Article

Exogenous EBR Ameliorates Endogenous Hormone Contents in Tomato Species under Low-Temperature Stress

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Abstract: Low-temperature stress is a type of abiotic stress that limits plant growth and production in both subtropical and tropical climate conditions. In the current study, the effects of 24-epi-brassinolide (EBR) as analogs of brassinosteroids (BRs) were investigated, in terms of hormone content, antioxidant enzyme activity, and transcription of several cold-responsive genes, under low-temperature stress (9 °C) in two different tomato species (cold-sensitive and cold-tolerant species). Results indicated that the treatment with exogenous EBR increases the content of gibberellic acid (GA3) and indole-3-acetic acid (IAA), whose accumulation is reduced by low temperatures in cold-sensitive species. Furthermore, the combination or contribution of BR and abscisic acid (ABA) as a synergetic interaction was recognized between BR and ABA in response to low temperatures. The content of malondialdehyde (MDA) and proline was significantly increased in both species, in response to low-temperature stress; however, EBR treatment did not affect the MDA and proline content. Moreover, in the present study, the effect of EBR application was different in the tomato species under low-temperature stress, which increased the catalase (CAT) activity in the cold-tolerant species and increased the glutathione peroxidase (GPX) activity in the cold-sensitive species. Furthermore, expression levels of cold-responsive genes were influenced by low-temperature stress and EBR treatment. Overall, our findings revealed that a low temperature causes oxidative stress while EBR treatment may decrease the reactive oxygen species (ROS) damage into increasing antioxidant enzymes, and improve the growth rate of the tomato by affecting auxin and gibberellin content. This study provides insight into the mechanism by which BRs regulate stress-dependent processes in tomatoes, and provides a theoretical basis for promoting cold resistance of the tomato.

Keywords: cold stress; cold-responsive genes; anti-oxidants; proline; malondialdehyde; hormone profiling

1. Introduction

Low-temperature stress in plants, categorized as freezing stress or chilling stress, is one of the main environmental stresses that adversely affects plant production across the world, especially in subtropical and tropical climate conditions. This environmental extreme is escalating due to global climate change and is, therefore, threatening sustainable crop production [1,2]. Cold stress impacts the photosynthetic system, impairing the cycle of carbon reduction, the thylakoid electron transport, and the stomatal control of CO_2, providing enhanced accumulation of sugars, lipids peroxidation, and water balance disturbance [3–6]. Moreover, a low temperature negatively impacted plants, especially in regards to macromolecules activity, altering the fluidity of the membrane, and reducing osmotic

potential of the cell [7]. Regarding the metabolic processes and pathways—cold stress affects antioxidant enzyme activities, membrane fatty acid compositions, and adjusting of the redox state and gene expression [8].

Once plants are exposed to stresses, such as cold stress, different kinds of reactive oxygen species (ROS) are generated, which can undertake a series of oxidation–reduction reactions. Plants defend themselves by enzymatic and non-enzymatic antioxidants [9]. An alternative defense strategy could be supplementing hormones, which could enhance antioxidant and detoxification ability in order to cope and tolerate stressful conditions [10,11]. Previous studies identified numerous hormones and signaling molecules associated with plant responses to particular stress. For instance: ethylene engaged in red light-induced anthocyanin biosynthesis in cabbage [12], the antioxidant system, and ABA in brassinosteroid-induced water stress tolerance of grapevines [13]. Coordination of signaling molecules and hormones positively influences the plant's responses to stress and ultimately its preservation in unfavorable conditions [14–16]. Moreover, brassinosteroids (BRs) as steroidal hormones are involved in an array of physiological and developmental processes via their active engagement in processes, such as antioxidant metabolism [4,17,18], photosynthesis [4,19], nitrogen metabolism [20,21], plant–water relations [22], and osmolyte accumulation [23] in various conditions [1,24–26]. Treatment with 24-epi-brassinolide (EBR) regulates the ascorbate–glutathione (AsA–GSH) component cycle in low-temperature stress on a temporal basis, leading to increased low-temperature tolerance in grapevines at the seedling stage [7]. Transcriptome analysis revealed that treatment with EBR in cold conditions raises the transcript levels of genes related to photosynthesis and chlorophyll biosynthesis, including those encoding for photosystem II (PSII) oxygen-evolving enhancer protein, photosystem I (PSI) subunit, light-harvesting chlorophyll protein complexes I and II, and ferredoxin [27].

Furthermore, BRs illustrated engagement in the regulation of ROS metabolism through the expression of many antioxidant genes that enhance the activity of antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD), and peroxidase (POX) [17]. Under low-temperature conditions, plants, by active BR signaling and accumulation of the activate Brassinazole resistant 1 (BZR1) (a BR signaling positive regulator protein), elevate the respiratory burst oxidase homolog 1 (*RBOH1*) transcript levels and the apoplastic H_2O_2 production [28]. The *RBOH1* encodes NADPH oxidase that is involved in ROS in the apoplast, mainly for signaling purposes [25] Moreover, crosstalk between the alternative oxidase (AOX) pathway and BR plays a pivotal role in ameliorating plant tolerance to cold stress, and it has been shown that BR-induced AOX synthesis protects photosystems by bounding ROS synthesis exposed to low-temperature stress [29]. In young grapevine seedlings, foliar application of 24-epi-brassinolide adjusted proteins, free proline contents, and soluble sugars activates the antioxidant machinery to increase chilling stress tolerance [30].

Tomato is a popular garden fruit worldwide because of its edible fruits, rich in antioxidants, and capable of fighting against ROS. Overexpression of DWARF or exogenous EBR application enhances low-temperature tolerance by diminishing oxidative damage in tomato plants [31]. It is worth noting that ROS may also act as a signal in mediating BR-adjusted responses in low-temperature tolerance [32]. A previous study showed that, to protect the plants from oxidative damage, glutaredoxin (GRX), 2-cysteine peroxiredoxin (2-Cys Prx), and RBOH1 participate in a signaling cascade to mediate BR-induced low-temperature tolerance in tomatoes [31]. It was shown that BRs can interact with auxin, salicylic acid, cytokinin, abscisic acid, jasmonic acid, gibberellin, and ethylene, in controlling several morpho-physiological processes in plants [33]. The objective of the current study was to evaluate the effects of 24-epi-brassinolide (EBR) treatment on hormone content, antioxidant activity, the content of malondialdehyde (MDA) and proline, and gene expression of cold-responsive genes on the tomato species under low-temperature stress. This study provides insight into the mechanisms by which BRs regulate stress-dependent processes in tomatoes and provides a theoretical basis for promoting cold resistance in tomato.

2. Materials and Methods

2.1. Plant Materials and Growth Condition

In this study, seeds of the two contrasting tomato species, cold-sensitive (*Solanum lycopersicum* cv. 'Moneymaker') and cold-tolerant (*S. habrochaites*, Accession 'LA1777') [34], were selected to investigate the effects of BR on tomato seedlings under low-temperature stress. Firstly, seeds were sterilized using 2% sodium hypochlorite solution for 12 min and then washed with double distilled water and dried. The three sterilized seeds of each species were sown in plates containing 50% vermicompost and 50% perlite. In this study, 30 plates were used for each tomato species. The plates were maintained in a growth chamber at 23 ± 2 °C with the 16 h light/8 h dark cycling. After 40 days, tomato plates of each species were divided two groups; half of them were sprayed with 5 mg/L of 24-epi-brassinolide (EBR) and repeated after 6 h. After 3 h from the last spraying, each treatment was divided into two groups; the first group was transferred to a growth chamber at 9 ± 1 °C and the second group was maintained at 23 ± 1 °C. After three days, the leaves of each sample were cut and stored in liquid nitrogen and transferred to −80 °C for the next analysis. In the present study, control plants were cultivated at 23 °C and without spraying EBR.

2.2. Hormone Profiling

To analyze the free forms of the hormones, including abscisic acid (ABA), indole-3-acetic acid (IAA), and gibberellin (GA3), the young leaves (2.0 g) of each treatment were well-powdered using liquid nitrogen and then samples were crushed by cold methanol. The extract was achieved using 30 mL of 80% cold aqueous methanol in darkness at 4 °C. To determine the hormone content, 10 µL of the extract was injected. The concentration of each hormone was determined using HPLC (Unicam, Cambridge, UK) with a C18 reverse-phase column (4.6 × 250 mm Diamonsic C18, 5 µm, PerkinElmer, Ohio, USA) and column temperature was 35 °C, gradient elution, mobile phase in methanol, and 1 mL/min flow rate at a wavelength of 254 nm. The peak area of the standard was considered to determine the sample concentration. Moreover, the standards of ABA, IAA, and GA3 were received from Sigma–Aldrich (Steinheim, Germany). The content of IAA and GA3 was measured based on the method defined by Tang et al. [35]. The content of ABA was determined according to the method characterized by Li et al. [36].

2.3. Lipid Peroxidation Assay and Proline Content

The malondialdehyde (MDA) content has been identified as a marker of lipid peroxidation rate associated with oxidative stress. In the current study, 200 mg of fresh leaves were homogenized using 1% TCA (*w/v*). The MDA content was measured according to the method defined by Campos et al. [37]. Moreover, 0.5 g of leaves were homogenized by 10 mL of 3% sulfosalicylic acid to determine the proline content in each sample. In the current study, the free proline content was analyzed using a method described by Zhang and Huang [38].

2.4. Enzyme Activity

The tomato leaves (300 mg) were ground to a powder in liquid nitrogen and mixed in 3 mL of 0.1 M extraction phosphate buffer (pH 7.5) and the mixed sample was shortly vortexed. The homogenized samples were centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant of each sample was transferred to determine the enzyme activities. The glutathione peroxidase (GPX; EC 1.11.1.9) activity was distinguished using a method described by Mittova et al. [39], and catalase (CAT; EC 1.11.1.6) activity was measured as described by Aebi [40].

2.5. RNA Extraction and Real Time PCR

The leaves of tomato seedlings were well powdered in liquid nitrogen and the total RNA of each sample was extracted using RNX [TM]-Plus (SinaClon, Tehran, Iran), based on

the manufacturer's protocols. The extracted RNA samples were then treated by RNase-free DNase I (Thermo Fisher Scientific, Wilmington, MA, USA). The Nano Photometer (Implen N50, Munich, Germany) and a 1% (*W/V*) agarose gel were used to check the quality and quantity of extracted RNA samples. The cDNA was synthesized using 1 µg total RNA and M-MULV reverse transcriptase (Thermo Fisher Scientific, Wilmington, MA, USA) based on the instructions of manufacture. The real-time PCR reactions were run using RealQ Plus 2x Master Mix Green High ROXTM (Ampliqon, Odense, Denmark) on an ABI StepOne system. In this study, four genes belonging to the apetala2/ethylene responsive factor (AP2/ERF) gene family, involved in response to low-temperature stress [41] and the inducer of CBF expression 1 (ICE1), as a key transcription factor gene involved in cold stress tolerance [42], were selected to study the expression patterns by real-time PCR. The *elongation factor 1α* (*EF-1α*; *Solyc06g005060*) gene, as an internal control gene, was used to calculate the relative expression of target genes. The specific real-time PCR primers of genes were designed and evaluated by the online Primer3 Plus tool (Table 1). Finally, the relative expression levels of selected genes were calculated using the $2^{-\Delta\Delta Ct}$ method [43].

Table 1. List of used primers in real-time PCR reactions.

Gene Name	Gene ID	Primer (5′-3′)	Product Size (bp)
ERF.B13	Solyc08g078190	F: GGTGAAGAAGTATGAATGGATCGA R: TCACAGGAACCGAAACAATCG	71
ERF2	Solyc01g090310	F: CTTATGACCAAGCCGCATTC R: ACCCGAGCCGATTAAATGAG	74
ERF52	Solyc03g117130	F: CATTGGGGATCTTGGGTTTC R: TTAGTGCGTGCTGTTGAACC	143
ERF13	Solyc04g054910	F: TCAAGTATGGCCTCCTGCAA R: GAGCAACCTTCACTATTACATGAC	88
ICE1	Solyc03g118310	F: ATGGAGGAACTGGTTCTTGG R: TCCACACCTCCATCATCAAC	139
EF-1α	Solyc06g005060	F: GGAACTTGAGAAGGAGCCTAAG R: CAACACCAACAGCAACAGTCT	158

2.6. Statistical Analyses

All experiments were run in triplicate with three technical replicates, and the effect of the low temperature and EBR treatments on analyzed variables within each species was analyzed by one-way ANOVA and Tukey test using Minitab software (version 17). The final graphs were created using Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) based on the average of each treatment and the standard deviation (SD).

3. Results

3.1. Effects of EBR on Endogenous Hormones in Tomato Leaves Exposed to a Low Temperature

After three days of exposure to low-temperature stress, the content of both GA3 and IAA hormones significantly decreased in cold-sensitive species, but not in cold-tolerant species, although a slight decrease of IAA content under stress was still observed (Figure 1). Exogenous EBR treatment could significantly increase the content of GA3 and IAA in cold-sensitive species in comparison to the control under low-temperature stress. Moreover, the ABA content in both tomato species significantly increased in response to low-temperature stress. A sharp increase in the ABA content was observed in cold-sensitive species that received EBR treatment compared with the control under the low-temperature stress. The treatment with EBR had different effects in cold-sensitive and cold-tolerant species. In fact, although the trend of accumulation in response to a low temperature was similar to that observed in an untreated plant (-EBR), in the cold sensitive species, the treatment with EBR did not significantly affect the GA3, ABA, and IAA content in unstressed conditions, with respect to untreated plants (-EBR). However, it led to a higher accumulation of hormones under stress with respect to untreated plants. The opposite was true for cold-tolerant

species: in all cases (IAA, ABA, GA3), treatment with EBR led to a higher accumulation of hormones in unstressed plants with respect to untreated ones, whereas it did not affect the hormone concentration in stressed plants.
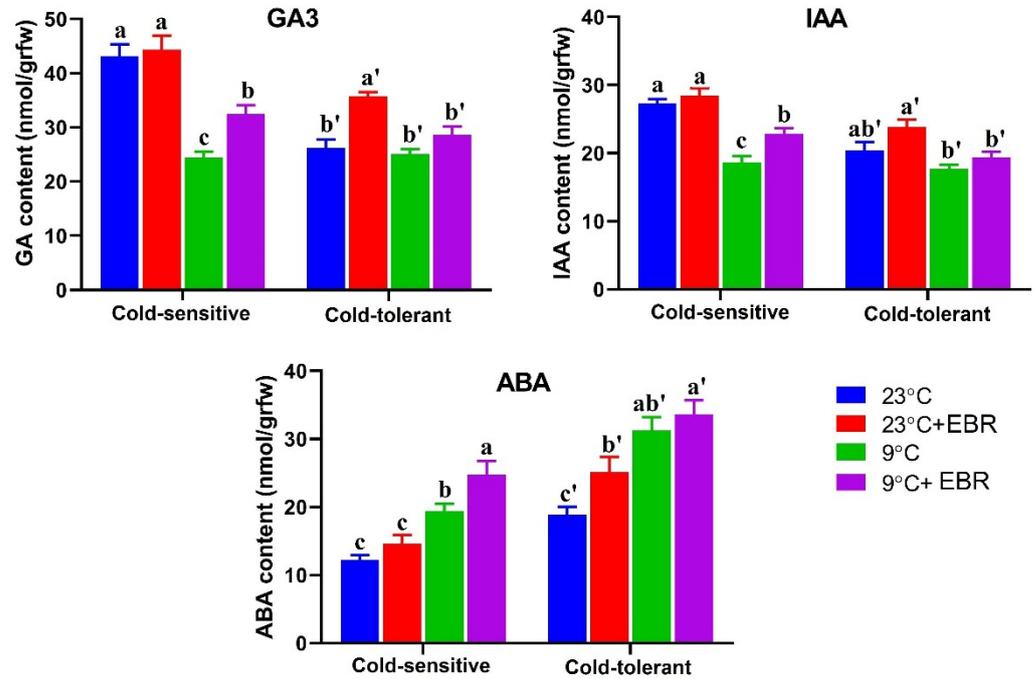

Figure 1. Profile of hormone content (in nanomoles per gram fresh weight (nmol/grfw)) of tomato leaves in response to temperature change and EBR application. Different letters above a bar show significant difference according to the Tukey's range test at $p < 0.05$.

The ABA/GA3 and ABA/IAA ratio increased in both species after three days of exposure to low-temperature stress (Figure 2). Interestingly, the ABA/GA3 and ABA/IAA ratio in cold-tolerant species were higher than the cold-sensitive species. However, the results of the current study revealed that EBR could not affect the ABA/GA3 and ABA/IAA ratio.
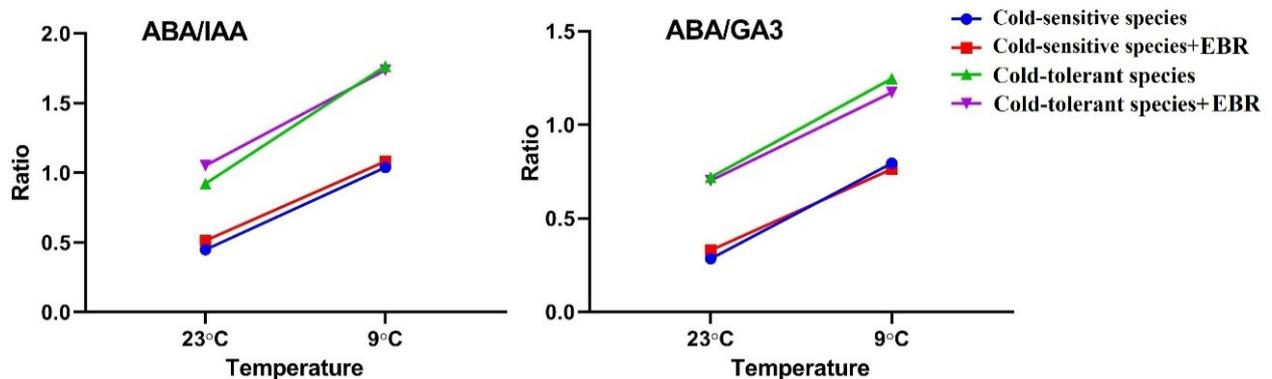

Figure 2. The ration of ABA/IAA and ABA/GA3 under low-temperature stress and EBR application.

3.2. MDA and Proline Are Increased By a Low Temperature

The content of MDA and proline significantly increased in both species, especially in the cold-tolerant species in response to low-temperature stress (Figure 3). Interestingly, EBR treatment could not affect the content of MDA and proline when compared with the control under normal temperature and low-temperature stress. However, in general, the EBR treatment slightly reduced the content of MDA and proline.
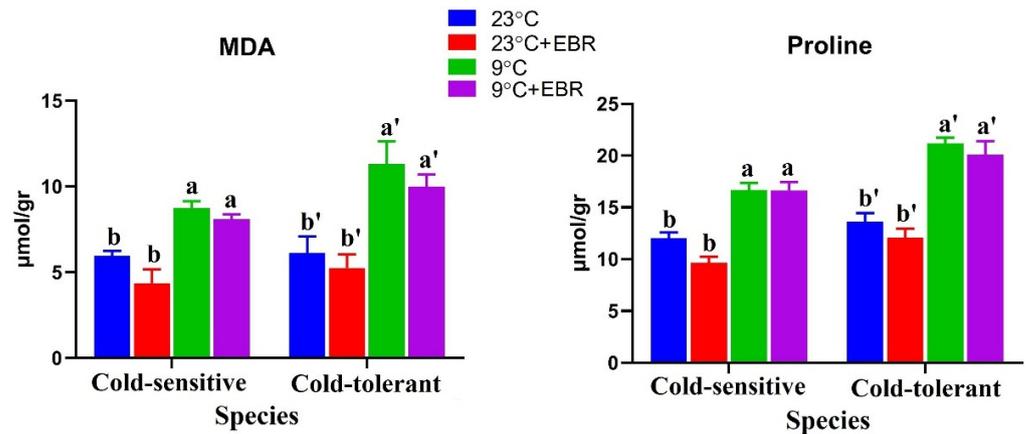
Figure 3. The profile of MDA and proline content under low-temperature stress and EBR treatment. Different letters above a bar show significant difference according to the Tukey's range test at $p < 0.05$.

3.3. Effects of EBR on Activity of Antioxidant Enzymes

According to the results of antioxidant activity, EBR treatment could affect the GPX activity in both tomato species (Figure 4). In normal conditions (23 °C), the EBR treatment increased the GPX activity compared to the untreated plants in both species, whereas in low-temperature stress, the treatment significantly enhanced GPX activity only in the cold-sensitive species. EBR treatment showed different effects in the tomato species under low-temperature stress (Figure 4). The cold-sensitive species, in plants not treated with EBR, showed an increase in CAT activity under cold stress, whereas the treatment with EBR seemed to impair the CAT response to cold. Interestingly, EBR treatment significantly increased the CAT activity in the cold-tolerant species.
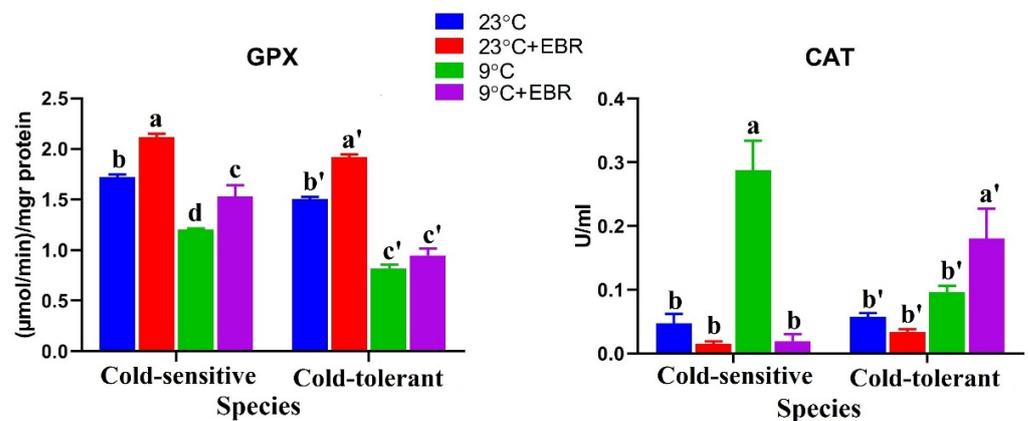

Figure 4. The profile of GPX and CAT activity under low-temperature stress and EBR treatment. Different letters above a bar show significant difference according to the Tukey's range test at $p < 0.05$.

3.4. Effect of EBR on Expression Pattern of ERF Genes

The expression pattern of four members of the ERF multigenic family, together with the MYC-like bHLH transcriptional activator ICE1, was investigated. The expression levels of ERF genes, as well as the ICE1 gene, were significantly affected by low temperatures compared to normal temperatures (Figure 5). Most studied genes were significantly upregulated in both species in response to low-temperature stress while the expression pattern of ERF13 was sharply downregulated in the cold-tolerant species. Furthermore, EBR treatment increased the expression levels of the ERF2, ERF13, ERF.B13, and ICE1 genes in the cold-sensitive genotype comparing to the control, while they were not affected by EBR treatment in the cold-tolerant species. Our results revealed that studied genes are involved in response to low-temperature stress and BR may associate with cold tolerance.
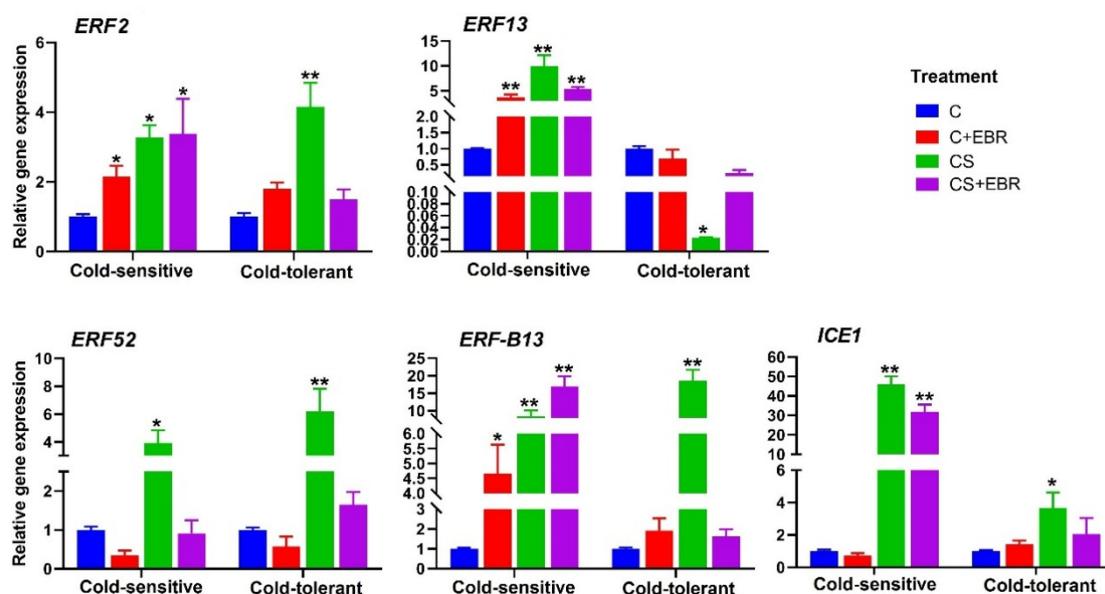
Figure 5. Expression patterns of studied genes under a low temperature (CS) and EBR treatment application; * and ** above a bar shows a significant difference between the applied treatments and normal temperature treatments as control (C) at *p*-value < 0.05 and *p*-value < 0.01, respectively (according to Student's *t*-test).

4. Discussion

4.1. EBR Improves Cold Tolerance by Affecting ABA Content

Various interactions between plant hormones induce a heterogeneous network of plant responses that make it challenging to predict plant performance in response to adverse conditions [44,45]. Moreover, BR can regulate stress responses by cross-talking with other phytohormones [33,46]. In this study, the synergetic interaction was observed between BR and ABA in response to low-temperature stress, where endogenous ABA content significantly increased in the cold-sensitive species under low-temperature stress and EBR treatment. However, the ABA/GA3 and ABA/IAA ratios were not influenced by EBR application. Abscisic acid (ABA) is known as a stress hormone that is influenced by stress and raises plant durability during abiotic stresses, such as drought and cold stress [47]. Moreover, ABA can decrease the damage of dehydration by closing the stomatal pore and maintaining the cellular water [48,49]. However, several antagonistic effects have been observed between signaling components of BRs and ABA under different stress conditions [47,50]. One well-known case of crosstalk occurs at the GSK3-like kinase BIN2 (BRASSINOSTEROID-INSENSITIVE 2), which inhibits the signaling components of the BR pathway, but can be activated by ABA [51]. Moreover, it was stated that ABA negatively controls the BR signaling pathway via phosphorylation of BES1 (bri1-EMS-SUPPRESSOR 1) as a BR signaling positive regulator [52]. Furthermore, Divi et al. found that BR effects are masked by ABA in *Arabidopsis* responses to heat stress, and only in the ABA-deficient *aba1-1* mutant, BR application could make the positive effect [53]. On the other hand, Bajguz stated that BR can enhance the ABA content in *Chlorella vulgaris* under stress conditions [54]. Overall, it seems that the interaction between ABA and BR plays important role in increasing stress tolerance through controlling the synthesizing antioxidants, photosynthesis, and expression of stress response genes [55]. Our results indicated that application of BR is involved in low-temperature stress tolerance by directly/indirectly affecting the ABA content of the tomato species.

4.2. EBR Application Affects the Auxin and GA Content under Low-Temperature Stress

The synergetic interactions are stated between BR and auxin in regulating the cellular processes related to growth, such as cell proliferation and cell expansion [53,56,57]. Furthermore, it was defined that BR and GA are involved in several common cellular

processes and BR can regulate cell elongation from GA metabolism [58]. In the current study, the content of IAA and GA was significantly decreased in cold-sensitive species under low-temperature stress. The decrease in the content of GA and IAA through cold stress limits the plant growth and lets it withstand adverse environmental conditions, such as cold, salt, and osmotic stress [59,60]. Moreover, previous studies stated that the IAA content is decreased in response to abiotic stresses, including salinity and cold stress [61,62]. Cold stress can inhibit the activity of acropetal auxin transport by controlling the PIN2 as an auxin efflux carrier [62]. It seems that the transport of auxin from root to shoot is reduced in tomato seedlings under cold stress. Furthermore, the ABA/GA3 and ABA/IAA ratio were increased under low-temperature stress that revealed an antagonist interaction between ABA with auxin and GA in response to low-temperature stress.

The content of GA3 and IAA was significantly increased by EBR application in the cold-sensitive species, compared with the control, under low-temperature stress. Various studies on the role of plant hormones in response to adverse conditions have been performed, but the exact interaction between BR with auxin and GA has not yet been determined, based on molecular information. The expression of many target genes that are involved in growth processes and stress response are commonly controlled by both BR and auxin [57,63,64]. Furthermore, BR and auxin may be involved in the induction of the phosphoinositide and calcium–calmodulin signaling as a second messenger in cellular signal transduction [57]. In the present study, the IAA content increased under low-temperature stress. It seems that BR might affect the polar transporter of auxin [65], and under low-temperature stress, the auxin transfer may increase from root to shoot. Moreover, BR can induce the expression of genes involved in GA biosynthesis, such as *GA3ox-2* [58]. Furthermore, BR can interact with DELLAs, as the GA suppressors, from BZR1, a BR signaling positive regulator [66–68]. In general, it seems that the application of EBR may affect the GA biosynthesis and increase the GA content in the tomato seedlings under low-temperature stress. Overall, the use of EBR treatment as a stimulant may induce some cellular signaling pathways associated with stress tolerance and reduce the adverse effects of stress on growth by increasing the content of growth-regulating hormones, such as GA and auxin.

4.3. MDA and Proline Are Not Affected by EBR Treatment

Abiotic stresses, such as low-temperature stress, hurt the cell membrane through enforced lipid peroxidation and membrane oxidation [11,69]. Antioxidant enzymes activity and the proline content were enhanced by the 28-homobrassinolide treatment in the *Brassica juncea* under cadmium stress. Moreover, the content of proline in roots was higher than in the leaves [70]. The content of MDA under salinity stress in rice seedlings was reduced by EBL treatment [71]. In this study, we discovered that MDA content significantly enhanced in the cold-sensitive species in low-temperature conditions, showing that the plasma membrane was affected and lipid peroxidation increased. In the same line, the increased activities of the antioxidant systems, as a result of BR applications, remarkably defeat the chilling injury of the tomato species by minimizing membrane lipid peroxidation in stress conditions. Moreover, proline content increased in response to low-temperature stress. During stress, proline, as an osmolyte, plays a critical role in controlling cell turgor and stability of membranes [72]. Furthermore, proline can reduce lipid peroxidation and acts as an antioxidant to overcome the oxidative stress created by cold stress [72,73]. Application of brassinosteroid in peppermint (*Mentha piperita* L.) under salinity hampered the death of the plant even at severe stress (150 mM) and prevented negative impact of salinity stress through elevating the activities of antioxidant enzymes and reducing the lipid peroxidation [74]. Moreover, our results revealed that the content of MDA and proline were not influenced by EBR treatment. Overall, it seems that BRs work from a proline-independent pathway to increase endurance to low-temperature stress, although more detailed studies are needed.

4.4. Effects of EBR on Activity of Antioxidant Enzymes

To enhance uncontrolled free radicals, plants respond by non-enzymatic and enzymatic antioxidants to regulate cellular homeostasis and mitigate oxidants [75,76]. Moreover, maintenance and regulation of redox homeostasis seem to be crucial to elevate chilling tolerance in tomato plants [77,78]. Thus, adjustment of the antioxidant system is remarked as a significant mechanism for increasing tomato chilling tolerance. It was demonstrated that using BRs induces antioxidant enzyme activity as well as non-enzymatic antioxidants. For instance, maize seedlings treated by brassinolide (BL) increased the activities of SOD, CAT, APX, carotenoid content, and ascorbic acid [10]. Antioxidative enzymes activity and mRNA expression of Cat A, Mn-SOD, Cat B, Cu/Zn-SOD, GR, and APX remarkably enhanced with EBL treatment under heavy metal stress in *Oryza sativa* L. [79]. In the present study, the effect of EBR application on the antioxidant activity, CAT, and GPX, in the tomato species, was different under low-temperature stress. Different behaviors of CAT were observed in both cold sensitive and cold tolerant species, or, in other words, the cold stress led to an increase in CAT activity in both species. However, the enhancement was much higher in cold sensitive compared to the cold tolerant species (Figure 4). There were substantial differences based on the genotype considered in BR treated plants; the CAT activity in the cold-tolerant species was increased by EBR treatment compared with untreated plants under low-temperature stress. In fact, cold tolerant plants treated with EBR showed an increase in CAT activity, which was higher than untreated plants. In cold sensitive genotypes the EBR treatment seem to impair the CAT activity, whereas in cold-sensitive species, the GPX activity was more influenced by EBR application (Figure 4). It seems that the effect of BR on the activity of antioxidant enzymes depends on the plant species, likely depending on the amount of stress received, the tomato species uses different mechanisms to reduce the induced oxidants. Previous studies on the effect of BR on elevated tolerance of resistant and susceptible tomato species in low temperatures indicated that EBR treatment enhanced the activities of the enzymes in pepper [80], cucumber [81], and eggplant [82] in low temperatures. It seems that oxidative stress is induced by low-temperature stress in the tomato species. Antioxidant enzymes, CAT, and GPX are induced to reduce the oxidants and keep cellular redox. From these results, it could be concluded that BR treatment could play a significant role in the alleviation of ROS damage by increasing antioxidant the defense system, resulting in elevating the tolerance of the tomato species to chilling stress.

4.5. Effects of Low-Temperature Stress on Cold-Related Genes

Ethylene responsive factor (*ERF*) genes belong to the *AP2/ERF* gene family, known as a large gene family of transcription factors [83,84]. The ERF gene family, as a key regulator, plays an important role in response to adverse conditions, such as cold stress in plant species [85,86]. In this study, the expression level of cold-responsive genes, *ERF* genes, and *ICE1*, selected based on previous studies [41,42], was significantly induced by low-temperature stress. *ICE1* as an upstream transcription factor can regulate cold-responsive genes, such as *CBF* genes [42]. Interestingly under EBR application in a cold-tolerant species, expression patterns of *ERF* genes and *ICE1* reversed to normal temperature conditions. However, Kagale et al. indicated that the EBL application could induce the expression of cold-related genes [87]. Extensive studies were performed on the role of *AP2/ERF* gene family in response to abiotic and biotic stresses as well as hormone treatments, but the effect of BR on this gene family has not yet been investigated. Recently, Xie et al. stated that TINY, an AP2/ERF transcription factor, may negatively affect the expression of BR-responsive genes while it positively controls drought-responsive genes in *Arabidopsis* [88]. In addition, previously, it has been revealed that EBR treatment increases the basic thermo-tolerance of *Brassica napus* [89]. The merit of EBR to grant tolerance in plants to different stresses was corroborated via expression analysis of a subset of cold and drought stress marker genes [87]. Brassinosteroid induced auxin-related genes and cell wall-modifying in soybeans, contrarily, transcriptome analysis demonstrated the twisted BR roles in various biological processes by suppressing some WRKY genes engaged in senescence and stress

responses [90]. Overall, our results disclosed that exogenous EBR application might interact with endogenous hormones and reduce the negative effects of low temperatures that induce the expression of cold-responsive genes, *ERFs* and *ICE1*, to return to a state similar to that without stress.

5. Conclusions

In the current study, the effect of the EBR application was investigated in two tomato species under low-temperature stress. The results depicted that low-temperature stress can create oxidative stress and reduce the content of growth-regulatory hormones, IAA and GA3. Moreover, the EBR application increased the content of endogenous ABA, and a synergetic interaction was observed between BR and ABA in response to low-temperature stress. Furthermore, our findings indicated that ABA/GA3 and ABA/IAA ratios are not affected by EBR treatment. In the current study, we found that EBR treatment could not affect the content of MDA and proline under low-temperature stress, but could increase the content of antioxidant enzymes to reduce ROS induced by low-temperature stress. Overall, we concluded that EBR diminishes the adverse effect of low-temperature stress by increasing the content of endogenous phytohormones, increasing the content of antioxidant enzymes, and controlling the gene expression. Furthermore, it seems that BR effects are dependent on the tomato species.

Author Contributions: P.H., M.E. and A.E. designed the study. P.H. and M.E. conducted the experiments and analyzed the data. P.H. and M.A. investigated. All authors contributed to writing the manuscript. A.V., F.P. and G.B. contributed to the interpretation, presentation, and discussion of the data. All authors contributed to writing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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