Identification of the cell wall proteins associated with the softening of *Lycium barbarum* L. fruit by using iTRAQ technology

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ABSTRACT

Excessive softening of *Lycium barbarum* L. (LBL) fruit can limit the storage and transportation of fresh fruit. To better understand the underlying molecular mechanisms of fruit softening in LBL, changes in the pre-climacteric (S1) and post-climacteric (S2) proteomes were investigated by iTRAQ methods. The 14-fold reduction in S2 fruit firmness compared to S1 was accompanied by increased respiratory intensity and degradation of cell wall components. A total of 258 differentially expressed proteins (DEPs) were identified, which were mainly associated with photosynthesis, carbohydrate, amino acids and fatty acids metabolism. From the functional proteomic analysis, enhanced energy metabolisms, such as glycolysis/glucogenic and TCA cycle contributed to cell wall degradation and conversion to substrates for respiratory metabolism, leading to fruit softening. These findings have provided new insights into the molecular pathways associated with fruit softening in LBL and the bioinformatics analyses provided insightful information for further transcriptional studies.

1. Introduction

*Lycium barbarum* L. (LBL) is a typical melting flesh fruit that undergoes rapid ripening prior to harvest. Unfortunately, this softening is negative for the LBL and can reduce the nutritional and sensory qualities of the fruit (Liu et al., 2020, 2021; Liu, Liu, Li, & Zhao, 2020). The characteristics of fruit softening differ among species and cultivars, and are largely determined by cell wall modifications that are generally attributed to the disassembly of the cellulose and hemicellulose network through depolymerization of pectin and hemicellulose (Chea et al., 2019). The fruit cell wall is composed of cellulose, non-cellulosic wall polysaccharide polymers, such as hemicellulose and pectin, and a small amount of protein (Bashline, Lei, & Gu, 2014). Hemicellulose attaches to cellulose microfibrils, forming the cellulose-matrix network that, together with lignin, gives the rigidity and strength to the cells. These cell wall components are synthesized at different locations and are assembled into a functional cell wall matrix that structurally supports cells and fruit (Xiao, Li, Jiang, Jiang, & Duan, 2019). The structure of the cell wall is required not only to be strong and rigid to provide the structural support for the fruit, but also to allow anisotropic cell expansion in a controlled manner. Therefore, various cell wall modifying enzymes and proteins are responsible for cell wall modifications during the ripening and softening of fruits, including polygalacturonase (PG), pectin methylesterase (PME), pectate lyase (PL), α-arabinofuranosidase (AF), β-galactosidase (GAL), α-mannosidase, β-xylanosidase and endo-1,4-β-xylanase (Defilippi, Ejsmontewicz, Covarrubias, & Gudenschwager, 2018; Goulao & Oliveira, 2008). Transcription factor-mediated regulation of softening-related gene expression is also involved in fruit softening (Gwanpua, Verlinden, Hertog, Nicolai, & Geeraerd, 2017). Thus, fruit softening is the result of cell wall modification caused by multiple factors, and the knowledge of cell wall mechanisms associated with these changes is fundamental for understanding how the cells control softening through cell wall synthesis and cell wall remodeling.

Given the complexity of the softening process, the use of tools that may allow an all-around evaluation of the molecular processes triggered within the fruit is important (Ricardo, Campos-Vargas, & Orellana, 2012). Proteomics may represent a prospective approach to revealing the complex physiological processes associated with fruit softening at the global protein level. An efficient and reliable quantitative method, isobaric tags for relative and absolute quantitation (iTRAQ), has been widely used for proteomic studies and facilitating more reproducible quantification and comprehensive elucidation of protein expression in an extremely complex biological system (Jiang, Kang, Feng, Yu, & Luo, 2012).

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2020). Recently, iTRAQ approaches have been generally used to explore the molecular mechanism underlying fruit ripening or softening in a range of species, including oriental melon (Guo, Xu, Cui, Chen, & Qi, 2017), Vitis vinifera (Martínez-Esteso, Vilella-Antó, Pedrezo, Valero, & Bru-Martínez, 2013) and pomegranate (Niu et al., 2018), leading to the characterization of proteins, enzymes, and physiological regulatory networks associated with ripening and softening. These studies have, however, mainly focused on multiple ripening and softening-related processes, no single process such as fruit softening caused by cell walls. Currently, the cell wall proteome has been studied in several fruit species, including Pyrus sinkiangensis Yu (Gong et al., 2020), banana (Xiao et al., 2019), Vitis vinifera (Martínez-Esteso et al., 2009). Limited information about the cell wall proteome concerning LBL fruit softening is available, and the cell wall proteomic approach has been generally recognized as a powerful tool for elucidating complex characteristics of fruit development, paving the way for its application in LBL.

In the study, a comparative proteomic analysis by iTRAQ technology was performed to investigate the differential expressed cell wall proteins between the pre-climacteric (S1) and post-climacteric (S2) stages. The study aimed to identify the biochemical processes associated with the softening process and proteins that may play important roles in the softening process of LBL fruit. This study presented the first cell wall proteome of LBL fruit, based on detailed proteomic data. We have also provided new insights into the dynamics of protein abundance changes, contributing to further understanding of the molecular mechanisms underlying regulation of softening-related genes.

2. Materials and methods

2.1. Fruit collection

*Lycium barbarum* L. (LBL) (Ningqi 1) fruit samples were obtained from a fruit orchard at the sixth team plantation of Helan Mountain Farm in Ningxia, China. We collected fruit from 20 trees (more than 10 years old), and approximately 50 fruits per tree, with about 1000 fruits per period, which were mixed well in perforated PE self-sealing bags. LBL was harvested in the morning and transported to the College of Agriculture, Ningxia University within 1 h after harvesting for the measurement of the fresh fruit firmness, respiration rate, and observation of the cell microstructure. Three portions of 50 g each of fruit from both stages of ripening were taken, frozen in liquid nitrogen, then stored at −80 ºC for the extraction of cell wall components and proteins.

2.2. Measurements of physiological parameters

Firmness was determined in 10 individual fruits on the lumbar side of the fruit using a TA.XT Plus firmness tester (Stable Micro Systems Manufacturing Co., London, UK) (Ren et al., 2020).

The respiration rate of fruit was determined using a fruit and vegetable respirometer (SYS-GH30A, Saiyas Technology Co., Dandong, China). Briefly, the respiration intensity meter was switched on and preheated for 30 min at room temperature (25 ºC). 30 g of LBL was placed in a 0.25 L cylindrical respiration chamber and the gas circulation pump was switched on; zeroed and the value x1 was recorded when the value was stable and x2 after 5 min. The respiration rate was calculated as follows.

\[
\text{Respiration rate \left[ \text{mg} \times (\text{kg} \cdot \text{h}) \right] = \frac{(x_2 - x_1) \times 0.25 \times 44 \times 1000 \times 60}{v \times m \times 5}
\]

where: \(x_1\), starting concentration of \(\text{CO}_2\) phase, \(\mu\text{mol}/\text{mol}\); \(x_2\), ending concentration of \(\text{CO}_2\) phase, \(\mu\text{mol}/\text{mol}\); 0.25, volume of cylindrical breathing chamber, L; 44, molar mass of \(\text{CO}_2\), g/mol; 24.45, molar volume of \(\text{CO}_2\) at 25 ºC, L/mol; m is the weight of the fruit used for the determination, kg; 1000, 60 and 5 are conversion factors.

Cell wall components were isolated in the form of alcohol-insoluble residue as described previously. Isolated cell wall components were fractionated into water-soluble pectins (WSP), CDTA-soluble pectins (ISP), Na2CO3-soluble pectins (CSP), KOH-soluble polymers (hemi-celluloses) and H2SO4-soluble polymers (celluloses) (Chea et al., 2019). Uronic acid contents in WSP, ISP and CSP fractions were determined by the them-hydroxy diphenyl method using galacturonic acid as a standard (Blumenkrantz & Asboe-Hansen, 1973). Hemicellulose and cellulose contents were measured using the anthrone method (D’Amour, Gosselin, Arul, Staighe, & Willemot, 2006) with glucose as a standard. The contents of galacturonic acid and glucose were measured using an UV-T6 spectrophotometer (Persee General Instruments Co., Ltd, Beijing, China).

2.3. Transmission electron microscopy (TEM)

Sample preparation of LBL for TEM was performed according to the method described by He et al. (2017). LBL flesh at the waist from the skin to endocarp was cut into slices of 3 mm × 2 mm. For TEM observations, all sections were examined under a H-7650 transmission electron microscope (Hitachi, Ltd. Tokyo, Japan) at 80 kV, and whole images were acquired.

2.4. Extraction of cell wall proteins

Cell wall proteins were extracted according to Xiao et al. (2019) with some modifications. Briefly, the samples of frozen LBL fruit tissue were finely powdered in liquid nitrogen and suspended sequentially with different concentrations of sucrose (0.4, 0.6 and 1.0 M) in ice-cold homogenizing acetate buffer (5 mM, pH 4.6) with mild stirring for 15 min. After each suspension, the mixture was centrifuged and the supernatant was then discarded. The grain was washed twice with the buffer and dried under a vacuum. Protein was extracted and purified from ground samples using a developed phenol extraction method, followed by ammonium TCA-acetone precipitation (Zheng et al., 2013). The protein yield was determined by Bradford protein assay, using bovine serum albumin as a standard. The protein samples were stored at −80 ºC before using.

2.5. iTRAQ labeling and SCX fractionation

Peptides were labeled with iTRAQ reagents according to the manufacturer’s instructions (SCIEX Pte. Ltd, Framingham, MA, USA). Each aliquot (100 µg of peptide equivalent) was reacted with one tube of iTRAQ reagent. After the sample was dissolved in 100 µL of 0.05 M TEAB solution, pH 8.5, the iTRAQ reagent was dissolved in 41 µL of anhydrous acetonitrile. The mixture was incubated at room temperature for 1 h. Then 8 µL of 5% hydroxylamine were added to the sample and incubated for 15 min to quench the reaction. The Multiplex labeled samples were pooled and lyophilized. The iTRAQ-labeled peptide mixture was fractionated using a Strata X (C18, 3.5 µm, 2.1 × 50 mm) (Thermo Fisher Scientific, Waltham, MA, USA) on an LC-20AB HPLC pump system (Shimadzu Corporation, Kyoto, Japan) at 0.3 mL/min. Buffer A consisted of 10 mM ammonium formate and buffer B consisted of 10 mM ammonium formate with 90% acetonitrile; both buffers were adjusted to pH 10 with ammonium hydroxide. A total of 30 fractions were collected for each peptide mixture, and then concatenated to 15 (pooling equal interval RPLC fractions). The fractions were dried for nano HPLC-MS/MS analysis.

After vacuum drying, the sample (100 µg) was digested with trypsin (SCIEX Pte. Ltd, Framingham, MA, USA) at 37 ºC for 16 h. The tryptic peptides were reconstituted in 0.5 M TEAB and peptide labeling was performed by iTRAQ reagent (SCIEX Pte. Ltd, Framingham, MA, USA). After 2 h of labeling reactions, the peptides were further purified using Strata X C18 (Thermo Fisher Scientific, Waltham, MA, USA), the labeled peptide mixtures were then multiplexed and vacuum dried. Strong cation exchange (SCX) chromatography was performed with an LC-
2.6. HPLC-MS analysis

The peptide mixture (5 μg) was resuspended in buffer A (0.1% formic acid, 84% acetonitrile) and centrifuged for 10 min at 20,000 g, with the final concentration at approximately 0.5 μg/μl. The mobile phase was acetonitrile and 0.1% formic acid-water solution, 10 μL supernatant was loaded onto an Easy LC HPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) by the autosampler onto a C18 trap column (Thermo Scientific EASY column, 10 cm, ID75 μm, 3 μm, C18-A2). The peptides were then subjected to nanoelectrospray ionization, followed by tandem mass spectrometry (MS/MS) in a Q-Exactive system (Thermo Fisher Scientific Inc, Waltham, MA, USA) coupled online to the HPLC.

2.7. Bioinformatics analysis

Raw data were converted to RAW files for further bioinformatics analysis, and the exported RAW files were searched by the local Mascot server using the Mascot 2.2 (Matrix Science Inc, London, UK) and Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc, Waltham, MA, USA). To reduce the probability of false peptide identification, after a Mascot
probability analysis using NCBI BLAST+ (NCBI-blast-2.2.28+-win32.exe), only those peptides at the E-value < 1e-3 confidence interval were counted as having been identified. Each protein identified with high confidence included at least two unique peptides. All proteins with a false discovery rate (FDR) < 1% were subjected to functional classification by the Clusters of Orthologous Groups of proteins UniProtKB (https://www.uniprot.org, FASTA database). Functional annotation and category analysis of the DEPs were performed using the online software Blast2GO Command Line (Version: go database_201608.obo download address: https://www.geneontology.org). Furthermore, the COG database (https://www.ncbi.nlm.nih.gov/COG/) and KEGG database (https://www.genome.jp/kegg/) were used to classify the identified proteins.

2.8. Quantitative real-time PCR (RT-PCR) analysis

The RT-PCR experiment was conducted using an CFX fluorescent quantitative PCR instrument (Bio-Rad Laboratories, Richmond, CA,
Table 1
Quantification of DEPs (mean ± SD) and fold change (FC) associated with fruit softening were screened for quantification.

| UniProt ID | Protein Name | S1    | S2    | P-value | Fold Change |
|------------|--------------|-------|-------|---------|-------------|
| A0A097P6G61 | Geranylgeranyl reductase | 0.865 ± 0.049 | 0.585 ± 0.016 | 0.032 | 0.676 |
| A0A0K12AP9  | Photosystem II CP47 reaction center protein | 0.860 ± 0.060 | 0.597 ± 0.038 | 0.006 | 0.694 |
| A0A0V0H5G8  | Ribulose biphosphate carboxylase small chain | 1.125 ± 0.093 | 0.613 ± 0.026 | 0.002 | 0.545 |
| A0A0V0H0G3  | Putative oxygen-evolving enhancer protein 3, chloroplastic-like | 0.744 ± 0.044 | 0.573 ± 0.059 | 0.030 | 0.770 |
| A0A0V0HHG7  | Cytochrome b6-f complex iron-sulfur subunit | 0.894 ± 0.049 | 0.636 ± 0.017 | 0.002 | 0.712 |
| A0A0V0UIJ1  | Cytochrome b559 subunit alpha | 0.691 ± 0.040 | 0.445 ± 0.028 | 0.002 | 0.644 |
| A0A0V0HPI2  | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial | 1.254 ± 0.045 | 1.563 ± 0.041 | 0.002 | 1.246 |
| A0A0V0HUH1  | Aldose 1-epimerase | 1.013 ± 0.004 | 0.727 ± 0.034 | 0.014 | 0.718 |
| A0A0V0FP8  | Putative pyruvate decarboxylase 1-like | 1.146 ± 0.068 | 1.690 ± 0.078 | 0.002 | 1.474 |
| A0A0V0IZL1  | Photosystem II D2 protein | 0.812 ± 0.051 | 0.428 ± 0.022 | 0.001 | 0.527 |
| A0A0V0J060  | Putative photosystem 1 chlorophyll A apoprotein-like | 0.805 ± 0.057 | 0.614 ± 0.066 | 0.037 | 0.763 |
| A0A142BLJ1  | ATP synthase subunit alpha, chloroplastic | 0.827 ± 0.016 | 0.630 ± 0.013 | 0.000 | 0.762 |
| B3RF2       | Chloroplast chlorophyll a/b-binding protein (Fragment) | 0.951 ± 0.130 | 0.629 ± 0.062 | 0.034 | 0.661 |
| C5MR70      | Chloroplast manganese stabilizing protein II (Fragment) | 0.747 ± 0.055 | 0.517 ± 0.032 | 0.007 | 0.692 |
| K4BB47      | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | 1.203 ± 0.028 | 1.564 ± 0.122 | 0.015 | 1.300 |
| K4CR9       | Chlorophyll a-b binding protein, chloroplastic | 0.565 ± 0.053 | 0.308 ± 0.072 | 0.015 | 0.546 |
| M1B9T8      | Aspartate aminotransferase | 1.030 ± 0.049 | 1.237 ± 0.079 | 0.034 | 1.201 |
| M1CD6       | Fructose-bisphosphate aldolase | 0.889 ± 0.034 | 0.703 ± 0.057 | 0.017 | 0.791 |
| P50433      | Serine hydroxymethyltransferase, mitochondrial | 1.107 ± 0.070 | 0.745 ± 0.085 | 0.010 | 0.673 |
| Q2VEF1      | Photosystem II reaction center protein H | 0.641 ± 0.069 | 0.495 ± 0.089 | 0.021 | 0.772 |
| Q70PN9      | Putative photosystem 1 reaction centre PSI-D subunit | 0.581 ± 0.039 | 0.435 ± 0.031 | 0.014 | 0.748 |
| Q85B4W      | Cytochrome f | 0.853 ± 0.117 | 0.698 ± 0.036 | 0.039 | 0.819 |
| Q99CA4      | Putative ferredoxin (Fragment) | 1.036 ± 0.024 | 2.110 ± 0.181 | 0.001 | 2.036 |

Continued on next page
| UniProt ID | Protein Name                                          | S1          | S2          | P-value | Fold Change |
|------------|-------------------------------------------------------|-------------|-------------|---------|-------------|
| M1B044     | Beta-hexosaminidase                                   | 0.877 ± 0.014 | 0.724 ± 0.056 | 0.020 | 0.826       |
| M1D0V6     | Fructose-bisphosphate aldolase                       | 0.889 ± 0.034 | 0.703 ± 0.057 | 0.017 | 0.791       |
| P50433     | Serine hydroxymethyltransferase, mitochondrial        | 1.107 ± 0.070 | 0.745 ± 0.085 | 0.010 | 0.673       |

**Amino acids biosynthesis and metabolism**

| UniProt ID | Protein Name                                          | S1          | S2          | P-value | Fold Change |
|------------|-------------------------------------------------------|-------------|-------------|---------|-------------|
| A0A0V08D11 | Putative 3-ketoacyl-CoA thiolase 2, peroxisomal-like  | 1.073 ± 0.034 | 1.448 ± 0.031 | 0.000 | 1.349       |
| F1DBB9     | Chloroplast polyphenol oxidase                       | 1.169 ± 0.078 | 0.741 ± 0.021 | 0.034 | 0.634       |
| K7QK65     | Adenosylhomocysteinase                               | 1.001 ± 0.022 | 1.256 ± 0.026 | 0.000 | 1.255       |
| M1A1T2     | D-3-phosphoglycerate dehydrogenase                   | 1.029 ± 0.029 | 0.801 ± 0.022 | 0.001 | 0.779       |
| M1B9T8     | Aspartate aminotransferase                           | 1.030 ± 0.049 | 0.727 ± 0.079 | 0.034 | 1.201       |
| M1C0V6     | Fructose-bisphosphate aldolase                       | 0.889 ± 0.034 | 0.703 ± 0.057 | 0.017 | 0.791       |
| P50433     | Serine hydroxymethyltransferase, mitochondrial        | 1.107 ± 0.070 | 0.745 ± 0.085 | 0.010 | 0.673       |

**Fatty acid biosynthesis and metabolism**

| UniProt ID | Protein Name                                          | S1          | S2          | P-value | Fold Change |
|------------|-------------------------------------------------------|-------------|-------------|---------|-------------|
| A0A0K1ZAP9 | Photosystem II CP47 reaction center protein           | 0.860 ± 0.060 | 0.597 ± 0.038 | 0.006 | 0.694       |
| A0A0V089B8 | Putative fatty acid hydroperoxide lyase-like          | 1.178 ± 0.007 | 1.518 ± 0.003 | 0.002 | 1.289       |
| A0A0V08D11 | Putative 3-ketoacyl-CoA thiolase 2, peroxisomal-like  | 1.073 ± 0.034 | 1.448 ± 0.031 | 0.000 | 1.349       |
| A0A0V08DV2 | Putative allene oxide synthase-like                   | 0.986 ± 0.090 | 0.691 ± 0.074 | 0.015 | 0.701       |
| K4ASM0     | Lipoygenase                                           | 0.905 ± 0.138 | 0.579 ± 0.065 | 0.039 | 0.640       |
| K4BP29     | Alpha-galactosidase                                   | 0.919 ± 0.041 | 0.726 ± 0.047 | 0.005 | 0.790       |

**Gene transcription translation and protein modification**

| UniProt ID | Protein Name                                          | S1          | S2          | P-value | Fold Change |
|------------|-------------------------------------------------------|-------------|-------------|---------|-------------|
| A0A0V08H61 | GTP-binding nuclear protein                          | 1.143 ± 0.040 | 1.432 ± 0.087 | 0.013 | 1.253       |
| A0A0V08NK1 | Putative ovule protein                                | 1.147 ± 0.064 | 1.477 ± 0.126 | 0.030 | 1.287       |
| A0A0V08SX1 | Putative cell division cycle protein 48-like          | 1.025 ± 0.061 | 1.434 ± 0.055 | 0.001 | 1.399       |
| O82013     | 17.3 kDa class II heat shock protein                 | 0.960 ± 0.048 | 1.416 ± 0.121 | 0.008 | 1.476       |
| Q6WHC0     | Chloroplast small heat shock protein class I          | 0.881 ± 0.016 | 1.528 ± 0.212 | 0.019 | 1.734       |
| V5K655     | Heat shock protein 70                                 | 1.054 ± 0.004 | 1.591 ± 0.048 | 0.000 | 1.510       |

USA), and the Solanaceae Actin gene (Gene symbol, LOC107840006) (Genomic Sequence: XM_016683691.1) was used as the internal reference gene. The \(2^{-\Delta\Delta CT}\) method was used to calculate each gene’s relative expression level (Liu et al., 2022). The corresponding gene primer sequences were presented in Table S1.

### 2.9. Statistical analysis

Experiments were performed in a completely randomized design with three replicates.

The analysis of statistically significant differences in firmness and cell wall components was performed by the independent samples t-test analysis at \(P < 0.05\) using SPSS 24.0 software (SPSS Inc., Chicago, IL, USA). Graphs were constructed using Origin 2018 (OriginLab Inc., Northampton, Ma, USA).

### 3. Results and discussion

#### 3.1. Changes in LBL firmness, cell wall fractions and microstructure

The reduction in firmness is considered to be a hallmark event of fruit softening, and the measurements of firmness and physiological parameters in LBL allowed us to evaluate suitable materials to reveal the proteome changes associated with softening during ripening of LBL. Fruit firmness declined nearly 14-fold between the pre-climacteric (S1) (3.83 ± 0.17 N) and post-climacteric (S2) (0.27 ± 0.17 N) stages (Fig. 1a). As shown in Fig. 1a, fruit softening is accompanied by an increase in respiratory intensity and cell wall degradation. Fruit respiratory intensity increased from 103.80 ± 4.65 mg/(kg h) at S1 to 137.33 ± 4.18 mg/(kg h) at S2, with a percentage increase of 32.30%. Parenchyma tissues of LBL were extensively altered from S1 to S2. The contents of cell wall components, including propectins (WSP, CSP and ISP), cellulose and hemicellulose, showed significant decreases \((P < 0.05)\) (Fig. 1b, c). These cell wall materials structurally support cells and organs, and their solubilization or depolymerization may alter cell wall structure and cell-to-cell adhesion. The parenchyma tissue and cell wall were intact at the S1 stage, but as the ripening progressed, they underwent a large deformation, particularly at the S2 stage (Fig. 1e, f).
3.2. Protein identification, quantification and expression profiles

To identify the key proteins associated with the modification of cell wall components, structures and function, we sampled S1 and S2 before harvest, corresponding to fruits at the post-climacteric and post-climacteric stages, respectively, for comparative analysis of the LBL fruit cell wall proteome using iTRAQ technology. Extraction and identification of S1 and S2 cell wall proteins resulted in the identification of 2811 cell wall proteins (Table S2). Protein abundance values of $P < 0.05$ and fold change (FC) $> 1.2$ were considered to be significantly different between the S1 and S2. The volcano plot revealed the asymmetry of differentially expressed proteins (DEPs) between up-regulated and down-regulated proteins (Fig. 2a). Two hundred and eighty-five proteins were identified as DEPs (Fig. 2b), and the clustering heat map showed the clustering of the DEPs, with the two groups of samples showing good intra-group similarity and inter-group variability. The heat map also showed that 133 and 125 proteins were up-regulated and down-regulated at S1 and S2 respectively. Previous studies (Jiang et al., 2020; Jiang, Feng, Zhang, Luo, & Yu, 2020) reported that the process of fruit softening was related to energy production and conversion, amino acid transport and metabolism, carbohydrate metabolism, cell wall membrane, and secondary metabolism. Hence, we further analysed the involvement of these DEPs in the metabolic processes associated with fruit softening.

A total of 147 proteins were involved in the physiological regulation of fruit by enriching the screened DEPs into the KEGG pathway (Table S2). Excluding uncharacterised proteins, 44 DEPs were correlated with fruit softening by their involvement in metabolic processes, including photosynthesis and energy production, carbon biosynthesis and metabolism, amino acids biosynthesis and metabolism, fatty acid biosynthesis and metabolism, gene transcription translation and protein modification and redox regulation (Table 1, Fig. 2c). The correlation of these 44 DEPs with fruit softening was analysed using Spearman correlations (Fig. 2d). As expected, these proteins showed a clear association with firmness. This may also verify that the metabolic processes involved in these proteins lead to fruit softening. Of these, 23 proteins were involved in the process of photosynthesis and energy production, mainly associated with the photosystem, chloroplast and cytochromes. The down-regulation of these proteins indicates a reduced photosynthesis, and that the fruit may ripe and soften.

Metabolic activity is dominated by carbohydrates, amino acids and fatty acids metabolism. Jiang et al. (2020) reported that the metabolism of amino acids and fatty acids produced the flavour and aromatic substances of the fruit and was not directly related to fruit softening. In contrast, the metabolism of carbohydrate compounds was thought to be the underlying cause of fruit softening. Carbohydrates are the basic substances for fruit energy metabolism and storage, as well as the basic skeletal unit of fruit and tissues (Wang et al., 2021), and glycan degradation leads to a weakening of the cell wall support, resulting in fruit softening. The β-hexosaminidase involved in glycan degradation is responsible for the degradation of glycans by hydrolyzing β-N-acetylgalactosamine glucose or β-N-acetylgalactosamine at the ends of glycosides, oligosaccharides, polysaccharides and complex sugars (e.g. glycolipids, glycoproteins) (He et al., 2017). However, the synergistic action of cell wall degrading enzymes, including α-galactosidase, β-galactosidase, pectate lyase and pectinesterase is known to promote the degradation and depolymerisation of cell wall materials. In this study, although some of the cell wall degrading enzymes were identified (Table S1), the differences in their protein expression levels were not significant at the S1 and S2 stages. Degradation of the cell walls is necessitated by a combination of enzymatic reactions, and the lack of significant differences in their expression levels suggests that the abundance of these cell wall degrading enzymes remains at high levels. Recent works have shown that the softening of fruit was closely linked to the degradation of starch (Zhu et al., 2021). Starch, the glycogen of the plant, is the first to be broken down during carbohydrate metabolism. As shown Fig. 1, the starch granules disappeared at the S2 stage, suggesting that the degradation of polysaccharides was converted from glycogen to structural polysaccharides.

3.3. Gene expression of proteins associated with fruit softening

Next, RT-PCR was used to verify the mRNA expression of the proteins associated with cell wall degradation (Fig. 3). Except for pectate lyase, polygalacturonase, pectate lyase, pectinesterase, α-galactosidase, β-galactosidase and α-arabinofuranosidase were significantly up-regulated. The mRNA expression levels of α-amylase and β-amylase involved in starch degradation were higher in S2 than S1. Pectinesterase can remove the methoxyl group in the xylooligosaccharides present in pectin and catalyzes the conversion of pectin ester acid to pectin acid, which can then be degraded by pectinesterase. In addition, α-galactosidase hydrolyses the terminal, non-reducing α-galactose residues in α-galactosides, including galacto-oligosaccharides, galactomannans and galactolipids, β-galactosidase cleaves the α-D-galactose residue in the side chain of rhamnogalacturonan I type pectin, and the two together promote the hydrolysis of pectin (Wen, Strom, Tasker, West, & Tucker, 2013). The contents of crude cell wall materials, cellulose, hemicellulose and pectin (WS, ISP, CSP) significantly decreased in the S2 compared to the S1 (Fig. 1). This is consistent with a previous study (Liu et al., 2021), and suggests that exploring the reasons for the reduction in cell wall materials is important in revealing fruit softening. In addition, glucose-6p isomerase, glucose-1-phosphate phosphodimidase, acotinate hydratase, citrate synthase, malate dehydrogenase and β-glucosidase involved in glycolysis/gluconeogenesis, pentose phosphate pathway, pyruvate metabolism, citrate cycle (TCA cycle) energy metabolism processes, were also significantly up-regulated ($P < 0.05$). The weakening of photosynthesis and the enhancement of respiration may lead to the depletion of macromolecules. As shown in Fig. 1a and Table 1, the fruit had a weakened energy synthesis during the S2 stage.
period and an enhanced energy metabolic activity, including the cell wall, starch, and other glycols being broken down and consumed as respiratory substrates, which weakened or even eliminated the cellular support structures, leading to softening of the fruit.

4. Conclusions

The degradation of cell wall components during fruit ripening is an intrinsic factor of LBL fruit softening, which was verified by the microstructure of the fruit tissue. The iTRAQ technology was used to identify 258 differentially expressed cell wall proteins (DEPs) when fruit underwent the transition from the mature pre-climacteric (S1) to the post-climacteric (S2) stage. The DEPs were related to photosynthesis and energy production, carbon biosynthesis and metabolism, amino acids biosynthesis and metabolism, fatty acid biosynthesis and metabolism, gene transcription/translation and protein modification, which were implicated in LBL fruit softening. Fruit softening was caused by cell wall degradation, where energy metabolism caused large molecules of cell wall materials to be broken down into substrates for respiratory metabolism, and the gene expression of these enzymes was verified using RT-PCR. The identification of these proteins and pathways associated with cell wall degradation may provide a good starting point for further dissection of the molecular mechanisms underlying LBL fruit softening.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability statement

Research data are not shared.

Appendix A. Supplementary data

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