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Hydrogen sulfide inhibits the browning of fresh-cut apple by regulating the antioxidant, energy and lipid metabolism

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Abstract: Surface browning is the primary limiting factor for the shelf-life of fresh-cut apple. Hydrogen sulfide (H_2S) treatment is known to effectively inhibit the browning, however, little is known about the underlying molecular mechanism. In the present paper RNA-Seq technology was used to analyse the transcript expression profiles of control and H_2S treated fresh-cut apple immediately after treatment (C0 and S0) and 6 d of storage (C6 and S6) at 4 °C. The results identified 3782 and 1164 differentially expressed unigenes (DEGs) in S0 *vs.* C0 and S6 *vs.* C6, respectively. Expression of most DEGs related to antioxidant systems and energy metabolism was up-regulated after H_2S treatment, whilst expression of genes encoding polyphenol oxidase, peroxidase, lipid-degrading enzymes, such as lipoxygenase and phospholipase D, was repressed. Further quantitative real-time PCR testing validated the reliability of our RNA-Seq results. We therefore propose that H_2S treatment inhibited the surface browning of fresh-cut apple by regulating antioxidant, energy and lipid metabolism to maintain the membrane integrity of the plant tissue.

Keywords: Fresh-cut apple; hydrogen sulfide, browning, transcriptome

1. Introduction

Fresh-cut apple, which contain phenolic components that have an antioxidant capacity in the fruit, have recently emerged as popular snacks in food service establishments, school lunch programs, and for family consumption (Guan and Fan, 2010). However, they are more perishable than intact produce because apple fruit undergo enzymatic browning during minimal processing. Surface browning is the major limiting factor for the shelf-life of fresh-cut apple, and its appearance strongly

and negatively affects the consumer's purchase decision (Shrestha et al., 2020; Toivonen and Brummell, 2008).

Enzymatic browning of fresh-cut fruit and vegetable is a complex and highly regulated process. It is generally considered that polyphenol oxidase (PPO) catalyses the oxidation of phenolic compounds to quinones which then condense to form brown polymers (Milani and Hamedi, 2004). In a typical plant cell PPO is associated with the plastid and phenolic substrates are located in the vacuole. Cellular and intracellular disruption by cutting allows the substrates to mix and hence react to form browning polymers (Landrigan et al., 1996). However, PPO activity and phenolic content are not the only factors affecting browning reaction. Li et al. (2017) indicated that the lipid membrane of cells may be an important factor in browning of fresh-cut pear as a higher lipoxygenase (LOX) activity, lower unsaturated fatty acid ratio and severe cell membrane damage accompanied a stronger degree of browning. Cysteine protease inhibitors inhibited the browning of fresh-cut potato through reducing the accumulation of free amino acids (Dong et al., 2020), implying that protein degradation is an integral component of the browning mechanism. After the browning of fresh-cut lotus tubers, the ROS metabolism homeostasis was damaged and energy efficiency decreased as the antioxidant enzymes (TPX and SOD) and energy metabolism related protein (H+-ATPase, PDC) were down-regulated (Jiang et al., 2012). Recent studies have used high-throughput transcriptome sequencing techniques to characterize the expression profile of the browning response in different types of fruit and vegetable after cutting. Several enzymatic browning-related differentially expressed genes are consistently implicated, including *PPO*, *phenylalanine ammonia lyase* (*PAL*), *peroxidase* (*POD*), *catalase* (*CAT)*, *superoxide dismutase* (*SOD*) (Docimo et al., 2016; Zhang et al., 2019; Zhu et al., 2017). However,

71 the transcriptomic events that occur during the browning process of fresh-cut apple is
72 still unknown.

73 Hydrogen sulfide (H_2S) is the third endogenous signaling molecule after carbon
74 monoxide and nitric oxide, which is traditionally known as a toxic gas. In the past
75 decade, studies showed that it can be generated in many types of mammalian cells and
76 might be beneficial to human health via multiple mechanisms (Giovinazzo et al., 2021;
77 Wang, 2002). In plant, H_2S has been revealed not only to regulate normal
78 physiological processes (Hancock and Whiteman, 2014; Jin and Pei, 2015), but also
79 to prevent the postharvest senescence of fruit and vegetable (Hu et al., 2012; Hu et al.,
80 2014a). Recent research has demonstrated H_2S treatment could inhibit surface
81 browning in many types of fresh-cut fruit and vegetable by regulating the phenolic
82 metabolism or antioxidant defense system (Sun et al., 2015; Zheng et al., 2016). In
83 these paper, the application concentration of H_2S were quite low. Furthermore, the
84 essential oils in organosulphur rich fruits and vegetables, such as onion, garlic, shallot,
85 leek, can be metabolized to generate H_2S in biological conditions and modulate cell
86 signaling (Liang et al., 2015). Thus we propose that trace H_2S gas used in storage and
87 preservation of fruits and vegetables could be safe. However, the underlying
88 molecular mechanism of browning inhition by H_2S is still unknown. In the present
89 study, transcriptome analysis was performed using a high-throughput sequencing
90 platform (Illumina HiSeqTM2000) to give new insights into the mechanism of
91 browning inhibition by H_2S treatment in fresh-cut apple.
92

93 **2. Materials and Methods**

2.1 Sample preparation and treatments

"Fuji" apple (*Malus domestica* cv. Red Fuji) were freshly harvested from a commercial farm in Dalian City, P. R. China in October 2017. Apples were selected based on uniform color, size, hardness, and the absence of any visible physical defects and fungal infections. After harvest, the fruit were immediately transported to the laboratory at Dalian Minzu University and stored at 4 °C prior to the experiments. In these apples, weight (312.9 ± 37.4 g), equatorial (9.1 ± 0.5 cm), longitudinal (8.1 ± 0.3 cm) diameters were monitored. These fruits were rinsed gently by hand, using tap water, and were dried naturally. After that, apples were peeled, cored, then cut into 2 cm-thick cubes with a sharp stainless-steel knife. The cubes of each apple were divided into two groups, one was used for the H_2S treatment and the other one was the control. Sodium hydrosulfide (NaHS, Sigma) solutions were used as the H_2S donor and 200 mL of 0.7 $mmol \cdot L^{-1}$ NaHS was prepared and placed into the bottom of sealed containers (desiccators) which themselves had a total volume 3 L. This solution could release H_2S gas into the container headspace rapidly and reached the peak concentration within 30 min, which was then maintained at a constant concentration of 0.30×10^{-10} $mol \cdot L^{-1}$ H_2S gas for the duration of sample treatment (Hu et al., 2012). Fresh-cut apple were placed on a grid above the NaHS solution and thereby exposed to H_2S treatment for 24 h. Water was used instead of NaHS solution in the control samples. Three independent containers were set up to generate H_2S and likewise there were three independent control samples. After treatment, samples were placed in a different plastic box (15 × 21 × 2.5 cm) for each replicate and each box was wrapped with polyethylene cling film to prevent further gas exchange. The time

when the control and H_2S treatment ended was defined as day 0 and from this time point on, all samples were stored for up to 12 d at 4 ± 1 °C.

2.2 Measurement of color

The color of fresh-cut apple was measured every two days using repeated measures of eight fresh cut apple cubes. Color data were captured using a Minolta Chroma Meter Model CR-300 (Minolta. Tokyo, Japan), using the CIELAB color parameters, L^* (lightness), a^* (green chromaticity) and b^* (yellow chromaticity). Browning index (BI) was calculated as follows (Palou et al., 1999):

$$BI = [100(x-0.31)]/0.172$$

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

2.3 Enzyme activities

All enzyme extracts were prepared by homogenizing 5 g of fresh-cut apple in a homogenizer (T-25, IKA, Germany), on ice, using the following extraction media: 20 mL of 0.2 mol L^{-1} phosphate buffer (pH 6.4) containing 0.1 g polyvinylpolypyrrolidone (PVPP) for PPO, POD, SOD, CAT, glutathione reductase (GR) and 20 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.5) containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) and 3 mmol L^{-1} ascorbic acid for ascorbate peroxidase (APX). For phenylalanine ammonia lyase (PAL): the extraction solution was 20 mL of 0.1 mol L^{-1} sodium borate buffer (pH 8.7) containing 0.037 % EDTA, 0.137 % β-mercaptoethanol and 3 % PVPP. Extracts were then centrifuged at 12 000 *g*, for 30 min, at 4 °C, and supernatants were collected for further analysis.

PPO and POD activities were examined using the method reported by Chen et al. (2016a). One unit of PPO and POD activities was defined as a decrease of OD value in absorbance per minute.

PAL activity was measured by using a method reported by Yin et al. (2012). One unit of PAL activity was defined as a decrease of 0.01 × OD value in absorbance per minute.

SOD activity was measured using p-nitro-blue tetrazolium chloride (NBT) according to Ren et al. (2012). One unit of SOD activity was defined as the amount of enzyme that caused 50 % inhibition of NBT.

CAT activity was determined according to Ren et al. (2012), by monitoring the disappearance of H_2O_2 by recording the decrease in absorbance at 240 nm. One unit of CAT activity was defined as a decrease of 0.01 × OD value in absorbance per minute.

APX activity was measured by determining the amount of oxidized ascorbate using the method of Ren et al. (2012). One unit of APX activity was defined as a decrease in the OD value in absorbance per minute.

GR activity was examined based on the oxidation of nicotinamide-adenine dinucleotide phosphate (NADPH) and the change of absorbance at 340 nm was monitored (Zhang et al., 2017). One unit of GR activity was defined as the amount of enzyme that oxidized 1 μmol NADPH per min.

All the enzymatic activities were expressed as U kg^{-1} FW.

2.4 H_2O_2 content

Three grams of fresh-cut apple were put into 5 mL of cold acetone (100 %) and

169 then homogenized. Subsequently, the mixture was centrifuged (Allegra X-30R,
170 Beckman Coulter, USA) for 20 min (10000 *g*, 4 °C). The supernatant was used to
171 analyze H_2O_2 content by the titanium peroxide method (Patterson et al., 1984). The
172 result was expressed as mmol kg^{-1}. There were three independent biological replicates
173 for the control and the H_2S treatment.
174
175 2.5 Ascorbic acid content
176
177 Ascorbic acid (AsA) content was determined according to AOAC (1990) by
178 using titration with 2,6-dichlorophenolindophenol. The result was calculated by
179 comparison to a standard AsA curve and expressed in mg of AsA per kg fresh weight.
180 There were three independent biological replicates for the control and the H_2S
181 treatment.
182
183 2.6 Phenolic content
184
185 Phenolic compounds were extracted into 75 % ethanol and the content of
186 individual phenolics was determined by using an Agilent Technologies (Waldbronn,
187 Germany) 1100 Series HPLC system according to Chen et al. (2016b). Ten μL of
188 each sample was filtered and then separated using a Hypersil BDS C18 column (250
189 mm × 4.6 mm, 5 μm; Thermo, Bellefonte, PA, USA). The phenolics were eluted by
190 using two mobile phases (A: 2 % v/v acetic acid in water, B: 2 % v/v acetic acid in
191 water and acetonitrile, 50:50, v/v) with a linear gradient from 10 to 55 % B in 50 min
192 and monitored at 254 nm (for quercetin, eugenol, epicatechin), 280 nm (for catechin,
193 tannic acid, vanillic acid) and 320 nm (for caffeic acid and chlorogenic acid). The

194 flow rate was 1 mL min^{-1}. The result was calculated by comparison to standard curves
195 constructed from dilutions of pure standards for each phenolic compound and
196 expressed as mg per kg of sample.
197
198 2.7 Transcriptome Analysis
199
200 2.7.1 mRNA library construction and sequencing
201 Total RNA was extracted from each biological replicate of the control and H_2S
202 treatment using Trizol reagent (Invitrogen, CA, USA) following the manufacturer's
203 procedure. The total RNA quantity and purity were analysed using a Bioanalyzer
204 2100 and RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0.
205 Approximately 10 μg of total RNA representing a specific adipose type was subjected
206 to isolation of Poly (A) mRNA with poly-T oligo attached magnetic beads
207 (Invitrogen). Following purification, the mRNA was fragmented into small pieces
208 using divalent cations under elevated temperature. Then the cleaved RNA fragments
209 were reverse-transcribed to create the final cDNA library in accordance with the
210 protocol for the mRNA Seq sample preparation kit (Illumina, San Diego, USA), the
211 average insert size for the paired-end libraries was 300 bp (±50 bp). We then
212 performed the paired-end sequencing on an IlluminaHiseq4000 at the LC Sciences
213 (Hangzhou, Zhejiang, China), following the vendor's recommended protocol.
214
215 2.7.2 RNA-seq reads mapping
216 The clean reads were mapped onto the apple genome GDDH13 Version 1.1
217 (https://iris.angers.inra.fr/gddh13/the-apple-genome-downloads.html) using HISAT
218 (Johns Hopkins University Center for Computational Biology, Baltimore, MD, USA.

https://daehwankimlab.github.io/hisat2/,version:hisat2-2.0.4), which initially removed a portion of the reads based on quality information accompanying each read and then mapped the reads to the reference genome (Kim et al., 2015). HISAT allows multiple alignments pre-read (up to 20 by default) and a maximum of two mismatches when mapping the reads to the reference. HISAT build a database of potential splice junctions and confirms these by comparing the previously unmapped reads against the database of putative junctions.

2.7.3 Transcript abundance estimation and differentially expressed testing

The mapped reads of each sample were assembled using StringTie (http://ccb.jhu.edu/software/stringtie/,version:stringtie-1.3.4d.Linux_x86_64, Pertea et al., 2015) with default parameters (command line: ~stringtie -p 4 -G genome.gtf -o output.gtf -l sample input.bam). Then, all transcriptomes from samples were merged to reconstruct a comprehensive transcriptome using perl scripts. After the final transcriptome was generated, StringTie and Ballgown (http://www.bioconductor.org/packages/release/bioc/html/ballgown.html) was used to estimate the expression levels of all transcripts (Frazee et al., 2015; Pertea et al., 2015). StringTie was used to perform expression level for mRNAs by calculating FPKM. The differentially expressed mRNAs and genes were selected with $|\log_2 FC| \geq 1$ and with statistical significance ($P < 0.05$) by R package-Ballgown

2.8 Quantitative Real-Time PCR (qRT-PCR)

To validate the RNA-Seq data, the expression of 10 selected DEGs were quantified by using qRT-PCR following the manufacturer's protocols (One Step

SYBR® PrimeScript™ RT-PCR Kit, TaKaRa, Japan). Total RNA was extracted from three independent biological replicates of both the control and H_2S treated apple material and purified by using TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan). The sequences of gene specific qRT-PCR primers are listed in Supplementary Table S1. *Md18sRNA w*as used as a housekeeping gene. Calculation of the relative quantification was performed by the comparative $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). Three biological replicates were used for qRT-PCR analysis.

3. Results and discussion

3.1 Effect of H_2S treatment on the browning of fresh-cut apple

Surface browning is one of the major limiting factors for the shelf life of fresh-cut apple and strongly and negatively affects the consumers' decision whether or not to purchase the product. As shown in Fig. 1A, the initial surface color of H_2S-treated fresh-cut apple was a clean, pale yellow. The control samples had already started to discolor from the time spent in the incubator post-cutting. As the storage time progressed, the color of control samples turned to a pale brown. H_2S treatment effectively inhibited the surface browning of fresh-cut apple over the 12 d of storage, with discoloration remaining less than the browning index (BI) of the control at day 0 throughout the experiment. The BI refers to the intensity of the brown color and has been considered an important indicator of the browning degree. A continual increase in BI of fresh-cut apple was observed during storage (Fig. 1B). H_2S treatment clearly suppressed the increase in browning degree over the entire storage period.

3.2 Physiological responses of fresh-cut apple to H_2S treatment

To assess the physiological responses of fresh-cut apple to H_2S treatment, we monitored the activities of PPO, POD, PAL, SOD, CAT, APX and GR, the contents of H_2O_2, AsA and phenolics during storage at 4 °C (Fig. 2). H_2S treatment significantly increased the enzyme activities of SOD, CAT, GR, PAL and increased the contents of AsA and phenolics, whilst it inhibited the accumulation of H_2O_2 and PPO, POD and APX activities, as compared with the control ($P < 0.05$). It is interesting to note that the activities of five enzymes (PPO, PAL, CAT, SOD and APX) reached their maximum or minimum on the 6th day of storage. Therefore, fresh-cut apple samples that were taken immediately after H_2S treatment (day 0) and after storage at 4 °C for 6 d were used for transcriptome analysis to explore the molecular mechanism of browning inhibition.

3.3 Transcriptome gene expression analysis

In order to investigate the molecular mechanism initiated by H_2S treatment in browning inhibition of fresh-cut apple, RNA-Seq libraries were designed including control and H_2S treated samples at two stage (day 0 and day 6). Supplementary Table S2 and S3 provide a quality summary of the sequence data and the number of unigene and transcript identified in each sample. There were 21, 26, 6 and 12 uniquely expressed genes were found in C0, S0, C6 and S6, respectively (Table S4). We totally identified 3782 (1638 up-regulated and 2144 down-regulated) and 1164 DEGs (529 up-regulated and 635 down-regulated) in S0 *vs.* C0 and S6 *vs.* C6, respectively, only 406 genes in common (Fig. 3A, 3B). There are more DEGs in S0 vs. C0 than that in

S6 vs. C6, indicating that a majority of transcriptional responses were manifested soon after the treatments. The top up-regulated DEG in S0 *vs.* C0 and S6 *vs.* C6 were *zinc finger AN1 domain-containing stress-associated protein 12* and *glutathione S-transferase (GST)*, respectively. *Polygalacturonase inhibitor 2* was the top down-regulated DEG in both S0 *vs.* C0 and S6 *vs.* C6 (Table S5). Then we analyze the possible browning-related genes in the DEGs of two samples. In the control, there were 775 and 879 DEGs up- and down-regulated respectively at day 6 relative to day 0. While in the H_2S treatment, 1171 and 1144 DEGs up- and down-regulated were identified respectively after 6 d storage, only 312 were in common (Fig, 3C). This limited overlap in transcriptomic events indicating that H_2S treatment altered the gene expression patterns of fresh-cut apple during storage and this change might be related to the browning inhibition.

GO term enrichment analyses were conducted for DEGs in S0 *vs.* C0, S6 *vs.* C6, C6 *vs.* C0 and S6 *vs.* S0, respectively, to evaluate the potential functions of these genes. The top 15 enriched GO terms ($P < 0.05$) of biological process, cellular component and molecular function are shown in Fig. 4A-D. When comparing the GO terms in S0 *vs.* C0 to S6 *vs.* C6, 18 out of 45 GO term were found in common. Within the biological process category, “oxidation-reduction process” was the most enriched term in both S0 *vs.* C0 and S6 *vs.* C6. Besides, several GO terms related to response to abiotic stresses, such as salt stress, cold, heat, water deprivation, were significantly enriched in both two comparable pairs. For cellular component, GO terms related to membrane, such as “plasma membrane”, “integral component of plasma membrane”, “vacuolar membrane”, dominated in both S0 *vs.* C0 and S6 *vs.* C6. In S0 *vs.* C0, DEGs associated with “transferase activity” was the most dominated GO term in molecular function, while that was “metal ion binding” in S6 *vs.* C6. There were five

common GO terms in S0 v*s.* C0 and S6 *vs.* C6, including genes coding proteins with "oxidoreductase", "glutathione transferase activity", "pyridoxal phosphate binding" etc.

Next, we compared the function of DEGs in C6 *vs.* C0 and S6 *vs.* S0, only 11 GO terms were found in common, indicating that H_2S treatment changed the metabolism of fresh-cut apple, which might be involved in the browning inhibition (Fig 4C, 4D). For biological process, the most dominated GO term was "oxidation-reduction process" in the control, while that was "protein ubiquitination" in the H_2S treatment. Six common GO terms were found in C6 *vs.* C0 and S6 *vs.* S0. Within the cellular component category, "cytoplasm", "integral component of plasma membrane" and "nuclear speck" were significantly enriched in both control and H_2S treatment. "cytoplasm" was the most dominated GO terms in the control, which was the second enriched GO terms in the H_2S treatment. The largest group number of DEGs in the H_2S treatment were enriched in "nucleus". Besides, several GO terms related to membrane, such as "plasma membrane", "vacuolar membrane", "endoplasmic reticulum membrane", "Golgi membrane" and "endosome membrane" were only enriched in the H_2S treatment. The top three GO terms for molecular function in the control and H_2S treatment were "nucleic acid binding", "oxidoreductase activity" "ubiquitin-protein transferase activity" and "protein serine/threonine kinase activity", "nucleotide binding", "transferase activity", respectively. To further understand the changes induced by H_2S related to the browning process, the gene expression pattern of DEGs in the control and H_2S treatment within the common GO terms were analyzed (Fig. S1). H_2S treatment increased the number of up-regulated DEGs in "glucose import" and "glucose transmembrane transporter activity", while increased the down-regulated DEGs in

344 “integral component of plasma membrane”, “lipid metabolic process”, “negative
345 regulation of transcription”, “protein ubiquitination” and “response to cold”.
346 Therefore, these DEGs expression pattern changes together with the involvement of
347 “membrane”, “nucleus” induced by H_2S might be related to the browning inhibition.
348 In order to characterize the pathways that related to the browning inhibition by
349 H_2S treatment, we performed pathway enrichment analysis on DEGs based on the
350 KEGG database with $P < 0.05$ as the threshold. We found that 25/102, 11/98, 11/125
351 and 6/123 pathways demonstrated significant changes in S0 *vs.*C0, S6 *vs.* C6, C6 *vs.*
352 C0 and S6 *vs.* S0, respectively (Table S6). There were seven pathways that were all
353 enriched in the H_2S treatment versus the control at both stages of shelf life, including
354 two within carbohydrate metabolism and two representing lipid metabolism. The
355 pathways that were specifically enriched in S0 v*s.*C0 included five of carbohydrate
356 metabolism and six of amino acid metabolism. However, three pathways representing
357 biosynthesis of secondary metabolites (phenylpropanoid biosynthesis, flavonoid
358 biosynthesis and anthocyanin biosynthesis) were only found in S6 *vs.* C6. Two
359 pathways “Circadian rhythm-plant” and “Biotin metabolism” were found in both
360 control and H_2S treatment during storage. “Ubiquitin mediated proteolysis” had the
361 largest number of DEGs in S6 *vs.* S0.
362
363 3.4 Differentially expressed genes related to phenol peroxidases and phenolics
364
365 Surface browning of fresh-cut products results from the oxidation of phenolic
366 substances to quinones catalyzed by phenol oxidases (such as PPO and/or POD),
367 which subsequently condense to form browning polymers (Degl'Innocenti et al., 2005;
368 Saltveit, 2000). Suppression of *PPO* genes was an effective approach to reduce the

browning of potato tuber (Chi et al., 2014; Coetzer et al., 2001). In the same way, numerous studies have shown that the inhibition of surface browning of fresh-cut apple was accompanied by a decrease in PPO activity (Hemachandran et al., 2017; Saba and Sogvar, 2016). In the present study, one gene encoding PPO was up-regulated (*MSTRG.17066*, a 1.72 log_2FC significant increase) in the control at day 6 relative to day 0, but no significant change in *PPO* expression was observed in H_2S treatment during storage (Fig. 5). H_2S treatment showed significant reductions in gene expression of *PPO* relative to the control at day 0 (*MSTRG.17063*, a 2.2 log_2FC significant reduction) and day 6 (*MSTRG.9035*, a 1.9 log_2FC significant reduction). This result fits with the observed pattern of PPO activity of fresh-cut apple (Fig. 2). The PPO activity in the control samples at day 6 was higher than that at day 0 and H_2S treated samples showed consistently lower PPO activity than the control.

The POD enzyme, which uses H_2O_2 as a catalyst for the oxidation of phenolic compounds (Reyes et al., 2007), could also enhance browning reactions in the presence of ongoing PPO-mediated browning (Richard-Forget and Gauillard, 1997). In the present study, two *POD* genes (*MSTRG.11943, MSTRG.22268*) were induced, while one *POD* (*MSTRG.27890*) was repressed by H_2S treatment. *MSTRG.22268* was up-regulated and *MSTRG.27890* was down-regulated in the control at day 6 relative to day 0, but they did not differentially expressed in the H_2S treatment during storage. *MSTRG.11943* was down-regulated in the H_2S treatment but was not differentially expressed in the control during storage. Three genes encoding POD (*MSTRG.28170, MSTRG.6438, MSTRG.898*) were repressed in S6 *vs.* C6. Only *MSTRG.6438* was found differentially expressed in both control (a 2.11 log_2FC significant increase) and H_2S treatment (a 1.78 log_2FC significant increase) during storage (Fig. 5). Therefore, H_2S treatment was effective at repressing induction of *MSTRG.6438* that would

394 normally be expressed at a later stage post-cutting, which might be related to the
395 browning inhibition. It is unclear why fresh-cut apple has different copies of POD
396 genes and showed different expression patterns in H_2S treatment relative to the
397 control at two stages. We speculated that the cutting process induced the production of
398 ROS, but H_2S treatment stopped the induction of the POD that would normally use
399 H_2O_2 as a catalyst for oxidation of phenolic compounds. Six-day storage led to
400 accumulation of ROS in control tissues, providing a higher concentration of H_2O_2 and
401 enabling significant levels of POD-mediated polyphenol browning. However, H_2S
402 treatment was not able to completely inhibit either the expression of *POD* genes or the
403 enzymic activity of POD. There is also a debate on the correlation between POD
404 activity and browning reactions of fresh-cut fruit and vegetable in previous reports
405 (Liu et al., 2019; Sun et al., 2015) and leads to the conclusion that the function of the
406 *POD* gene family needs to be further studied.

407 The biosynthesis of phenolics, which are the substrates of enzymatic browning
408 reactions, involves a complex network of routes based on the shikimate and
409 phenylpropanoid pathways. Genes encoding chorismate mutase (CM), chorismate
410 synthase (CS), PAL and shikimate O-hydroxycinnamoyltransferase (HCT), which
411 involved in the shikimic acid pathway and subsequent conversion of chorismate to
412 phenylalanine, and phenylpropanoid pathways were all up-regulated in S0 vs. C0 or
413 S6 vs. C0, while *bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase*
414 (*3DD, SD*), *4-coumarate-CoA ligase* (*4CL*), were down-regulated. The implication of
415 these findings is that H_2S treatment actually induces the synthesis of phenolic
416 compounds and this is further supported by the biochemical assays which
417 demonstrated the significantly higher accumulation of several phenolic compounds
418 (chlorogenic acid, vanillic acid, catechin, tannic acid, quercetin), in H_2S treated

samples compared to the control during the storage. Chlorogenic acid and catechin were reported to be good substrates for PPO in apple (Amaki et al., 2011). Therefore, we concluded that H_2S mediated delay of browning of fresh-cut apple is not through reduction of the phenolic substrates.

3.5 Differentially expressed genes related to lipid metabolism

Although the eventual browning symptoms occur because of the interactions between PPO and/or POD enzymes and phenolic substrates, the enzymes and substrates are ordinarily separated from each other by intracellular membranes. Docimo et al. (2016) has reported that there was a lack of correlation between flesh browning and PPO activity or phenolic content in cut eggplant. Similarly, Cantos et al. (2002) has found that PPO activity and phenolic compounds are not rate-limiting in browning development of fresh-cut potatoes. Li et al. (2017) has reported that the membrane may be an important factor in browning of fresh-cut pear and the browning was caused by the cell membrane degradation which damaged the cell compartmentalization. Therefore, the progress of the browning reaction was thought to be related to the processes that affect the membrane integrity. Besides the loss of cell integrity due to processing, the breakdown of membranes within cells of fruit tissues during storage also causes the loss of cellular compartmentalization, and subsequently increases the degree of browning (Toivonen and Brummell, 2008, Li et al., 2017). Since lipids are essential constituents of the cell membrane, any alteration of membrane lipid composition such as the decrease of unsaturated fatty acids, the increase of saturated fatty acids, or degradation of membrane phospholipids, may change the biophysical and/or biochemical membrane properties and damage the

integrity of membrane (Saquet et al., 2003; Zhang et al., 2018)). In the present study, of the 13 DEGs related to the fatty aicid metabolism in the control samples, three were up-regulated at day 6 compared to day 0, whereas ten were down-regulated over the same storage period. With the addition of H_2S treatment, 12 genes were differentially expressed, with ten up-regulated at day 6 compared to day 0, and two down-regulated over the same storage period. Among the DEGs in the control and H_2S treatment during storage, only two genes were in common, which were up-regulated in both samples. Comparing the DEGs in H_2S treatment to the control found that most genes (19 out of 21) were down-regulated at day 0, but three out of four genes were up-regulated at day 6 (Table S7). Glycerophospholipid metabolism also changed after H_2S treatment, 11 (six genes were up-regulated and five genes were down-regulated) and 20 DEGs (eight genes were up-regulated and 12 genes were down-regulated) were found in the control and H_2S treatment at day 6 relative to day 0, respectively. There were 37 and 10 genes differentially expressed in S0 *vs.* C0 and S6 *vs.* C6, respectively (Table S8).

LOX and PLD are two important lipids-degrading enzymes, which induce the degradation of unsaturated fatty acids and phospholipids, respectively. Mellidou et al. (2014) revealed that the expression of genes encoding LOX and PLD increased when browning developed during the storage of apple. In the present study, we did not find the same result as the literature shown, one *LOX* and one *PLD* were down-regulated in the control at day 6 relative to day 0, which was not differentially expressed in the H_2S treatment during storage. But comparing the H_2S treatment to the control, three genes encoding PLD and two genes encoding LOX were down-regulated at day 0 and two of these *PLD*s and one *LOX* maintained low expression by day 6 of storage (Fig. 5). The down-regulation of these genes might increase the content of unsaturated fatty

acids and phospholipids in H_2S treated material, thus retarding the damage of cellular membrane structure that would otherwise occur in the control. H_2S treatment reduced LOX activity and lipid peroxidation has also been observed in other fresh-cut fruit (Gao et al., 2013; Zheng et al., 2016). It can thus be suggested that H_2S treatment could suppress the activation of membrane lipids-degrading enzymes, possibly at a level of transcriptional regulation by inhibiting the expression of genes *LOX* and *PLD*, which in turn might retard the degradation of cell membrane lipids and maintain the integrity of cell membrane structure.

3.6 Differentially expressed genes related to antioxidant systems

Mechanical injury by cutting processing increased the production of reactive oxygen species (ROS), which induce membrane lipid peroxidation and promote browning reactions. Antioxidant enzymes and antioxidants inside the fruit tissues are necessary for ROS detoxification (Møller, 2001). Enhancing enzymatic and non-enzymatic antioxidant capacities to alleviate the oxidative damage is one way to inhibit the browning of fresh-cut fruit and vegetable during storage (Hu et al., 2014b; Zheng et al., 2016). H_2S treatment induced the expression of genes encoding GST, as 16 and 6 *GSTs* were found up-regulated in H_2S treatment relative to the control at day 0 and day 6, respectively. Six and two *GSTs* were up-regulated and down-regulated in the control at day 6 relative to day 0, respectively. Seven of these genes remained unchanged in the H_2S treated samples over the same storage period, but five detected *GSTs* in the H_2S treatment were all down-regulated. These results indicated that H_2S treatment activated the GSTs expression at an earlier stage, but most of them have less longevity (Fig. 5). Besides, one gene encoding CAT and one gene encoding SOD all

494 showed significantly higher expression, while two *APXs* showed significantly lower
495 expression in tissue from the H_2S treatment compared to control at day 0.
496 Physiological results supports this, the activities of SOD and CAT increased while
497 APX activity decreased after H_2S treatment (Fig. 2). After 6 d of storage, only one
498 APX-encoding gene was up-regulated, whilst one gene encoding APX and one
499 encoding SOD were down-regulated in H_2S treatment relative to the control. There
500 were three and two *SOD*s down-regulated in the control and H_2S treatment,
501 respectively. Therefore, it is reasonable to see that the number of up-regulated DEGs
502 involved in antioxidant systems was greater in S0 *vs*. C0 compared to those in S6 *vs.*
503 C6 (Fig. 5), implying that the antioxidant capacities of H_2S treated sample at day 0
504 were higher than at day 6.

505 AsA is an antioxidant that is able to interact with ROS directly or together with
506 antioxidant enzymes. AsA plays an important role in the development of browning.
507 Exogenous application of AsA effectively inhibited the browning of fresh-cut fruit
508 (Yan et al., 2017) and browning does not occur unless the endogenous AsA
509 concentration falls below a certain threshold value (Franck et al., 2007). In the plant
510 cell, AsA can be continuously oxidized through enzymatic (APX and AO) or
511 non-enzymatic reactions and can be reduced back to AsA by recycling reductases
512 (DHAR, MDHAR) (Cocetta et al., 2014). The AsA recycling pathway has been
513 evidenced as important for the re-establishment of AsA in horticultural crops
514 (Mellidou and Kanellis, 2007). Although *APX* in fresh-cut apple was down-regulated
515 in S0 *vs.* C0, the expression of *AO* and *MDHAR* was up-regulated in S0 *vs.* C0, while
516 they were unchanged after 6 d of storage. In the control sample, one *AO* was
517 up-regulated at day 6 relative to day 0, but three *AOs* were down-regulated and two
518 *MDHARs* were up-regulated in the H_2S treatment during storage (Fig. 5). This result

519 suggested that AsA recycling pathway was triggered by H_2S, but it did not work
520 efficiently after storage. The transcriptome results corresponded well to the content of
521 AsA. We observed a decrease of AsA in fresh-cut apple during storage, regardless of
522 treatment, but H_2S treatment significantly inhibited the decline. Overall, the
523 up-regulation of antioxidant enzymes and AsA recycling-related gene expression
524 aided ROS scavenging, as we observed that H_2S significantly alleviated the
525 accumulation of H_2O_2 and maintained it at a lower level compared with the control
526 throughout storage. The ability of H_2S treatment to increase the antioxidant capacity
527 and reduce the accumulation of ROS has also been reported in various fresh-cut fruit
528 and vegetable (Sun et al., 2015; Zheng et al., 2016). Therefore, we concluded that H_2S
529 treatment delayed browning of fresh-cut apple might be associated with its effect on
530 enhancing the antioxidant defense capacity, which reduced the oxidative damage from
531 ROS.
532
533 3.7 Differentially expressed genes related to energy metabolism
534
535 An increasing body of evidence has demonstrated that the browning of
536 postharvest horticultural crops is often accompanied by lower levels of ATP content
537 and energy charge (Franck et al., 2007; Lin et al., 2018). ATP, as an "energy flux",
538 plays an important role in lipid synthesis and cell membrane restoration. Once ATP
539 production rate is lower than a certain threshold, free fatty acids will be released from
540 phospholipids and the cell membrane structure will be damaged (Rawyler et al., 1999),
541 which allows PPO to mix with phenolics and react to produce browning compounds.
542 Futhermore, ATP also plays an important role in AsA cycling. ATP deficiency can
543 lead to ROS accumulation, which resulted in membrane lipid peroxidation and the

membrane structural disruption. Adequate ATP is beneficial to inhibit flesh and peel browning of fruit (Saquet et al., 2003). In postharvest fruit, ATP is mainly generated by oxidative phosphorylation. In our work, 62.5% of DEGs related to oxidative phosphorylation were down-regulated in the control at day 6 relative to day 0. However, in H_2S treatment, that percent of down-regulated DEGs was reduced to 31.8% (Table S9), indicating that H_2S treatment retained oxidative phosphorylation capacity, which might be related to promoting browning inhibition. ATPase and NAD are key enzymes for oxidative phosphorylation (Wang et al., 2018). Jiang et al. (2012) has reported ATPase was down-regulated after fresh-cut lotus tissue browning. Transcript levels of the genes encoding the *Ph*ATPase γ and *Ph*ATPase α subunits decreased during *Phalaenopsis* explant browning (Xu et al., 2015). Seven genes encoding ATPase (include calcium-transporting ATPase, copper-transporting ATPase etc.) and three genes encoding NAD were induced after H_2S treatment (S0 *vs.* C0). With the prolonged storage time, the energy production capacity was gradually reduced. However, the expression of two *ATPase* and one *NAD* gene remained up-regulated in H_2S treated tissue relative to control after six days of storage (Fig. 5). Therefore, the up-regulation of these genes encoding energy metabolism enzymes involved in oxidative phosphorylation by H_2S treatment might increase the synthesis of ATP and thus provide enough energy for maintaining the cell membrane structure of fresh-cut apple.

The respiratory pathways, such as glycolysis, tricarboxylic acid (TCA) cycle, mitochondrial electron transport chain (ETC) also supply energy to maintain normal metabolism in postharvest fruit and vegetables. Most DEGs related to the respiratory pathway were down-regulated in the control fresh-cut apple during storage, while in the H_2S treated sample, the number of up-regulated DEGs in glycolysis and TCA

569 increased (Table S10 and Table S11). H_2S treatment therefore appears to promote the
570 retention of metabolic processes linked to energy generation in stored fresh-cut apple.
571 At day 0, H_2S treatment induced the expression of genes encoding sucrose synthase
572 (*SUS*) while it repressed the expression of genes encoding fructokinase as compared
573 to the control (Fig. 5). It is suggested that SUS was used to cleave sucrose to provide
574 hexose-phosphates instead of using hexokinases, which is an ATP consuming process.
575 This alteration could save energy by using only one molecule of PPi instead of two
576 molecules of ATP (Boeckx et al., 2019). Three genes encoding
577 6-phosphofructokinase, one of the rate-limiting enzymes in glycolysis, were
578 up-regulated by H_2S treatment. Aldehyde dehydrogenase genes, which encode a
579 critical enzyme for anaerobic respiration, were repressed by H_2S treatment. Thus, we
580 propose that H_2S regulated glycolysis provides substrate for ATP production, rather
581 than being consumed by anaerobic respiration. In the TCA cycle most of the DEGs
582 (13/15) were down-regulated in S0 *vs.* C0, indicating that TCA pathway of fresh-cut
583 apple was repressed by H_2S treatment (Table S11). Furthermore, genes encoding
584 mitochondrial respiratory chain complex I, II and III were induced in S0 *vs.* C0 or S6
585 *vs.* C6 (Fig. 5), which might be expected to result in the elevated synthesis of
586 energy-generating molecules. It is worth noting that excessive respiration would also
587 consume metabolic substances and lead to aging and senescence. Therefore,
588 maintaining proper respiration intensity and simultaneously supplying sufficient
589 energy play important roles in the inhibition of browning. It can be assumed that
590 H_2S-inhibited browning of fresh-cut apple could be related to the reduction of
591 respiratory intensity via conserving higher levels of energy in the form of ATP. This
592 was supported by the previous reports, which confirmed that exogenous H_2S
593 treatment significantly reduced respiration rates whilst concomitantly inducing higher

energy production (Hu et al., 2012).

3.8 Validation of RNA-Seq by qRT-PCR

A total of 10 genes with differing expression patterns were selected to validate the RNA-Seq data by qRT-PCR evaluation using specific primers (Table S1). As shown in Fig. 6, correlation analysis of the gene expression ratios showed a good correlation (R^2 = 0.92886) between qRT-PCR and RNASeq, indicating the high reliability of the RNA-Seq data obtained in our study.

4. Conclusions

Surface browning, induced by mechanical damage, is a complex and highly regulated process. H_2S treatment effectively inhibited surface browning and maintained the initial color of fresh-cut apple during the storage. Transcriptome analysis revealed that the browning inhibition by H_2S was mainly related to the regulation of lipid metabolism, antioxidant system, and energy metabolism. The results are helpful to understand the browning inhibition mechanism conferred by H_2S on fresh-cut apple, which is beneficial for the technological innovation of browning prevention and amelioration for fresh-cut fruit.

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Notes:

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822 **Figures**

823 Fig. 1 Effects of H_2S treatment on the (A) appearance and (B) browning index of
824 fresh-cut apple during storage at 4 °C.

825 Fig. 2 Physiological changes of fresh-cut apple after H_2S treatment during storage at
826 4 °C. (A) PPO, (B) POD, (C) PAL, (D) SOD, (E) CAT, (F) APX and (G) GR activities,
827 (H) H_2O_2 content, (I) AsA content, (J) phenolic content.

828 Fig. 3 Differentially Expressed Genes (DEGs) between samples. (A) Numbers of up-
829 and down-regulated DEGs in S0 *vs.* C0, S6 *vs.* C6, C6 *vs.* C0 and S6 *vs.* S0. Venn
830 diagrams showing common DEGs in S0 *vs*. C0 and S6 *vs.* C6 (B), C6 *vs*. C0 and S6
831 *vs.* S0 (C).

832 Fig. 4 The top 15 enriched GO terms ($P < 0.05$) of biological process, cellular
833 component and molecular function in S0 *vs.* C0 (A), S6 *vs.* C6 (B), C6 *vs*. C0 (C) and
834 S6 *vs.* S0 (D).

835 Fig. 5 Proposed model for the browning inhibition of fresh-cut apple by H_2S.

836 Fig. 6 Correlation analysis between RNA-seq and qRT-PCR data.

837 **Supplementary material**

838 Fig. S1 Gene expression pattern of the DEGs of the control and H_2S treatment at day
839 6 relative to day 0 in the common GO terms.

840 Table S1 Primers used in qRT-PCR for the validation of RNA-Seq data.

841 Table S2 Quality summary of sequencing data.

842 Table S3 The number of unigene and transcript identified in each sample.

843 Table S4 Uniquely expressed genes in each samples.

844 Table S5 Top 10 up- and down-regulated genes after H2S treatment.

845 Table S6 KEGG pathway classification of the DEGs in S0 vs. C0, S6 vs. C6, C6 vs.
846 C0 and S6 vs. S0

847 Table S7 DEGs involved in fatty acid metabolism.

848 Table S8 DEGs involved in Glycerophospholipid metabolism.

849 Table S9 DEGs involved in oxidative phosphorylation.

850 Table S10 DEGs involved in glycolysis.

851 Table S11 DEGs involved in TCA.

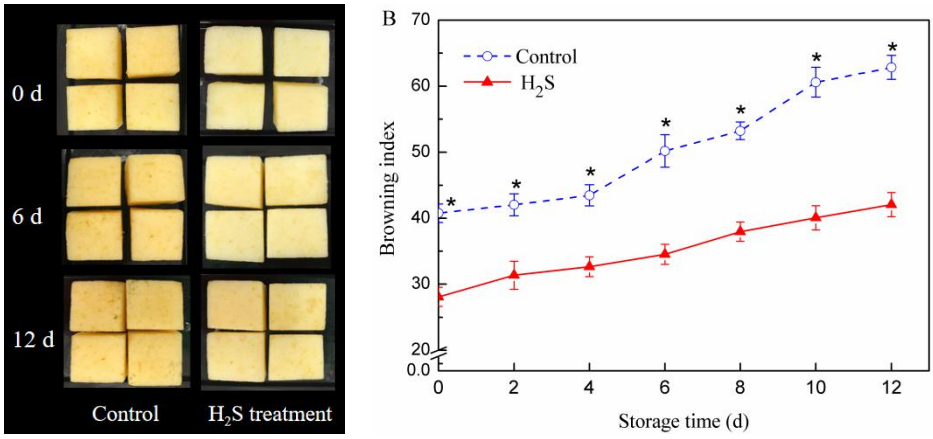


Fig. 1 Effects of H_2S treatment on the (A) appearance and (B) browning index of fresh-cut apple during storage at 4 °C. The error bars represent standard deviation (SD, n = 3). *: statistically significant differences ($P < 0.05$).

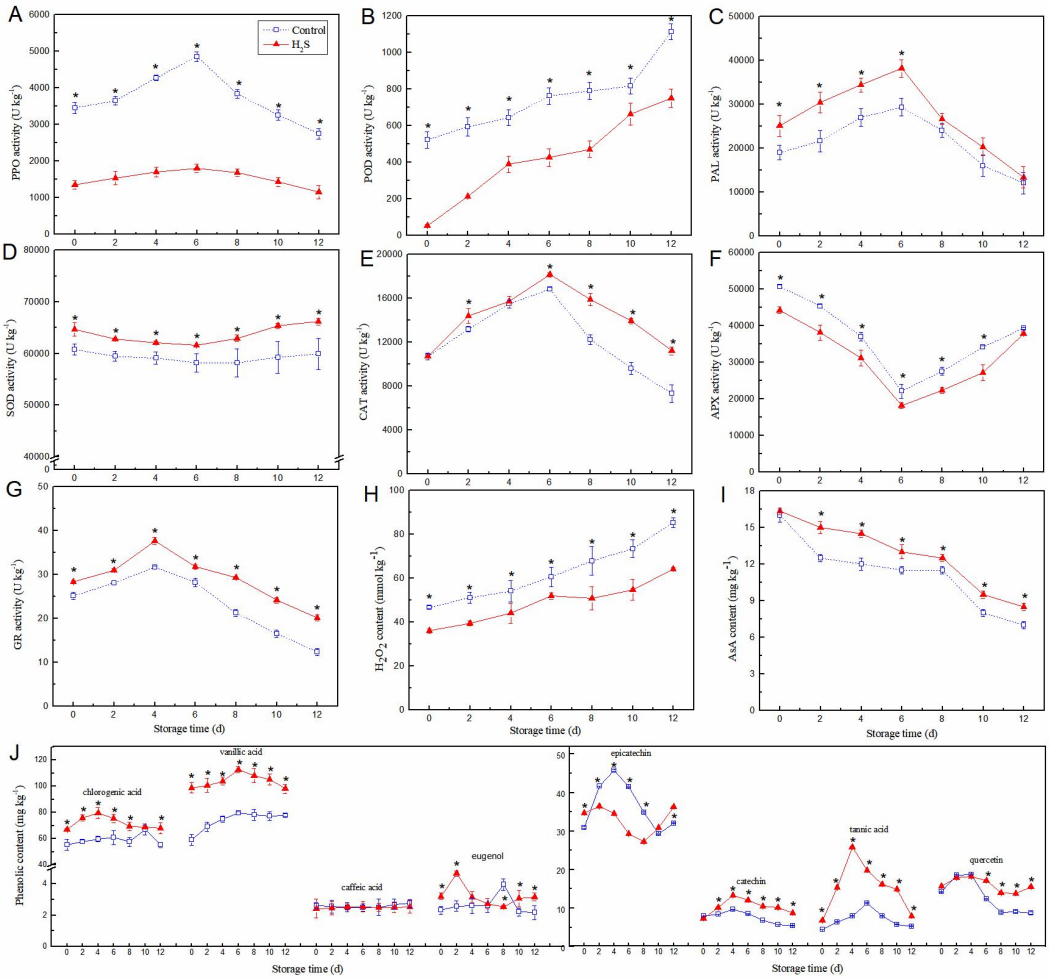


Fig. 2 Physiological changes of fresh-cut apple after H_2S treatment during storage at 4 °C. (A) PPO, (B) POD, (C) PAL, (D) SOD, (E) CAT, (F) APX and (G) GR activities, (H) H_2O_2 content, (I) AsA content, (J) phenolic content. The error bars represent standard deviation (SD, n = 3). *: statistically significant differences ($P < 0.05$).

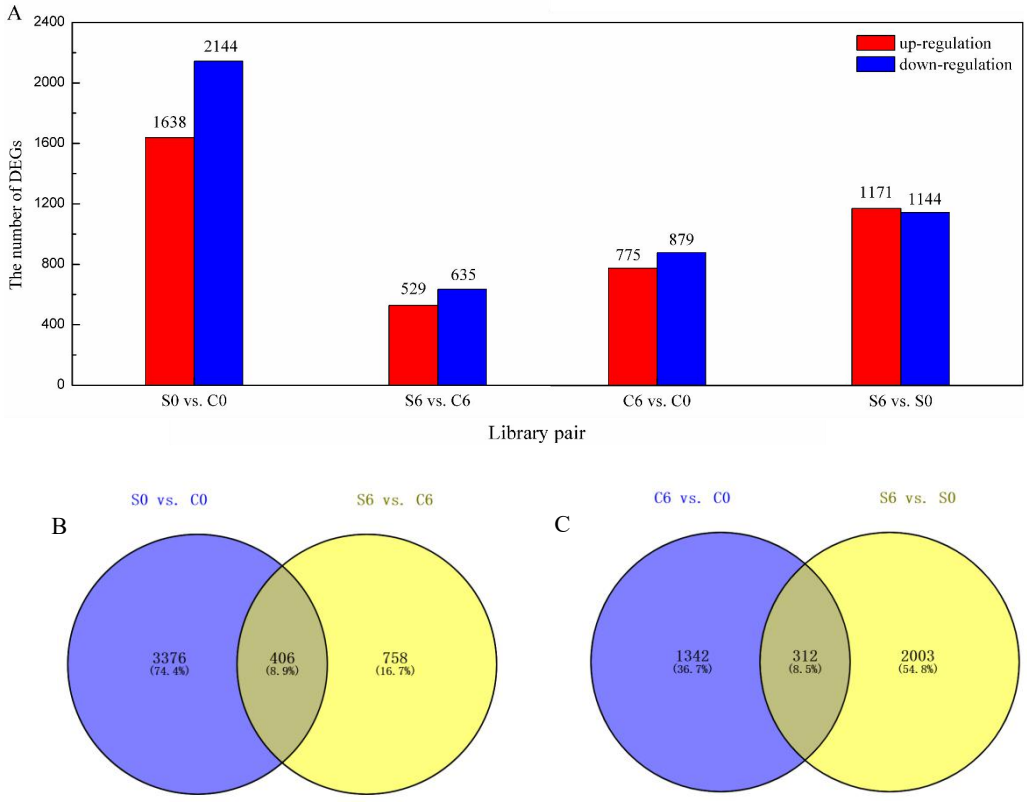
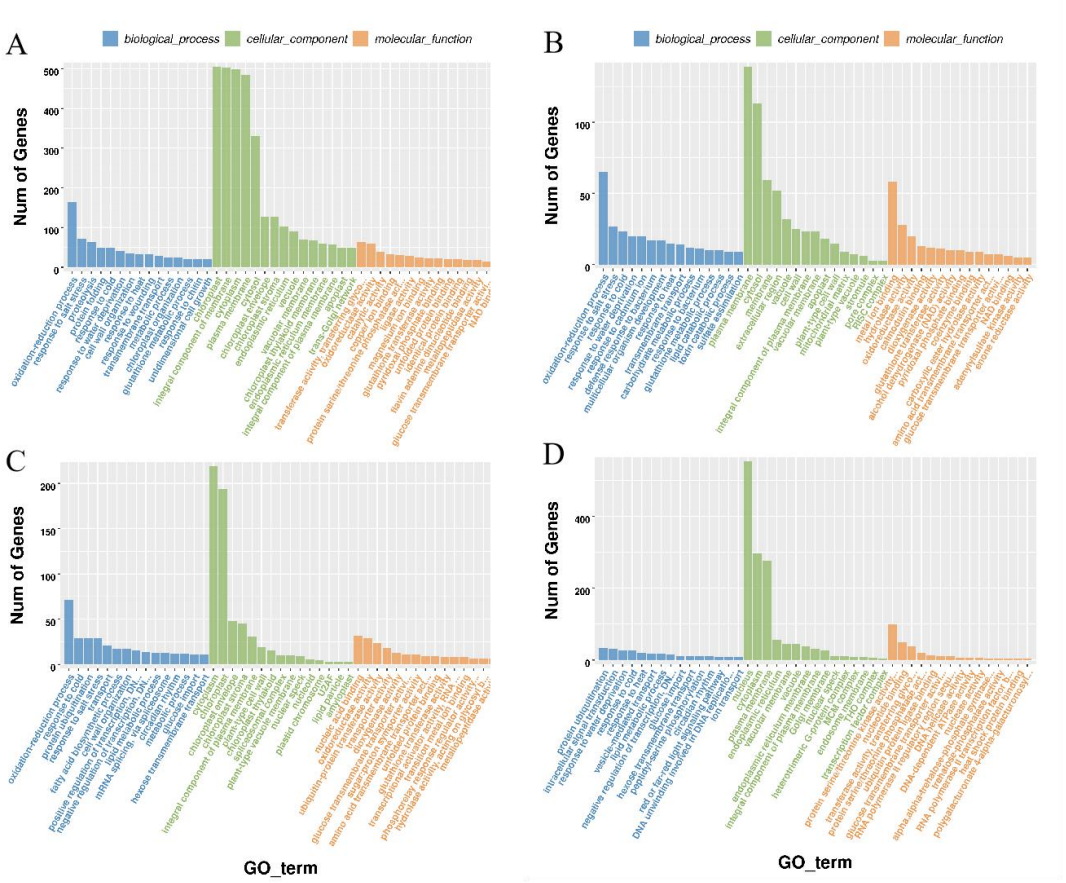


Fig. 3 Differentially Expressed Genes (DEGs) between samples. (A) Numbers of up- and down-regulated DEGs in S0 *vs.* C0, S6 *vs.* C6, C6 *vs.* C0 and S6 *vs.* S0. Venn diagrams showing common DEGs in (B) S0 *vs.* C0 and S6 *vs.* C6, (C) C6 *vs.* C0 and S6 *vs.* S0. DEGs were filtered with a cut-off of $|\log_2 FC| \geq 1$ and $P < 0.05$.



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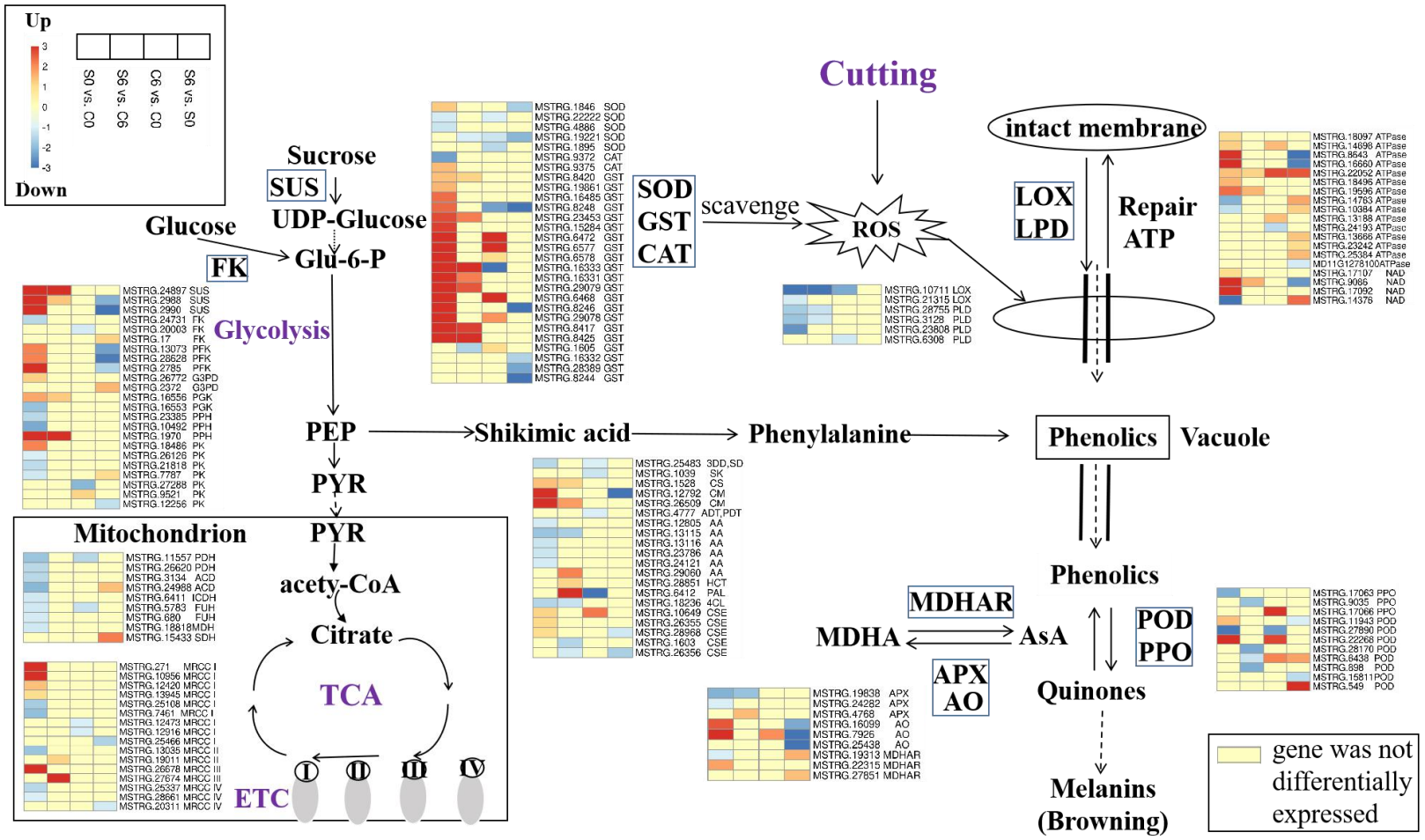

Fig. 5 Proposed model for the browning inhibition of fresh-cut apple by H_2S. Heat maps representing the expression patterns of DEGs, yellow

means gene was not differentially expressed ($P \geq 0.05$ or $-1 < \log_2FC < 1$). 3DD,SD: bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, 4CL: 4-coumarate-CoA ligase, AA: aspartate aminotransferase, ACD: aconitate hydratase, ADT,PDT: arogenate dehydratase/prephenate dehydratase, AO: ascorbate oxidase, APX: ascorbate peroxidase, AsA: ascorbic acid, CAT: catalase, CM: chorismate mutase, CS: chorismate synthase, FK: fructokinase, FUH: fumarate hydratase, G3PD: glyceraldehyde-3-phosphate dehydrogenase, GST: glutathione S-transferase, HCT: shikimate O-hydroxycinnamoyltransferase, ICDH: isocitrate dehydrogenase, LOX: lipoxidase, LPD: phospholipase D, MDH: malate dehydrogenase, MDHAR: monodehydroascorbate reductase, NAD: NADH dehydrogenase, PAL: phenylalanine ammonia-lyase, PDH: pyruvate dehydrogenase, PFK: 6-phosphofructokinase, PGK: phosphoglycerate kinase, PK: pyruvate kinase, POD: peroxidase, PPH: phosphopyruvate hydratase, PPO: polyphenol oxidase, SOD: superoxide dismutase, SDH: succinate dehydrogenase, SUS: sucrose synthase, SK: shikimate kinase.

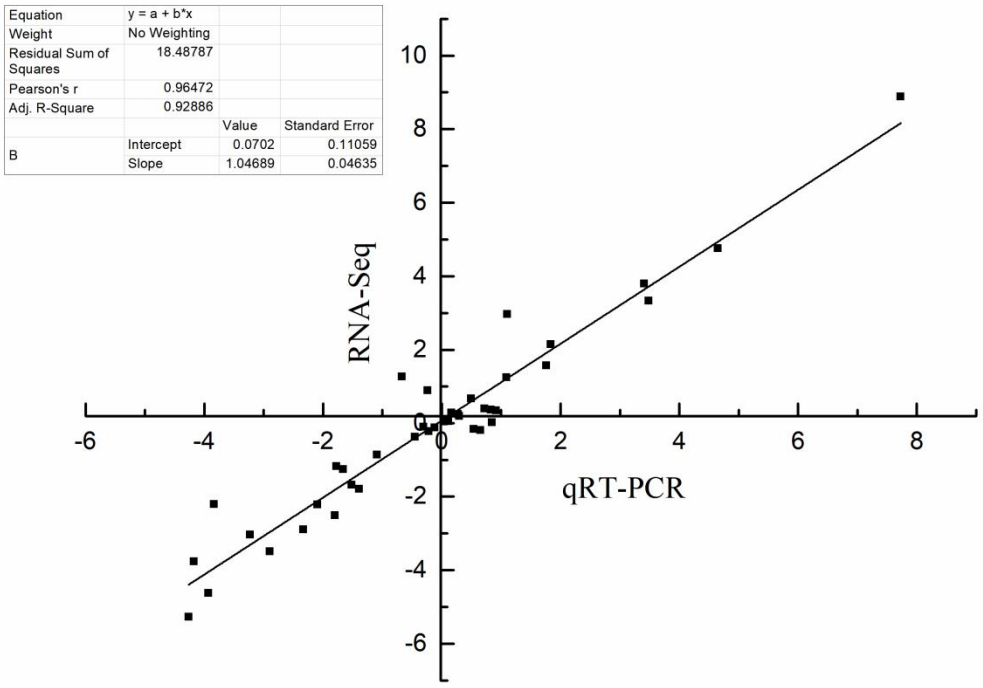

Fig. 6 Correlation analysis between RNA-seq and qRT-PCR data. Ten DEGs from the RNA-seq assay were used for qRT-PCR assay. The log_2FC obtained by qRT-PCR (X-axis) was plotted against log_2FC by RNA-seq (Y-axis).