Metabolomic Insights into the Browning of the Peel of Bagging ‘Rui Xue’ Apple Fruit

zhengyang zhao (✉ zhaozy@nwsuaf.edu.cn)

Northwest Agriculture and Forestry University

Hui Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Lulong Sun

Northwest A&F University: Northwest Agriculture and Forestry University

Shuang Wang

Northwest A&F University: Northwest Agriculture and Forestry University

Miaomiao Fan

Northwest A&F University: Northwest Agriculture and Forestry University

Shuhui Zhang

Shandong Agricultural University

Research article

Keywords: Malus domestica Borkth, Fruit bagging, ‘Rui Xue’, Browning, Metabolomic

Posted Date: December 16th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-126179/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Bagging is one of the most important techniques for the production of high-quality fruits. In the actual of cultivating, we found a new kind of browning in peel of apple fruit that occurs before harvest and worsen during storage period. There are many studies on metabonomic analysis of browning about storage fruits, but few studies on the mechanism of browning before harvest. This experiment was based on metabonomic studies to explore the effect of bagging on fruit peel browning.

Results: In this study, five-year-old trees of 'Rui Xue' were used as materials. Bagging fruits without browning (BFW) and bagging fruits with browning (BFB) were set as the experimental groups, non-bagging fruits (NBF) were set as control (CT). Our results show that 131 metabolites of BFW were significantly up-regulation and 217 metabolites were significantly down-regulation compared with NBF; 198 metabolites of BFB were significantly up-regulation and 178 metabolites were significantly down-regulation compared with BFW (Variable important in projection (VIP) > 1 and p-value < 0.05). The synthesis and accumulation of flavonoids and lipids were significantly changed after bagging; compared with NBF, 11 flavonoids and 6 lipids molecules were significantly decreased in the BFW (top 50 metabolites). Compared with BFW, 11 flavonoids and 7 lipids were further decreased in BFB, but, 12 triterpenes were significantly accumulated in BFB. As the peel browning, galactose metabolism, ABC membrane transporter protein, flavonoid biosynthesis and linoleic acid metabolism pathways were significantly changed in BFW and BFB compared with NBF. Physiological indicators show that, compared with NBF, the MDA in peel of BFW and BFB significantly increased by 0.46 times and 2.94 times, respectively; and the cell membrane permeability of BFW and BFB were significantly increased by 15.7% and 28.2%, respectively. We analyzed the expression of related genes in the metabolic pathways of flavonoids, compared with NBF, the flavonoid synthesis genes *MdLAR* were significantly up-regulated by 2.55 and 9.37 times in BFW and BFB, and the *MdANR* genes were also significantly up-regulated by 2.33 and 2.69 times, respectively. In addition to this, the downstream flavonoids-related polymeric genes *MdLAC7* and *MdLAC14* were the most significantly expressed. Compared with NBF, *MdLAC7* was significantly up-regulated by 0.43 and 13.88 times in BFW and BFB, and *MdLAC14* was also significantly up-regulated by 7.13 and 3.17 times, respectively.

Conclusions: Our findings demonstrated that the microenvironment of fruit was changed by bagging, the destruction of cell structure, the increase of lipid peroxidation of cell membrane, the decrease of flavonoids and the increase of triterpenoids were the main reasons for the browning of peel about bagging 'Rui Xue'.

Background

Apple is one of the most produced fruits in the world, which occupies an important position in the world fruit trade [1]. China has become the largest producer of apple in the world (Food and Agriculture Organization of the United Nations, FAO). In 2018, China's apple cultivation area has reached

2.072 million hm², accounting for 57.7% of the world's total cultivation area, and the total production reached 39.235 million t, accounting for 54.5% of the world's total apple production.

Bagging is one of the most important techniques for the production of high-quality fruits, which can prevent damages from disease, insects, and birds, reduce sunburn and decrease pesticide residues, improve the appearance, quality, and commodity value of the fruits. Bagging has been widely used in China, Australia, Japan, and the United States for the cultivation of peach, apple, pear, grape, and loquat [2, 3]. However, bagging also has some negative effects on the growth and development of fruits. The dark condition inside the bag inhibits the photosynthetic capacity in peel, decreases the layer number of cells in the fruit epidermis, and a lack of liposomes, leading to a thinner cuticle and decreased resistance [4]. In addition, bagging reduces the content of citric acid and total soluble solids (TSS), increases the content of titratable acid, and results in a significantly decreased fruit maturity [5].

One interesting phenomenon was observed that browning on peel was easily occurred in bagging fruits, especially some late-ripening varieties, including 'Fuji', 'Golden Delicious', 'Pink Lady', 'Envy', 'Rui Xue', and so on, which occurred at the late stage of fruit growth and development (from September to October) in the field (Fig. 1). While no browning occurred in unbagging fruits. At before harvest, browning started at the position of stalk cavity and then expand to the whole surface of fruit. Meanwhile, the stalk cavity and other expanded part changes from pale brown to brown and dark brown (Fig. 2). During storage, the healthy surface of the fruit deteriorates gradually by outward diffusion from the stalk cavity stalk cavity. Seriously, the brown part could penetrate into the interior of fruit from the peel, without any peculiar smell. Although this kind of browning does not affect the taste, the appearance of fruit was influenced which reduces its value [6, 7]. This kind of browning is different from the superficial scald which only occurs during storage.

The browning in apple peel affects fruit quality [4]. However, how bagging induced browning in apple peel has not been thoroughly studied. Metabolomic analysis is useful for investigating the relationships between genotypes and phenotypes [8]. Metabolomic analysis has been used to study the browning about fruits, the results showed that a large number of terpenes could induce the litchi peel browning [9]. Analysis revealed metabolic variations in tricarboxylic acid cycle and gamma-aminobutyric acid shunt generating reactive oxygen species, which causes stressed conditions inside the mesocarp. Further, reduced levels of antioxidants and enzymes dissipating reactive oxygen species in mesocarp deteriorate the fruit physiology [10, 11]. Comparative metabolomics helped to reveal the overall qualitative and quantitative changes in metabolites in biological systems [12]. However, few studies have attempted to use comparative metabolomics to investigate the mechanism of browning in peel of apple fruit before harvest.

We hypothesized that bagging changed the microenvironment of apple fruit, reduced the antioxidant capacity of peel, leading to a thinner peel, and in the regions with large differences in temperature between day and night, the antioxidant content in fruit is reduced, the structure of the peel cells is altered, and enzymatic browning of the fruit on the tree is induced after the cell membrane is broken. Here, we

conducted a metabolomic analysis to study the browning mechanism of apple peel using the sensitive variety 'Rui Xue'. The findings were helpful for our understanding in the physiological changes of peel before harvesting, refining the cultivation techniques before harvesting and storage techniques.

Results

The Effects of Bagging on Peel Thickness

The peel plays an important role in protecting the fruit from outside stimulations [13,14]. We measured the thickness of the peel to determine whether bagging alters the cellular structures of peel. The epithelial cells of the NBF included 5-6 layers of subcells (Figure 3A), while the upper epidermal cells of the bagging fruit (BFW and BFB) had only 4-5 layers (Figure 3B-C), indicating that bagging changed the microenvironment around the fruit and reduced the number of cell layers in the peel.

The peel thickness of the BFW was only 0.031 mm, which was significantly reduced (by 16.2%) compared with that of NBF (Figure 3D). However, the peel thickness of the BFB did not decreased compared with NBF, but increased significantly (by 16.0%) compared with the BFW, indicating that the cell walls of the peel were thickened.

The Effects of Bagging on the Microstructure of Peel Cells

We used transmission electron microscopy to determine the microstructure of the peel cells in the BFW and BFB. The BFW fruits maintained the original tissue structure, with a uniform, complete and clear honeycomb structure, smooth and flat cell surface (Figure 4A). The cellular structure of the BFB was obviously deformed and shriveled, the subcutaneous cells had begun to sag, the structure was loose and fluffy, the cellular structure was no longer honeycombed and complete, the surface tissue was broken, and the cellular structure was destroyed (Figure 4B).

Metabolomic Analysis

Principal Component Analysis and Metabolite Intensity Distribution Assessment of QC Samples

To test the stability of the mass spectrometry system, we mixed (in equal proportions) samples to create QC samples. Firstly, we analyzed the principal components of QC samples; the PCA model diagram obtained through 7-fold cross-validation (seven cycles of cross-validation) is shown in Figure 5A. The QC samples were closely clustered together, indicating that the experiment was stable and repeatable. Hierarchical clustering for the expression levels of all metabolites intuitively demonstrate the stability of the relationship between QC and other samples (Figure 5B). There was no significant differences in the intensity distribution of metabolites between the QC and the other three samples, indicating a stable and accurate data.

Determination of Metabolites from the Browning Part of the Peel

We analyzed the differences of all metabolites in the peel of NBF, BFW, and BFB. Compared with NBF, 1775 and 2089 metabolites were significantly up-regulated and down-regulated in BFW, respectively (Figure 6A). Compared with BFW, 2211 and 2534 metabolites were significantly up-regulated and down-regulated in BFB (Figure 6B). To screen the marker metabolites, a combination of multi-dimensional and single-dimensional analysis was used to screen the differential metabolites within groups. Differential metabolites with a VIP > 1 and p-value < 0.05 were regarded as significantly differential metabolites. 131 metabolites of BFW were significantly up-regulation and 217 metabolites were significantly down-regulation compared with NBF; 198 metabolites of BFB were significantly up-regulation and 178 metabolites were significantly down-regulation compared with BFW.

Screening of Differential Metabolites

To investigate the relationship among samples and the differences in metabolites among different samples, a hierarchical clustering analysis was conducted on the expression levels of all those metabolites where a significant difference had been detected. According to the VIP value, a visual analysis was performed for the expression levels of the first top 50 differential metabolites. The results showed that, compared with NBF, 13 flavonoids (including apigenin, rutin, myricetin 3-glucoside, isoquercitrin, quercetin, quercetin 3-O-glucoside, phloridzin, 6-hydroxyluteolin 6-xyloside, procyanidin B2, quercetin 3-arabinoside and morin were significantly decrease, clausarinol and gingerenone B were significantly increase), ten carbohydrate compounds (including lactulose, d-mannitol, sucrose, d-galactose, d-maltose, maltotriose, glucose 1-phosphate, fructofuranosy and 3-fucosyllactose were significantly increase, d-glucopyranoside was significantly decrease), ten lipid molecules (d-glucopyranoside, hydroxypregn sulfate, 1-hexanol arabinosylglucoside, corchoionol C 9-glucoside and vomifoliol were significantly decrease, 2-hydroxyadipic acid, SM(d18:1/18:1(9Z)), scillirosidin, PI(15:0/0:0) and C16 sphinganine were significantly increase), nine benzene ring-type compounds (including eugenol, anthraquinone and buclizine were significantly decrease, benzene-1,3,5-triol, thiabendazole, triphenyl-phosphate-thiabendazole, pandamarilactam-3x, citbismine C and pterisin H were significantly increase), five organic acids and their derivatives (including raltitrexed, isocitrate and 1-O-p-coumaroyl-beta-D-glucose were significantly increase, ustiloxin D and oxane-2-carboxylic acid were significantly decrease), one scatterbrained lipid (armillaripin was significantly increase) and two other types metabolites (including 2,2-dichloro-1,1-ethanediol was significantly increase, atorvastatin was significantly decrease) had significantly changes in BFW (Figure S1A, Table S1).

The differential metabolites were further subdivided. The down-regulated differential metabolites were mainly flavonoids in BFW compared with NBF. It is well known that most plants contain flavonoids [15], which play an important role in plant growth and development. As a kind of secondary metabolite, flavonoids can remove free radicals and delay the normal tissue cell apoptosis [16]. However, flavonoids were not included among the up-regulated metabolites in BFW. To determine the causes of browning, or the substances produced by browning, the metabolites detected in the BFB and BFW groups were compared. Among the significantly changed metabolites (Figure S1B), we identified 12 flavonoids (including procyanidin-B2, phloridzin, hydroxyluteolin, quercetin 3-arabinoside, gingerenone B, quercetin-3-

O-glucoside, isoquercitrin, quercitrin, morin and clausarinol were significantly decrease, eurycomanone and marmesin galactoside were significantly increase), 13 triterpenes (including ganosporeric acid A was significantly decrease, medicagenic acid, 12-oleanadien-28-oic acid, esculentic acid, oxane-2-carboxylic acid, phytolaccinic acid, ganolucidic acid B, protobassic acid, ganolucidic acid E, 16alpha-hydroxygypsogenic acidid, pokeberrygenin and corosin were significantly increase), 11 lipids and their associated molecules (including SM(d18:1/18:1(9Z)), C16-sphinganine, scillirosidin, estradiol-17beta-3-sulfate, 2-Hydroxyadipic acid and vomifoliol, tetranor-PGDM were significantly decrease, physalolactone B, EB-1213, Pentanorcholecalciferol and cyclo-cholest were significantly increase), 7 organic acids and their derivatives (including chlorogenic acid, feruloyl C1-glucuronide, cis-5-caffeoylquinic acid, raltitrexed and quinic acid were significantly decrease, acetyl tributyl citrate and isocitrate were significantly increase,), 4 sugar compounds (including D-maltose, D-Fructofuranosyl, 3-fucosyllactose and sucrose were significantly decrease), 2 benzene ring-type compound derivatives (including citbismine C was significantly decrease, C2-(4-Methyl-1,3-pentadienyl) anthraquinone was significantly increase), 1 scatterbrained lipid (armillaripin was significantly decrease), and 1 other compound ((17Z)-1 α , 25-dihydroxy-26, 27-dimethyl-17, 20, 22, 22, 23, 23-hexadehydrovitamin-D3 was significantly increase) had significantly changes in BFB (Figure S1B, Table S2).

Enrichment Analysis of Differential Metabolites

An enrichment analysis was carried out to understand the differential metabolic pathways of various samples by comparing the differential metabolites to the Kyoto Encyclopedia of Genes and Genomes (KEGG, <https://www.kegg.jp/>) database. The top 20 metabolic pathways with significant enrichment were selected for bubble mapping (Figure 7). Galactose metabolism, ABC membrane transporter protein, starch and sucrose metabolism, glyceride/phospholipid metabolism, and fructose and mannose metabolism were significantly changed in BFW compared with NBF (Figure 7A-C).

To better understand the final major metabolites and metabolic pathways of bagging-induced fruit browning, we conducted enrichment analysis of differential metabolites and metabolic pathways in BFW and BFB fruits. Multiple metabolic pathways were significantly changed in BFB compared with BFW: ABC membrane transporter, galactose metabolism, flavonoid biosynthesis, carbon metabolism, fructose and mannose metabolism, and linoleic acid metabolism (Figure 7B-D). Based on the top 50 differential metabolites, the inhibition of flavonoid biosynthesis leads to the decrease of antioxidant substances, which is directly responsible for fruit browning, while the damage of the cell membrane might be the direct cause of the inhibition of flavonoid biosynthesis and transport.

Membrane Lipid Peroxidation

By measuring the microstructure of the peel, we determined that the browning after bagging was closely related to the integrity of the cell structure. We further measured the degree of membrane lipid peroxidation in the cells. Compared with NBF, the MDA content in peels of BFW and BFB were significantly increased by 0.46 times and 2.94 times, respectively (Figure 8A). This finding indicates that the peroxidation of the cell membrane is accelerated by the microenvironment changes following

bagging. We also measured the cell membrane permeability and found that, compared with NBF, the cell membrane permeability of BFW and BFB were significantly increased by 15.7% and 28.2%, respectively (Figure 8B).

Antioxidant Capacity of the Peel

Based on metabolomics analysis, bagging significantly reduced the accumulation of antioxidant substances in the peel, inhibited the synthesis of flavonoids after browning. To further verify whether the decrease in antioxidant leads to the browning of 'Rui Xue' peel, we examined the content of total phenols (TP), total flavanols (TFA), and total flavonoids (TFO) in the peel. As shown in Figure 9, after bagging, the accumulation of antioxidant substances in the peel was significantly reduced, and the reduction degree of BFB was more significant. Compared with NBF, BFW significantly reduced the content of TP and TFO by 12.6% and 5.1%, respectively, and the content of TFA showed a decreasing but not significant trend. Compared with NBF, the contents of TP, TFO, and TFA in the BFB group were significantly reduced (by 38.6%, 35.1%, and 28.0% respectively), indicating that bagging affected the accumulation of antioxidant in the peel.

Analysis the Gene Expression of Related-Flavonoids

As shown in Figure 10, compared with NBF, the flavonoid synthesis genes *MdCHS*, *MdF3H*, *MdDFR*, *MdANS* and *MdUFGT* were significantly down-regulated in BFW and BFB, while the *MdLAR* genes were significantly up-regulated by 2.55 and 9.37 times in BFW and BFB, and the *MdANR* genes were significantly up-regulated by 2.33 and 2.69 times in BFW and BFB, respectively. Interestingly, with the high expression of flavonoids-related synthetic genes, the downstream flavonoids-related polymeric genes were also highly expressed, among which *MdLAC7* and *MdLAC14* were the most significantly expressed. Compared with NBF, *MdLAC7* was significantly up-regulated by 0.43 and 13.88 times in BFW and BFB, and *MdLAC14* was significantly up-regulated by 7.13 and 3.17 times in BFW and BFB, respectively.

Discussion

Bagging typically improves apple fruit quality. Bagging can promote fruit coloring and improve fruit surface smoothness. In addition, bagging can also reduce the direct pollution of pesticides, dust, and prevent diseases and attack by pests and birds [17]. Bagging has become an important technique in the production of high-quality and pollution-free fruits. However, bagging can also induce some negative effects on fruits. The microenvironment after bagging will affect the thickness of the peel and the content of sugar, acid, mineral elements, and other internal soluble substances in fruits, which inhibits the synthesis and accumulation of antioxidant substances, such as flavonoids and phenols in fruits [18, 19, 20], thereby reducing the fruits' resistance to stress.

Browning in fruit, including enzymatic and non-enzymatic, could be induced by a series of physiological and biochemical processes under aging or adverse conditions. Enzymatic browning is closely related to the integrity of cell membrane. When cells suffer from stress, the ability of the fruit to clear free radicals

decreases, which leads to the accumulation of reactive oxygen species, accelerated membrane lipid peroxidation, and increased membrane permeability [21]. Enzymatic browning is induced by enzymes distributed in the elastoplast and substrates [21]. Mechanisms of non-enzymatic browning include the Maillard reaction, ascorbic acid oxidation, caramelization, and autooxidation of phenols [22].

Glycerol phospholipid compounds are important components of the cell membrane [23], and d-galactose metabolism will induce oxidative damage to the cell membrane [24], and the ABC transporters are involved in controlling the transport of secondary metabolites [25]. These three pathways are crucial for cell membrane structure and function. After bagging, these three metabolic pathways were obviously altered. Therefore, we believe that bagging can change the structure of the cell membrane, causing damage to the cell membrane and leading to changes in membrane permeability and function. In conclusion, we believe that bagging-induced browning in peel is induced by changed microdomain environment, which caused a thinner peel, reduced the ability to cope with abiotic stress (e.g., day-night temperature differences), aggravated damage to the cell membrane (increasing the cell membrane permeability), and ultimately lead to peel browning.

Flavonoids are synthesized in plants through the phenylephrine metabolic pathway, starting from phenylalanine and catalyzed by the phenylalanine ammonia-lyase (*PAL*) to form cinnamic acid, which then forms p-coumaroyl-CoA catalyzed by cinnamate 4-hydroxylase (*C4H*) and 4-Coumarate: CoA ligase (*4CL*). Under the joint action of chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), and flavanone 3-hydroxylase (*DFR*), p-coumaroyl-CoA is transformed into flavonoid products [26]. Flavonoids are major secondary metabolites, including anthocyanin, flavone, flavonol, procyanidins, isoflavone, and orange ketone different subgroups [27]. Flavonoids play a series of roles in cells, inducing color changes and improving the antioxidant ability of the plant [28, 29]. *LAC* is a downstream gene of flavonoid metabolism. Laccase as an intracellular enzyme, which is involved in the biosynthesis of lignin, capable of monolol oxidative polymerization, and participates in the polymerization of flavonoid substances [30]. The laccase encoded by *TT10* in the model plant *Arabidopsis*, as a flavonoid oxidase to catalyze oxidative polymerization of epicatechin to produce yellow and brown pigments different from colorless proanthocyanidins [31]. Epicatechin and procyanidin secreted into plasmids can be interpreted that in the process of seed dry cell death caused by broken vacuole, and epicatechin and former cyanine glycosides interact with *TT10*, originally colorless former cyanine glycosides and cell wall polysaccharides and other phenolic compounds form oxide compounds, can easily lead to organization dim [32]. Some laccase genes (such as *LAC14* and *LAC15*) also had a higher expression in the peel of fruit rust [33]. Wang et al [34] studied, such as in the no browning peel of apple, its high expression of chlorogenic acid content, may be associated with inhibition of peel browning, exogenous chlorogenic acid treatment significantly enhanced the expression of genes involved in the biosynthesis of flavonoids, lignin synthesis gene was inhibited expression, showed that laccase and phenolic compounds in fruit growth and postharvest browning phenomenon plays an important role [30]. Disruption of laccase expression will lead to reorientation of substrate flux through phenylpropane metabolic pathways [35]. In litchi, *ADE/LAC* is located in vacuoles and transported to the cell wall to

function, and oxidative polymerization with catechin in peel occurs, accelerating degradation of procyanidins and promoting browning of peel [32]. Figure 11 shows the metabolic pathway of flavonoids.

After bagging, more triterpenes enriched in BFB, while the accumulation of triterpenes was not obvious in the peel of NBF or BFW, indicating that the accumulation of triterpenes in the peel was caused by browning. The specific accumulation process needs to be further studied. Terpenoids were summarized all of isoprene or isopentane, the floorboard of the polymers and their derivatives. These oxygen-containing derivatives include alcohol, aldehyde, ketone, and carboxylic acid. It is derived from a methylene-dicarboxylic acid, and the basic frame for the five-carbon unit, carbon for a single ring, multiple rings, open-chain terpene, open-chain terpene formula (C_5H_8) general formula, including steroid, carotenoids, plant hormones, and quinones [36]. Terpenoids are widely found in nature and are the main components of some plant essences, resins, and pigments (such as rose oil, eucalyptus oil, turpentine) [37]. But triterpene compound is a kind of basic by 30 carbon atoms in the parent nucleus of terpenoids, in the form of free or combined with sugar as the form of glycosides or ester in plants, with a variety of biological activities [38], which is made up of a number of isoprene material after removing hydroxyl end to end, most of the 30 carbon atoms, a small group of 27 carbon atoms terpenoids [29]. For instance, ganolucidic acid, first proposed by Kubota et al [39], which is a highly oxidized lanolin triterpenoid, a triterpenoid commonly found in *ganoderma lucidum*. It is generally yellow-brown and has a very bitter taste. The excessive expression of triterpenes in the peel after bagging might be principally responsible for peel browning. Could the excessive accumulation of triterpenes result in changes in the peel thickness, the appearance quality, and the taste of fruit? The effect of bagging on the synthesis of triterpenes remains to be studied.

Conclusions

In summary, the results of this study indicate that the destruction of cell structure, the increase of lipid peroxidation of cell membrane, the decrease of flavonoids and the increase of triterpenoids were the main reasons for the browning of peel about bagging 'Rui Xue'. What's more, whether there is a direct relationship between the high *MdLAC* expression and the decrease of flavonoids and the increase of triterpenes, which requires further investigations.

Materials And Methods

Experimental Design

The experiment was conducted at the Bai Shui County Apple Test Station of Northwest A & F University (35°2' N, 109°6' E) from May to October in 2019. The test station owns a warm temperate continental monsoon climate with an altitude of 915 m, annual mean rainfall of 578 mm, and an annual mean temperature of 11.4 °C. In the middle and late stage of fruit growth, the maximum temperature of atmosphere and inner bag in the daytime was 31.7°C, the relative humidity was 40.7% and 35.9%, respectively, the minimum temperature at night was 4.1°C and 4.6°C, respectively, and the relative

humidity was 100%, the details of the date are given in Supplemental Figure S2. Scions of 'Rui Xue' were grafted to M26 roots stocks. 5-year-old fruit trees of 'Rui Xue' were used as materials. The trees were cultivated as spindle-shaped (1.5 × 3.0 m). Bagging was conducted in middle of May 2019. The external bag was brown and the inner was red (the specification of fruit bags was 155 mm × 180 mm, the outer paper was 57.0 g wood pulp composite paper, the outer color was pulp color, and the inner color was black, the inner paper was 32.0 g red paper coated with wax on both sides). Three rows of bagging and without bagging fruits were set as biological repeats, with ten plants in each line. The fruits were harvested with bags at the mature stage (October, 2019). The bagging fruits without browning (BFW) and bagging fruits with browning (BFB) were randomly selected as the experimental group, non-bagging fruits (NBF) were used as the control (CT). Thirty fruits with the same size, maturity, and without any mechanical damage were selected for analysis for each group. The fruit peels of the stalk cavity were sampled with a sterilized scalpel, and then immediately frozen in liquid nitrogen and stored at -80°C.

Methods

Classification of Apple Peel Browning

According to the methods of Chen et al [9] (slightly modified), the bagged fruits were classified into five levels according to the browning area (expressed by S) of the peel (Figure 1):

0, there was no brown spot on the surface of fruit;

Ⅰ, the browning area was $0 < S \leq 1/5$;

Ⅱ, the browning area was $1/5 < S \leq 1/4$;

Ⅲ, the browning area was $1/4 < S \leq 1/3$;

Ⅳ, the browning area was $1/3 < S \leq 1/2$;

Ⅴ, the browning area was $1/2 < S \leq 1$.

Peel Thickness

The thickness of the peel was determined by the paraffin section method [40]. Each peel section in every group was randomly selected at three different positions of the stalk cavity peel. The whole area of tissues was pictured under a 100-fold field of view, so as to ensure the consistent background light of each photo. We chose the outer three layers of cells as the critical for measuring the thickness of the peel. Image-pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the thickness (mm) of [epidermis](#) at five points of each slice with a 100-fold scale as the standard.

Microstructure of the Peel Cells

The ultrastructure of cells was determined according to the method described by Chen et al [9]. The peel flakes were fixed on the glass-covered with 4% glutaraldehyde for 5 h and washed with ethanol with an increasing gradient (10-100%) for 10 min. Each piece of tissue was soaked in 100% ethanol for 10 min and then stored overnight in a dryer. The completely dehydrated tissues were transferred to the double-sided carbon-belted aluminum pile in a vacuum, then sputtered and gilded, and finely sliced. The visualized 3D model was analyzed at 20 kV using a scanning electron microscope (FEI Quanta 200, Thermo Fisher Scientific, Bedford, MA, USA).

Metabolites Profiling

An ACQUITY UPLC ultra-high-performance liquid phase series AB Triple TOF 5600 high-resolution mass spectrometer was used to determine liquid-mass coupling. For the determination, 80 mg samples were taken. Then, the internal standard (20 μ L) (L-2-chlorophenylalanine, 0.3 mg/mL; Lyso PC17:0; 0.01 mg/mL, all solutions were configured with methanol) and 1.0 mL methanol:water (V:V=7:3, contains 0.01 mol/L BHT (2,6-di-tert-butyl-p-cresol)) were mixed. Then, two small steel balls were added, and the sample pre-cooled at -20 °C for 2 min before being placed in the grinding machine (60 Hz, 2 min). After 30 min of ultrasonic extraction, the sample was left to stand at -20 °C for 20 min, then centrifuged for 10 min (13000 rpm, 4 °C). The supernatant (300 μ L) and shake it dry, then use 400 μ L methanol:water (V:V=1:4) to redissolve it, vortex for 30 s, and ultrasound for 2 min. After centrifugation for 10 min (13000 rpm, 4 °C), using a syringe to absorb 150 μ L of the superfluid, and using 0.22 μ L of the organic phase pinhole filter to filter it. Then, the filtered organic phase was transferred to the LC injection vial and stored at -80 °C until LC-MS analysis. Three biological repeats were tested in each group. The quality control sample (QC) was prepared by mixing an equal volume of the extract of all samples; the final volume of each QC was the same as the sample volumes. All extracted reagents were pre-cooled at -20 °C before use.

Membrane Lipid Peroxidation

MDA determination was conducted according to the method described by Zhao [41] with minor modifications. Briefly, we took 1.0 mL of the sample supernatant extracted above and added 2.0 mL 0.67% thiobarbituric acid (TBA). Samples containing only 1.0 mL water were set as a negative control. Next, the samples were paced in a boiling water bath for 15 min, and then rapidly cooled by immersion in cold water, and poured them into 10 mL centrifuge tubes. All tubes were centrifuged at 400 rpm for 20 min and determined the absorbance of all samples at 600 nm, 532 nm, and 450 nm using a spectrophotometer.

Cell membrane permeability was measured according to a simplified method [41]. Briefly, fruits were cleand with tap water, and then rinsed twice with distilled water. 0.5 g peel of stalk cavity was taken into 50 ml centrifugal tube, 20 ml of distilled water added, shaking. Then the conductivity was measured using DDS-307 conductivity meter (Shanghai Precision Scientific Instrument Co.Ltd, Shanghai, China) .The nozzle seals were put in a boiling water bath for 10 min, cooled by tap water, shaken and conductance measured according to the formula to calculate the cell membrane permeability.

Total Antioxidant Capacity

According to the methods of Lee and Wicker [42], 1.0 g peel was grounded in liquid nitrogen. Then, a 1.5 ml alcohol-acetone mixture (v:v = 7:3) was added and incubated at 37 °C for 1 h. The homogenate was then centrifuged at 13,000 rpm at 20 °C for 10 min. The supernatant was immediately stored in the -20 °C refrigerator, which used to determine the total antioxidant activity and content of antioxidant substances.

Total phenol content was spectrophotometrically determined according to the Folin-Ciocalteu method [43]. Flavonoids were determined by nitrite-aluminum chloride [44], using rutin as standard, and the results were calculated using the rutin calibration curve. Flavanol content was detected with p-DMACA [32]. The total antioxidant capacity of peel was determined by iron reduction ability method.

Total RNA extraction and qPCR analysis

Total RNA was extracted from the apple of peel, using the RNA Plant Plus Reagent Kit (TIANGEN, China) and reverse transcribed using the PrimeScript First-Strand cDNA Synthesis Kit (Takara, Dalian, China). Quantitative polymerase chain reactions (qPCRs) were carried out in the ABI7500 System using SYBR Premix Ex Taq (Takara). The calculation method for qRT-PCR was $2^{-\Delta\Delta CT}$. At least three replicates for each sample were used for qPCR. The primers used for qPCR are listed in Table1.

Statistical Analysis

Three biological repeats were taken from the experimental data. Microsoft Excel 2010, SigmaPlot 13, and Progenesis QI V2.3 software were used to process and plot the data, and SPSS 24.0 software was used to conduct a one-way analysis of variance (ANOVA). The data were analyzed using the t-test. The data were expressed as mean $\pm$ standard error. Significance was defined at $p < 0.05$

Declarations

Authors' Contributions

ZYZ, HW and LLS conceived and designed the experiments. HW, SW, MMF and SHZ performed experiments. HW, SW and MMF contributed reagents, materials, and data analysis. ZYZ, HW and LLS wrote the paper.

Funding

This work was supported by the earmarked fund for the Special Project for Building Industrial Technology System of National Modern Agriculture (Apple) (CARS-28), the Modern Agro-industry Technology Research System, China (CARS-27); the Northwest A&F University of Science and Technology (2014 YB 086).

Conflict Interest

The authors have no conflict of interest to declare.

Acknowledgement

We appreciate the linguistic assistance provided by TopEdit (www.topeditsci.com) during the preparation of this manuscript.

References

1. Zhao J, Quan PK, Liu HK, Li LL, Qi SY, Zhang MS, Zhang B, Li H, Zhao YR, Ma BQ, Han MY, Zhang HH, Xing LB. Transcriptomic and metabolic analyses provide new insights into the apple fruit quality decline during long-term cold storage. *J Agric Food Chem*. 2020;68:4699–716.
2. Sharma RR, Pal RK, Sagar VR, Parmanick KK, Rana MR, Gupta VK, Kumar K, Rana MR. Impact of pre-harvest fruit-bagging with different coloured bags on peel colour and the incidence of insect pests, disease and storage disorders in 'Royal Delicious' apple. *J Hortic Sci Biotech*. 2014;89:603–18.
3. Tran DH, Yen CR, Chen YKH. Effects of bagging on fruit characteristics and physical fruit protection in red pitaya (*hylocereus* spp.). *Biological Agriculture Horticulture*. 2015;31:158–66.
4. Amarante C, Banks NH, Max S. Effect of preharvest bagging on fruit quality and postharvest physiology of pears (*Pyrus communis*). *N Z J Crop Hortic Sci*. 2002;30:99–107.
5. Shlomo M. Efficiency of bagging pomegranate fruits. *Acta Hortic*. 2015;1089:485–8.
6. Zhao CR Analysis of the unknown disease on 'Rui xue' apple fruit [D]. Northwest A&F University. 2018.
7. Li J. Screening of special fruit-growing bags for new apple variety 'Ruixue' [D]. Northwest A&F University. 2019.
8. Zhao SY, Liu ZL, Shu YS, Wang ML, He D, Song ZQ, Lu AP. Chemotaxonomic classification applied to the identification of two closely-related Citrus TCMs using UPLC-Q-TOF-MS-based metabolomics. *Molecules*. 2017;22:1721.
9. Chen X, Wu QX, Chen ZSZ, Li TT, Zhang ZK, Gao HJ, Yun Z, Jiang YM. Changes in pericarp metabolite profiling of four litchi cultivars during browning. *Food Res Int*. 2019;120:339–51.
10. Oak P, Deshpande A, Giri A, Gupta V. Metabolomic dynamics reveals oxidative stress in spongy tissue disorder during ripening of *mangifera indica* L. fruit. *Metabolites*. 2019;9:255.
11. Jordi GB, Busatto N, Christian L, Violeta LG, Costa F. Investigation of the transcriptomic and metabolic changes associated with superficial scald physiology impaired by lovastatin and 1-methylcyclopropene in pear fruit (cv. "blanquilla"). *Hort Res*. 2020;7:49.
12. Lim S, Lee JG, Lee EJ. Comparison of fruit quality and GC-MS-based metabolite profiling of kiwifruit 'Jecy green': Natural and exogenous ethylene-induced ripening. *Food Chem*. 2017;234:81–92.
13. Liu DC, Zeng Q, Ji QX, Liu CF, Liu SB, Liu Y. A comparison of the ultrastructure and composition of fruits' cuticular wax from the wild-type 'newhall' navel orange (*citrus sinensis*[L] osbeck cv. newhall) and its glossy mutant. *Plant Cell Rep*. 2012;31:2239–46.

14. He YZ, Han JW, Liu RS, Ding YD, Wang JQ, Sun L, Yang XM, Zeng YL, Wen WW, Xu J, Zhang HM, Yan X, Chen ZX, Gu ZL, Chen H, Tang H, Deng Q, Cheng XX. Y. J. Integrated transcriptomic and metabolomic analyses of a wax deficient citrus mutant exhibiting jasmonic acid-mediated defense against fungal pathogens. *Hort Res.* 2018;5:43.
15. Zhang K, Wang HT, Cong DP. A high-quality apple genome assembly reveals the association of a retrotransposon and red fruit colour. *Nat Commun.* 2019;10:1494.
16. Baderschneider B, Winterhager P. Isolation and characterization of novel benzoates, cinnamates, flavonoids and longanins from Riesling wine and screening for antioxidant activity. *J Agric Food Chem.* 2001;49:2788–98.
17. Karara H, Ahmada M, Ullaha H, Wajidb M, Zubairb M, Razab H. Effectiveness of Fruit Bagging for the Control of Insect-Pests Complex and its Impact on Quality of Mango Fruits. *Korean J Hortic Sci Technol.* 2019;2:45–8.
18. Chen CS, Zhang D, Wang YQ, Li PM, Ma FW. Effects of fruit bagging on the contents of phenolic compounds in the peel and flesh of ‘Golden Delicious’, ‘Red Delicious’, and ‘Royal Gala’ apples. *Sci Hortic.* 2012;142:68–73.
19. Wang L, Zhang X, Liu Y, Shi X, Wang Y, Zhang C, Zhao Z. The effect of fruit bagging on the color, phenolic compounds and expression of the anthocyanin biosynthetic and regulatory genes on the ‘Granny Smith’ apples. *Eur Food Res Technol.* 2013;237:875–85.
20. Feng F, Li M, Ma F, Cheng L. The effects of bagging and debagging on external fruit quality, metabolites, and the expression of anthocyanin biosynthetic genes in ‘Jonagold’ apple (*Malus domestica* Borkh.). *Sci Hortic.* 2014;165:123–31.
21. Zhu H, Chen C, Zeng J, et al. MicroRNA528, a hub regulator modulating ROS homeostasis via targeting of a diverse set of genes encoding copper-containing proteins in monocots. *New Phytol.* 2020, 225, (1).
22. Martins SIFS, Jongen WMF, Boekel MA. J. S. V. A review of maillard reaction in food and implications to kinetic modelling. *Trends Food Sci Technol.* 2000;11:364–73.
23. Wang H, Chen YH, Sun JZ, Lin YF, Lin YX, Lin MS, Hung YC, Ritenour MA, Lin HT. The Changes in Metabolisms of Membrane Lipids and Phenolics Induced by *Phomopsis longanae* Chi Infection in Association with Pericarp Browning and Disease Occurrence of Postharvest Longan Fruit. *J Agric Food Chem.* 2018;9:1454.
24. Song X, Bao M, Li D, Li YM. Advanced glycation in D-galactose induced mouse aging model. *Mech Ageing Dev.* 1999, 108, (3).
25. Badri DV, Quintana N, El KEG, Kim HK, Choi YH, Sugiyama A, Verpoorte R, Martinoia E, Manter DK, Vivanco JM. An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. *Plant Physiol.* 2009;151:2006–17.
26. Telias A, Bradeen JM, Luby JJ, Hoover EE, Allan AC. Regulation of anthocyanin accumulation in apple peel. *Hortic Rev.* 2011a;38:357–91.

27. Tanaka Y, Sasaki N, Ohmiya A. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant J.* 2008;54:733–49.
28. Lin YX, Jiang LY, Chen Q, Li YL, Zhang YT, Luo Y, Zhang Y, Sun B, Wang XR, Tang HR. Comparative transcriptome profiling analysis of red- and white-fleshed strawberry (*Fragaria x ananassa*) provides new insight into the regulation of the anthocyanin pathway. *Plant Cell Physiol.* 2018;59:1844–59.
29. Zhang XX, Zhang S, Yang YR, Wang D, Gao HY. Natural barrigenol-like triterpenoids: a comprehensive review of their contributions to medicinal chemistry. *Phytochemistry.* 2019;161:41–74.
30. Gong YH, Song J, Du LN, Vinqvist M, Palmer LC, Fillmore S, Pang X, Zhang Q. Z. Q. Characterization of laccase from apple fruit during postharvest storage and its response to diphenylamine and 1-methylcyclopropene treatments. *Food Chem.* 2018;253:314–21.
31. Lucille P, Lucien RJean-Marc,K, Michel C, Loi`c L, Isabelle D. TRANSPARENT TESTA10 Encodes a Laccase-Like Enzyme Involved in Oxidative Polymerization of Flavonoids in Arabidopsis Seed Coat. *Plant Cell.* 2005;17:2966–80.
32. Fang YL, Meng JF, Zhang A, Liu JC, Xu TF, Yu WL, Chen SX, Li H. Influence of shriveling on berry composition and antioxidant activity of Cabernet Sauvignon grapes from Shanxi vineyards. *J Sci Food Agric.* 2011;91:749–57.
33. Legay S, Guerriero G, Deleruelle A, Lateur M, Evers D, André CM, Hausman JF. Apple russetting as seen through the RNA-seq lens: strong alterations in the exocarp cell wall. *Plant Mol Biol.* 2015;88(1–2):21–40.
34. Wang, L. J., Li, J. H., Gao, J. J., Feng, X. X., Shi, Z. X., Gao, F. Y., ... Yang, L.Y. Inhibitory effect of chlorogenic acid on fruit russetting in 'Golden Delicious'apple. *Scientia Horticulturae*, 2014, 178, 14–22.
35. Zhao Q, Nakashima J, Chen F, Yin Y, Fu C, Yun J, Dixon RA. LACCASE is necessary and nonredundant with PEROXIDASE for lignin polymerization during vascular development in Arabidopsis. *Plant Cell.* 2013;25(10):3976–87.
36. Boba A, Kostyn K, Kozak B, Wojtasik W, Kulma A. Fusarium oxysporum infection activates the plastidial branch of the terpenoid biosynthesis pathway in flax, leading to increased ABA synthesis. *Planta.* 2020;251:50.
37. Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. *Nat Chem Biol.* 2007;3:408–14.
38. Haralampidis K, Trojanowska M, Osbourn AE. Biosynthesis of triterpenoid saponins in plants. *Adv Biochem Eng Biotechnol.* 2002;75:31–49.
39. Kubota T, Asaka Y, Miura I, Mori H. Structures of ganoderic acid a and b, two new lanostane type bitter triterpenes from ganoderma lucidum (fr.) karst. *Helv Chim Acta.* 1982;65:611–9.
40. Ban QY, Han Y, Meng K, Hou YL, Rao JP. 2016. Characterization of β -Galactosidase Genes Involved in Persimmon Growth and Fruit Ripening and in Response to Propylene and 1-Methylcyclopropene. *Journal of Plant Growth Regulation*, 2016, 35, 1025–1035.

41. Zhao SJ, Shi GA, Dong XC. Experimental guidance of plant physiology. Beijing: China agriculture press; 2015. 55–7, 142–143.

42. Lee HS, Wicker L. Anthocyanin pigments in the skin of lychee fruit. J Food Sci. 1991;56:466–8.

43. Liu Y, Qiu NX, Yang XY. Assessment of total phenolic content and in vitro antimicrobial activities of ethanol extract from grape residue left after making wine. J Food Sci. 2011;32:5–9.

44. Koyama K, Gotoyamamoto N. Bunch shading during different developmental stages affects the phenolic biosynthesis in berry skins of ‘Cabernet Sauvignon’ grapes. J AmerSocHortSci. 2008;133:743–53.

Tables

Table 1 The primer sequences for real-time PCR.

Gene name	Gene ID	Forward primer sequence	Reverse primer sequence
<i>MdActin</i>	MD12G1140800	TGACCGAATGAGCAAGGAAATTACT	TACTCAGCTTTGGCAATCCACATC
<i>MdCHS</i>	MD13G1285100	ACCCAGGTGGCCCTGCTATTCT	TCCCTCCCGGTCGTTTTGA
<i>MdF3H</i>	MD15G1246200	GGAAGGAGCTTTTGTGGTCAA	GGTTCGTGAATGTGGCTATGGA
<i>MdDFR</i>	MD15G1024100	AGGAACTGTGAATGTGGAGGAGC	AGGAACTGTGAATGTGGAGGAGC
<i>MdANS</i>	MD06G1071600	TCCAAAAGAGTACATCAGACCTAA	ACACCCAGTCCACAGTTGC
<i>MdLAR</i>	MD16G1048500	TCTTGGCCCTACTTTGACAA C	AGAGTTCCCACTTCCACATCA
<i>MdANR</i>	MD10G1311100	CCACCTCACAGCACTACAAGAG	GCAAATTTCCAAGCTGTCCTTCT
<i>MdUFGT</i>	MD01G1234400	GCAAGCCG CAGGAGGACATA	CGGAGATCCAGAAAGTGACCCA
<i>MdLAC7</i>	MD04G1142300	ATGGCACGGTTAGCCTTT	CTAACATTTCCGCAGATCAAGT
<i>MdLAC12</i>	MD01G1159400	TGGAGGCACTCAACGCATTT	GATCTATAGATTCTTAACAA
<i>MdLAC14</i>	MD02G1264400	ATGGCCTCAGACAAGAAAATAC	CTAAGACTTGAAACAAGGAGGCA
<i>MdLAC15</i>	MD07G1308000	ATGAGTATTTTAATTAGCTTGC	ACATGGAGGCATTCTTG TGG
<i>MdLAC16</i>	MD07G1307400	ATGCATCTCCACGGATT CAG	TGTTAACATGGAGGCATTCC

Figures

Figure 1

Investigation on different late-ripening varieties about peel browning.

Figure 2

The classification in apple peel browning about bagging ‘Rui Xue’.

Figure 3

The effect of bagging on peel thickness. Note: A: Unbagging fruit; B: Bagging fruit without browning; C: Bagging fruit with browning; D: Thickness of apple peel

Figure 4

Effects of bagging on the microstructure of peel cells. A: Bagging fruit without browning; B: Bagging fruit with browning

Figure 5

Principal component analysis of QC samples and analysis of metabolite strength distribution. Note: A: PCA model figure; B: Hierarchical clustering figure QC: Mixed samples; BFB: Bagging fruit with browning; BFW: Bagging fruit without browning; NBF: Unbagging fruit

Figure 6

The effect of fruit peel browning on metabolites. A: BFW-NBF group; B: BFW-BFB group Note: The red origin represents the significantly up-regulated differential metabolites in the experimental group, the blue

origin represents the significantly down-regulated differential metabolites, and the gray point represents no significant difference.

Figure 7

Enrichment analysis of metabolic pathways of different metabolites. A,C: BFW-NBF group; B,D: BFW-BFB group Note: The ordinate is the name of metabolic pathway; the abscissa is the enrichment factor (Rich factor = the number of metabolites with significant difference/the number of total metabolites in the pathway). The color from red to green means that the p-value decreases successively.

Figure 8

The relationship between membrane lipid peroxidation and peel browning. Error bars represent the averages of three biological replicates $\pm$ SD. Different letters represent differences between different processes. (Significance was defined as $P < 0.05$).

Figure 9

Relationship between antioxidant capacity and browning of peel. A: Total phenols content; B: Total flavonoids content; C: Total flavonoid content; D: Total antioxidant activity. Error bars represent the averages of three biological replicates $\pm$ SD. Different letters represent differences between different processes. (Significance was defined as $P < 0.05$).

Figure 10

Analysis the Gene Expression of Related-Flavonoids. Error bars represent the averages of three biological replicates $\pm$ SD. Different letters represent differences between different processes. (Significance was defined as $P < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FIG.S1A.pdf](#)
- [FIG.S1B.pdf](#)
- [FIGS2.png](#)
- [TablesSupp.docx](#)