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Selenium Treatment Regulated the Accumulation of Reactive Oxygen Species and the Expressions of Related Genes in Postharvest Broccoli

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Abstract: This study aimed to investigate the impact of selenium (Se) treatment on the accumulation of reactive oxygen species (ROS) and the expressions of related genes in broccoli. To achieve this, one group of broccoli heads was treated with a selenite solution of 2 mg L^{-1}, while another group was soaked in distilled water, serving as the control. The effects of these treatments were evaluated by analyzing the browning, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents, enzyme activity, and gene expression levels of *WARK* and *RBOH*. Our results show that the Se treatment effectively inhibited H_2O_2 accumulation in the broccoli and reduced harmful MDA levels. The inhibition of ROS accumulation following the Se treatment was associated with enhanced activity of the CAT and SOD enzymes, increased expression levels of *BoCAT* and *BoSOD*, and decreased expression levels of the *WRKY* and *RBOH* transcription factors. Our study provides insights into the mechanism of action of selenium and its potential application in vegetable storage.

Keywords: selenium treatment; broccoli; ROS; browning

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1. Introduction

Broccoli (*Brassica oleracea* L. var. *Italica*) is an important global vegetable, with extensive cultivation covering approximately 140,000 hectares in China alone [1]. Its popularity stems from being a rich source of essential minerals, amino acids, dietary fiber, and various phytochemicals, making it a highly nutritious choice [2]. However, postharvest broccoli faces a challenge due to its susceptibility to oxidative damage, leading to browning at the incision site and significantly reducing its shelf-life. The degree of browning in cut broccoli directly impacts its commercial value as it affects consumers' willingness to purchase.

ROS are natural byproducts of plant metabolism, serving as both signaling molecules and potential toxins [3]. Under chilling stress, plants experience excessive ROS accumulation in cells, including superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2) [4]. However, elevated ROS levels can lead to oxidative damage to macromolecules and contribute to cell death pathways [5]. Generally, plants possess intricate mechanisms to maintain a delicate balance of ROS as they can have both beneficial and harmful effects. Enzymes such as catalase (CAT), ascorbate peroxidase (APX), and superoxide dismutase (SOD) function in living organisms to regulate ROS concentrations by scavenging H_2O_2 and O^{2-} [6,7]. Among the various pathways of ROS production, the NADPH oxidase encoded by the respiratory burst oxidase homolog (*RBOH*) gene has been extensively studied [8]. Under abiotic stress conditions, NADPH oxidase is activated by calcium and phosphorylation, leading to ROS production in plants [9]. The release of ROS is closely associated with the activity of specific cis-acting elements in the upstream regions of target genes. Among

those elements, the W-Box has been identified as a main ROS-specific element [10]. Interestingly, the W-Box (TTGACC) has been characterized in several members of the *ROBHs* gene family [11]. Previous research by [12] demonstrated that the mutation of *AtRBOHd* in *Arabidopsis* limited ROS accumulation, while [13] showed that the accumulation of RBOHd protein increased through the mutation of *PIRE* and *PBL13*, leading to enhanced ROS production. Additionally, *WRKY* TFs have been identified as positive regulators of *ROBH* genes. For instance, Adachi et al. [14] reported that the silencing of *WRKYs* resulted in the downregulation of *ROBHb*, impairing ROS burst, with the W-Box identified as a binding site for *WRKYs* in the cis-acting elements of *ROBH* genes.

Selenium is widely recognized for its potential antioxidant properties and has been extensively studied [15]. Studies investigating selenium's antioxidant effects date back to the 1970s and 1980s, during which it was proposed that selenium occupies active sites in glutathione peroxidase [16]. Subsequent research has established a consensus that selenium, as a component of various selenoproteins with antioxidative activity, plays a crucial role in the response to oxidative stress [17]. These selenoproteins include glutathione peroxidase and thioredoxin reductase [18]. Of particular importance, selenium has been reported to enhance stress resistance, antioxidant capacity, and enzyme activity in plants [19]. Despite these promising findings, there is a limited information on the application of selenium in postharvest fruit and vegetable preservation.

In our previous study, we employed transcriptome sequencing (RNA-seq) and observed a significant upregulation of genes encoding *BoPOD*, *BoSOD*, *BoAPX*, and *BoCAT* after selenium treatment. In contrast, the expressions of *BoRBOHd* and *BoRBOHf*, which are closely associated with ROS concentration, were downregulated. These findings led us to speculate that selenium may regulate ROS levels by modulating the expressions of ROS metabolism-related genes in broccoli. Therefore, the main objective of this study was to investigate the impact of selenium on antioxidation in broccoli. Through our investigation, we aimed to gain deeper insights into the potential mechanisms underlying selenium's biological functions in controlling antioxidation in broccoli.

2. Materials and Methods

2.1. Plant Material, Processing, and Storage Conditions

For this experiment, we collected fresh broccoli samples from the Juxin Entrepreneurship Park of Shanxi Agricultural University, ensuring that they were free from any surface bruises, pest infestation, or mechanical damage. The selected broccoli heads were washed with distilled water and left to naturally air dry. Subsequently, the broccoli heads were randomly divided into two groups, with each group containing 35 broccoli heads. One group was immersed in water containing 2 mg L^{-1} of selenite (Se treatment, Damao chemical reagent factory, Tianjin, China), while the other group was immersed in distilled water (control) for 20 min. Following the dipping process, the broccoli heads were air-dried and carefully placed in polyethylene film bags (0.02 mm thickness, 80 cm × 60 cm in size) before being stored at 0 °C.

During the storage period, flower buds were excised from the broccoli at different time intervals, immediately frozen in liquid nitrogen, and stored at −80 °C until further analysis. Sampling of the flower buds was conducted randomly from five broccoli samples every 10 days, up to a storage duration of 60 days. For each parameter analysis, three biological replicates were conducted.

2.2. Malondialdehyde (MDA) and H_2O_2 Content Analysis

The measurement of the MDA content was conducted in accordance with the thiobarbituric acid (TBA) method [20]. One gram of flower buds from five broccoli heads was utilized. The MDA content was expressed as μ mol kg^{-1} on a fresh weight (FW) basis.

To determine the H_2O_2 content, 0.1 g of broccoli head was employed, and the Hydrogen Peroxide Assay Kit (Solarbio, Beijing, China) was used. The H_2O_2 content was subsequently quantified spectrophotometrically using an H_2O_2-specific reagent, titanium sulfate.

2.3. Enzyme Activities Assays

The activities of the POD, CAT, SOD, and APX enzymes were measured using an enzyme activity assay kit (Solarbio, Beijing, China), following the manufacturer's instructions. We strictly adhered to all steps and procedures outlined in the provided instructions to ensure accurate and reliable enzyme activity measurements.

2.4. RNA-seq Analysis

The broccoli samples were analyzed by RNA-seq following the established research methods as described in [19].

2.5. RT-qPCR Analysis

The total RNA was extracted from the broccoli florets using the TransZol Up Plus RNA Kit (TransGen, Beijing, China). Gene-specific primers, as listed in the Supplementary Materials Table S1, were employed for the qRT-PCR analysis, and the qRT-PCR reactions were performed using the PerfectStart Green qPCR SuperMix (TransGen, Beijing, China). The gene-specific primer sequences for qRT-PCR were provided in Table S1. To ensure accurate normalization, the tomato *Actin* gene (LOC) was used as the internal control. The relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method, and the results were normalized using the *Actin* internal reference gene. The analysis was conducted with three independent biological replicates.

2.6. Evolutionary Tree Construction of BoRBOH and BoWRKY

The standalone BLAST package (NCBI, www.ncbi.nlm.nih.gov/blast/, accessed on 12 March 2023) was used for the homolog search analysis of the *BoRBOH* and *BoWRKY* genes. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates in MEGA 7.0 software (Mega Limited, Auckland, New Zealand).

2.7. Statistical Analysis

Statistical and linear regression analyses were performed using Origin Pro 2021 (Origin Lab Inc., Northampton, MA, USA). Standard deviation calculations, one-way ANOVA, and Pearson correlation analysis were performed using SPSS v.20 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Browning, H_2O_2 Content, and MDA Content

As shown in Figure 1A, the browning of broccoli increased during storage. However, the Se treatment significantly reduced the browning compared to the control group throughout the storage period. The H_2O_2 content increased during the first twenty days of storage in the control group, followed by a decrease after twenty days (Figure 1B). A similar trend was observed in the Se-treated broccoli head group. However, from day 40 to 60, the Se treatment significantly inhibited the accumulation of H_2O_2 compared to the control group. Additionally, the H_2O_2 content in the Se-treated broccoli head was higher than in the control broccoli head at 20 d.

The MDA content in the broccoli head of the control group reached its highest peak at 20 d, after which it decreased gradually (Figure 1C). While the MDA content in the Se treatment group showed a similar trend to the control, it was consistently significantly lower than that in the control broccoli head throughout the entire storage period.

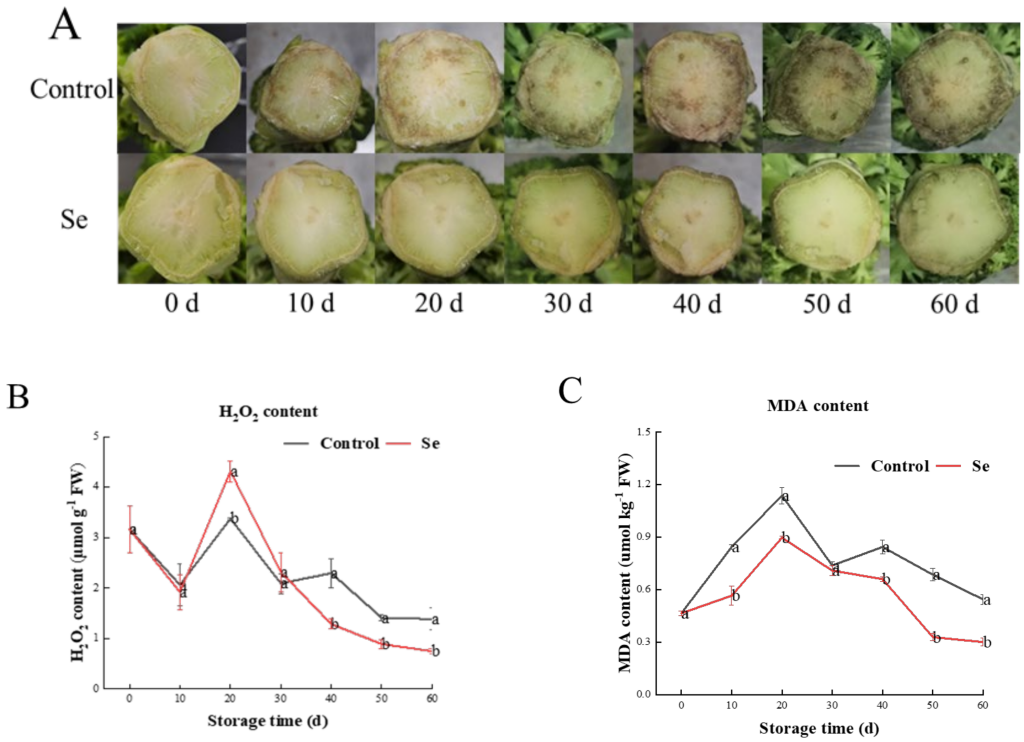

Figure 1. Effects of Se treatment on browning (**A**), H_2O_2 content (**B**), and MDA content (**C**) of broccoli during storage. Each data point represents the mean of three replicate assays ± standard error. Different letters represent significant differences within the same group during storage ($p < 0.05$), as determined by Duncan's multiple range test.

3.2. POD, CAT, SOD, and APX Activity

In general, the POD activity of Se−treated broccoli head was lower than that of the control group during the first thirty days of storage (Figure 2A). Conversely, the Se treatment resulted in higher CAT activity and SOD activity than observed in the control group throughout the entire storage period (Figure 2B,C). However, the APX activity in the control group increased during storage, while the Se treatment resulted in lower APX activity compared to the control group during most of the storage period (except at 50 days) (Figure 2D).

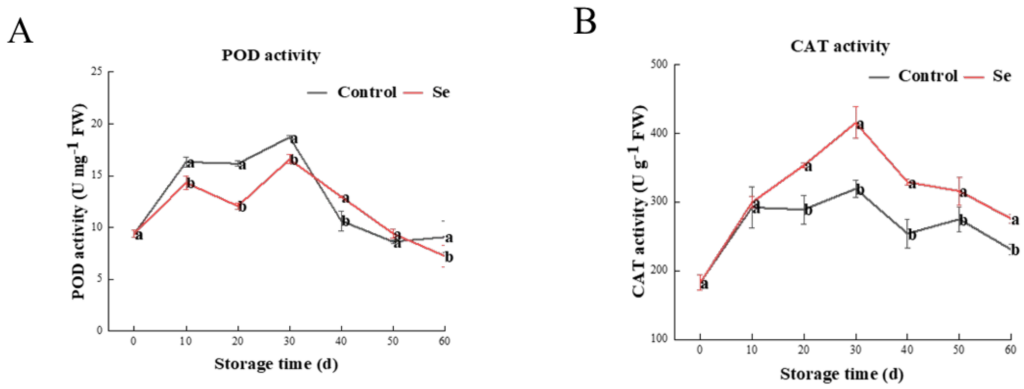

Figure 2. *Cont.*

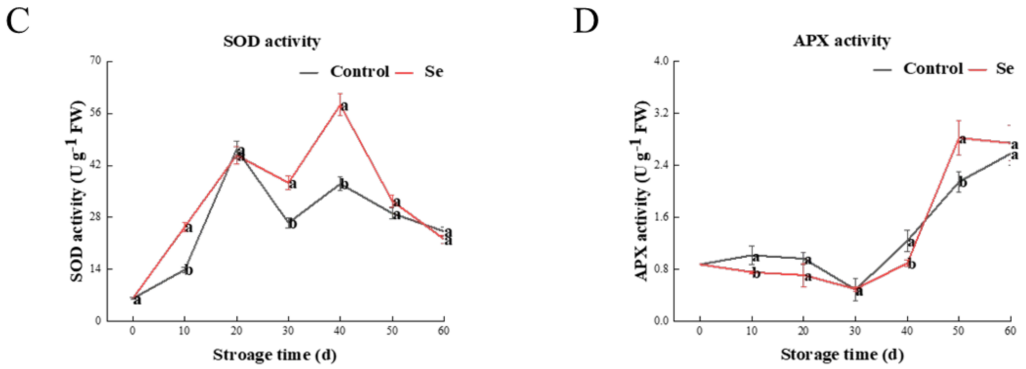

Figure 2. Effects of Se treatment on the enzymatic activity of POD (**A**), CAT (**B**), SOD (**C**), and APX (**D**) in broccoli during storage. Each data point represents the mean of three replicate assays ± standard error. Different letters indicate significant differences within the same group during storage ($p < 0.05$), as determined by Duncan's multiple range test.

3.3. RNA-seq Results Analysis

The RNA-seq results show that there were two and five differentially expressed genes related to ROS metabolism for the plant *RBOH* gene and *WRKY* gene families, respectively. These genes participate in multiple biological processes, including the MAPK signaling pathway and plant–pathogen interaction (Figure S1).

3.4. Genes Expression Level

As shown in Figure 3A, the *BoPOD* gene expression in the Se-treated broccoli head was generally lower than in the control during storage, except at 30 d and 40 d. The expression of *BoPOD* rapidly peaked at 40 d in the Se treatment, while it appeared at 50 d in the control. In the control broccoli heads, the expression of *BoSOD* rapidly increased within the first ten days of storage, followed by a decrease, maintaining a lower level. In contrast, the expression of *BoSOD* in the Se-treated broccoli head remained relatively stable level and was higher compared to the control group from 20 d to 40 d of storage, although its expression level was lower than the control at 10 d (Figure 3B). Similarly, the *BoAPX* expression in the control group rapidly increased at 10 d and then gradually decreased. Conversely, in the Se-treated group, the expression of *BoAPX* showed a rapid decrease at 10 d. Unlike *BoSOD*, the *BoAPX* expression in the Se-treated broccoli heads was higher than in the control from 40 d to 60 d of storage (Figure 3C). Moreover, the *BoCAT* expression in the Se-treated broccoli heads was higher than that in the control broccoli heads for most of the storage time (Figure 3D).

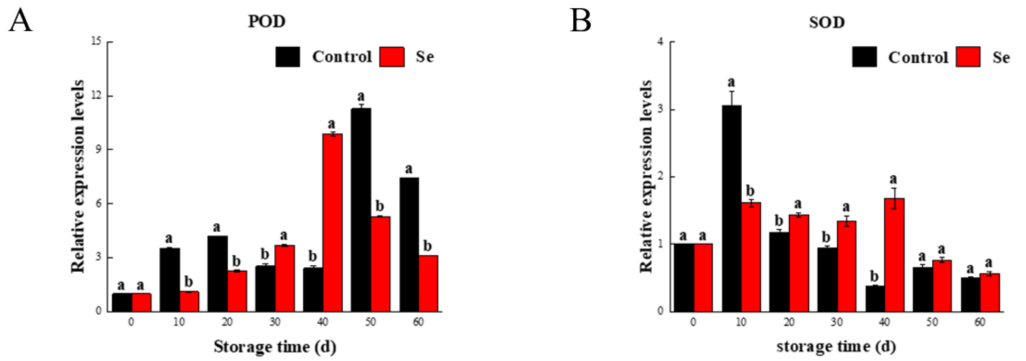

Figure 3. *Cont.*

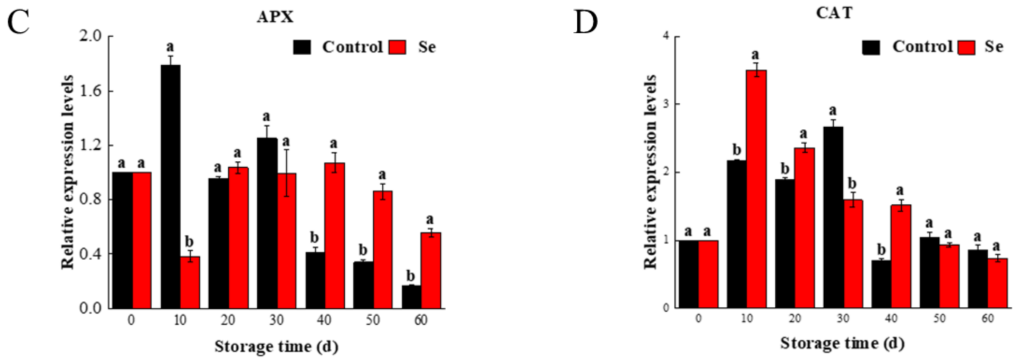

Figure 3. Effects of Se treatment on the expression levels of genes involved in ROS metabolism, including *BoPOD* (**A**), *BoSOD* (**B**), *BoAPX* (**C**) and *BoCAT* (**D**). Each data point represents the mean of three replicate assays ± standard error. Different letters represent significant differences within the same group during storage ($p < 0.05$), as determined by Duncan's multiple range test.

3.5. Expression Patterns of BoRBOHd and BoRBOHf

Based on similarity searches using broccoli's RBOHd and RBOHf amino acid sequences, two homologous *RBOH* genes were identified from *Arabidopsis* (Figure S2). From a general perspective, the expression patterns of *RBOH*-related genes were found to be similar. Specifically, for *BoRBOHf*, its expression level in the Se-treated broccoli heads was lower than that in the control broccoli heads after 20 days of storage (Figure 4A). Additionally, the expression of *BoRBOHf* was induced at low temperatures, particularly at 0 and 20 days of storage. On the other hand, the expression of *BoRBOHd* showed a durable induction, especially at 0 and 40 days of storage (Figure 4B). Overall, the data in Figure 4 demonstrate that the Se treatment effectively inhibited the expressions of both *BoRBOHf* and *BoRBOHd* during cold storage.

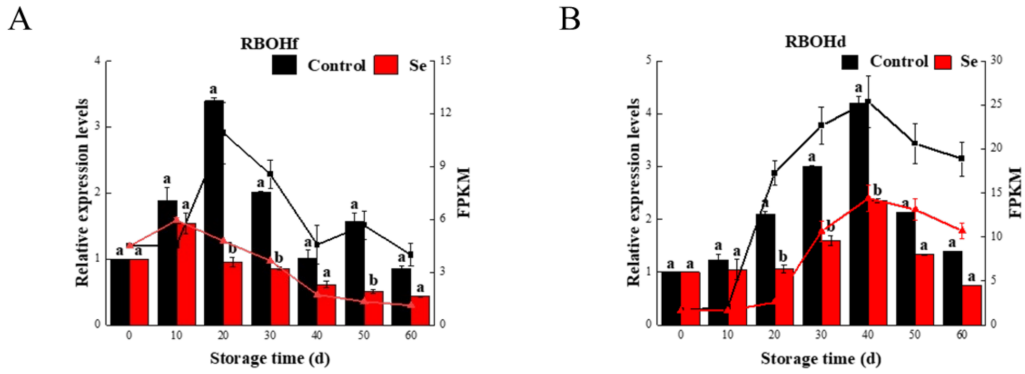

Figure 4. Effects of Se treatment on the expression levels of *RBOH* transcription factors involved in ROS metabolism, including *BoRBOHf* (**A**) and *BoRBOHd* (**B**). Each data point represents a mean of three replicate assays ± standard error. Different letters represent significant differences in the same group during storage ($p < 0.05$) by Duncan's multiple range test.

3.6. Expression Patterns of BoWRKYs

The homology analysis results revealed a striking similarity between *BoWRKY33*, *BoWRKY25*, *BoWRKY15*, and *BoWRKY6*, and their respective homologs in *Arabidopsis*, namely, *AtWRKY33*, *AtWRKY25*, *AtWRKY15*, and *AtWRKY6* (Figure S3). Throughout the storage period, except *BoWRKY6*, the expression levels of all *WRKY*-related genes exhibited an initial increase, followed by a subsequent decrease. Notably, the expression of *BoWRKY33* was significantly inhibited upon treatment with Se during storage (Figure 5A). Additionally, the expressions of *BoWRKY25* and *BoWRKY15* were inhibited by the Se treatment at 0 and 40 days of storage, respectively (Figure 5C,E). Interestingly, *BoWRKY6* expression showed an upward trend, and there were significant differences in its expression

level between the Se treatment and the control groups (Figure 5B). Importantly, the qPCR results demonstrate a high level of concordance with the RNA-Seq data, underscoring the reliability of our RNA-Seq findings.

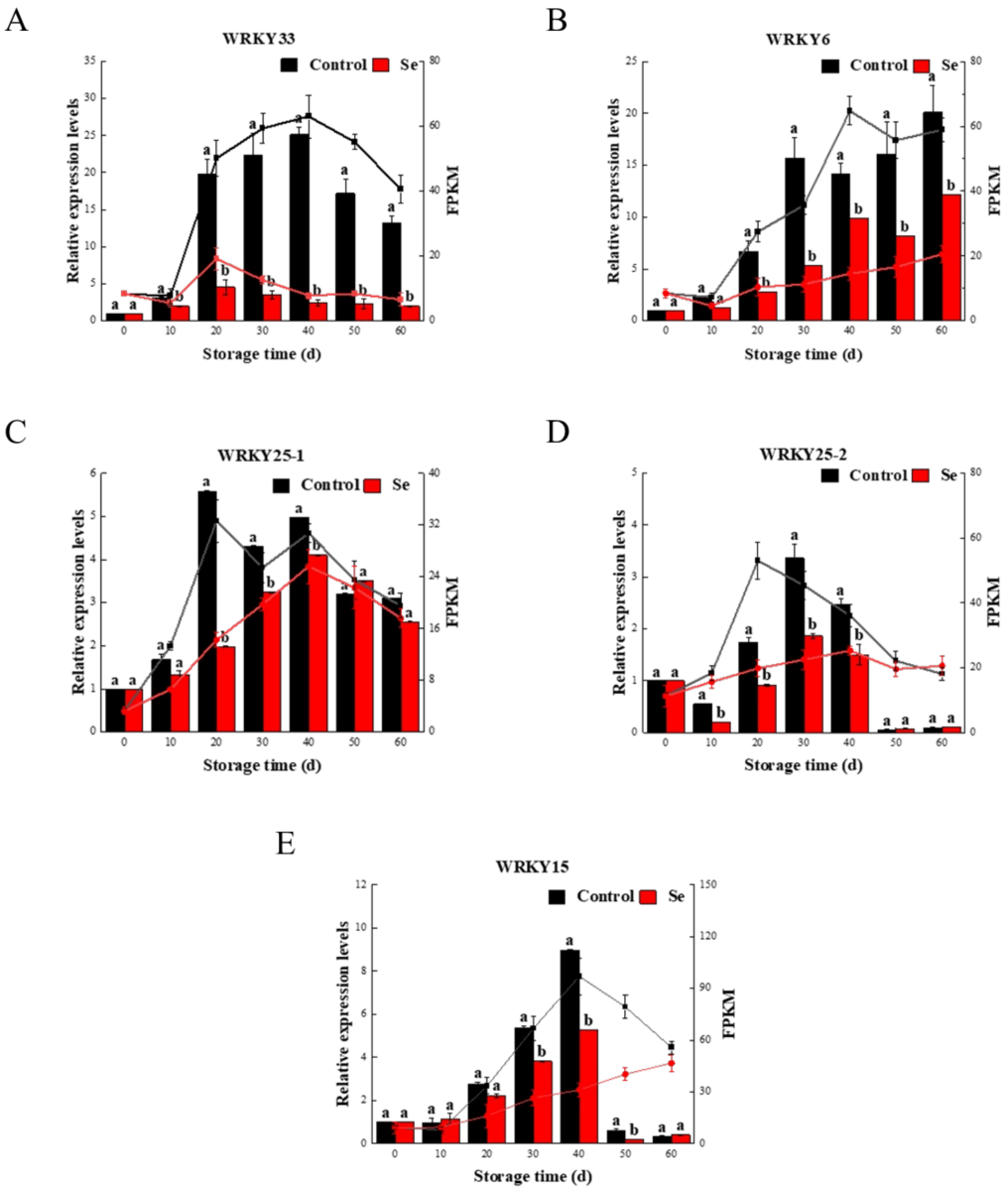

Figure 5. Effects of Se treatment on the expression levels of *WRKY* transcription factors involved in ROS metabolism, including *BoWRKY33* (**A**), *BoWRKY6* (**B**), *BoWRKY25-1* (**C**), *Bo25-2* (**D**) and *BoWRKY15* (**E**). Each data point represents a mean of three replicate assays ± standard error. Different letters represent significant differences in the same group during storage ($p < 0.05$) by Duncan's multiple range test.

4. Discussion

In recent years, cruciferous vegetables have gained significant attention for their ability to accumulate Se, making them stand out among other vegetation in this regard [21]. Studies have demonstrated that cruciferous vegetables can accumulate Se at a rate 100 times higher than other plants in the vicinity [22]. Selenium is a beneficial element known to enhance plant growth and development by inducing secondary metabolism and improving the antioxidant capacity of enzymes [23]. While there are numerous studies focusing on the effects of Se biofortification on crop growth and development, there is limited research on

the impact of postharvest Se treatment on the storage quality of broccoli. In our previous studies, we discovered that Se treatment not only preserved the green phenotype of broccoli heads but also inhibited the loss of aromatic compounds during storage, resulting in improved storage quality [19,24]. However, a comprehensive understanding of the storage quality effects of Se treatment requires further exploration. Therefore, in the present study, we examined the impact of Se treatment on the accumulation of ROS in broccoli heads during storage. The results revealed that Se application at concentrations of 2 mg L^{-1} enhanced the total antioxidant capacity of the broccoli heads, reduced ROS accumulation, and effectively alleviated the browning of wounds in the broccoli heads (Figure 1). To gain insights into the metabolic mechanisms underlying ROS regulation under Se treatment, we conducted transcription analysis. Our findings indicate that the expressions of genes belonging to the *BoWRKY* and *BoRBOH* transcription factor families were influenced by the Se treatment, thereby affecting the concentration of ROS in broccoli heads.

The cellular presence of ROS, such as H_2O_2, O^{2-}, singlet oxygen (O_2), hydroxyl radical (HO), and various forms of organic and inorganic peroxides [25], can lead to damage in lipids, proteins, RNA, DNA, and other cellular molecules. One major form of damage is membrane lipid peroxidation, leading to the accumulation of MDA [26]. However, a dynamic equilibrium exists between ROS and the antioxidant defense system in plants. Within this equilibrium, to mitigate the harmful effects of MDA, the activity of antioxidant enzymes, such as CAT, SOD, and PPO, is modulated [27]. In this study, postharvest selenium treatment was found to significantly reduce the H_2O_2 and MDA contents in the broccoli heads compared to the control group (Figure 1B,C). Meanwhile, we observed increases in the activities of the CAT, SOD, and POD enzymes following the Se treatment, which facilitated the breakdown of H_2O_2 and O^{2-} (Figure 2A,C). Additionally, the metabolism of ROS was closely linked to the process of browning, and increasing the antioxidant capacity has been shown to delay browning [28]. This observation was supported by a study on sweet potatoes, which revealed the accumulation of a significant amount of H_2O_2 in cells from browning regions [29], suggesting a direct association between ROS and browning. Our experimental results further substantiate these findings, demonstrating a positive correlation between browning and H_2O_2 content, as well as MDA content and H_2O_2 content. Moreover, our study highlights that the postharvest Se treatment effectively delayed browning by scavenging H_2O_2, thus contributing to improved storage quality in broccoli heads.

Extensive insights into the metabolic pathway (KEGG) were obtained from the analysis of the transcriptomic data, revealing the involvement of pathways related to ROS metabolism, including the plant–pathogen Interaction and MAPK signaling pathways. The critical sources of ROS in plants are NADPH oxidases (*RBOHs*) [30]. These transmembrane proteins play a highly regulated role, utilizing cytosolic NADPH to generate O_2^- in the apoplast, which is subsequently converted to H_2O_2 [6]. Emerging evidence indicates that *RBOH* responds to various biotic and abiotic stresses primarily by modulating ROS generation [31]. For example, in tomato, exposure to high-temperature and oxidative stress leads to an increase in the expression level of *SlRBOHf*, resulting in the accumulation of ROS [32]. Similarly, low-oxygen and low-temperature stresses trigger an upregulation of *RBOHd* [33]. In this study, we identified two *BoRBOHs*, *BoRBOHd* and *BoRBOHf*, through our transcriptomics analysis of broccoli. Subsequent RT-qPCR analysis demonstrated that *BoRBOHd* and *BoRBOHf* were significantly induced by low temperature but inhibited by the Se treatment during storage. Although low-temperature storage is a common preservation method, it renders fruits and vegetables susceptible to cold stress during storage. Furthermore, we observed high homology between *BoRBOHd* and *AtRBOHd*, as well as between *BoRBOHf* and *AtRBOHf*, suggesting similar functions between these pairs. It is noteworthy that *AtRBOHd* and *AtRBOHf* have been verified as primary contributors to ROS generation under abiotic stresses [34]. In summary, the application of Se has proven effective in alleviating cold stress in broccoli by inhibiting the overexpression of *BoRBOHd* and *BoRBOHf* during low-temperature storage.

WRKY genes play a crucial and widespread role in higher plants, influencing various aspects of plant growth and development, metabolic processes, morphological and structural formation, and signal transduction [35]. These transcription factors are an integral part of the plant's response to environmental stresses, regulating the expressions of hormone-associated genes either positively or negatively, and reacting to signals from plant pathogen infestation [36]. In addition, *WRKY* genes are implicated in the plant's response to abiotic stresses. For instance, in *Arabidopsis*, *AtWRKY25*, *AtWRKY26*, and *AtWRKY33* are induced under cold stress [37]. Furthermore, *AtWRKY25* and *AtWRKY33* have been identified as substrates of the protein kinase MPK4, which stimulates H_2O_2 production [38]. By conducting a sequence alignment of *BoWRKY* and their homologs, we observed a high degree of similarity between *BoWRKY33* and *AtWRKY33*, as well as between *BoWRKY25-1*, *BoWRKY25-2* and *AtWRKY25*. Additionally, our investigation revealed that *BoWRKY33* and *BoWRKY25* are induced under low-temperature conditions. Surprisingly, their expression levels decreased following the Se treatment. Furthermore, existing studies have indicated that the expression of *RBOHd* is regulated by *WRKY33*, which consequently affects ROS production indirectly [14]. Similarly, *AtWRKY6* and *AtWRKY15*, which share high homology with *BoWRKY6* and *BoWRKY15*, respectively, have been reported to be induced by ROS. Decreasing the expression of *AtWRKY15* was found to enhance resistance to oxidative and osmotic stress [39]. In our experiments, we observed that the expression levels of *BoWRKY6* and *BoWRKY15* were lower compared to the control group. This could potentially be attributed to the relatively low concentration of ROS in the samples. Overall, the Se treatment appears to effectively inhibit the accumulation of ROS by modulating the expressions of the *BoWRKY33* and *BoWRKY25* transcription factors.

5. Conclusions

Our experimental findings demonstrate that Se treatment effectively mitigated ROS accumulation in postharvest broccoli during cold storage. Compared to the control group, the Se-treated broccoli exhibited lower levels of excessive ROS accumulation and membrane lipid peroxidation. Notably, the expression levels of *BoRBOHd* and *BoRBOHf*, which are known to be associated with ROS production, were reduced in response to the Se treatment. Additionally, the Se treatment seemed to regulate the activity of the *BoWRKY33* and *BoWRKY25* transcription factors, contributing to the inhibition of ROS production. We speculate that the expression of *BoRBOH* might be affected by the levels of the *BoWRKY* transcription factors, warranting further investigation. Our findings provide novel insights into the regulatory mechanisms of Se treatment in scavenging ROS in postharvest broccoli at the transcriptional level. Understanding these molecular interactions and signaling pathways could lead to the development of strategies to improve the postharvest storage quality and stress tolerance of broccoli and other fruits or vegetables.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14051047/s1, Table S1. Name and sequence of primers designed for genes related to ROS metabolism. Figure S1. Plant-Pathogen interaction and MAPK signaling pathways. Figure S2. Phylogenetic relationships among *RBOHs*. Phylogenetic tree of *RBOH* sequences of broccoli (*BoRBOH*), Arabidopsis thaliana (*AtRBOH*), Solanum tuberosum (*StRBOH*), and Oryza sativa (*OsRBOH*) based on a conservative approximate alignment method. Figure S3. Phylogenetic relationships among *WRKYs*. Phylogenetic tree of *WRKY* sequences of broccoli (*BoWRKY*), Arabidopsis thaliana (*AtWRKY*), Hordeum vulgare (*HvWRKY*), zea mays (*ZmWRKY*) and Oryza sativa (*OsWRKY*) based on a conservative approximate alignment.

Author Contributions: Conceptualization, W.W., G.R. and Y.L.; methodology, W.W. and G.R.; software, W.W., G.R. and Y.L.; validation, W.W., Y.L. and Y.C.; formal analysis, Y.L. and J.D.; writing—original draft preparation, W.W.; writing—review and editing, Y.L., Y.W. and G.R.; project administration, Y.L. and L.Z. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented are contained within the article.

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

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