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Effect of $CaCl_2$ Treatment on Enzymatic Browning of Fresh-Cut Luffa (*Luffa cylindrica*)

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Abstract: Enzymatic browning is a major issue that reduces the commercial value of *Luffa cylindrica* during storage, processing, and transportation. Our results showed that 1% $CaCl_2$ treatment was optimal for reducing the surface browning of fresh-cut luffa. After storage at 25 °C for four days, the treated luffa had a significantly higher total phenolic (TP) content than the untreated luffa. At the end of the storage period, the calcium treatment showed low malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) accumulation in the luffa. The treated luffa maintained higher superoxide dismutase (SOD), catalase (CAT), and phenylalanine ammonia lyase (PAL) activities and lower polyphenol oxidase (PPO) activity as compared to the untreated luffa. Furthermore, the genes regulating SOD (e.g., *SOD1*, *SOD2*, and *SOD3*), CAT (e.g., *LcCAT1* and *CAT2*), and PAL (e.g., *PAL1* and *PAL2*) in calcium-treated luffa were upregulated to varying degrees, suggesting that Ca^{2+} inhibited the browning of fresh-cut tissue by regulating the activities of those enzymes. Ultrastructure images showed that the treated luffa could maintain the relative integrity of the cell membrane and organelles. Therefore, Ca^{2+} might act as a second messenger to reduce ROS oxidative damage and maintain the cell membrane integrity. This study provides new insights into the breeding of new luffa varieties that are resistant to browning and post-harvest treatments to reduce the browning of luffa tissue.

Keywords: luffa; calcium chloride; fresh-cut browning; gene expression; ultrastructure; lipid peroxidation

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1. Introduction

Luffa (*Luffa cylindrica*) is an annual vegetable crop of the family Cucurbitaceae. The young fruits of luffa are edible and the high-fiber, dried mature fruits are generally used for washing ships and decks, in the manufacture of slippers or baskets, shoe mats, and as an inner lining for vehicle bonnets. In oriental medicine, luffa is used as a treatment for fever, enteritis, and swelling, etc. Extracts from living vines are used as an ingredient in cosmetics and medicine [1]. Browning that occurs during storage, processing, and transportation is a major problem affecting the commercial value of luffa [1], and both the quality of fresh-cut luffa and its acceptance by consumers depend on the degree to which its surface turns brown during shelf life. Increasingly, people are starting to pay attention to diet, nutrition, and health. Because luffa is nutritious, delicious, and has beneficial effects on human health, the land area on which it is cultivated continues to increase in China. There are two luffa species, including the towel gourd (*Luffa cylindrica Roem.*) and the sinkwa towel sponge gourd (*Luffa acutangula Roxb.*). The former tends to brown, which results in decreasing

flavor, odor, nutritional value, and shelf life [2]. Therefore, determining the physiological and molecular mechanisms underlying luffa browning is an important research direction for luffa breeding. Phenol enzymatic oxidation, membrane lipid peroxidation, excess reactive oxygen species (ROS) production, and scavenging system failure have been identified as causes of the browning of fresh-cut luffa [3–5].

Luffa must be sold almost immediately after harvesting. Long-term storage and processing commonly result in browning, which has adverse effects on flavor, odor, and nutritional value [6]. Factors causing browning reactions in pears, apples, taros, and lotus roots have been thoroughly studied [7–10]. Various enzymes affect the browning of fresh-cut fruits and vegetables. Many chemical additives, such as ascorbic acid and $CaCl_2$, are used to inhibit the activity of such enzymes to maintain the bright color of fruits and vegetables [6]. Even mild processing can stimulate the activity of browning-related enzymes, thereby promoting enzymatic reactions [11]. The key enzyme involved in enzymatic browning is the oxidative enzyme PPO [12], which catalyzes the oxidation of phenolics to o-quinones, which are then polymerized into dark compounds, causing surface browning [13]. PAL participates in the synthesis of phenolic compounds, and PAL activity affects the increase in phenolic content [14]. Therefore, antibrowning agents often function by regulating enzyme activity. It is important that SOD and CAT antioxidant enzymes are able to remove excessive reactive oxygen species (ROS), such as H_2O_2 and O^{2-}, thereby protecting cells from oxidative damage, maintaining cell membrane structure and function, enhancing plant stress resistance, and delaying the senescence and browning of fruits and vegetables [15–17]. When fresh lotus roots are cut, SOD expression in the roots decreases, and the ability of antioxidant enzymes to scavenge free radicals weakens, resulting in browning [18]. In Laiyang pears, increases in the activity of endogenous antioxidant enzymes, such as SOD, can prevent ROS accumulation and membrane lipid peroxidation, thereby maintaining cell membrane structure and function and inhibiting browning [19,20]. Hou et al. studied the effect of $CaCl_2$ on chilling damage in loquat fruits stored at 1 °C for 35 days and determined that $CaCl_2$ could significantly inhibit browning and hardening and promote SOD and CAT activity and gene expression [21].

PAL is the most crucial enzyme in the composition of phenolic compounds. It can promote the catalytic conversion of L-phenylalanine into cinnamic acid, which can then be converted to phenol through a series of enzymatic reactions [22]. PAL resynthesis and increased PAL activity are the initial responses of fruits and vegetables to biotic and abiotic stress. Soluble phenolic compounds produced by PAL are isolated in vacuoles and participate in browning reactions only when mixed with enzymes (PPO or peroxidase) after cell membrane rupture. Maria D. C. Antunes demonstrated that treating fresh-cut kiwifruit with $CaCl_2$ could significantly inhibit browning without affecting the total phenol content of the fruit [23].

Ca^{2+} can prevent the browning of fresh-cut vegetables by protecting cell membrane structure, preventing cell wall rupture and stabilizing the membrane system [24,25]. Ca^{2+} is an important second messenger in plant signal transduction. Many developmental and environmental stimuli induce increases in cytoplasmic Ca^{2+}, which lead to various physiological and downstream responses. Some studies have investigated the use of Ca^{2+} treatments in preventing the browning of fruits and vegetables, but the effect of calcium chloride on enzymatic browning has not yet been studied in relation to luffa [26,27]. Our study explored the effect of Ca^{2+} on fresh-cut luffa and the physiological mechanisms underlying this effect. This enabled us to screen out the main genes affecting the browning of fresh-cut luffa, thereby providing ideas for the use of gene editing to breed new varieties of luffa with resistance to browning. We analyzed the browning index (BI) values; monitored the activity and genetic expression of PPO, SOD, CAT, and PAL; measured the contents of MDA, polyphenols, and H_2O_2; and observed the changes in organelles. The results may serve as a reference for the browning-resistant breeding, processing, and transportation of luffa.

2. Materials and Methods

2.1. Sampling and Treatment

Easy-browning luffa '256' was planted in a greenhouse in Jurong, Zhenjiang, Jiangsu Province. From May to August 2021, luffa fruits that had intact surfaces, no mechanical damage, and were of uniform size (at 9 days after fruit setting) were harvested.

2.2. Treatment Method

The surface of each fruit, the planer, kitchen knife, and cutting board were all disinfected with 0.2% sodium hypochlorite. Three groups of fruits underwent treatment with $CaCl_2$ at different concentrations (0.1% $CaCl_2$; 1% $CaCl_2$; 2% $CaCl_2$), and the control group was treated with sterilized distilled water. Each replication was made up of 4 trays and 20 slices were put into each tray. Both sides of the slices were sprayed every 24 h. The first tray was sprayed with 20 mL sterilized distilled water and was used as the control. The second, third, and fourth trays were sprayed with 20 mL 0.1% $CaCl_2$, 1% $CaCl_2$, and 2% $CaCl_2$, respectively. Then, the treated slices were dried using sterile air and placed in trays lined with filter paper, which were then covered with plastic wrap. The trays were stored at 25 ± 1 °C and 85–90% relative humidity, which was measured using a digital humidity meter. Three replications were performed for each treatment, and each treatment consisted of 80 slices. The samples were collected every day for further study.

2.3. Measurement of Browning

The colors of the cut surfaces were measured with a chromameter (TS7700, 3nh Technology Co., Ltd., Shenzhen, China) at different time points (days 1, 2, 3, and 4) in order to extract their color values (L^*, a^*, and b^*). Fifteen slices were taken for color measurement. The measurements were performed on six points in the center, three points on one side and three points on the other side. We calculated the BI using the following formula [28,29]:

(1) $BI = (x - 0.31) \times 100/0.172$,

(2) where $x = a^* + 1.75 \times L^*/5.645 \times L^* + a^* - 3.012 \times b^*$

(3) $\%BI\ increase = (BI_x - BI_1)/BI_1 \times 100$

BI_x represents the BI on day x, and BI_1 represents the BI on the treatment day.

2.4. Measurement of Antioxidant Capacity

The antioxidant capacity was determined according to the free radical scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) with slight modifications, and 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) [30–32]. Luffa extract was prepared by heating 2 g of luffa in 200 mL deionized water at 80 °C for 5 min. After extraction, the sample was cooled to room temperature and then filtered using filter paper. Referring to the kit instructions, 2.5 mL of luffa extract solution was mixed with 1 mL of DPPH (0.3 mol/L) to react at room temperature in the dark for 30 min. The absorbance of the reaction solution was measured using a UV–VIS spectrophotometer at 517 nm with a 1 cm glass cuvette. As the control, 2.5 mL of T deionized water was mixed with 1 mL of DPPH (0.3 mol/L). The DPPH scavenging capacity was calculated using the following formula: DPPH scavenging capacity (%) = $(Ac - As)/Ac \times 100\%$, where Ac is the absorbance of the mixture of DPPH solution and deionized water, and As is the absorbance of reaction between the luffa solution and the DPPH solution.

Then, 5 g of luffa tissue was thoroughly ground, 20 mL of the extract (Vethanol:Vacetone = 7:3) was added to the liquid, and this mixture was incubated at 37 °C for 1 h. The reaction solution was filtered using filter paper (Waterman 42), and the filter paper was rinsed with 5 mL of the extract (Vethanol:Vacetone = 7:3). The filter residue was extracted again using the above method, and the combined filtrate was stored at −20 °C before use. The working solution configuration was to mix ABTS (7.4 mmol/L) and potassium persulphate (2.6 mmol/L) in equal quantities and store them in the dark at room temperature. The solution had to be prepared and used on the same day. We added 50 µL filtrate to 950 µL

of the working solution, which was then mixed and measured for absorbance at 734 nm. The above operations were completed within 10 min. The formula used to calculate ABTS scavenging activity was the same as that used for DPPH.

2.5. Measurement of Activity of Browning Enzymes (PAL, PPO, SOD, and CAT)

The activity of PAL and PPO were measured following the procedure described in a previous study [10]. PAL activity was measured as follows: a 0.1 g sample of luffa and 0.9 mL of borate buffer (2 M, pH 8.8) were mixed and centrifuged at 10,000 rpm at 4 °C for 10 min. Thereafter, 0.2 mL of supernatant, 1.2 mL of borate buffer (0.2 M, pH 8.8), and 0.4 mL of phenylalanine (50 mM) were incubated at 30 °C for 30 min. Subsequently, 80 µL of HCl (6 M) was added to terminate the reaction, and the absorbance was measured at 290 nm. The activity of the PAL was expressed in units per gram of protein. PPO activity was measured by mixing a 0.2 g luffa sample with 1 mL of PBS (pH 6.5) and centrifuging the mixture at 8000 rpm at 4 °C for 10 min. Then, 0.2 mL of supernatant, 0.6 mL of PBS (0.1 M, pH 6.5), and 0.2 mL of catechol (0.2 M) were combined and incubated at 37 °C for 10 min and at 95 °C for 5 min. The absorbance was measured at 420 nm. The activity of the PPO was expressed in units per gram of protein.

The determination of CAT activity was based on the method used by Gao et al., with slight changes [33]. The test mixture consisted of 2.9 mL of H_2O_2 (20 mM) and 100 µL of crude enzyme extract. One unit of CAT activity was defined as 1 µmol of H_2O_2 degradation per gram of tissue per min.

SOD activity was determined by photoreduction inhibition of nitrocyantetroxazole as reported by Maghoumi (2013) [34]. One unit of SOD activity was equivalent to a 50% inhibition of NBT reduction.

2.6. Determination of MDA, H_2O_2, and Total Phenol Content

The MDA content of the pulp of the fresh-cut luffa was measured on the basis of the method previously described by Wang et al., with a slight modification [35]. A sample (2 g) of pulp was ground in 8 mL of phosphate buffer (50 mM), and the mixture was centrifuged at 12,000 rpm at 4 °C for 4 min. The supernatant (1 mL) was added to 3 mL of thiobarbituric acid (5 g/L) in trichloroacetic acid (100 g/L). The mixture was placed in boiling water for 15 min before being rapidly cooled and centrifuged at 12,000 rpm at 4 °C for 10 min. The unit of MDA content was nmol/g FW. The H_2O_2 content was determined by grinding a 3 g sample into 5 mL of 100% cooled acetone and centrifuging the homogenate at 10,000 rpm at 4 °C for 20 min. After the supernatant was removed, the H_2O_2 content (in µmol/g FW) was measured immediately. The total phenol content of the fresh-cut luffa was evaluated using the Folin–Ciocalteu method with some modifications [36–38]. We homogenized 1 g of luffa in 4 mL 80% (*v*/*v*) methanol solution, and then this was sonicated (Digital ultrasonic cleaner, LC-JY96-IIN, China) for 30 min and centrifuged at 10000 rpm for 15 min at 4 °C. The supernatants (2 mL) were then centrifuged for 3 min at 7000 rpm. Extracts were used for the analysis of the total phenol content. The extracts (10 µL) were added to 115 µL deionized water and 125 µL Folin–Ciocalteau reagent. The extracts were stirred and, after 6 min, 1.25 mL 7% (*w*/*v*) Na_2CO_3 was added. The mixtures were incubated for 90 min at room temperature and the increase in absorbance at 760 nm was measured using an UV–VIS spectrophotometer against a blank solution. Using a gallic acid standard curve, the results were expressed as mg gallic acid equivalents per g FW (mg/g FW).

2.7. Ultrastructural Observation

Ten slices of treated luffa samples and untreated controls were randomly selected from each group on days 1 and day 4, respectively. Before sampling, Petri dishes with fixative for TEM were prepared in advance. Small blocks of tissue were removed from the luffa slices and immediately put into the Petri dishes, and then cut to a smaller size of 1 mm^3 in the fixative. The 1 mm^3 tissue blocks were transferred into an EP tube with fresh TEM fixative for further fixation, using vacuum extraction until the samples sank to

the bottom. The samples were fixed for 2 h at room temperature and then fixed at 4 °C for preservation and transportation. Then, the tissues were washed using 0.1 M phosphate buffer (PB, pH 7.4) 3 times for 15 min each time. The tissues were fixed with 1% OsO_4 in PB for 7 h at room temperature avoiding exposure to light. After removing 1%OsO_4, the tissues were rinsed in 0.1 M PB (pH 7.4) 3 times, for 15 min each time. The slices were dehydrated using a graded ethanol series (30, 50, 70, 80, 90, 100, and 100%, 7 min each) into pure acetone (2 × 10 min), then were infiltrated in graded mixtures (3:1, 1:1, and 1:3) of acetone and soaked in glyceryl aliphatic epoxy resin. Finally, the slices were embedded in pure resin with 1.5%N, N-dimethylbenzylamine and polymerized for 12 h at 45 °C and for 48 h at 60 °C. The ultrathin sections (70 nm thick) were sectioned on the ultramicrotome (Leica UC7) and placed on the 150 meshes cuprum grids with formvar film. These were double-stained with uranyl acetate and lead citrate and examined using a Hitachi 700 transmission electron microscope (Pannoramic MIDI/250, 3D HISTECH) [39].

2.8. Real-Time Quantitative PCR Analysis of Enzymatic Browning-Related Genes

The RNA of treated luffa pulp samples was extracted using a Plant RNA Extraction Kit (Takara Biotechnology, Dalian, China). The concentration and quality of the RNA were evaluated using an ultramicro nucleic acid protein analyzer and 1% agarose gel electrophoresis. The RNA was reverse-transcribed into cDNA using a Takara reverse transcription kit in accordance with the manufacturer's instructions.

Fluorescent quantitative primers were designed for the following genes related to enzymatic browning in luffa: *LcPAL1*, *LcPAL2*, *LcCAT1*, *LcCAT2*, *LcSOD1*, *LcSOD2*, and *LcSOD3* (Table 1). Luffa 18S rRNA was selected as the internal reference gene. Three technical replicates and three biological replicates were used.

Table 1. Primers used for gene expression analysis by qRT-PCR.

Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Description
LcPAL1	CGCTGGACCCAAGTACAGG	GGTTGACAGTGTCGACTGTGTCC	Phenylalanine ammonia-lyase 1
LcPAL2	GCTCAATTTTCTGAGCTTGT	GGACGTGGCTTGTTAC	
LcCAT1	CTAGTGGGAAACTGCTAACT	GGATAACAGTGGAGAAACGT	Catalase isozyme 1
LcCAT2	TCACCATAACAACCACATGAAG	CACACACCTTTCTCTCTTTCCG	Catalase
LcSOD1	CACAGGAAGATGGTGAAGG	CCAGCAGGGTTGAAATGT	Superoxide dismutase [Cu-Zn]-like
LcSOD2	CCACGCTCTTGGCGATACA	CCATGGTCCTTCTTCAATGGA	Predicted: superoxide dismutase [Cu-Zn] 2
LcSOD3	CACTTCTCCATAGCAAATGC	GGTCAGGGAAGGCG	Predicted: superoxide dismutase [Cu-Zn]
ACTIN	GTGTTCTTCGGAATGACTGG	ATCGTTTACGGCATGGACTA	reference gene

2.9. Data Analysis

To determine the effect of the different storage times, all data are presented as the mean ± standard errors (SEs) of the three replications of each treatment. One-way analysis of variance was conducted using Tukey's HSD test and SPSS Statistics, version 19.0 (SPSS Inc., Chicago, IL, USA). The level of significance was set at 5%. The relative expression of all the genes was calculated using the $2^{-\Delta\Delta Ct}$ method [40].

3. Results

3.1. Effect of $CaCl_2$ Treatment on Browning of Fresh-Cut Luffa

To determine the optimal concentration of exogenous $CaCl_2$ for maintaining the quality of fresh-cut luffa, we tested three concentrations of $CaCl_2$. There was no browning in the treatment groups on day 1. From day 2 onwards, the samples exhibited browning in varying degrees. The browning in the control group was the most obvious, and the browning index (BI) increased day by day. The BI in the treatment groups also increased over time; however, browning occurred more slowly in the treatment groups than in the control group. On day 3, the BI reached 75% in the control group and was approximately 40% in the 0.1% and 2% $CaCl_2$ treatment groups, and was only 30% in the 1% $CaCl_2$ treatment group. The BI of the 1% $CaCl_2$ treatment group was the lowest, indicating that

the effect of the 1% $CaCl_2$ treatment was the most significant of all the treatments (Figure 1). The control group reached the highest value, whereas the 0.1% and 2% $CaCl_2$ treatment reached 49% and 46%, respectively, at the end of the storage time, and the 1%$CaCl_2$ group had the lowest value. Therefore, the browning mechanism of fresh cut luffa treated with 1% $CaCl_2$ was studied.

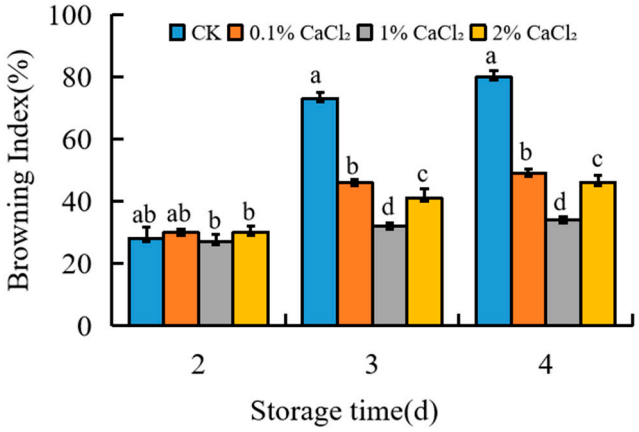

Figure 1. Effect of $CaCl_2$ treatment on BI of fresh-cut luffa. Each value is presented as a mean ± SE (n = 3). Statistically significant differences ($p \leq 0.05$) are indicated by different letters above bars.

3.2. Effect of $CaCl_2$ Treatment on MDA and H_2O_2 in Fresh-Cut Luffa

Figure 2a presents the changes in the MDA content of fresh-cut luffa samples. The MDA content in both untreated luffa and treated luffa increased over the first 3 days. On day 4, the MDA content in treated luffa remained mostly unchanged, whereas that in untreated luffa continued to increase. The MDA content of the fresh-cut luffa samples that had been treated was significantly lower than that of untreated luffa (37% and 51.5% lower on the third and fourth days, respectively). These results suggest that treatment may improve the antioxidant capacity of fresh-cut luffa.

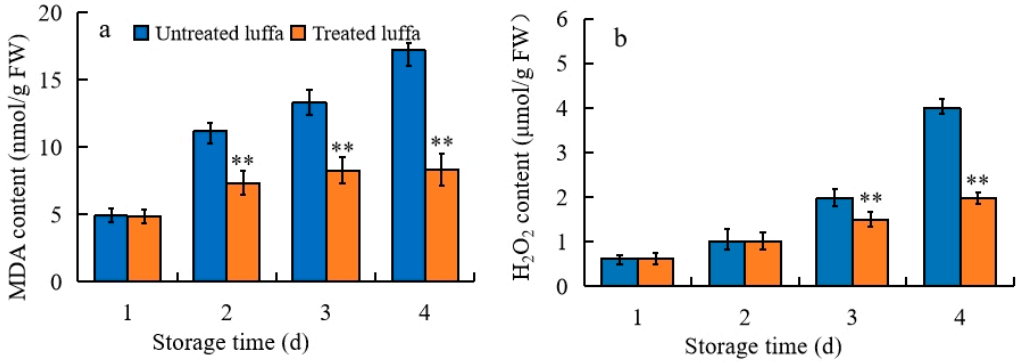

Figure 2. Effect of $CaCl_2$ treatment on (**a**) MDA and (**b**) H_2O_2 contents in fresh-cut luffa. Each value is presented as mean ± SE (n = 3). ** represent extremely significant differences ($p < 0.05$). MDA, malondialdehyde; H_2O_2, hydrogen peroxide.

The H_2O_2 content in untreated luffa gradually increased over the first 3 days and rapidly increased on day 4, whereas that in treated luffa increased gradually over the whole storage period. Compared with untreated luffa, the H_2O_2 content in treated luffa was significantly lower on days 3 and 4 (24.25% and 50.4% lower, respectively; Figure 2b).

3.3. Effect of $CaCl_2$ Treatment on Antioxidant Capacity of Fresh-Cut Luffa

ABTS and DPPH measurement were used to evaluate the antioxidant capacity of the samples. The changes in the ABTS radical scavenging capacity of untreated luffa

and treated luffa during the storage period are presented in Figure 3a. Compared with untreated luffa, ABTS radical scavenging activity in treated luffa was significantly higher on days 3 and 4 ($p < 0.05$). The DPPH scavenging activity in treated luffa was significantly higher than that in untreated luffa on day 3 ($p < 0.1$) and day 4 ($p < 0.05$). This change in the ABTS and DPPH scavenging activity was in line with the change in total phenolic content (Figure 3a,b). The results suggest that $CaCl_2$ treatment can maintain a high total phenol content, which may be related to its ability to promote retention of ABTS and DPPH scavenging activity. Higher ABTS and DPPH scavenging activity is associated with higher total phenol content, which inhibits the conversion of polyphenols into quinones.

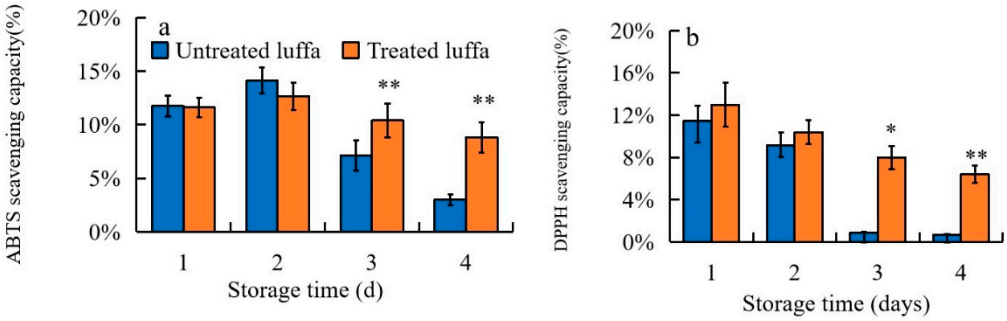

Figure 3. Effect of $CaCl_2$ treatment on (**a**) ABTS and (**b**) DPPH scavenging activity in fresh-cut luffa. Each value is presented as mean ± SE ($n = 3$). ** and * represent extremely significant differences and significant differences, respectively ($p < 0.05$ and $p < 0.01$, respectively). ABTS, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

3.4. $CaCl_2$ Treatment Prevented Phenolic Oxidation by Inhibiting PPO Activity

The total phenol (TP) content in both untreated luffa and treated luffa decreased over the whole storage period, but it decreased more significantly in untreated luffa, and the decrease in TP content in treated luffa was small.

The TP content in treated luffa was consistently higher than that in untreated luffa from day 2 onwards (Figure 4a), and in particular on days 3 and 4, suggesting that $CaCl_2$ treatment can maintain the total phenol content of luffa.

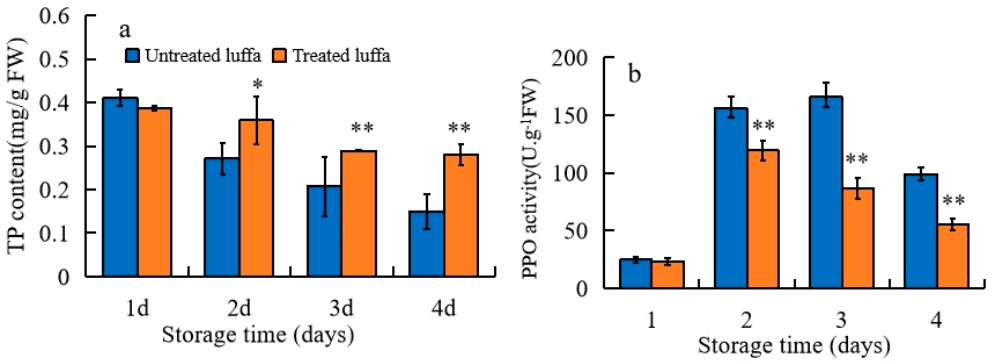

Figure 4. Effect of $CaCl_2$ treatment on (**a**) TP and (**b**) PPO activity in fresh-cut luffa. Each value is presented as mean ± SE ($n = 3$). ** and * represent extremely significant differences and significant differences, respectively ($p < 0.05$ and $p < 0.01$, respectively). TP, total phenol; PPO, polyphenol oxidase.

As indicated in Figure 4b, PPO activity initially increased and subsequently decreased in both untreated luffa and treated luffa over the storage period. However, the PPO activity in treated luffa was significantly lower than that in untreated luffa from day 3 onwards ($p < 0.05$), indicating that $CaCl_2$ inhibited PPO activity and, in turn, the oxidative polymerization of phenols to quinones.

3.5. Effect of $CaCl_2$ Treatment on Enzyme Activity and Gene Expression of SOD and CAT

As indicated in Figure 5a, although SOD activity initially increased and subsequently decreased in both untreated luffa and treated luffa, the SOD activity in treated luffa was significantly higher than that in untreated luffa from day 2 onwards. In particular, the SOD activity in treated luffa increased sharply on the second day and decreased on day 3. $CaCl_2$ treatment upregulated the expression of *LcSOD2* on day 2 and of *LcSOD3* and *LcSOD1* on day 3 (Figure 5b–d). $CaCl_2$ treatment also significantly upregulated the expression of the two CAT genes, indicating that Ca^{2+} promotes the encoding and translation of SOD genes into SOD enzymes. As indicated in Figure 5e, the CAT activity in treated luffa was significantly higher than that in untreated luffa on day 2, after which it decreased slightly but still remained higher than that in untreated luffa. The CAT activity in untreated luffa increased on day 2 and decreased continually thereafter (Figure 5e). $CaCl_2$ treatment significantly upregulated the expression of *LcCAT1* on days 3 and 4 and of *LcCAT2* on days 2 and 3 (Figure 5f,g). The results demonstrate that $CaCl_2$ treatment can significantly inhibit the production of H_2O_2; promote SOD and CAT activity; and upregulate the expression of *SOD1*, *SOD2*, *SOD3*, *CAT1*, and *CAT2*.

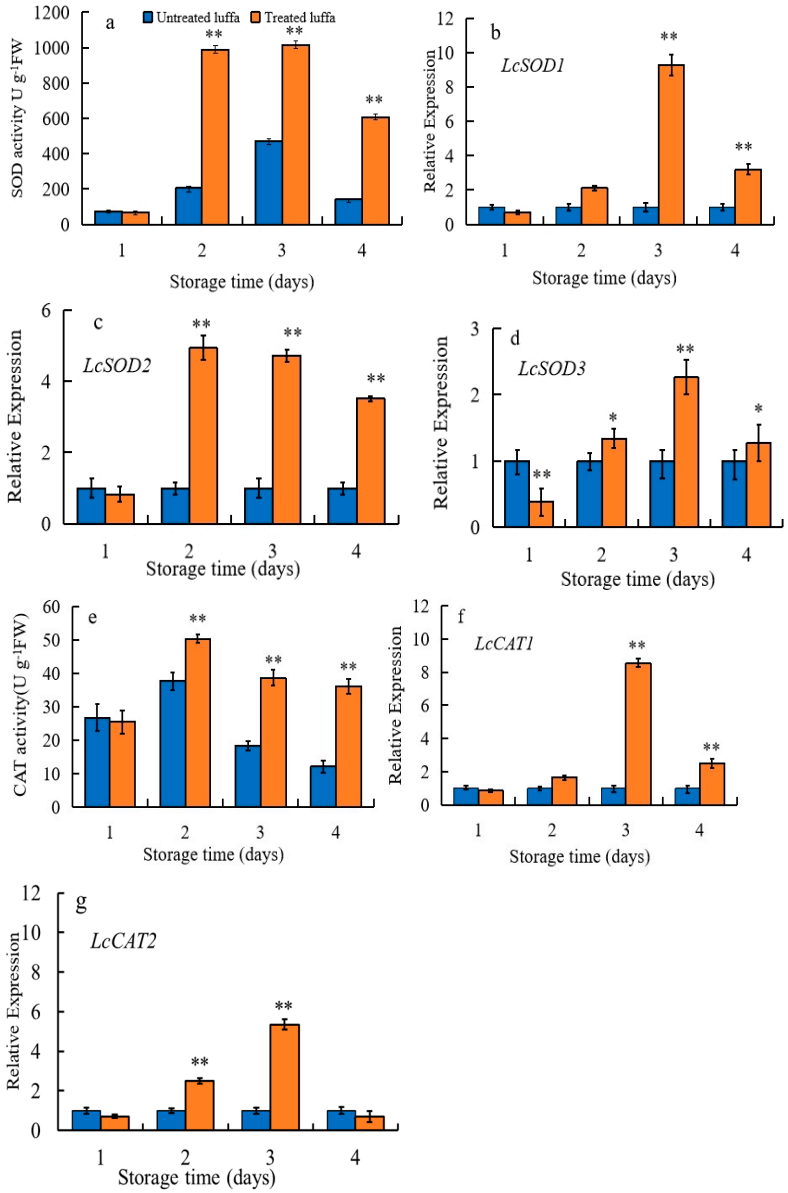

Figure 5. Effect of $CaCl_2$ treatment on antioxidant enzyme activity and related gene expression in

fresh-cut luffa. (**a**–**d**) SOD activity and gene expression; (**e**–**g**) CAT activity and gene expression. Each value is presented as mean ± SE (n = 3). ** and * represent extremely significant differences and significant differences, respectively ($p < 0.05$ and $p < 0.01$, respectively). SOD, superoxide dismutase; CAT, catalase.

3.6. Effect of $CaCl_2$ Treatment on Enzyme Activity and Gene Expression of PAL

As indicated in Figure 6a, although PAL activity decreased during storage time in both untreated and treated luffa, the PAL activity in untreated luffa was consistently lower than that in treated luffa (Figure 6a), which is consistent with the trend of total phenol content. In addition, $CaCl_2$ treatment significantly upregulated *LcPAL1* expression throughout the treatment period but only upregulated *LcPAL2* on days 3 and 4 (Figure 6b,c). $CaCl_2$ treatment significantly enhanced PAL activity, significantly upregulated *LcPAL1* and *LcPAL2* expression, and significantly promoted the retention of total phenol content in the luffa. Many phenolic compounds have antioxidant properties, and $CaCl_2$ treatment may enhance the antioxidant ability and promote the accumulation of phenolic substances in luffa.

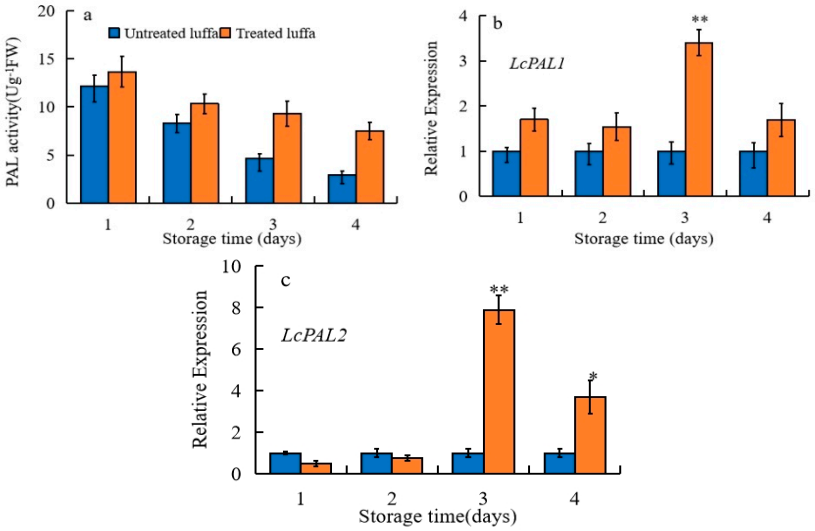

Figure 6. Effect of $CaCl_2$ treatment on (**a**) phenylalanine ammonia lyase (PAL) activity and (**b**,**c**) *PAL* gene expression in fresh-cut luffa. Each value is presented as mean ± SE (n = 3). ** and * represent extremely significant differences and significant differences, respectively ($p < 0.05$ and $p < 0.01$, respectively). PAL, phenylalanine ammonia lyase.

3.7. Effect of $CaCl_2$ Treatment on the Ultrastructure of Fresh-Cut Luffa Cells

To further verify that 1% $CaCl_2$ treatment can prevent the browning of fresh-cut luffa, we observed the changes in cell ultrastructure with a transmission electron microscope. On day 1, in both untreated luffa and treated luffa, the cell walls were clearly visible, the cell membranes were intact, the cytoplasm was uniform, and no plasmolysis was observed (Figure 7a,b). In addition, the morphologies of the ribosomes in the cells were intact, the chloroplasts adhered closely to the cell edges, the structures of the thylakoids were clear, many spherical mitochondria were present, the internal tubular cristae were evenly distributed, the endoplasmic reticula were distributed throughout the cytoplasm, and the vacuoles contained a large amount of high-density electronic substances (Figure 7e,f). On day 4, the cells in untreated luffa exhibited obvious plasmolysis, intercellular spaces, and thinning cell membranes. The rupture of the chloroplast outer membranes produced medullary substances, the mitochondria decreased in number or even disappeared, the tubular cristae disappeared, the endoplasmic reticula broke, the nuclei were fragmented, and some organelles were severely damaged, resulting in the outflow of internal proteins and enzymes and the vacuolation of the cells (Figure 7g). In the treated luffa, no plasmolysis occurred, the cell morphologies remained intact, the cell walls and cell membranes were

smooth, and the nuclei were intact (Figure 7c,d). Oval chloroplasts were clearly visible in the cells, in some of which thylakoids were visible. The chloroplasts were distributed close to the cell wall, the endoplasmic reticula were broken, the vacuolar matrix was evenly distributed, many spherical mitochondria were present, and the density of the tubular cristae had decreased (Figure 7h). These results suggest that 1% $CaCl_2$ treatment can indeed prevent the browning of luffa.

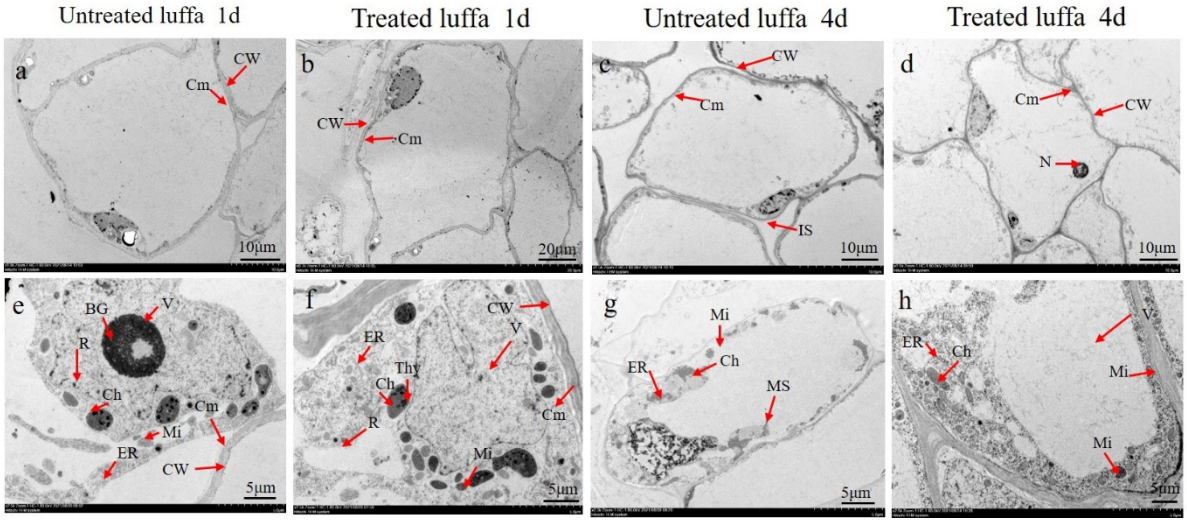

Figure 7. Subcellular structure of fresh-cut luffa. (**a**,**e**) Ultrastructure of cells in untreated luffa on day 1. (**b**,**f**) Ultrastructure of cells in treated luffa on day 1. (**c**,**g**) Ultrastructure of cells in untreated luffa on day 4. (**d**,**h**) Ultrastructure of cells in treated luffa on day 4. CW, cell wall; Cm, cell membrane; IS, intercellular space; N, nucleus; V, vacuole; BG, high electron-density substances; R, ribosome; Ch, chloroplast; Mi, mitochondrion; ER, endoplasmic reticulum; Thy, thylakoid; MS, myelinic structure.

4. Discussion

Tissue browning is a complex process. Many factors, such as mechanical damage, enzymatic browning and interact to cause browning of fruits and vegetables [41]. Many methods for prevention of browning have been studied, covering the physical, chemical, and biotechnology fields [9,42]. In previous studies, fresh-cut potatoes were soaked in 0.05% CAT for 5 min and stored at 4 °C for 8 days. The result showed that the H_2O_2 and O^{2-} content was significantly lower than in the control [43]. Taro soaked samples in citronella hydrosol (CH) or rose hydrosol (RH) for 30 min, and these were then packed in plastic bags and stored at 4 °C for 12 days. The results showed that the values of L, a, and b were significantly reduced, and PAL, POD, and PPO activities were inhibited [44]. However, the molecular genetic mechanisms involved are rarely mentioned. This lack of in-depth understanding of luffa germplasm restricts breeding progress. Therefore, there is an urgent need to identify an efficient and harmless drug to alleviate the browning of fresh cut luffa and further explain the original genetic causes of browning.

Ca^{2+} is an important second messenger that plays a critical role in protecting fruits and vegetables from browning [45]. Zia Ullah Khan et al. observed that exogenous $CaCl_2$ treatment can prevent the browning of fresh-cut mushrooms, thereby prolonging their shelf life and acceptability to consumers [46]. Hou et al. reported that $CaCl_2$ treatment could prevent chilling damage to loquats and reduce their browning rate [21]. In our study, on day 3, BI reached 75% in untreated luffa and approximately 40% in the 0.1% and 2% $CaCl_2$ treatment groups, but only 30% in the 1% $CaCl_2$ treatment group (Figure 1), which is in line with the results of previous studies investigating the effect of $CaCl_2$ on the browning rate of *Pyrus bretschneideri* Rehd. and *Ziziphus jujuba* Mill. cv. Dongzao [45,47]. Harvested cauliflowers were dipped in 2% $CaCl_2$ at 40 °C to extend their shelf life and maintain freshness [48]. The 'Rojo Brillante' persimmon exhibited increased antioxidant levels and

antioxidase activity, as well as reduced browning, when treated with 10 g/L citric acid together with 10 g/L $CaCl_2$ [27].

Membrane system injury and abnormal cell metabolism are the factors affecting lipid peroxidation. MDA and H_2O_2 are considered products of lipid peroxidation in plant cell membranes [49]. In our study, the levels of MDA and H_2O_2 increased gradually over time, but were significantly lower in the treated luffa compared with the untreated at the end of the storage period (Figure 2a,b). The slow accumulation of MDA and H_2O_2 protects the plasma membrane system in plants from damage. Reactive oxygen scavenger enzymes in cells remove excessive MDA and H_2O_2 in time to prevent cell damage [50,51]. $CaCl_2$ significantly inhibited the accumulation of MDA and H_2O_2 (Figure 2a,b). ABTS radical scavenging activity in treated luffa was significantly higher than it was in untreated luffa on days 3 and 4 ($p < 0.05$). The DPPH scavenging activity in treated luffa was significantly higher than it was in untreated luffa on day 3 ($p < 0.1$) and day 4 ($p < 0.05$). This result is consistent with the results reported by Li et al., who soaked loquats in exogenous $CaCl_2$ to prevent chilling damage and internal browning [52]. Maria et al. observed that kiwifruit soaked in 2% $CaCl_2$ significantly increased ABTS free radical scavenging capacity for 8 days and DPPH free radical scavenging capacity for 4 days [23]. The total phenol content in treated luffa was consistently higher than that of untreated luffa from day 2 onwards (Figure 4a), in particular on days 3 and 4. Zhang et al. discovered that $CaCl_2$ treatment of pears effectively reduced the appearance of peel browning (PB) and increased firmness and polyphenol during storage. This accords with our results [53]. $CaCl_2$ treatment can maintain a high total phenol content, which may be related to its ability to promote retention of ABTS and DPPH scavenging activity. Higher ABTS and DPPH scavenging activity was associated with higher total phenol content, which inhibited the conversion of polyphenols into quinones. This is consistent with the research results of Chun et al. and Kim et al. (Figures 3a,b and 4a) [54,55].

PPO is generally considered to play a key role in the browning of fresh-cut products by inducing generation of dark compounds [56]. Numerous studies have reported that various compounds can prevent the browning of fresh-cut products by inhibiting PPO activity. For example, Sun et al. reported that fumigating lotus roots in a closed glass bottle with H_2S could significantly inhibit PPO activity [57]. Li et al. studied the effects of polysaccharide coatings (alginate, chitosan, and carrageenan) on fresh-cut lettuce refrigerated at 4 °C for 15 days and identified three types of coating that could inhibit enzymatic browning by inhibiting PPO activity [58]. Xiao observed that pears dipped in $CaCl_2$ limited the occurrence of brown spots, reduced the activities of PPO and POD, increased CAT and SOD activity, and retarded the loss of phenolic compounds [47]. The decrease in PPO enzyme activity during storage was attributed to the reduction in cell damage by Ca^{2+} [59]. In our study, the PPO activity in treated luffa was significantly lower than in the untreated luffa from day 3 onwards (Figure 4b). Additionally, the $CaCl_2$-treated luffa slices accumulated more phenols than the untreated slices (Figure 4a). Because the $CaCl_2$ treatment inhibited PPO activity, thereby inhibiting the biosynthesis of phenolic compounds, the treatment group may have experienced less oxidation of phenolic compounds and greater accumulation of phenolic compounds than the untreated group. Deng and Xiu suggested that spraying Chinese winter jujube fruit with $CaCl_2$ (4%) decreased PPO activity and increased SOD and CAT activity. This observation is consistent with our results [45].

ROS production and scavenging system failure have been identified as causes of the browning of fresh-cut products [10,60]. CAT and SOD are peroxide-scavenging enzymes that may play a role in preventing browning [61]. SOD can convert excess O^{2-} into H_2O_2, and CAT converts H_2O_2 into H_2O and O_2 [62]. Studies have reported that pears [63], blood oranges [64], and bananas [65] contain highly active antioxidant enzymes, and the comprehensive effect of such enzymes is one of the mechanisms underlying the suppression of oxidative damage and prevention of browning. In our study, $CaCl_2$ treatment significantly increased the activities of CAT and SOD on the second day of the storage period (Figure 5a,e). *LcCAT1* and *LcCAT2* genes were significantly expressed on the second and

third day, respectively, indicating that *LcCAT1* and *LcCAT2* regulated CAT enzyme activity at the transcriptional level (Figure 5f,g). Ya Wang et al. sequenced the fresh-cut luffa with browning resistance and browning susceptibility and analyzed the expression of the *LcCAT* gene family, which also confirmed our results [66]. Zhu et al. sequenced fresh-cut luffa and found that CAT enzyme activity was regulated by the *LcCAT1* and *LcCAT2* genes [2]. SOD activity began to increase on the second day of the storage period, and decreased slightly on the fourth day, which was consistent with the expression time of the *LcSOD2* gene, indicating that the activation of the *LcSOD2* gene by $CaCl_2$ treatment improved the activity of antioxidant enzymes in fresh-cut luffa (Figure 5c). Both *LcSOD1* and *LcSOD2* genes increased from the second day and decreased on the fourth day, which was consistent with the change trend of the SOD enzyme, but the range of the change was different. Thus, *LcSOD1* and *LcSOD2* may not be the key genes regulating the SOD enzyme (Figure 5b,d). The most effective concentration of $CaCl_2$ to treat the browning of fresh-cut luffa was 1%. This inhibited the excessive accumulation of MDA and H_2O_2, activated the expression of the *LcCATs* and *LcSODs* genes, and improved the activities of the SOD and CAT antioxidant enzymes. Mei et al. indicated that the pretreatment of fresh-cut strawberries with alcohol vapor could improve the SOD and CAT activity and the activation-related gene expression, and suggested that *LcSOD1* was not the key gene of the SOD enzyme. This is consistent with the results of this study [67].

PAL is essential to the synthesis of phenols. When fruits undergo stress induced by mechanical damage, PAL activity increases, enabling the fruits to synthesize phenols to reduce mechanical damage [68]. *LcPAL* genes are important to the synthesis of phenolic compounds [56]. In this study, $CaCl_2$ treatment significantly upregulated *LcPAL1* throughout the storage period but only upregulated *LcPAL2* on days 3 and 4 (Figure 6b,c). $CaCl_2$ treatment significantly enhanced PAL activity, significantly upregulated *LcPAL1* and *LcPAL2* expression, and promoted the retention of total phenol content in the luffa. Morteza Soleimani Aghdam found that $CaCl_2$ maintained higher total phenols and DPPH and ABTS scavenging activity in the cornelian cherry (*Cornus mas*). Additionally, PAL enzyme activity in the cornelian cherry fruits was significantly increased [69]. This is line with our results although the study did not mention the *PAL* gene. Pannipa Youryon reported that $CaCl_2$ and CaGlu could alleviate internal browning, retard PAL activity, and increase total phenols in the Queen pineapple. These findings are not entirely consistent with our research [64].

ROS production (including H_2O_2, superoxide anions and free radicals) increases with the impact of external pressure. This disturbs the balance of the ROS metabolism, leading to membrane lipid peroxidation and membrane protein polymerization, which destroys the ultrastructure of organelles, including the mitochondria, chloroplasts, and endoplasmic reticula of plant cells [70,71]. In our research, on day 4, the cells of the untreated luffa samples exhibited obvious plasmolysis with large intercellular spaces and thin cell membranes. The rupture of the chloroplast outer membranes produced medullary substances, the mitochondria decreased in number or even disappeared, the tubular cristae disappeared, the endoplasmic reticula broke, the nuclei were fragmented, and some organelles were severely damaged, resulting in the outflow of internal proteins and enzymes and the vacuolation of the cells (Figure 7g). In treated luffa, no plasmolysis occurred, the cell morphologies were intact, the cell walls and cell membranes were smooth, and the nuclei were intact (Figure 7c,d). Oval chloroplasts were clearly visible in the cells, in some of which thylakoids were also visible. The chloroplasts were distributed close to the cell walls, the endoplasmic reticula were broken, the vacuolar matrix was evenly distributed, many spherical mitochondria were present, and the density of the tubular cristae had decreased (Figure 7h). This study indicated that spraying $CaCl_2$, especially 1% $CaCl_2$ treatment, can effectively maintain the relative integrity of cells and reduce the binding of cell contents with external oxygen, thereby alleviating browning. Similar results have been reported on fruits, such as pears, grapes, and peaches [70,72,73].

5. Conclusions

Spraying fresh-cut Luffa with $CaCl_2$ can significantly inhibit surface browning and restrain the accumulation of MDA and H_2O_2. In addition, $CaCl_2$ treatment increases the retention of phenols; promotes antioxidant activity; enhances the activity of SOD, CAT, and PAL; suppresses PPO activity; and prevents the oxidative polymerization of phenols to quinones during storage. Such treatment can also upregulate the expression of genes related to the synthesis of *LcSOD*, *LcCAT*, and *LcPAL* to varying degrees. Regarding cell ultrastructure, in cells treated with $CaCl_2$, no plasmolysis occurs, the cell morphologies and nuclei remain intact, and the cell walls and cell membranes remain smooth. Within the treated cells, chloroplasts are clearly visible, the vacuolar matrix is evenly distributed, many spherical mitochondria are present, and the density of the tubular cristae decreases.

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