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Exogenous Gibberellic Acid Ameliorates Chilling Injury in Peach (*Prunus persica* L.) by Improving the Antioxidant System

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Abstract: Currently, several studies have demonstrated that cold stress can cause the accumulation of reactive oxygen species (ROS) in fruit. However, little is known about the roles of gibberellic acid (GA_3) on the antioxidant system in the mitochondria of fruit. To explore the molecular basis of GA_3 interference with the chilling tolerance of fruit, 'Jinqiuhongmi' peach fruit was treated with 0.1 mmol L^{-1} GA_3 after harvest. Exogenous GA_3 treatment relieved the chilling injury of postharvest peach fruit with a lower cold injury index and higher antioxidant level. In addition, GA_3 delayed the senescence of peaches by reducing the firmness, respiratory action, and ethylene production. The antioxidant enzyme activities were elevated, including superoxide dismutase (SOD) and catalase (CAT). Moreover, GA_3-treated peaches exhibited lower hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) in comparison with the control. These results showed that the application of 0.1 mmol L^{-1} of GA_3 enhanced the chilling resistance of peach fruit by regulating the antioxidant system.

Keywords: gibberellic acid; chilling injury; antioxidant system; peach

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1. Introduction

Peach (*Prunus persica* L.) is a cold-sensitive fruit that matures rapidly after harvest, and the fruit is perishable due to rot. A low temperature can slow down microbial growth on the pericarp and the activities of various enzymes, and it has been widely used to maintain nutritional quality. However, peach is vulnerable to developing chilling injury (CI) during 2.2~7.6 °C storage. The internal browning (IB) of the flesh, sap loss, and pulp flocculation—which happens inside the fruit—are the primary signs of CI in peaches [1]. Therefore, these issues are difficult to detect in time at the beginning of CI. So far, numerous research works have been available to mitigate the CI of peaches. Therefore, investigating proper methods to prevent the deterioration of fruit quality is crucial to preserving the commercial value of peach fruit.

As signaling molecules, reactive oxygen species (ROS) play a critical role in the postharvest preservation of fruits. In addition, ROS are also accountable for responding to biotic and/or abiotic stresses [2]. However, the accumulation of excessive ROS causes oxidative damage to plant cells such as lipid peroxidation, protein denaturation, and DNA damage [3]. There exists a natural ROS scavenging system in plant cells that includes

antioxidant enzymes (SOD, POD, and CAT) and non-enzymatic antioxidants (polyphenols and flavonoids). SOD could convert $O_2^{-\cdot}$ into H_2O_2. POD and CAT are responsible for decomposing H_2O_2. Previous studies have shown that the modulation of antioxidant capacity could promote plant resistance within various stress conditions such as cold [4], drought [5], and salinity [6]. For post-harvest fruits and vegetables such as pomegranate [7], hardy kiwifruit [8], eggplant [9] grapefruit [10], and peach [11], increasing the antioxidant capacity can have a substantial impact on alleviating chilling injury. Membrane lipid peroxidation is caused due to the accumulation of ROS, which are produced in plants that have suffered from low temperatures [12]. Gradually, the accumulation of malondialdehyde (MDA), reflecting membrane damage, may lead to tissue deterioration, because of which metabolic activities in plants will be seriously hindered or even maladjusted. Three significant antioxidant-protective enzymes found in plant cells—catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD)—are assumed to be the main mechanisms behind the resistance to cold stress in plants [13].

As a critical phytohormone, gibberellic acid (GA_3) is an important regulator of many physiological processes in plant defense against adversity stress. Used on many fruits and vegetables during the pre-harvest stage, GA_3 has a direct impact on the quality and postharvest permanence of products [14]. Recent studies have shown that spraying GA_3 after harvest had a positive influence on CI in fruit during long-term storage [15]. For instance, exogenous GA_3 treatment effectively modulates the activities of many enzymes in fruit cells, alleviates chilling injury, and reduces ethylene biosynthesis, thus improving the storage quality of orchard products [16,17]. The same results were demonstrated using tomato [18], loquat [19], and green chili [20]. To explore the complicated postharvest physiology process of peaches, a further study of the influence of exogenous GA_3 on peaches is still needed.

Little was known about using GA_3 to prevent cold-stored fruits and vegetables from being damaged due to chilling, and a satisfactory explanation of potential mechanisms in the application of GA_3 was especially lacking. We measured important enzymes involved in ROS metabolism and analyzed corresponding transcript abundance. We aimed to provide insights to explore the mechanism of GA_3 in mitigating CI in harvested fruit and present a practical method for preserving peach fruit.

2. Materials and Methods

2.1. Fruit Material and Post-Harvest Treatments

'Jinqiuhongmi' peaches were harvested at a maturity stage of eight in October 2020 from a local orchard in Beijing and promptly transported at an ambient temperature to the laboratory on the same day for subsequent selection. Peaches that were fresh, in good health, with a uniform size and color, with no pests or diseases and no mechanical damage were stored at a temperature of 0 °C for pre-cooling to discharge their field heat. Then, we randomly divided them into two groups, each with 400 fruits for three replicates:

(1) Control group (CK): These peaches were immersed in sterile, deionized water.
(2) GA_3 group: These peaches were immersed in 0.1 mmol/L of GA_3.

The peaches from both the control group and the treatment group were submerged for 10 min, and then they were allowed to dry for 40 min at room temperature afterward. Then, for 21 days, both groups were kept at 4 °C and 90% relative humidity. During that time, 27 peaches from each group were removed every 7 days for analysis. After 21 days of refrigeration, the peaches were shifted to 26 °C for 3 days to simulate shelf storage. After measuring the internal browning (IB) index, firmness, ethylene production, and respiration rate at all time points, samples of fruit flesh from five fruits per replicate were pooled, rapidly frozen in liquid nitrogen, and thereafter stored at −80 °C for further analysis. Each treatment was replicated thrice.

2.2. Measurement of IB Index

The peaches' IB index was determined according to the method described by Wang et al. with little modifications and assessed according to the symptoms of flesh browning on each fruit surface using the following scale [21]: 0: no visible flesh browning; 1: ≤15% flesh browning; 2: 15–30% flesh browning; 3: 30–45% flesh browning; and 4: ≥45% flesh browning.

The index was calculated using the following formula:

IB index (%) = Σ [(flesh browning scale) × (number of peach with that flesh browning scale)]/(4 × total number of fruit evaluated) × 100.

2.3. Measurement of Firmness

Nine peach fruits were selected from each group for the firmness determination. Two opposite points near the maximum cross diameter of the peeled peach were used, and their firmness was determined with a fruit firmness tester (GY-4, Zhejiang TOP Instrument Co., Ltd., Hangzhou, China) equipped with an 11.1-mm-diameter flat probe [22]. The measurements were repeatedly taken three times for each group, and the results were expressed in N force.

2.4. Measurements of Ethylene Production and Respiration Rate

The ethylene production and respiration rate were assayed by consulting the method of [23,24] with minimal revisions. Three replicates of 2 peaches each were enclosed in 1.5 L glass chambers for 2 h at each sampling time. Then, the ethylene production and respiration rate of the fruit were detected using an F950 hand-held gas analyzer (Felix Instruments, Camas, WA, USA). The results were calculated based on the following formulas:

$$Re = \frac{(V_1 - V_2) \times C}{m \times t} \quad (1)$$

$$Rr = \frac{w \times (V_1 - V_2) \times M \times 1000}{V_0 \times m \times t} \quad (2)$$

where V_1 is the volume of the respiratory chamber, 1.5 L; V_2 is the volume of samples, L; C is the ethylene concentration released from the sample, $\mu L \cdot L^{-1}$; m is the sample weight, g; t is the determination time, h; w is the CO_2 concentration released from the sample, %; M is the molar mass of CO_2, $g \cdot mol^{-1}$; and V_0 is the molar volume of CO_2, $L \cdot mol^{-1}$.

2.5. Measurements of H_2O_2 and MDA Content

The H_2O_2 and MDA content was conducted in keeping with the operating instructions from an H_2O_2 and MDA detection kit [24] (BC3595-100T/96S; BC0025-100T/96S, Solarbio, Beijing, China). The H_2O_2 content was expressed as $mmol \cdot kg^{-1}$. The MDA content was expressed as $nmol \cdot g^{-1}$.

2.6. Measurements of SOD and CAT Activity

The SOD and CAT activity were assayed according to an SOD and CAT activity detection kit (BC5165-100T/48; BC0205-100T/96S, Solarbio, Beijing, China). Approximately 0.1 g of peach tissue from each group was weighed, added to 1 mL of extracting solution to homogenize in an ice bath, and centrifuged it at 8000× *g* at 4 °C for 10 min, and the supernatant was taken and put on ice for measurement. The activity was expressed as $U \cdot g^{-1}$. The SOD activity was expressed as $U \cdot g^{-1}$. The CAT activity was expressed as $U \cdot g^{-1}$, for which one unit of CAT activity indicates that each gram of tissue catalyzes the degradation of 1 μmol of H_2O_2 per minute in the reaction system.

2.7. Extraction of Total RNA and RNA-Seq

Total RNA was extracted according to the cetyltrimethylammonium bromide (CTAB) method described by Zhao et al. [25]. RNA-seq was performed by Ouyi Corporation (Shanghai, China). Briefly, three biological replicates with RNA integrity greater than 8 were selected from 0 d, 21 d, and 21 + 3 d frozen samples from each set of treatments for library preparation and sequenced at a 150 bp paired end on the Illumina HiSeq platform [26]. Transcript abundance was calculated as fragments per kilobase per million (FPKM).

2.8. Real-Time Quantitative PCR (RT-qPCR) Analysis

The method was performed according to the study by Zhao et al. [25]. The primers used in our research are listed in Table S1.

2.9. Statistical Analysis

All data came from the means of 3 biological replicates. A completely randomized design was used in the trial. The results were statistically analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Statistical comparisons between the treatment and control groups were conducted using Duncan's multiple range test ($p < 0.05$). For differential gene expression, a comparative $2^{-\Delta\Delta CT}$ method was imposed.

3. Results

3.1. Effect of GA_3 Treatment on IB Index and Fruit Firmness

IB is a typical symptom of CI during the cold storage of peach fruit, with the enzymatic oxidation of phenols being the main cause. And pulp flocculation is another symptom of cold injury [27]. As shown in Figure 1A, the IB occurred after 14 d. After 21 d of 4 °C storage, the peaches were transferred to 26 °C, and the chilling injury symptoms became severe. The IB was effectively alleviated using exogenous GA_3.

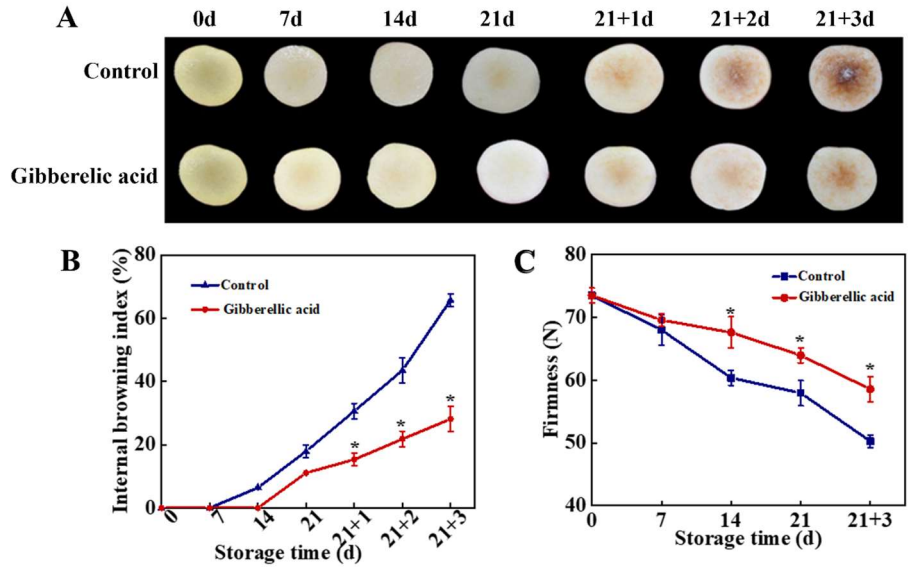

Figure 1. The effects of GA_3 treatment on the appearance of peach fruit (**A**), internal browning index (**B**), and firmness (**C**) during cold storage at 4 °C. The vertical bars represent the standard deviation of the means of triplicate samples ($n = 9$). The * symbol indicates significant differences among different treatments at $p < 0.05$.

Figure 1B showed that the IB symptom was delayed due to the treatment with 0.1 mmol/L of GA_3 by 11.1% over 21 d and 28.1% over 21 + 3 d. The IB index of the control group was 17.9% over 21 days of 4 °C, and it soared to 65.7% after these fruits were transferred to 26 °C for 3 days.

The firmness exhibited a decreasing trend during the entire period. The peach firmness showed a declining pattern over the course of storage, with a minor decrease during the first 21 days and a substantial reduction after 21 d, which was linked to the temperature (Figure 1C). Exogenous GA_3 can ensure the normal softening of fruit, but it slightly delays the decrease in fruit hardness. As the temperature rose after 21 d, the effect of delaying became more obvious. The firmness of the control group was 50.24 N, while that of GA_3 was 58.56 N.

3.2. Effect of GA_3 on the Respiration and Ethylene Production Rate of Peach Fruit

The respiration rate showed an increase in the control group at 14 d after harvest, while it underwent a minor fall in the first week and rapid growth over 7–14 d in the GA_3 group. Next, the respiration rate of both groups declined over 14–21 days; subsequently, it rose again, reached the second peak, respectively, and then fell again (Figure 2A). The first respiration peak of the control group was 483.50 $\mu L \cdot (kg \cdot h)^{-1}$, which was 1.05 times higher than that of the peaches treated with GA_3 in the second week of 4 °C storage.

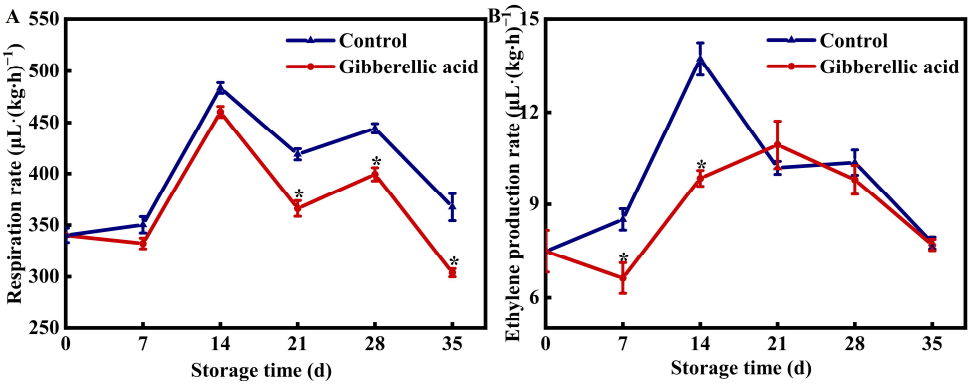

Figure 2. The effects of GA_3 treatment on respiration rate (**A**) and ethylene production (**B**) during cold storage at 4 °C. The vertical bars represent the standard deviation of the means of triplicate samples (n = 3). The * symbol indicates significant differences among different treatments at $p < 0.05$.

It is worth mentioning that the tendency toward ethylene production in the control and treated peaches was comparable to that of the respiration rate of these two groups (Figure 2B). Compared with the ethylene production peak of the control group, which occurred after 14 d, the treatment samples went up to the top of their ethylene production after 21 d. The peak of the GA_3 peaches (10.94 $\mu L \cdot (kg \cdot h)^{-1}$) exhibited a lower level compared to that of the control group, which was 13.72 $\mu L \cdot (kg \cdot h)^{-1}$.

3.3. Effects of Exogenous GA_3 on Enzymes Related to Antioxidant Activity

The SOD activity in GA_3-treated fruit exhibited a peak within the first 7 days, as depicted in Figure 3A, followed by a subsequent decline throughout the entire storage period. In comparison with the control fruit, the SOD activity showed a slightly increasing trend in the first week and gradually decreased until the end of storage. GA_3 treatment can improve the SOD activity, especially on day 7 and day 21 ($p < 0.05$).

The CAT activity quickly decreased in the first 7 days in both the GA_3-treated fruit and the control fruit, but during the subsequent storage time, the activity of CAT exhibited different patterns. The CAT activity maintained a steady trend. CAT is a crucial component of the biological defense system, playing a key role in decomposing H_2O_2 into H_2O and safeguarding cells against the toxic effect of H_2O_2. In this investigation, the maximal enzyme activity in the treated group with 0.1 mmol/L GA3 was found after 21 d (Figure 3B). The treated fruits exhibited considerably higher enzyme activity than the control (21 d and 21 + 3 d).

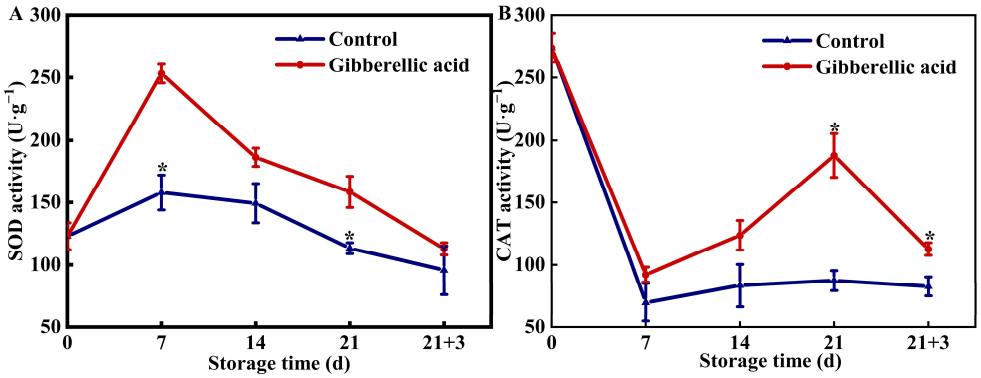

Figure 3. The effects of GA3 treatment on superoxide dismutase (SOD) activity (**A**) and catalase (CAT) activity (**B**) during cold storage at 4 °C. The vertical bars represent the standard deviation of the means of triplicate samples (n = 3). The * symbol indicates significant differences among different treatments at $p < 0.05$.

3.4. Effects of Exogenous GA_3 on MDA and H_2O_2 Contents

MDA and H_2O_2 are consistently recognized as markers of plant oxidative stress levels and plant membrane structural integrity at low temperatures. According to Figure 4A,B, the increases in both MDA and H_2O_2 contents were observed throughout the whole preservation stage at 4 °C. As the samples were moved to 26 °C for the following shelf-life storage, there were surges in MDA and H_2O_2 contents ($p < 0.05$). Treatment with GA_3 resulted in dramatic declines in MDA and H_2O_2 contents under cold stress, which clearly indicated that post-GA_3 treatment could significantly shield plants from oxidative damage and, thus, enhance their cold tolerance.

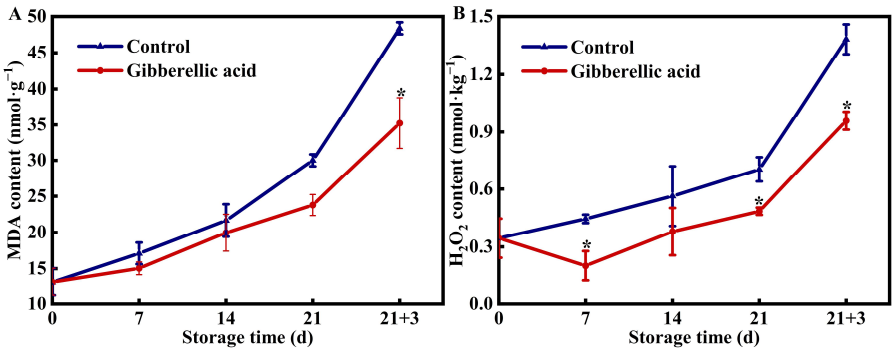

Figure 4. The effects of GA_3 treatment on malondialdehyde (MDA) content (**A**) and hydrogen peroxide (H_2O_2) content (**B**) during cold storage at 4 °C. The vertical bars represent the standard deviation of the means of triplicate samples (n = 3). The * symbol indicates significant differences among different treatments at $p < 0.05$.

3.5. Gene Expression Analysis

To illuminate the mechanisms for the improvement of the antioxidant capacity in the GA_3-treated samples, we assessed the expression profiling of antioxidant system-related genes on different storage days, including 4 *PpSODs*, 2 *PpCATs*, 6 *PpPODs*, and 3 *PpPPOs*. Striking changes in the expression of some genes were noticed in Figure 5. Except for *PpSOD2*, of which the expression was downregulated during room-temperature storage, the GA_3 treatment upregulated the expression of 4 *PpSODs* over the whole course of preservation, corresponding to the activity of SOD. As Figure 3A shows, the SOD activity was consistently higher in the treated fruits than in the untreated fruits. The relative expression of 2 *PpCATs* in the GA_3-treated peaches was markedly higher than it was in the untreated peaches, which is similar to the tendency for the CAT activity. Of the analyzed 6 *PpPODs* and 3 *PpPPOs*, the transcript abundance of *PpPOD21* and *PpPPO1/3/4* was

diminished, and the transcript abundance of *PpPODA2/12/17/44/51* and *PpPPO1/2* was promoted due to GA_3 in comparison with the control samples.

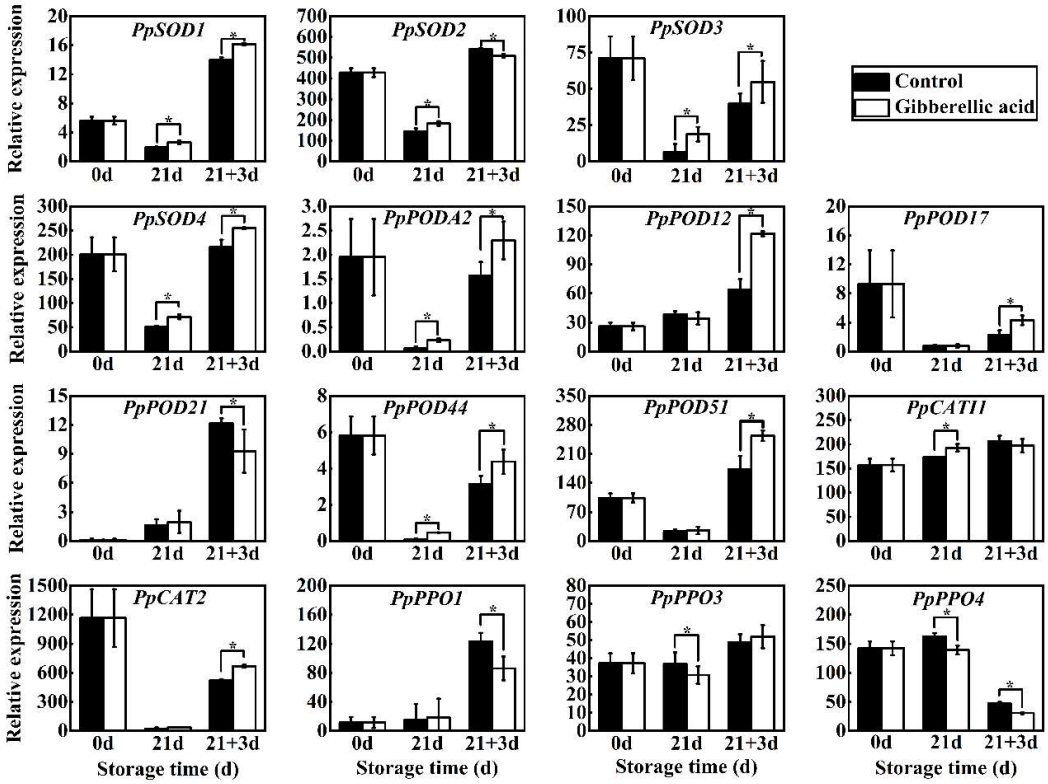

Figure 5. The effect of the GA_3 treatment on the mRNA abundance of genes related to ethylene biosynthesis and the ethylene signaling pathway of fruit stored at 4 °C. The validation of mRNA abundances was determined using RT-qPCR. The data are the means of three biological replicates ±SE. The * symbol indicates significant differences among different treatments at $p < 0.05$.

4. Discussion

Generally, peaches stored at 2.2~7.6 °C are prone to cold chilling, especially after they are transferred to room-temperature surroundings. When the fruit is exposed to cold stress, it results in the disruption of the cell membrane integrity and the accumulation of reactive oxygen species (ROS), including H_2O_2, O_2^-, nitric oxide, and peroxynitrite [28]. Excessive ROS can induce lipid peroxidation and membrane degradation, thereby accelerating fruit ripening and senescence processes, leading to various symptoms of CI, such as internal tissues turning brown and powdery, as well as flocculation. The protective enzymes in plants, such as SOD, POD, CAT, and other enzymes involved in active oxygen metabolism, undergo corresponding alterations simultaneously [29]. These changes are crucial because of their role in scavenging oxygen free radicals and reactive oxygen species. Many studies have demonstrated that regulating the antioxidant system eliminates excessive ROS accumulation and induces chilling tolerance in postharvest fruit and vegetables [30,31].

Several studies have shown the beneficial influence of GA_3 on the CI of harvested fruits [32]. GA_3 has been involved in the signaling pathways modulating multiple defense responses induced via biotic and abiotic stresses, especially chilling stresses [18]. However, only a few studies have concentrated on the effect of postharvest GA_3 on fruit quality, while most treatments have been applied to trees or fruits during pre-harvest [33]. Moreover, the fundamental mechanisms through which GA_3 enhances fruits' chilling resistance remained unclear. In this research, we discovered that GA_3 treatment effectively alleviated the chilling injury in peaches, as observed with the lower respiration rate, ethylene production, and MDA and H_2O_2 production, as well as the higher expression of antioxidant-related genes.

When the chilling symptom of peaches became more serious after they were transferred to 26 °C, the application of GA_3 could also maintain a good character.

When fruits and vegetables are subjected to low temperatures, their lipid composition and structure change, which influences modifications in cell membrane function [34]. IB is a typical symptom of CI, and the peroxidation of membrane fatty acids is the main reason [35]. Internal browning is generally considered to be enzymatic browning. The cell membrane structure is destroyed due to the low temperature, and a large number of phenols are oxidized via PPO to quinone, which is further polymerized to a brown pigment. The decline in *PpPPO1/3/4* resulting from a GA_3 treatment can be observed in Figure 5, but the effects on phenolics and the activity of PPO are variable, with unchanged, upward, or downward trends revealed in different reports, depending on tissues [36]. GA_3 can significantly alleviate the chilling injury of peaches with a better internal appearance, a lower IB index, and higher firmness (Figure 1). Thus, the stabilization of the membrane structure and function can improve plant resistance to low-temperature stress. Changes in MDA, a biomarker of membrane lipid peroxidation, are consistent with the IB index. The exogenous GA_3 treatment of peach fruit decreased the MDA content (Figure 4). Moreover, the imbalance in the ROS metabolism is the main factor of chilling-induced lipid peroxidation. We also observed a decline in H_2O_2 content in GA_3-treated fruit (Figure 4). Hence, chilling injury is associated with oxidative stress. Previous studies have shown that exogenous GA_3 treatment can alleviate the chilling injury of tomato [18], toon sprout, and peach [36] by increasing the antioxidant capacity during cold storage. Furthermore, an increasing amount of evidence demonstrates that phytohormones can reduce the development of chilling injury, and antioxidant-related enzymes play important roles in this process [37,38]. This is partly similar to our experiment, in which the respiration and ethylene levels of GA_3-treated peaches during the whole preservation stage were lower than those of the control peaches. These results align with the study of Martínez et al. [39]. This illustrates that GA_3 has a positive effect on maintaining post-harvest peaches' quality.

Redox equilibrium plays a considerable role in plants' cold adaptation, normal physiological metabolism, and various vital movements [40]. A low temperature will weaken the hydrophobic bond of protein molecules and strengthen the interaction between the hydrogen bond and electrostatic attraction, resulting in the change of protein conformation. Subsequently, the function of antioxidant enzymes in the free radical scavenging system is affected, and the balance of the generation and metabolism of active oxygen free radical is broken. Reactive oxygen species (ROS), which accumulate in cells, cause intense damage to lipids, proteins, and DNA, leading to membrane lipid peroxidation [41]. Meanwhile, the growth in MDA content damages the cell membrane, resulting in electrolyte leakage and an increase in cell membrane permeability. Figure 4 showed a gradual upward trend in the MDA content of peaches, which illustrated that an inappropriate low-temperature environment promoted membrane lipid peroxidation in fruit. A lower accumulation of MDA suggested that GA_3 can effectively reduce the membrane lipid peroxidation of fruit, maintain cellular integrity, and then enhance the chilling resistance of fruit. The tendency of H_2O_2 content was similar to that of MDA, indicating that intracellular ROS homeostasis was disrupted due to cold. It is widely known that SOD, POD, and CAT are key antioxidant enzymes to eliminate ROS and H_2O_2 [41,42]. GA_3 can boost the activities of SOD and CAT, which were obviously a consequence of the rise in the transcript abundance of *PpSOD1-4*, *PpCATI1*, and *PpCAT2*. Due to the higher expression of *PpPOD12*, *PpPOD17*, *PpPOD44*, and *PpPOD51* in treated peaches, the activity of POD was promoted via GA_3 [43]. Combined with higher SOD and CAT activities, it thereby reduces the accumulation of H_2O_2. Previous studies have shown that the regulation of antioxidant capacity is better for alleviating cold injury and maintaining post-harvest fruit quality, and our results are in alignment with previous work [44–46]. The results (Figure 6) suggest that the antioxidant system plays a significant role in preventing membrane damage that leads to IB.

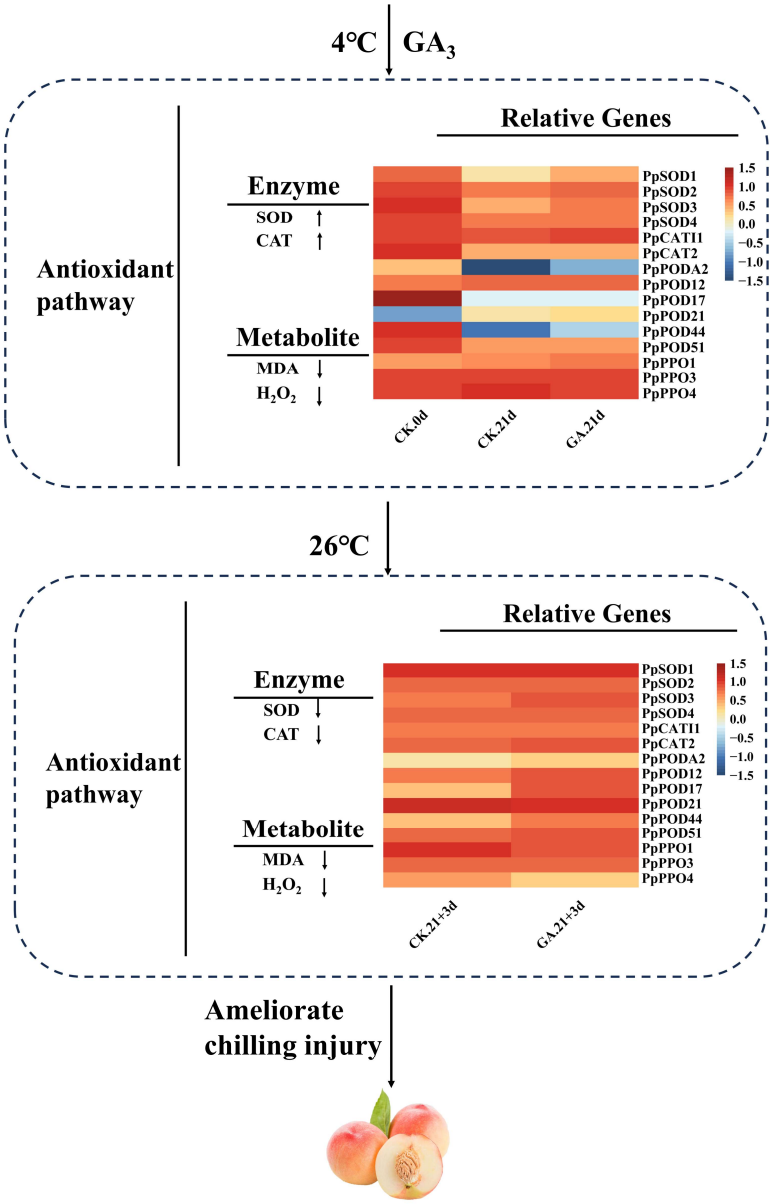

Figure 6. The effects of GA_3 treatment on the expression profiles of genes associated with reactive oxygen species' metabolic pathways in peach fruit stored at 4 °C and then transferred to 26 °C. The rows in each heat map represent the indicated genes, and the six columns indicate the following storage times (left to right): CK_0d, CK_21d, GA_21 + 3d, CK_21 + 3d, and GA_21 + 3d. The colors represent the FPKM values between different samples in the heat map. Each value represents the mean for three replicates. *SOD*, *superoxide dismutase*; *POD*; *CAT*; *PPO*.

5. Conclusions

This study has shown that GA_3 treatment alleviated the CI symptoms of peach fruit and inhibited the internal browning and the decrease in firmness in cold-stored peach fruit. The application of GA_3 also resulted in a suppression of the respiration rate and ethylene production during the late storage stage of peach fruit while causing a delay in the occurrence of the ethylene peak. In addition, GA_3 treatment significantly increased the gene expression of *PpSOD*, *PpCAT*, *PpPODA2/12/17/44/51*, and *PpPPO1/2*. At the same time, GA_3 treatment enhanced the activity of antioxidant enzymes, such as SOD and CAT, while suppressing the production of MDA and H_2O_2. In conclusion, the application of GA_3 could alleviate CI in peach fruit mainly through enhancing the antioxidant capacity and reducing the ethylene production under cold storage. Thus, the findings presented here

provide a new perspective on the development and quality improvement of post-harvest peach fruit.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14040816/s1: Table S1: Primers used for analyzing gene expression via qPCR.

Author Contributions: H.S.: conceptualization, investigation, methodology, formal analysis, visualization, writing—original draft, writing—review & editing. X.R.: investigation, methodology, writing—original draft. H.H.: formal analysis, investigation, methodology. Z.P.: formal analysis, methodology, investigation, validation. J.Z.: formal analysis, investigation, visualization. Z.Z.: conceptualization, investigation, validation. J.L.: project administration, resources, writing—review & editing. P.Z.: methodology, project administration, resources, writing—review & editing. Y.Z.: conceptualization, methodology, writing—review & editing, supervision. Y.D.: conceptualization, funding acquisition, project administration, supervision, writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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