pathway. In this study, the ABA content and expression of the *ICE1* gene increased in response to low temperature in both tomato species; however, they were more abundant in the susceptible species. Additionally, the expression of the *CBF1* gene was reduced under low temperature. Overexpression of the tomato-*CBF1* (*LeCBF1*) gene in *Arabidopsis* showed that *LeCBF1* is induced by cold stress and increases freezing tolerance [15]. Interestingly, the overexpression of *LeCBF1* did not improve freezing tolerance in transgenic tomato plants, and *LeCBF1* was not induced by drought and salinity stress or by exogenous application of ABA [15]. Accordingly, *CBF* genes are involved in responses to cold stress, but their functions differ little in different plant species. Overall, the lack of correlation between ABA content and the expression pattern of the *CBF1* gene reinforces the hypothesis that abscisic acid probably controls *COR* genes through a CBF-independent pathway in tomato species in response to long-term, low-temperature stress.

#### 4.3. Effects of Low-Temperature Stress on IAA Content

There is little information on the role of auxin in the response to cold stress or low-temperature stress. Shibasaki et al. [30] found that cold stress has little effect on auxin signaling and inhibits acropetal auxin transport by affecting the auxin efflux carrier PIN2. In this study, the IAA content decreased in response to low-temperature stress in the susceptible species. Additionally, Albacete et al. [56] reported that the IAA content decreased in the leaves of tomato in response to salt stress but increased in the roots. In contrast, Du et al. [69] found that the endogenous IAA content of rice seedlings slightly increased under cold stress (4 °C). Previous studies have also revealed that the auxin response factor (ARF) and auxin/indole-3-acetic acid (Aux/IAA) proteins, which are key elements of auxin signaling, are involved in the response to environmental stress [70,71]. ARF proteins are repressed by Aux/IAA proteins. In the presence of auxin, Aux/IAA repressors are degraded by the 26S proteasome pathway [72]. Interestingly, two *Aux/IAA* genes, *IAA5* and *IAA19*, which are involved in stress tolerance, are directly regulated by a CBF1 transcription factor [71]. It can be stated that the interaction between auxin and Aux/IAA proteins plays an important role in plant responses to abiotic stress, such as low-temperature stress. Overall, under long-term low temperatures, it is possible that auxin content is reduced in susceptible species for lowering the growth rate, which increases the adaptation.

#### 4.4. Effects of Low-Temperature Stress on GA Content

In this study, the GA<sub>3</sub> content was significantly reduced in the susceptible species in response to low temperature. According to previous studies, cold stress can affect GA biosynthesis and GA signaling, and CBFs are known as the point of interaction between cold stress and GA metabolism [32,73,74]. Overexpression of *CBF1* in *Arabidopsis* causes a reduction in GA by controlling the expression of *DELLA* genes, which are negative regulators of GA signaling [32]. Additionally, Shan et al. [74] found that the expression of the *DREB1/CBF1* gene in cotton is repressed by GA<sub>3</sub> treatment and that overexpression of *DREB1* in cotton could enhance the chilling tolerance of transgenic tobacco. In addition, *Arabidopsis* GA-insensitive mutant lines showed higher freezing tolerance than did the wild type [32]. However, Alonso-Ramírez et al. [75] reported that GA<sub>3</sub> application could reverse the negative effects of heat and salt stress in the early stages of *Arabidopsis* seedling growth. In this study, the expression level of the *DELLA-like* gene was significantly downregulated in both tomato species in response to low temperature. However, freezing tolerance was reduced in mutant *Arabidopsis* lines of *DELLA* genes [32]. Recently, Lantzouni et al. [76] found that *DELLA* proteins can affect cold-regulated genes by interacting with members of the *GRF* (*GROWTH REGULATORY FACTOR*) gene family in response to cold stress. Additionally, it was found that *DELLA-like* genes are involved in cell elongation and also regulate the stress resistance [77]. *RGL2*, as a *DELLA-like* gene, negatively regulates the GA responses and specifically controls the seed germination [78]. All these data indicate that GA is involved in the plant response to long-term, low-temperature stress, and reduction of GA content is probably linked with adaptation mechanisms of the susceptible tomato species. However, the interaction between GA signaling pathways and responses to cold stress is unclear.

#### 4.5. Interactions between Hormones in Response to Low-Temperature Stress

In the current study, the content of GA, auxin, and ZT was significantly reduced in susceptible species in response to low-temperature stress. Furthermore, CK was identified as an important hormone associated with low-temperature stress in tomato. The reduction in auxin and GA contents under cold stress limits the plant growth and causes it to face unsuitable conditions [31,37]. The reduced CK content can increase apical dominance, which aids to adapt to unfavorable conditions [79].

The different interactions between plant hormones cause synergistic or antagonistic interactions, affecting the downstream networks of hormone signaling [19,47]. *CBF* genes constitute a central point of plant hormone interactions that play an important role during

cold stress [6,47]. Plant hormones can control the key downstream genes related to cold responses via CBF-dependent or CBF-independent regulons [47]. For instance, CK, GA, and ABA mainly control the pathways of cold responses from CBF-independent pathways [80,81]. However, Lee and Seo [82] found that ABA can induce the CBF-COR pathway in Arabidopsis via *MYB96* as an ABA-induced transcription factor. On the other hand, BRs, ethylene, and JA regulate the downstream pathways via CBF-dependent pathways [47,83]. According to hormone profile and *CBF1* expression in tolerant species, our results suggested that CK and ABA control *COR* genes via CBF-independent pathways. Additionally, in Arabidopsis, the external application of CK could increase freezing tolerance, while the expression of the *CBF1* gene was not induced [57]. In our study, a strong negative correlation was observed between the ABA and IAA in both tomato species. ABA and auxin mostly have antagonistic interaction, and together are involved in many aspects of plant growth and development [84]. Several interaction nodes between auxin and ABA signaling pathways have been identified. For instance, AUXIN RESPONSE FACTOR 2 (ARF2), involved in auxin signaling, can be induced by ABA [84]. Interestingly, in the current study, a positive correlation was found between ABA and ZT in tolerant species. ABA is known as a stress hormone and regulates cell-signaling pathways in response to abiotic stresses. The positive correlation between CK and ABA supports the hypothesis, according to which CK is involved in increasing tolerance to low-temperature stress in *S. habrochaites* accession 'LA1777'. However, different results on the interaction between CK and ABA have been reported in previous studies. ABA and CK also have antagonistic interaction, and the ethylene biosynthesis pathway is identified as an interaction node between ABA and CK signaling pathways [85]. In addition, we observed a negative correlation between ABA and ZT content in susceptible species, confirming an antagonistic interaction between ABA and CK in response to low-temperature stress. Overall, it seems that the interaction between ABA and CK plays a critical role in low-temperature tolerance in tomato species. However, conditions such as the type and severity of stress and plant genetics may affect the interaction between phytohormones. Besides, antioxidants as upstream factors play a critical role in regulating other stress response components in response to low-temperature stress.

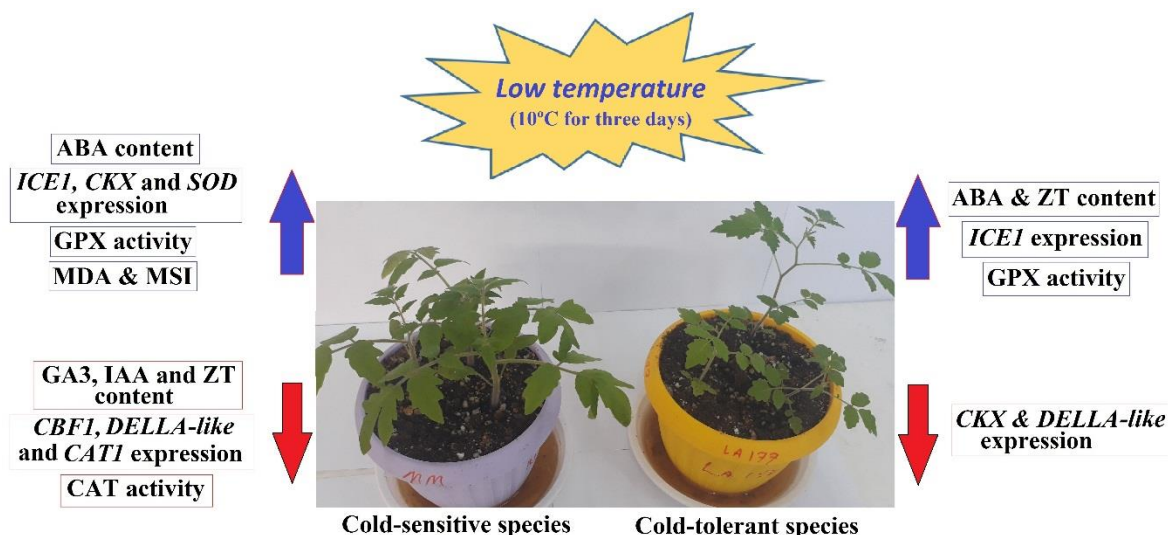
#### 4.6. Effects of Low Temperature on Lipid Peroxidation and Antioxidant Activity

Abiotic stresses, such as cold stress, damage the cell membrane by inducing oxidative and lipid peroxidation of the membrane [64,86]. In the current study, we found that both the MDA content and the MSI significantly increased in the cold-susceptible species under low temperature, indicating that lipid peroxidation was enhanced, and that the plasma membrane was injured. Under oxidative conditions, uncontrolled free radicals increase, and plants use enzymatic and nonenzymatic antioxidants to reduce oxidants and regulate cellular homeostasis [87,88]. Catalase (CAT) and glutathione peroxidase (GPX), which are enzymatic antioxidants, degrade hydrogen peroxide ( $H_2O_2$ ) into  $H_2O$  and  $O_2$  [87]. In this study, glutathione peroxidase was more induced than catalase by low-temperature stress in the tomato species. The increase in GPX activity indicates that low temperature induces oxidative stress in tomato. Additionally, both CAT activity and *CAT1* expression levels decreased in the susceptible species under low-temperature conditions. This result was consistent with the results of Park et al. [89]. Ntatsi et al. [90] found that in response to low temperature, the activity of antioxidant compounds are increased in *S. lycopersicum* Monemaker grafted onto *S. habrochaitis* LA1777, which may increase adaptation to low temperature. Besides, Munir et al. [91] reported that a  $Ca^{2+}$  sensor in *S. habrochaites*, calmodulin-like (*ShCML44*), is involved in tolerance to abiotic stress in tomato, and the overexpression of *ShCML44* could increase antioxidants content, maintaining lower MDA content and cell membrane damage. In addition, Guan et al. [92] found that the gene expression of *CAT1* in maize is induced by ABA and  $H_2O_2$ . Additionally, we found that the *SOD1* transcript was upregulated in response to low temperature, indicating that superoxide dismutase is also involved in ROS scavenging induced by low temperature. The SOD gene plays a critical role in cold tolerance in tomato [93]. Additionally, SOD is

known as a stable biochemical marker for tolerance of environmental stress in plants [94]. It seems that low temperature causes oxidative stress in tomato, and antioxidant enzymes such as GPX and SOD are induced to control the oxidants and maintain cellular redox.

## 5. Conclusions

In the current study, we evaluated tomato responses under long-term low temperatures based on hormone content and antioxidant activity. A summary of the significant effects of low-temperature stress on the measured characteristics is shown in Figure 5.



**Figure 5.** Effects of low temperature on hormone contents, antioxidant activity, and gene expression in tomato species. The red arrows indicate decreasing characteristics, and the blue arrows indicate increasing characteristics.

Our results show that long-term, low-temperature stress can affect the membrane integrity, which enhances lipid peroxidation in the susceptible species. In response to the negative effects of low temperature, GPX activity is induced and both ABA and ZT contents increase to regulate downstream signaling and cellular redox. It seems that *S. habrochaites*, as a cold-tolerant species, uses the different mechanisms related to antioxidants and hormone concentration, which can be used to improve the cold tolerance of commercial tomato cultivars. In this study, we stated that modification in the content of CK, IAA, and GA is probably associated with cold tolerance in tomato species. Increasing the CK content and not changing the content of auxin and GA can increase the cold tolerance of cultivated tomato species. Overall, our findings can be used in future programs related to introducing new lines with the ability to withstand low temperatures.

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