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Antisense Overexpression of Gγ Subunit CsGG3.1-2 Reduces Soluble Sugar Content and Chilling Tolerance in Cucumber

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Abstract: Plant G protein γ subunits have multiple functions in growth and development processes and in abiotic stress responses. Few functions of Gγ in horticultural crops have been revealed thus far. In this study, the potential function of *CsGG3.1-2*, one of the two alternative splice variants of Gγ gene *CsGG3.1* in cucumber (*Cucumis sativus* L.), was investigated using transgenic plants overexpressing antisense *CsGG3.1-2* under the control of the 35S promoter. The tolerance to chilling stress in transgenic plants was significantly decreased. Cold stress-related physiological parameters and the expression of *CBF*s and their downstream target genes were then measured. Compared with WT, the maximum efficiency of photosystem II (Fv/Fm), antioxidative enzymes activities, soluble protein, and proline accumulation decreased significantly in transgenic plants treated with cold stress, whereas the malonaldehyde (MDA) content increased. However, the overexpression of antisense *CsGG3.1-2* did not affect the induction of cold-inducible genes. Quantitative real-time PCR (qPCR) analysis showed the increased expression of *CBF* genes and their downstream target genes in transgenic plants, suggesting that CsGG3.1-2 affects cold responses via CBF-independent pathways in cucumber. At the same time, the sucrose and fructose contents decreased in transgenic plants under both normal and cold conditions. These findings suggest that soluble sugar deficiency is associated with chilling sensitivity in transgenic plants, and CsGG3.1-2 may have a role in regulating carbohydrate metabolism in cucumber.

Keywords: cucumber; G protein γ subunit; soluble sugar; cold stress

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1. Introduction

Heterotrimeric GTP-binding proteins (G proteins) are essential signaling molecules that mediate extracellular signal transduction. Each G protein has three subunits, Gα, Gβ, and Gγ [1]. The replacement of guanosine diphosphate (GDP) with guanosine triphosphate (GTP) on the Gα subunit induces the separation of Gα from the associated Gβγ dimer, which happens when stimuli decouple the RGS (regulator of G protein signaling) protein (a protein that has the function of promoting the GTP hydrolysis of Gα) from the Gα subunit [1]. Then, extracellular signaling is transduced to downstream effectors by the GTP-bound Gα and Gβγ subunits, respectively [2]. The genes encoding putative Gα, Gβ, and Gγ have been identified in various plant species. While animal G proteins are abundant in the available set of subunits, the number of each subunit in plants is much lower. The Gγ subunit is essential for the heterotrimer mediated signaling pathway since the amount of Gγ genes is greater than that of the Gα and Gβ genes, and moreover, splice variants for Gγ also exist [3]. Plant Gγ subunits belong to three distinct groups which are classified according to the structures of their C-terminals [4]. Those with a putative prenylation domain belong to Type A. Those lack a C-terminal prenylation domain and cysteine residues in the C-terminal region belong to Type B. Those possessing an extended

Cysteine-rich C-terminus domain belong to Type C, which is a plant-specific group. Trusov et al. showed that the expression patterns of Arabidopsis (*Arabidopsis thaliana*) *AGG1* and *AGG2* are distinct, and they suggested that the functional selectivity in Gβγ dimer signaling is provided by Gγ [5]. It has been shown that plant Gγ subunits have important functions in development and abiotic stress responses (e.g., salt, cold, drought, and heat) [2]. Moreover, the type C Gγ subunit seven has high application potential for crop yield improvements [6]. In rice (*Oryza sativa*), GS3 and DEP1/qPE9–1, two type C Gγ subunits, are involved in controlling the size of the grain and the panicle [6]. Over-expressing Arabidopsis type C Gγ (AGG3) promotes the growth of seeds and organs in Arabidopsis [7], and it increased seed yield and stress endurance in Camelina (*Camelina sativa*) [8]. Although the function of type C Gγ subunits has been well studied in Arabidopsis and rice, there is still a lack of comprehensive knowledge of the important roles played by type C Gγ subunits in horticultural crops. Improving the understanding of type C Gγ in horticultural crops is important for improving stress resistance and yield.

Cold stress adversely suppresses plant growth and development, and thus, it reduces the productivity and quality of crops [9]. In response to cold stress, signaling pathways begin with stress perception, followed by Ca^{2+} signal activation, which often stimulates a protein phosphorylation cascade that rapidly induces adjustments in the expression/activity of cold-responsive transcription factors (TFs), and, finally, they initiate tolerance mechanisms on the basis of the biochemical and physiological changes, including alterations in the activities of antioxidant enzymes [10] and the accumulation of soluble sugar and proline [11]. Among these cold-inducible TFs, CBFs (C-repeat Binding Factors) play essential functions in the plant response to cold. They regulate a large number of *COR* (cold-regulated) genes that can improve plant cold tolerance [12]. Moreover, studies have suggested that the CBF pathway occupies one point of cross-talk for cold stress signaling pathways and phytohormone regulated plant cold responses [13,14]. BZR1 (Brassinazole-resistant 1), one of the key TFs of Brassinosteroid (BR) signaling, positively modulates plant low temperature tolerance through acting upstream of *CBFs* to regulate their expression [15]. In Arabidopsis, the cytokinin (CK) receptors AHK2 (Arabidopsis histidine kinase2) and AHK3 double-mutants *ahk2* and *ahk3* showed increased *MYB15* (coding R2R3-MYB Transcription Factor MYB15) expression and decreased *CBF3* (target of MYB15) expression after cold treatment [16]. Miura and Ohta reported that the salicylic acid (SA) accumulating mutant *siz1*, which is sensitive to cold stress, showed the decreased expression of *CBF3* and its target genes [17]. Ma et al. reported that the Gα subunit RGA1 activated the Ca^{2+} signal for low-temperature sensing to cope with chilling stress by interacting with the cold sensor COLD1 in rice [18]. Most recently, Yan et al. reported that the CBF pathway was regulated by the Gα subunit through the *CsGPA1-CsCOR413PM2-* Ca^{2+} axis in cucumber [19]. Gβ and Gγ have also been found to be involved in low temperature responses. The over-expression of *TaGBPL*, a Gβ-like gene in wheat (*Triticum aestivum*), reduced the tolerance of Arabidopsis to low temperatures (16 °C) [20]. In rice, the Gγ genes *RGG1* and *RGG2* were upregulated by low temperature (4 °C) treatment, but they showed different expression patterns [21], suggesting that Gγ subunits may have distinct roles in low temperature responses.

Cucumber is one of the most widely cultivated horticultural crops in China. In a previous study, we found that there were six Gγ proteins (CsGG1, CsGG2.1, CsGG2.2, CsGG3.1-1, CsGG3.1-2, and CsGG3.2) in cucumber [22]. The first three Gγ proteins belonged to Type A, while the remaining three proteins belonged to Type C as they contained an extended Cysteine-rich C-terminus domain [22], and CsGG3.2 was found to positively regulate the cucumber cold response through the CBF pathway [22]. During our research, we also found that the over-expression of antisense *CsGG3.1-2* reduced the chilling tolerance of transgenic cucumber plants. To reveal the underling mechanism, we measured the cold stress-related physiological parameters and the expression of *CBFs* and their regulon in antisense *CsGG3.1-2* transgenic cucumber plants. We found that the over-expression of antisense *CsGG3.1-2* reduced the activities of antioxidative enzymes and the accumulation of soluble protein, proline, and soluble sugars, but it did not affect the gene expression of

the CBF pathway under cold stress. We concluded that CsGG3.1-2 affected cold responses via a CBF-independent pathway in cucumber.

2. Materials and Methods

2.1. Vector Construction and Plant Transformation

The antisense sequence of *CsGG3.1-2* was amplified with gene-specific primers (Table S1) and ligated into the *NcoI/PmacI* sites of the pCAMBIA-1305 vector to construct the antisense *CsGG3.1-2* expression vector under the control of the 35S promoter. After confirmation by sequencing, constructs with antisense *CsGG3.1-2* were introduced into 'Xintai Mici', a pure line cucumber cultivar that uses the *Agrobacterium* LB4404 strain for transformation. The transformation was conducted according to the method described previously [22]. The presence of transgenes was confirmed by employing the polymerase chain reaction (PCR) method with the gene-specific primers listed in Table S1.

2.2. Plant Materials and Cold Treatments

Cucumber seeds were germinated at 28 °C in darkness and sowed in pots filled with a vermiculite–peat mixture (1:1, volume/volume (*v*/*v*)) in a growth chamber where the temperature was maintained at 25 °C/17 °C (day/night) under a 12 h photoperiod with the light intensity maintained at 400 μmol m^{-2} s^{-1}. The cold stress treatment was carried out as previously described [22]. The T2 transgenic lines and wild type (WT) plants with two true leaves were treated with cold stress at 9 ± 1 °C/8 ± 1 °C (day/night) for 7 days with 400 μmol $m^{-2}s^{-1}$ light for 12 h every day. Plants kept at normal conditions were used as the control.

2.3. Leaf Chlorophyll Fluorescence Imaging

Cold injury in cucumber seedlings was determined by measuring the chlorophyll fluorescence of leaves. The chlorophyll fluorescence of the second true leaf (from the bottom) of the cold-treated plants was monitored using a portable chlorophyll fluorometer (PEA, Hansatech Instruments, Ltd., King's Lynn, UK). The maximum efficiency of the photosystem II (PSII) index Fv/Fm (Fm is the maximal fluorescence and Fv is the variable fluorescence) was monitored during the cold treatment (0 d, 1 d, 3 d, 5 d, and 7 d). Measurements of the leaves were taken during the light cycle, with 30 min of dark treatment, using the saturation pulse method [23].

2.4. Biochemical Analysis Assays

The second leaf (from the bottom) was sampled from the WT and antisense plants with/without cold treatment. The content of malondialdehyde (MDA) was determined using the TBA (thiobarbituric acid) colorimetric method [24]. The H_2O_2 content was determined using assay kits (Beijing Boxbio Science & Technology Co., Ltd., Beijing, China) with a UV-2600i spectrophotometer (Shimadzu (Shanghai) Experimental Equipment Co., Ltd., Shanghai, China) following the manufacturer's instructions. The soluble protein (SP) content was determined using the G520 Bradford method [25]. The proline content was determined via a reaction with ninhydrin [26]. The activities of the antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were detected using the nitrogen blue tetrazolium (NBT) method [27], the guaiacol method [28], and the UV spectrophotometric method [29], respectively. The sucrose and fructose contents were determined using the UV spectrophotometric method as described by Shen et al. [30].

2.5. Quantitative Real-Time PCR

Previous studies have proven that the induction of *CBF*s plays an important role in improving plant stress resistance [31]. Therefore, we analyzed the expression levels of genes in the CBF pathway (*CsCBFs*, *CsCOR15b,* and *CsKIN1* [22]) in antisense *CsGG3.1-2* transgenic plants, as well as WT plants, during cold treatment. The second leaf (from the bottom) was sampled from the WT and transgenic plants during the cold treatment (at

0 h, 4 h, 8 h, 24 h, and 168 h). Total RNA isolation was completed using the Plant Total RNA Isolation Kit RC401 (Nanjing Vazyme Biotechnology Co., Ltd., Nanjing, China), and first-strand cDNA was synthesized using HiScript III RT SuperMix for the qPCR (+gDNA wiper) RC323-01 (Nanjing Vazyme Biotechnology Co., Ltd., Nanjing, China) following the manufacturer's instructions. Then, quantitative real-time PCR (qPCR) was carried out using the gene-specific primers listed in Supplementary Table S1 and the ChamQ Universal SYBR qPCR Master Mix Q771 (Nanjing Vazyme Biotechnology Co., Ltd., Nanjing, China) with an Mx3000p Real-time PCR System (Agilent Technology (China) Co., Ltd., Beijing, China) following the manufacturer's instructions. Each sample had three biological replicates. The $2^{-\Delta\Delta Ct}$ [32] method was employed, and the relative expression levels of the genes were normalized to *CsActin*.

2.6. Chilling Tolerance Assessment

WT and T2 transgenic seedlings with two true leaves were exposed to chilling stress (8 ± 1 °C/5 ± 1 °C (day/night)) for 7 days, with 400 μmol $m^{-2}s^{-1}$ light for 12 h every day. Chilling injury (CI) was assessed according to the method described previously [22].

2.7. Data Analyses

Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) was used for data processing, and GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) was used for graphing. The data were statistically analyzed using Data Processing System (DPS) 17.0 (Refine Information Tech. Co., Hangzhou, China) software, and Duncan's new complex polar difference method was employed to test the significance of the differences (* $p < 0.05$ and ** $p < 0.01$). Data are presented as means ± SEs ($n = 3$).

3. Results

3.1. Overexpression of Antisense CsGG3.1-2 Reduced the Chilling Tolerance of the Transgenic Cucumbers

To evaluate the biological functions of CsGG3.1-2, transgenic plants that over-expressed antisense *CsGG3.1-2* were constructed. Three of those generated transgenic lines were confirmed to contain the antisense sequence of *CsGG3.1-2* (Figure 1A). Two lines (L1 and L2) that showed a small organ morphology similar to that of Arabidopsis mutants (loss of *AGG3* function [7] and *CsGG3.1-2* expression levels of approximately 1-fold lower than those of the WT plants (Figure 1B)) were selected for the experiments. For the chilling tolerance test, after 7 days of low temperature (8 °C/5 °C, day/night) treatment, two transgenic lines exhibited severe wilt and necrosis in the cotyledons and in the true leaves. The WT plants showed lighter dehydration and wilt in the cotyledons and at the edges of the true leaves (Figure 2A). The CI indices of the two transgenic lines were significantly higher than those of the WT plants, and they were increased by 24.33% and 22.33%, respectively (Figure 2B).

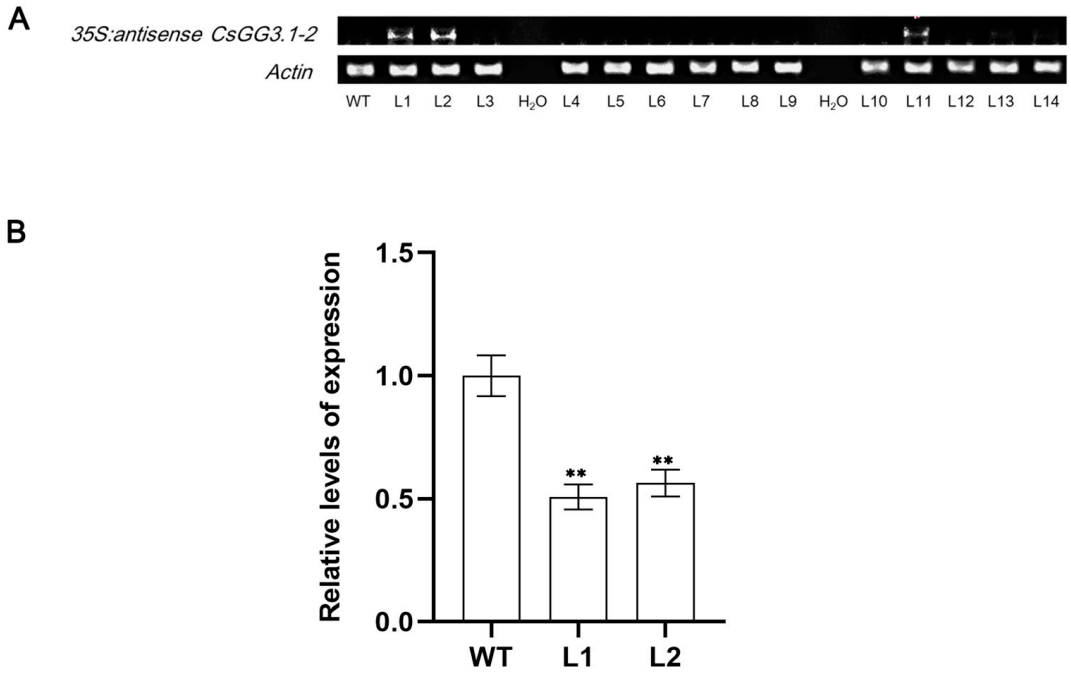

Figure 1. Expression identification of the transgenic cucumber plants. (**A**) PCR analysis of the T0 transgenic cucumber plants. (**B**) Expression levels of *CsGG3.1-2* mRNA in the antisense *CsGG3.1-2*-expressing T2-transgenic plants. The data shown are the means of three independent replicates (using 10 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (** $p < 0.01$).

Figure 2. Antisense *CsGG3.1-2*-expression reduced the chilling tolerance of cucumber. (**A**) Chilling sensitivity of the antisense *CsGG3.1-2*-expressing T2-transgenic and WT plants. (**B**) Chilling injury (CI) indexes of the antisense *CsGG3.1-2*-expressing T2-transgenic and WT plants. The data shown are the means of three independent replicates (using 10 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (** $p < 0.01$). Bar = 2 cm.

3.2. Overexpression of Antisense CsGG3.1-2 Reduces Chlorophyll Fluorescence Parameters (Fv/Fm)

The maximum efficiency of PSII, measured as Fv/Fm, is one of the most stress-affected fluorescence parameters, and chilling stress typically leads to a decrease in Fv/Fm [33]. In the leaves of the WT plants, there was a very small decrease in Fv/Fm at the edges after 3 days of cold treatment. The Fv/Fm decreased rapidly from then on, but primarily at the edges of the leaves of the WT plants (Figure 3A,B). In the transgenic lines, however, the Fv/Fm decreased at the edges of the leaves as early as 1 day after cold treatment, and after 5 days of cold treatment, the Fv/Fm was reduced severely in nearly whole leaf areas on the transgenic plants (Figure 3A,B), indicating a serious loss of activity of PSII.

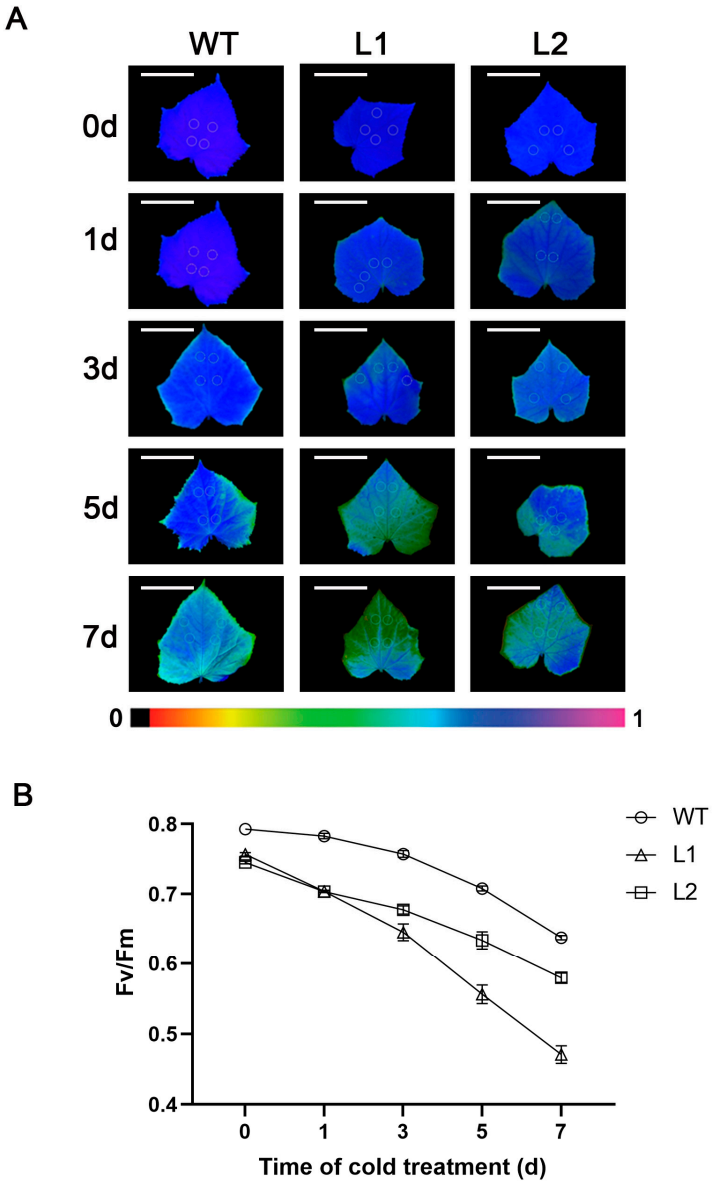

Figure 3. Potential photosynthetic efficiency (Fv/Fm) of antisense *CsGG3.1-2* transgenic and WT plants under cold treatment (9 °C/8 °C, day/night). Images (**A**) and quantitative analysis (**B**) of Fv/Fm at different times during cold treatment. The images show a representative measurement. The color bar at the bottom indicates the Fv/Fm values. The circles in the images shows the regions that have been quantitatively analyzed. The values in (**B**) represent the means ± SEs (n = 3) of the Fv/Fm of three independent replicates (using 10 seedlings each). Bar = 5 cm.

3.3. Activities of Antioxidant Enzymes and Accumulation of H_2O_2 and MDA under Cold Stress

The activities of antioxidant enzymes (SOD, POD, and CAT) in the leaves of the transgenic and WT cucumber seedlings with/without cold stress were measured. The overexpression of antisense *CsGG3.1-2* led to a decrease in the activities of SOD and POD in the transgenic plants with and without cold treatment (Figure 4A,B). There was no difference in the activity of CAT in the transgenic plants and the WT plants under both normal and cold conditions (Figure 4C), and the reduction in antioxidant enzyme activities led to the increased accumulation of H_2O_2 in the transgenic plants compared with the WT plants under cold conditions (Figure 5A).

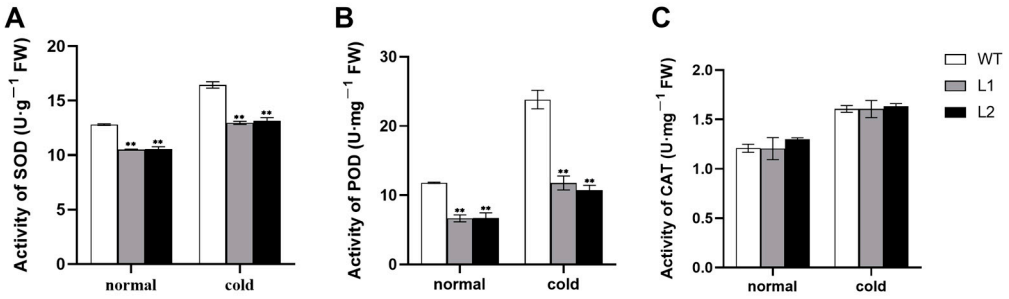

Figure 4. Antioxidant activities of antisense *CsGG3.1-2* transgenic and WT cucumber plants under cold stress. (**A**) Superoxide dismutase (SOD) activity. (**B**) Guaiacol peroxidase (POD) activity. (**C**) Catalase (CAT) activity. The data shown are the means of three independent replicates (using 20 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (** $p < 0.01$).

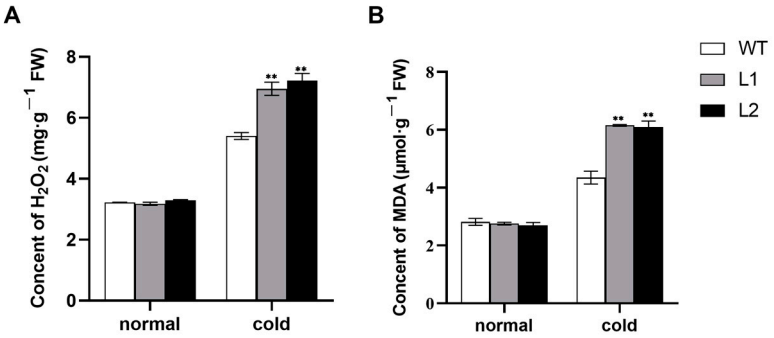

Figure 5. Hydrogen peroxide (H_2O_2) content (**A**) and malondialdehyde (MDA) content (**B**) in the antisense *CsGG3.1-2* transgenic and WT cucumber plants under cold stress. The data shown are the mean averages ± SEs ($n = 3$) of three independent replicates (using 20 seedlings each). Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (** $p < 0.01$).

The accumulation of MDA, an ROS (reactive oxygen species)-associated lipid peroxidation product, is always used to gauge cell membrane permeability [34]. The accumulation of MDA in the transgenic cucumber plants and the WT cucumber plants showed no difference under normal conditions (Figure 5B). After 7 days of cold treatment, the MDA contents in the transgenic and WT plants had increased, and an increase of approximately 40.17~41.54% higher in both transgenic lines than in the WT was seen (Figure 5B), indicating a more serious membrane permeability in the transgenic plants.

3.4. The Accumulation of Soluble Protein and Proline under Cold Stress

Under low temperature stress, many plants accumulate more permeable proline and soluble protein to protect against cellular damage [35]. Compared with the WT plants, the overexpression of antisense *CsGG3.1-2* in the transgenic cucumber plants resulted in a decrease in soluble protein content under both normal and cold conditions (Figure 6A). Proline contents showed no differences between the transgenic cucumber plants and the WT cucumber plants under normal conditions. However, a significant decrease in proline content (of approximately 14.66~16.94% lower than that in the WT plants) was observed in the transgenic cucumber lines upon exposure to cold stress (Figure 6B).

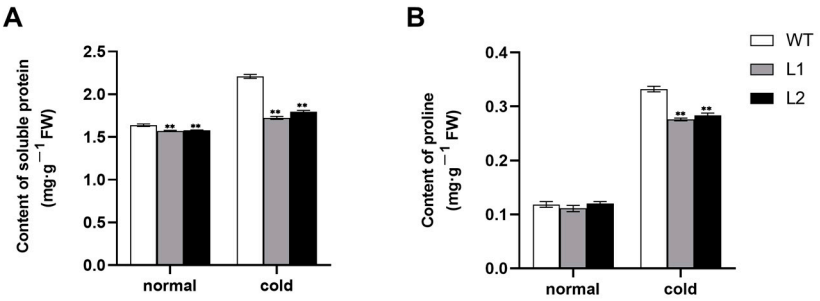

Figure 6. The content of soluble protein (**A**) and proline (**B**) in *CsGG3.1-2* transgenic and WT cucumber plants under cold stress. The data shown are the means of three independent replicates (using 20 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (** $p < 0.01$).

3.5. The Accumulation of Soluble Sugars under Cold Stress

Cold stress decreased soluble sugar (both sucrose and fructose) content in all plants (Figure 7). Under cold conditions, the sucrose and fructose content in the WT plants decreased by approximately 48.76% and 32.74% more, respectively, than that of the control. (Figure 7). Compared with the WT plants, the overexpression of antisense *CsGG3.1-2* decreased the sucrose and fructose content in cucumber seedling leaves under both normal and cold conditions. Under cold conditions, the sucrose contents of the transgenic lines were 29.26% and 35.37% lower than that of the WT plants, respectively, and the fructose contents were 40.71% and 40.12% lower than that of the WT plants, respectively.

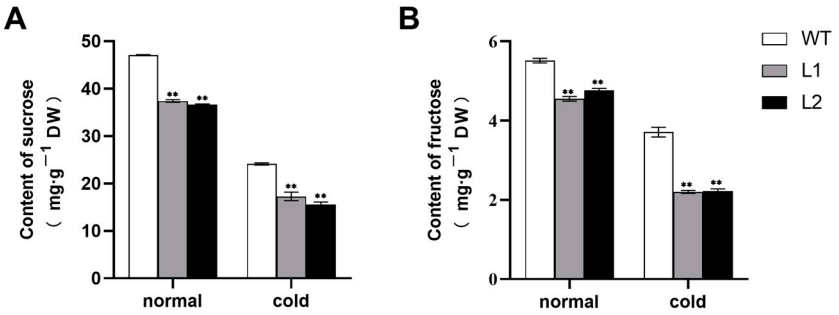

Figure 7. The contents of sucrose (**A**) and fructose (**B**) in the antisense *CsGG3.1-2* transgenic and WT cucumber plants under cold stress. The data shown are the means of three independent replicates (using 20 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (** $p < 0.01$).

3.6. The Expression of CBF and COR Genes

The results showed that the overexpression of antisense *CsGG3.1-2* improved the expression of three *CBF* genes and two *COR* genes (Figures 8 and 9).

The relative expression levels of the *CBF* genes in all the plants exposed to cold stress increased within 4 h, and the expression levels of all the three *CBF* genes were even higher in the antisense *CsGG3.1-2* transgenic cucumber plants compared to the WT plants during cold treatment (Figure 8). Cold treatment also increased the relative expression levels of *CsCOR15b* and *CsKIN1* in the WT plants and the transgenic cucumber plants. Moreover, the expression levels of *CsCOR15b* and *CsKIN1* in the antisense *CsGG3.1-2* transgenic lines also showed significant increases compared to the WT plants during cold treatment (Figure 9).

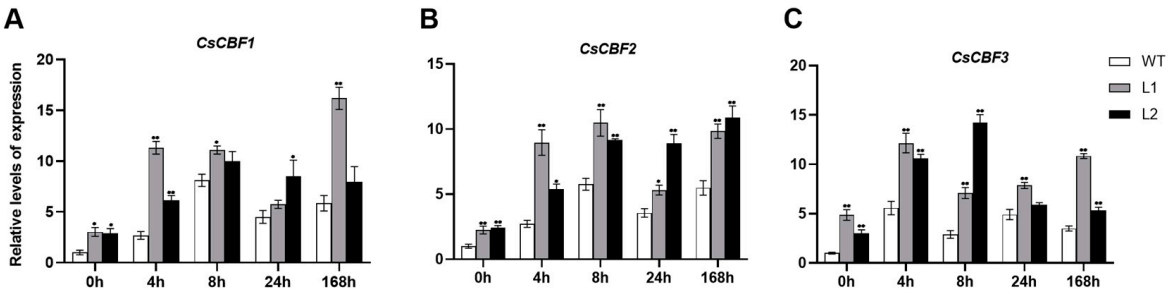

Figure 8. Relative expression levels of *CsCBFs* in the antisense *CsGG3.1-2* transgenic cucumber plants and WT cucumber plants treated with cold stress. (**A**) *CsCBF1*. (**B**) *CsCBF2*. (**C**) *CsCBF3*. The data shown are the means of three independent replicates (using 20 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (* $p < 0.05$ and ** $p < 0.01$).

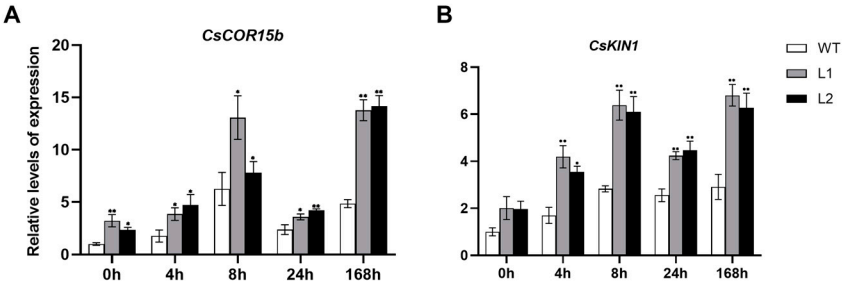

Figure 9. Relative expression levels of *CsCOR15b* (**A**) and *CsKIN1* (**B**) in the antisense *CsGG3.1-2* transgenic cucumber plants and WT cucumber plants treated with cold stress. The data shown are the means of three independent replicates (using 20 seedlings each) ± the SEs. Significant differences between the transgenic cucumber lines and the WT plants are indicated by asterisks (* $p < 0.05$ and ** $p < 0.01$).

4. Discussion

Gγ subunits are linked to several growth and development processes [6], and they also participate in the regulation of the responses to abiotic stressors such as salinity and low temperature [21]. The expression of RGG1 and RGG2 in rice was strongly induced by cold stress, while *BnGG2* in oilseed rape (*Brassica napus*) was downregulated during cold stress, which suggests that Gγ plays a role in a plant's cold stress response [21,36]. Photosynthesis is susceptible to low temperature stress. Blocking the photosynthetic electron transport rate, which is the result of damage to PSII, is an important mechanism of photosynthesis inhibition caused by cold stress [37]. Chlorophyll fluorescence imaging is always used to determine PSII activities, and chilling stress typically leads to a decrease in Fv/Fm [33,38]. In this study, the transgenic plants exhibited reduced Fv/Fm during cold treatment (Figure 3), which suggested that the overexpression of antisense *CsGG3.1-2* increased the cold sensitivity of the transgenic plants.

Antioxidative enzymes are important for enabling plants to protect themselves against oxidative stress under abiotic stressors. We found that the activities of SOD, POD, and CAT were upregulated in both the WT and transgenic plants under cold stress conditions (Figure 4), which was in line with the results of previous reports [27,39]. In a previous study, we found that CsGG3.2 participated in the low temperature response of cucumber by adjusting the activities of antioxidant enzymes [22]. Here, we showed that the overexpression of antisense *CsGG3.1-2* caused a significant decrease in the activities of antioxidant enzymes in the transgenic cucumber plants (Figure 4), which may account for the accumulation of H_2O_2 and the increased membrane lipid peroxidation (Figure 5).

It is well known that soluble sugars are accumulated following low temperature stress [11]. Moreover, studies have shown that increased sugar levels from the overexpres-

sion of carbohydrate metabolism enzymes and soluble sugar transporters that affect the subcellular distribution of sugars or overall cellular sugar homeostasis, such as β-Amylase and SWEET16 (Sugars Will Eventually Be Exported Transporters 16), improved the freezing tolerance of plants [40–43]. On the contrary, the GIGANTEA (a key component of photoperiodic flowering) mutants and plastidic sugar transporter (pSuT) mutants of Arabidopsis exhibited impaired freezing tolerance that was associated with sugar deficiency [44–46]. Soluble sugars play multifunctional roles in enhancing cold tolerance, including maintaining the osmotic potential, stabilizing biological components, and scavenging ROS. Cellular membranes are fluid structures. However, chilling stress negatively affects membrane properties, causing disruption of the membrane structure and function, which then leads to tissue necrosis [47]. The stabilizing effect of soluble sugars during freezing is well-known. Sucrose can increase cell membrane stability by directly interacting with the lipid headgroups of the phosphate [48]. Fructans can increase the stability of a membrane through direct insertion between the polar headgroups [49]. On the other hand, ROS are accumulated during low temperature stress, and they cause damage to macromolecules and cellular structures. It was hypothesized that soluble sugars also play significant roles in cellular ROS scavenging. Anthocyanins are proposed to be important components of the ROS scavenging system in plants, and thus, they play roles in facilitating plant survival under abiotic stress. Sucrose can stabilize DELLA proteins, which can promote anthocyanin synthesis by activating the MYB/bHLH/WD40 complex [50]. It was proposed that CAT, SOD, and POD can be protected by soluble sugars under chilling stress since soluble sugars interact with proteins through hydrogen bonding, and eventually, they prevent the cold denaturation of these enzymes [24]. Peshev et al. reported that fructans can directly scavenge $^{\cdot}OH$ through a Fento reaction in vitro, leading to the formation of oxidized sugars [51] which can also be detected in vivo [52]. In this study, we found that the accumulation of sucrose and fructose in transgenic cucumber plants was significantly lower than that of the WT cucumber plants (Figure 7), which may have contributed to the reduction in antioxidant enzyme activities and cold tolerance.

The CBF pathway has important roles in physiological and biochemical adjustments induced by cold. When plants are exposed to cold stress, CBFs are employed to interact with CRT (C-repeat)/DRE (dehydration-responsive element) cis-elements in the promoters of COR genes, such as *COR15b* and *KIN1*, and they regulate their transcription, which results in biochemical and physiological changes [53,54]. It has been demonstrated that cold-dependent sugar accumulation partially depends on the CBF pathway. Peng et al. reported that *PtrBAM1* (coding β-amylase), participating in the accumulation of soluble sugar under cold stress, is a member of the CBF regulon owing to the interaction between PtrCBF and the promoter of *PtrBAM1* [42]. It is well established that G proteins participate in a plant's low temperature signal transduction by activating the Ca^{2+} signal [18,19]. Recently, Guo et al. reported that tomato *LeGPA1* conferred cold tolerance by modulating the gene expression of CBF regulon and increasing soluble sugar accumulation and the activities of antioxidant enzymes [55]. Transgenic rice plants simultaneously over-expressing both *RGB1* and *RGG1* genes (the coding rice Gβ and Gγ subunits, respectively) showed increased sugar (glucose and fructose) contents in leaves, along with enhanced stress tolerance [56]. Rice Gβ and Gγ genes have also been strongly induced by cold stress [21,57], suggesting they may also play roles in cold resistance. In our previous work, we found that cucumber CsGG3.2 positively regulated the expression of *CsCBFs* and chilling tolerance [22]. Here, our results demonstrated that under cold stress, *CBF* and its target genes had even higher expression levels in the transgenic cucumber plants compared to the WT cucumber plants (Figures 8 and 9), indicating that the reduced soluble sugar contents and chilling tolerance of transgenic plants may not be associated with the CBF cold stress signaling pathway. However, it is noteworthy that type C Gγ subunits, which are known to be major quantitative trait loci for crop improvements [6,8,58], regulate carbohydrate metabolism. Recently, Zhang et al. proved that EDP1/qPE9-1 regulated carbohydrate metabolism by regulating the expression of the genes that encode soluble sucrose synthase (SSS) and sucrose synthase

(SUS), as well as their activity in rice grains during grain filling [59]. Moreover, Wang et al. reported that qPE9-1 affected photoassimilate (sucrose and starch) contents in rice leaves via moderating the Rubisco content and gene expression [60]. Besides sucrose and fructose, the accumulation of starch was lower in the transgenic cucumber plants (Figure S1). Because the rate and magnitude of sugar accumulation induced by cold acclimation largely relies on photosynthetic activity [61], it was speculated that CsGG3.1-2 may participate in regulating carbohydrate metabolism, and that it indirectly affects the chilling stress tolerance of cucumber (Figure 10).

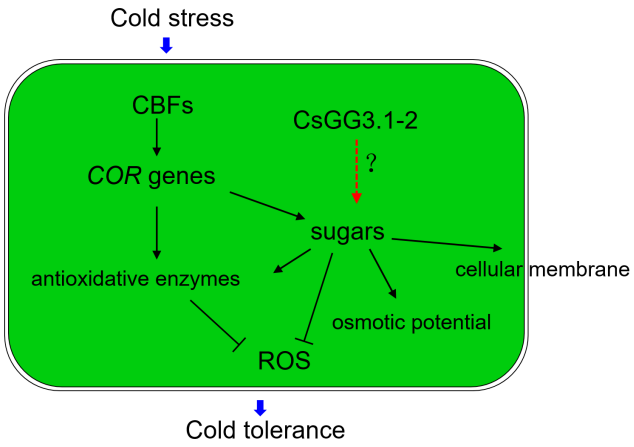

Figure 10. Mechanistic illustration summarizing the influence of CsGG3.1-2 on the cold tolerance of cucumber. CBFs regulate the transcription of *COR* genes, which results in adjustments to the activities of antioxidant enzymes and the accumulation of soluble sugars. Sugars help to maintain osmotic potential, stabilize proteins and cellular membranes, and scavenge ROS. CsGG3.1-2 may influence cellular sugar accumulation through regulating carbohydrate metabolism.

5. Conclusions

In this work, we found that transgenic cucumber seedlings constitutively overexpressing antisense *CsGG3.1-2* exhibited decreased detoxification of ROS, a higher accumulation of H_2O_2 and MDA, and increased chilling sensitivity. However, antisense overexpression did not repress the expression pattern of *CBFs* and their downstream target genes induced by cold stress, suggesting that CsGG3.1-2 affects cucumber cold responses via CBF-independent pathways. The accumulation of soluble sugars decreased in the transgenic cucumber plants under both normal and cold conditions, indicating that the increased chilling sensitivity of the transgenic cucumber plants was primarily associated with soluble sugar deficiency and that cucumber CsGG3.1-2 may play a role in regulating carbohydrate metabolism, which requires more research to be proven.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020240/s1, Figure S1: The starch content in antisense *CsGG3.1-2* transgenic and WT cucumber plants under cold stress; Table S1: Primer sequences used in this work.

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