Transcriptome and biochemical analyses of glutathione-dependent regulation of tomato fruit ripening

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ABSTRACT
Delay the ripening can improve fruit shelf life. Reduced glutathione (GSH) is an antioxidant that delays the ripening of fruits, though the GSH-mediated mechanism involved in fruit-ripening processes is currently unclear. This study used RNA sequencing to assess the GSH-induced transcriptional and biochemical alterations observed in tomato fruit during the post-harvest process. We found 970 differentially expressed genes (DEGs) after GSH treatment, and 124 were found to be candidate genes related to the ripening of GSH-mediated fruit. In addition, the expression levels of several candidate DEGs observed in ripe tomato fruit after GSH treatments were confirmed using quantitative real-time PCR. Biochemical analyses revealed that the GSH treatment decreased the proline content and the lipid peroxidation and ascorbate peroxidase activity levels. In contrast, it increased the superoxide dismutase, peroxidase, and catalase activity levels, as well as endogenous glutathione and ascorbic acid contents. These results confirm the important role played by GSH during the process of ripening tomato fruit.

1. Introduction
Glutathione (GSH), a thiol compound containing cysteine residues, functions in alleviating antioxidant stress-related toxicity, improving immunity, transporting and storing reduced sulfur, and maintaining the cell redox balance (Gill et al. 2013; Uys et al. 2014; Ge et al. 2019; Yao et al. 2021). Whereas GSH has roles in alleviating abiotic stresses, such as high salt (Zhou et al. 2019), high temperature (Nahar et al. 2015), and toxic metals (Anjum et al. 2012), recent studies have found it is also related to the regulation of genes. RNA sequencing (RNA-seq) can reveal GSH-responsive genes in various systems subjected to various physiological and stress environments (Hacham et al. 2014).

Tomato (Solanum lycopersicum L.) is an important economic and agricultural crop around the globe. Fruit ripening is an important process that makes the fruit appear attractive, which increases seed liberation and dispersal. Therefore, it is a genetic and closely coordinated process that can change flavor, appearance, smell, and texture (Srivastava et al. 2010). Fruit ripening is an oxidative phenomenon that involves alterations in the redox homeostasis of reactive oxygen species (ROS), including superoxide anion (O$_2^-$), hydroxyl radical (OH$^-$), and hydrogen peroxide (H$_2$O$_2$) (Huan et al. 2016). Recent biochemical findings have found that tomato fruits have a sulfhydryl-oxidative metabolism due to changes in key reactive ROS parts when the plant ripens from green to red (Morscher et al. 2015). Antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX), and non-enzymatic antioxidants, such as ascorbic acid (AsA) and GSH, are involved in ROS scavenging (Yao et al. 2021). Dehydrogenases, including malic enzyme (ME), 6-phosphogluconate dehydrogenase (6PGDH), glucose-6-phosphate dehydrogenase (G6PD), and isocitrate dehydrogenase (ICDH), produce the NADPH needed for the AsA-GSH cycle and increasing the antioxidant capacity (Yao et al. 2021). Other possible regulators, including abscisic acid (ABA), hydrogen sulfide, and nitric oxide, have been used for fruit ripening (Wang et al. 2013; Muñoz-Vargas et al. 2018; Luo et al. 2020). Research assessing how particular antioxidants affect the ripening process is still scant. In this study, the transcriptome profiles of ripening tomatoes were identified using high-throughput RNA-seq to analyze the GSH mechanisms involved in ripening. Candidate genes involved in GSH-mediated tomato fruit ripening are related to the following biological
processes: cell wall metabolism and modification, ripening-associated transcription factors, plant hormone metabolism and response, and antioxidant systems. These findings lay the groundwork for additional study of postharvest changes in color during tomato fruit ripening due to GSH.

2. Materials and methods

2.1. Plant material

The tomato (Solanum lycopersicum Mill. cv. MicroTom) plants were grown in an incubator under the following conditions: 16-h day (25°C) and 8-h night (17°C) at 70% relative humidity. Fruit from 80 different plants was harvested at the green immature stage. These fruits were all the same shape, size, and color.

Reduced GSH was purchased from Sigma (Chemical Co.St.Louis, MO, USA) and used as a tomato green immature stage treatment. Briefly, 150 fruit were immersed in an aqueous GSH solution (5 mM) in a box for 30 min, and 150 fruit were immersed in distilled water as the control. They were then stored at 25°C for 21 days (Figure 1). The concentration of GSH was selected according to the results of preliminary experiments (Table S1; Fig.S1). Three replicates for each treatment. Afterward, the samples were frozen in liquid nitrogen and maintained at −80°C until analyzed.

2.2. RNA extraction, library construction, and sequencing

According to the manufacturer’s instructions, a TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. An Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used to analyze the quality of the RNA, which was then confirmed with RNase-free agarose gel electrophoresis. Following extraction, Oligo (dT) beads were used to enrich eukaryotic mRNA, while a Ribo-Zero™ Magnetic Kit (Epicenter, Madison, WI, USA) was used to remove rRNA and enrich prokaryotic mRNA. A fragmentation buffer was used to fragment the enriched mRNA, which was reverse transcribed, using random primers, into cDNA. DNA polymerase I, RNase H, and dNTPs were used in a buffer to synthesize second-strand cDNAs, after which cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands). They were then end-repaired, poly(A) was added, and they were ligated to Illumina sequencing adapters. The resulting ligation products were chosen using agarose gel electrophoresis based on size, PCR amplified and sequenced by Gene De novo Biotechnology Co. (Guangzhou, China) using an Illumina HiSeq2500 system.

2.3. Sequence pre-processing and assembly

The reads produced by the sequencing system were raw reads with adapters or low-quality bases affecting their assembly and subsequent analyses. Thus, reads were further filtered using fastq software (version 0.18.0) to maintain high-quality clean reads (Chen et al. 2018) according to the following: 1) remove reads with adapters; 2) remove reads with >10% unknown nucleotides (N); and 3) remove reads with >50% low quality (Q-value ≤ 20) bases. A reference genome index was constructed, while HISAT2. 2.4 (Kim et al. 2015) was used to map paired-end clean reads to the reference genome; ‘-rna-strandness RF’ and other parameters were set to default. StringTie v1.3.1 (Pertea et al. 2015; Pertea et al. 2016) was used to assemble mapped reads from each sample using a reference-based approach. A fragment per kilobase of transcript per million mapped reads value was produced using StringTie software for each transcriptional location to analyze the variation and abundance of expression. The raw reads generated in this study were deposited in the NCBI database under accession number PRJNA787978.

2.4. Mapping and expression analysis

DESeq2 software was used to perform gene differential expression analyses between two groups, while edgeR was used for two samples (Love et al. 2014). In this study, genes and transcripts with absolute fold change ≥ 2 and false discovery rate (FDR) < 0.05 were differentially expressed genes (DEGs)/transcripts. GO terms that were significantly enriched in DEGs were identified using a gene ontology (GO) enrichment analysis, which was then classified by biological function (Ashburner et al. 2000). Each DEG was mapped to a GO term in the Gene Ontology database (http://www.geneontology.org/), while gene numbers were generated for each term. A hypergeometric test was used to identify GO terms significantly enriched in DEGs compared to genomic background. FDR correction was applied to the resulting p-values, with FDR ≤ 0.05 as the cutoff. Metabolic pathways or signal transduction pathways with significantly enriched DEGs compared to the whole genome were found using a KEGG pathway.
enrichment analysis (Kanehisa 2000). FDR correction was applied to the resulting p-values, with FDR ≤ 0.05 as the cutoff. Pathways that met all of these criteria were considered significantly enriched with DEGs.

To determine putative target genes for the ripening-associated TFs, promoter sequences for tomato were obtained by extracting a genomic sequence of 1000 bp upstream from the transcription start site (TSS) of each gene. PWMs for each TF were then used to scan promoter sequences using the Find Individual Motif Occurrences (FIMO) tool from the MEME Suite (v 4.11.2) (Olivares-Yañez et al. 2021), employing default parameters (p-value < 1×10e-4). The co-expression network analysis of ripening-associated TFs were differentially expressed in ripening tomato fruit using Cytoscape software.

2.5. DEG validation by a quantitative real-time PCR (qRT-PCR) analysis

To validate the transcriptomic results, 16 DEGs were detected in immature green fruit (Green) vs mature-period fruit without the GSH treatment (MP-GSH), Green vs mature-period fruit with the GSH treatment (MP + GSH), and MP-GSH vs MP + GSH were selected for a qRT-PCR analysis that used similar plant materials as in the RNA-seq. Genes were randomly selected based on their functions identified by this study and their high fold changes. Three biological replicates were performed for each sample, while three technical replicates were performed for each gene. A PrimeScript® II First-Strand cDNA Synthesis Kit (TaKaRa, Japan) was used to synthesize cDNA. Primer Premier 5.0 was used to design specific primers, while the length of the resulting amplified PCR products varied from 100 to 245 bp (Table S2). A CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) was used to conduct qRT-PCR, with SYBR Premix Ex Taq™ II (TaKaRa). Relative gene expression levels in various samples were assessed using the 2−ΔΔCt method. Relative DEG expression levels were assessed using green fruit as controls. Figures were constructed with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA).

2.6. Determination of proline, malondialdehyde (MDA), GSH, and AsA contents and enzyme activity assays

MDA concentration was measured to analyze lipid peroxidation, which formed thiobarbituric acid-reactive substances (Buege and Aust 1978). Ninhydrin assay (Bates et al. 1973) measured proline, spectrophotometrically at 520 nm. The ferricytochrome method (Yim et al. 1996) was used to assay total SOD activity (EC 1.15.1.1). POD (EC 1.11.1.7) activity was assayed according to the methods used by Ranieri et al. (Ranieri et al. 2000), while CAT activity (EC 1.11.1.6) was assayed by quantifying H2O2 levels (Aebi 1984). APX (EC 1.11.1.11) activity was assessed by measuring decreases in A290 owing to ASC oxidation by H2O2 (Mano et al. 2001). The GSH and GSH/GSSG contents in the fruit were determined in accordance with the procedure of Yu et al. 2002. AsA content was measured following the procedure of Costa et al. 2002.

2.7. Statistical analyses

One-way analysis of variance (ANOVA) was performed on the data, expressed as post-hoc multiple comparisons in SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) to assess the data. Means were analyzed at a P < 0.05 significance level using Duncan’s new multiple range test. The numbers used in the tables and figures are means ± SE (N = 3).

3. Results

3.1. Transcriptome sequencing analysis

After assessing the quality and filtering the data, 61.37 gigabytes (Gb) were obtained from the samples. Each sample had an average of approximately 6.82 Gb of clean data. Of the nine libraries, the GC% of the sequenced data varied from 41.77% to 42.19%, while more than 92.57% of reads had an average quality score exceeding 30 (Table S3). Based on this, the sequencing data was sufficiently accurate and of high enough quality for subsequent analyses. Table S3 displays general sequencing statistics, while the mapping efficiency of none tomato genome samples varied from 96.27%–96.98%. Later analysis of expression profiles only used uniquely mapped reads. Analysis of the correlation coefficients of each pair of biological replicates demonstrated highly consistent gene expression levels in each replicate (Fig. S2a), while replicates demonstrated consistent levels of expression (Fig. S2b), However, different samples were differentially expressed.

Figure 2. Analysis of gene expression during ripening and modulated by GSH in tomato fruits. (a) Analysis of differentially expressed genes (DEGs) between tomato fruits at immature stage (Green), mature period in untreated fruits (MP-GSH), and mature period in untreated fruits (MP + GSH). (b) Venn diagrams combining DEGs (up- and down regulated) among tomato fruits at different stages of ripening and under GSH treatment.
3.2. Transcriptomic analysis after GSH treatment

Assessing differences in gene expression in the MP-GSH and MP + GSH tomato fruit groups is one way of analyzing the effects of GSH treatment. However, our study used a more comprehensive approach by considering the green immature stage a reference (Figure 1). There were 8,316 DEGs (2,488 up-regulated and 5,828 down-regulated) identified in the Green vs. MP-GSH comparison. In the Green vs. MP + GSH comparison 8,210 DEGs (2,333 up-regulated and 5,877 down-regulated) were identified (Figure 2a). Additionally, DEGs can be classified based on their various expression patterns (Table 1), which allowed us to analyze the data and identify helpful biological information (Khatari et al. 2012) (Figure 2b).

3.3. Functions and processes influenced by the GSH treatment

GSH treatment effects can be difficult to identify based on genetic changes due to natural ripening. This varied with genes and groups. Genes that were up- or down-regulated in both comparisons (Green vs. MP-GSH and Green vs. MP + GSH) were considered to have expression not modulated by GSH. This was the case for 7,239 genes (1,981 up-regulated and 5,258 down-regulated) (Figure 2b). Some genes were up-regulated even without GSH application, leading us to assess biological processes, such as responses to stimuli, including redox state and oxidative stress, cell wall metabolism, and plant hormone metabolism. Additionally, this study identified important biological processes such as fruit ripening, oxidation-reduction, regulation of auxin-mediated signaling, and positive transcriptional regulation (Table S5). Conversely, we identified different categories among the down-regulated genes, which included carbohydrate, polysaccharide and cellular glucan metabolic processes, photosynthesis, and cytoskeletal protein binding (Table S6).

The genes identified from the Green vs. MP-GSH comparison, 507 up-regulated and 569 down-regulated, in which GSH constrained overexpression and repression, respectively. The application of GSH hid the overexpression of genes related to the following processes: protein metabolism-related regulation, negative organ and developmental growth regulation, oxidoreductase activity, and peroxiredoxin activity (Table S7). Furthermore, GSH mitigated the suppression of genes related to protein phosphorylation, lipid storage, response to oxygen-containing compounds, and calcium ion binding (Table S8).

3.4. Genes specifically affected by the GSH treatment

The expression of up- and down-regulated genes only located in MP + GSH (351 and 619, respectively) are modulated by GSH. Enrichment analysis of the 351 genes up-regulated by applying GSH is shown in Figure 3a. Closer analysis highlighted which genes relate to macromolecule modifications, such as phosphorylation, response to stimuli, such as kinase activity and WRKY proteins, responses to signal transduction, such as NADPH/NADP+ and GSH/GSSG, and regulation of defense responses to bacteria (Table S9).

Additionally, this study categorized 619 down-regulated genes (Figure 3b), including genes encoding proteins associated with biological processes such as metabolic processes, including gibberellin (GA), membrane lipid, and H2O2, as well as H2O2 catabolic processes. This study also identified molecular functions, such as binding and oxidoreductase activity (Table S10). Therefore, after 21 d, GSH application affected post-harvest storage more than gene expression patterns. This suggests that GSH regulates significant genetic alterations during tomatoes ripening process, which may occur in other crop fruit.

3.5. GSH treatment effects on genes involved in plant hormone metabolism and response, cell wall metabolism, antioxidant systems, and ripening-associated transcription factors (TFs)

GO and KEGG analyses demonstrated that most GSH-responsive DEGs were involved in plant hormone metabolism and response (Figure 4a, Table S11). GSH treatments affected the expression levels of plant genes related to hormone metabolism and response (Table S11; S12). Approximately 29 genes were related to hormone metabolism and response, of which 11, 1, 1, and 16 genes were related to the ethylene signal, GA, ABA and auxin signal pathways, respectively (Table S12). This study identified a variety of regulators essential to signal transduction and ethylene synthesis, such as ERS1, an ethylene receptor nerveripe (1), ETR2, an ethylene receptor (2), ERF3, an ethylene-responsive factor (1), LEA, an ethylene-responsive late embryogenesis-like protein (1), ACO, an ACC oxidase (3), and ACS, an ACC synthase (3) (Figure 4a). The expression levels of ERS1, ETR2, and ERF3, as well as ACS1/4, were significantly repressed after the GSH treatment, whereas LEA14-A, ACO1/4, and ACS2 were dramatically increased after the GSH treatment (Figure 4a). One GA-mediated signaling pathway gene was down-regulated and one salicylic acid (SA) synthesis gene was up-regulated after the GSH treatment. In total, 10 auxin response and transport genes were up-regulated after the GSH treatment, while auxin-responsive genes, including auxin response factors, PINs, auxin efflux carrier components, and IAA4, an auxin-responsive protein, were up-regulated following GSH application. The auxin-responsive protein SAUR76, auxin repressed/dormancy-associated protein DRMH1, auxin-responsive protein IAA27, and auxin-binding protein 1 T92 were down-regulated after the GSH treatment.

In total, 42 DEGs were involved in cell wall metabolism (Figure 4b), including tubulin alpha/beta chains (3), glycosyltransferases (G7s) (9), polygalacturonases (2), cellulose synthesis (3), xylanoglucan endotransglycosylase/hydrolases (XTHs) (7), expansins (2), O-fucosyltransferase 35 (1),

| Group | Description | Tendency | Gene number |
|-------|-------------|----------|-------------|
| 1     | Genes GSHt affected by the GSH treatment | Up-regulated | 1981        |
| 2     | Genes affected by the GSH treatment | Down-regulated | 5258        |
| 3     | Genes specifically affected by the GSH treatment | Up-regulated | 507         |
| 4     | Genes whose pattern of expression was changed by the GSH treatment | Down-regulated | 351         |
| 5     | Genes whose pattern of expression was changed by the GSH treatment | From down- to up-regulated | 619         |
| 6     | Genes whose pattern of expression was changed by the GSH treatment | Down-regulated | 1           |
dicting the binding site of belonging to analysis showed that in regulating tomato postharvest ripening. Co-expression further investigate how transcription factors were involved in regulating tomato fruit genes after the GSH treatment during fruit ripening. Conversely, HSFA5 demonstrated to be positive regulators after the GSH treatment during fruit ripening. Two bZIP factors, and two GATA factors, were down-regulated after the GSH treatment during fruit ripening (Figure 5b). Four MYB factors (two WRKYs, WRKY33, and WRKY70), two BHLHs (BHLH3 and BHLH47), three GATA factors (GATA5, GATA8, and GATA9), four HSFs (HSF3A, HSF3A, HSF3D, and HSF3C), two bZIPs (bZIP50 and HY5), NAC056, and MYB1, were demonstrated to be negative regulators after the GSH treatment during fruit ripening. Notably, several TFs were associated with fruit senescence, and most TFs were up-regulated after the GSH treatment during fruit ripening (Figure 5b).

As shown in Figure 4a, the proline content gradually increased at first and then decreased during the various fruit ripening stages assessed. The highest 2.20-fold increase happened in turning-color-period (TP) fruit, compared to green fruit. Importantly, MP + GSH and MP-GSH fruit each had proline contents like that of green tomato. However, the contents decreased in the GSH-treated fruit compared to the control, though this difference was not statistically significant.

MDA content slowly increased during stages of TP ripening; in yellow fruit, it increased 1.35-fold compared to green fruit (Figure 6b). Again, MP + GSH and MP-GSH fruit showed similar MDA contents, which were lower in the former than the latter. However, these differences were statistically not significant. MP + GSH and MP-GSH fruit both showed MDA contents similar to that of green fruit. These results confirmed that ripening is related to oxidative stress and that the application of GSH can decrease MDA content.

As shown in Figure 6c, the SOD activity level gradually increased as ripening proceeded, and MP + GSH fruit had slightly higher values than MP-GSH fruit. The POD activity was significantly lower in MP + GSH fruit than in the latter. However, these differences were statistically not significant. MP + GSH and MP-GSH fruit both showed MDA contents similar to that of green fruit. These results confirmed that ripening is related to oxidative stress and that the application of GSH can decrease MDA content.

As shown in Figure 6d, the CAT activity was significantly lower in MP + GSH fruit compared with MP-GSH fruit, but the difference was not statistically significant (Figure 6d). The CAT activity was significantly increased in MP compared with green fruit (Figure 6d), and there was a 1.46-fold increase in MP + GSH fruit compared with MP-GSH fruit.

As shown in Figure 6f, a maximum 5.49-fold increase in APX activity occurred in yellow fruit compared with green fruit. Intermediate APX activity values occurred in both MP + GSH and MP-GSH fruit. The APX activity was 2.47-
Figure 4. Differentially expressed genes (DEGs) with the annotated functions in plant hormone metabolism and response (a) and cell wall metabolism and modification (b) at different stages of ripening and under GSH treatment.

Figure 5. Differentially expressed genes (DEGs) with the annotated functions in antioxidant systems (a) and ripening-associated transcription factors (b) at different stages of ripening and under GSH treatment.
fold higher in MP + GSH fruit compared with green fruit. The values were slightly lower for MP + GSH fruit compared to the control, though these differences were not statistically significant.

As shown in Figure 6g, during ripening, the GSH content decreased at first and then increased during ripening, experiencing a 1.31-fold decrease in yellow (TP period) fruit to the control. Independent of the GSH treatment, MP fruit had the maximum GSH contents, with MP + GSH fruit having a slightly higher value. GSH/GSSG proceeded according to a similar pattern to GSH, though values were 10.77-fold lower in yellow (TP period) fruit compared to the control (Figure 6h). Independent of GSH treatment, the MP fruit, showed maximum AsA contents (Figure 6i).

### 3.7. Validation of RNA-seq-based gene expression

The expression trends in the two platforms for the 16 genes assessed using qRT-PCR and RNA-seq (Figure 7) were similar, suggesting that the expression profiles of genes in the RNA-seq assay were similar to the results obtained by the qRT-PCR analysis (Figure 7). Among the six genes involved in the plant hormone metabolism and response pathway, the increased expression levels of ACO1, ACS2, and IAA4 after the GSH treatment were positively correlated with fruit ripening. GSH treatments severely repressed ACS4, ERS1 and DRMH1 expression. Among the four genes related to cell wall metabolism, TUBA3 expression decreased but the levels of UGT91C1, TOG71, and β-GAL1 increased after GSH treatments, indicating that these genes play important parts during the fruit ripening process. Among the five genes involved in antioxidant systems, GSTU7, thioredoxin peroxidase IIB (PRXIIB), POD42, and APX1 decreased and glutathione peroxidase le-1 (GRXle-1) increased after the GSH treatment. The expression of the ripening-related TF NAC056 was positively correlated with fruit ripening after the GSH treatment.

### 4. Discussion

Plant antioxidants are essential for fruit ripening, while GSH is a plant antioxidant with an important role: delaying fruit ripening (Giovinazzo et al. 2004; Camejo et al. 2010; Palma et al. 2020). Therefore, a comprehensive understanding of the fruit post-harvest GSH-regulated ripening mechanism is both of scientific and economic importance. This study used RNA-seq to assess transcriptional and biochemical changes induced by GSH in post-harvest tomato fruit after a GSH treatment. The expression of 970 DEGs was exclusively modulated by GSH (Figure 3; Tables S9 and S10). 124 GSH-specific DEGs were involved in plant hormone metabolism and response, cell wall metabolism, antioxidant systems, and ripening-associated TFs (Figures 4 and 5; Table S12). qRT-PCR was used to confirm the expression levels of 16 candidate DEGs related to these pathways (Figure 7). In addition, GSH modulated the proline, glutathione, and ascorbic acid contents, lipid peroxidation level, and antioxidant enzyme activity level during ripening (Figure 6). These findings indicate that delays in post-harvest ripening due to GSH could be due to the direct or indirect regulation of genes related to cell wall metabolism, plant hormone metabolism and response, antioxidant systems, and ripening-associated TFs.

Plant hormones and antioxidants interact in regulating plant growth, development, and fruit ripening (Bahin et al. 2011; Datta et al. 2015). In this study, the GSH-regulated DEGs related to ethylene, GA, SA, and auxin pathways (Figure 4a; Table S12), indicating that these hormone-associated genes could regulate GSH-associated delay during the ripening of tomato fruits. Ethylene can trigger fruit ripening (Pech et al. 2012), which regulates fruit ripening by affecting ACS and ACO genes and fruit-specific polygalacturonase (Kou et al. 2021). GSH might be related to the ethylene biosynthesis pathway via positive modulation of ACO and ACS gene expression levels (Datta et al., 2015). However, our
findings demonstrate that applying GSH to tomato fruit reduced the expression levels of the ethylene biosynthetic genes ACS1/4, whereas ACO/ACO1/4 and ACS2 were dramatically up-regulated. These results suggest that these three homologous ACO genes, as well as the ACS gene, play essential but opposing roles in GSH response during tomato ripening, which could lower ethylene content following the application of GSH. We also found that three ethylene signaling genes, ERS1, ETR2, and ERF3, were down-regulated by the GSH application. At the receptor, ethylene action interference significantly represses the expression levels of several genes related to the ethylene signal pathway, including ERF, ETR, and ERS (Tadiello et al. 2016). In sweet cherry fruit, GAs is present during the onset of ripening, and exogenous GA3 applications delay ripening (Kuhn et al. 2020). Here, the gene encoding the key enzyme (RSI-1) involved in the GA-mediated signaling pathway was down-regulated, indicating that exogenous GSH treatment may decrease the GA level to delay tomato fruit ripening. The GSH treatment of tomatoes also changed levels of GSH-related gene expression, including PALA. PAL is involved in the phenylpropanoid pathway of SA synthesis, and SA can inhibit ethylene production in pear, thereby delaying fruit ripening (Chen et al. 2009; Shi et al. 2019). Auxin stimulates early receptacle expansion but inhibits later ripening, and involved in fruit development and inhibition of ripening (Iqbal et al. 2017; Guo et al. 2018). In a previous study, 12 auxin pathway genes were up-regulated by a GSH treatment, which suggested that exogenous GSH treatments may increase the auxin level to delay tomato fruit ripening.

Cell wall degradation is related to fruit quality and is differentially regulated during the development and ripening stages. At least 50 genes related to cell wall structure are expressed during tomato fruit development, which encode cellulose, hemicellulose, and pectins (The Tomato Genome Consortium 2012; Uluisik and Seymour 2020). Major transcriptomic changes were observed in DEGs related to cell wall metabolism when tomato fruit was treated with GSH treatment (Figure 4b; Table S12). In total, this study identified 42 DEGs related to cellulose synthesis, hemicellulose metabolism, cell wall metabolism, and sucrose metabolism, and 22 genes significantly increased during fruit ripening. The expression levels of GTs, which play important roles during the polysaccharide synthetic process, decrease as fruit maturation progresses (Keegstra and Raikhel 2001). GT expression levels in ripening apples are lower and more limited compared with those of genes involved in cell wall disassembly (Zhu et al. 2012). Similar to ripening delays in tomatoes following GSH application, there were significant increases in unigenes annotated as GTs in tomatoes.
treated with GSH compared to the control. Genes involved in hemicellulose showed expression profiles similar to those of genes involved in cellulose and sugar metabolism. Some genes, including five XTHs, EXLAI, O-fucosyltransferase 35, MUR3 and a KOR gene encoding hemicelluloses and β-GALI, which decreased during fruit ripening, were induced by the GSH treatments. In particular, expression of XTH23 dramatically increased after the GSH treatments, but showed a negative fold-change without the GSH treatment. Xyloglucan plays an important role in fruit softening, and it is expressed during fruit ripening (Keegstra and Raikhel 2001). These results suggest that GSH delays the ripening and decay of tomato plants by promoting cell wall assembly and preventing cell wall disassembly.

An increase in the proline content of fruit, including tomato, has been associated with various physiological processes, including senescence and ripening, indicating that it can be a proxy of fruit quality (Claussen et al. 2006). These results confirm that proline accumulates during the ripening stage and suggests that GSH is responsible for the negative regulation of proline biosynthesis. Our previous study suggested GSH decreases the proline content under salt-stress conditions to alleviate oxidative stress (Zhou et al. 2017). An increase in the MDA content indicates that lipid peroxidation increased and that plant cells were subjected to oxidative stress (Chou et al. 2012). Our findings confirmed that ripening is related to oxidative stress and that applying GSH can decrease the MDA content. Lipid peroxidation increases have been observed during the ripening process, which is related to decreased antioxidant enzyme activities (López-Huertas and Palma 2020). All these data agree with which is related to decreased antioxidant enzyme activities increases have been observed during the ripening process, GSH can decrease the MDA content. Lipid peroxidation that ripening is related to oxidative stress and that applying to oxidative stress (Chou et al. 2012). Our results suggest that GSH delays fruit ripening by regulating the ROS level and preventing cell wall disassembly.

Many TFs regulate fruit-ripening processes (Onik et al. 2018). After the GSH treatment, 21 ripening-associated TFs were differentially expressed in ripening tomato fruit. The expression levels of WRKY7/753/70, GATA8, HSFA3, bZIP50, and HY5 genes increased after the GSH treatments and decreased in the untreated fruit. WRKY53 regulates the expression of senescence-associated genes and acts at an upstream position in the WRKY-signaling cascade (Zentgraf et al. 2010). SA delays pear fruit senescence by up-regulating the WKY70 expression level (Changwal et al. 2021). The expression levels of HSFA5, TCP14, VSF-1, and GTE-1 genes decreased after the GSH treatments and increased in the untreated fruit. The tomato bZIP gene, as corroborated in this work, is highly expressed during ripening (Lovissetto et al. 2013). The FvTCP9 gene is transiently expressed in cultivated strawberries (Fragaria × vesca) and demonstrated that FvTCP9 overexpression promotes the ripening process in fruit (Xie et al. 2020). However, how TCP14 affects fruit ripening remains unclear. This study suggests that GSH delays tomato fruit ripening by up-regulating WRKY7/53/70, GATA8, HSFA3, bZIP50, and HY5 and down-regulating HSFA5, TCP14, VSF-1, and GTE-1. In apple, MdMYB1 is transcriptionally regulatedMdERF3, a key regulator of ethylene biosynthesis, and involved in anthocyanin biosynthesis during fruit ripening (Gao et al. 2020). In our study, most AP2/ERF genes were predicted to have MYB1 binding sites. This indicates that GSH treatment may regulate tomato fruit ripening through MYB1 binding to ethylene-related transcription factors.

5. Conclusions

RNA-seq was used to identify variations in the transcription and biochemical levels of tomato fruits treated with GSH and a control, which allowed us to assess how GSH affects ripening in tomato fruits after being harvested. There were 970 DEGs that were significantly responsive to GSH, including 619 down-regulated genes and 351 up-regulated genes. Bioinformatics analyses demonstrated that GSH could delay the ripening of tomato fruits by regulating genes related to antioxidant systems, ripening-associated TFs, plant hormone metabolism and response, and cell wall metabolism. This study lays the groundwork for subsequent identification of genes related to the ripening of tomato fruits after they have been harvested, though the additional study of these candidate genes is needed to identify the GSH-mediated molecular mechanisms governing this complex process.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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