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Transcriptome analysis reveals regulatory mechanism of postharvest softening in kiwiberry

Zhao Liu[1], Yang Sun[1], Zhenpan Liu[1], Jianyu Song[1], Weicong Yang[1], Zhannan Wang[1], Taiming Liang[2] and Dejun Liang[1*]

Abstract

Background Kiwiberry is an emerging edible fruit with market potential owing to its advantages of small size, thin and hairless skin, and sweet taste. However, kiwiberry is highly susceptible to softening after harvest, which poses a challenge for storage and transport. To reveal the underlying cause of kiwiberry softening, it is essential to investigate the characteristics of postharvest fruit and the molecular mechanisms that affect changes in fruit firmness.

Results Morphological observations and analysis of physical parameters showed that the skin of kiwiberry did not change markedly from the 1st to the 7th day after harvest, while the colour of the inner pericarp gradually turned yellow. By the 9th day of room temperature storage, the kiwiberries began to rot. The hardness decreased rapidly from the 1st to the 5th day postharvest, reaching the low level on the 5th day. The starch and pectin contents of kiwiberry showed a downward trend with increasing storage time. Transcriptome sequencing and weighted gene co-expression network analysis identified 29 key genes associated with the changes in the hardness of kiwiberry after harvest. Gene Ontology enrichment analysis indicated that these 29 genes are mainly involved in pectin metabolism, starch synthesis, starch decomposition, and starch metabolism. In addition, three transcription factors, *AGL31*, *HAT14*, and *ALC*, were identified to be strongly positively correlated with the 29 genes that affect the hardness changes of kiwiberry after harvest, and 28 of the 29 key genes were predicted to be regulated by *HAT14*.

Conclusions These results reveal the changes in morphological characteristics and physiological indicators during the postharvest ripening and softening of kiwiberry stored under room temperature conditions. Transcriptome analysis identified 29 key genes and three transcription factors that affect the firmness changes of postharvest kiwiberry. The results of this study thus provide insight into the transcriptional regulatory mechanism of kiwiberry softening during storage to improve the postharvest quality.

Keywords Kiwiberry, *Actinidia arguta*, Firmness, Postharvest, Transcription factor

Background

Actinidia arguta is a perennial deciduous vine plant in the family Actinidiaceae [1, 2]. The fruit of *A. arguta* is known as the kiwiberry, which is a strong economic, edible fruit crop owing to its small size, thin and hairless skin, and sweet flavour [3]. In the past few years, kiwiberry has become increasingly popular with consumers owing to its unique fruit characteristics, excellent flavour, and rich nutritional components [4–6]. However,

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compared with the Hayward kiwifruit (*Actinidia deliciosa*) that can be stored for 4–6 months, kiwiberry is more susceptible to softening under the same storage conditions [7, 8], making it difficult to store and transport, which has seriously restricted its market potential as an emerging fruit.

The softening of fruit texture is the result of the combined action of multiple factors [9]. Among them, changes in the structure of cell wall components play a major role in the softening of fruit [10, 11], especially the dissolution of pectin, which results in remodelling of the primary cell wall [12–14]. Previous studies have identified some primary cell wall pectin-degrading enzymes, including polygalacturonase, pectinate lyase, pectin methylesterase, pectin acetylesterase, rhamnogalacturonase lyase, and β-galactosidase, which synergistically promote the fruit ripening and softening processes [15, 16]. In addition, starch degradation contributes to the softening of fruits during storage, especially in fruits with a high starch content, such as bananas and kiwifruit [17]. In bananas, 27 of the 38 starch degradation-related genes were found to be significantly induced during fruit ripening, corresponding to the conversion of starch into soluble sugars [18]. In kiwifruit, 17 candidate genes related to starch degradation during fruit softening after harvest were identified based on whole-genome sequencing analysis [19]. As an emerging fruit, the specific pectin- and starch-degrading enzymes that play a role in the softening process of kiwiberry and the associated changes in the expression patterns of related genes during fruit storage remain unclear.

Transcription factors play important regulatory roles in various developmental processes, including fruit ripening, by interacting with specific DNA sequences in the promoters of target genes. Several transcription factors have been reported to be directly involved in the regulation of fruit ripening and softening. For example, the transcription factor MaERF9/11 regulates the ethylene biosynthesis genes *MaACS1* and *MaACO1* in banana [20]; in kiwifruit, two transcription factors in the ethylene signalling pathway, AdEIL and AdERF, affect fruit ripening by regulating the transcription of cell wall modification genes [21]; in tomato, RIN controls fruit ripening by regulating genes related to cell wall degradation [22, 23]; HY5 regulates starch degradation in the fruit by directly binding to the promoters of *SlPWD*, *SlBAM1*, *SlBAM3*, *SlBAM8*, *SlMEX1*, and *SlDPE1* [24]; Moreover, in banana, MaMYB3 inhibits the transcription of 10 starch degradation-related genes by directly binding to their promoters, further inhibiting starch degradation to delay fruit ripening and softening [18, 25]. However, the key regulatory transcription factors playing a role during the ripening and softening processes of kiwiberry and the associated regulatory pathways have not been analysed to date.

In this study, tetraploid kiwiberry (*A. arguta*) served as the material to investigate the changes in firmness and in pectin and starch contents during the postharvest ripening and softening process. Transcriptome sequencing was then used to study the changes in gene expression in kiwiberries collected at different storage times after harvest. Weighted gene co-expression network analysis (WGCNA) was used to identify the key genes that affect the postharvest firmness changes in kiwiberry and to screen the main transcription factors that regulate firmness-associated genes. This study provides a theoretical basis to gain a deeper understanding of the underlying molecular mechanisms contributing to the changes in the firmness of kiwiberry with increasing storage time after harvest.

Methods

Plant materials

The kiwiberry variety 'Longcheng No. 2' was selected as the experimental material due to its rapid softening process and the obvious phenotypic variation characteristics. The kiwiberries used in this study were collected from Songmudao Base, Dalian City, Liaoning Province, China (121°75′ E, 39°40′ N). On September 20, 2023, kiwiberries of consistent size and intact appearance were selected for unified harvesting. After that the kiwiberries were placed in a storage box, which is covered with a lid and kept in darkness. The harvested kiwiberries were placed in a single layer in box to avoid squeezing and stored at 24°C and 45% relative humidity for subsequent analysis.

Measurement of firmness, starch and pectin contents, and amylase and pectinase activities

The firmness test, starch and pectin content determination, and amylase and pectinase activity measurements of the fruit were performed on the 1st, 3rd, 5th, 7th, and 9th day after kiwiberry harvesting.Each test contained three biological replicates, and five fruits were measured in each replicate. The firmness was measured according to the method of Gan et al. [26]. The pericarp on the opposite sides was removed with a blade, and the firmness (in Newtons, N) was measured using a hand-held firmness tester. Assay kits from Jiangsu Maisha Inc. (Nanjing, China) were used to measure the contents of starch and pectin as well as the activities of amylase and pectinase (catalogue no. ADS-W-DF001-96, ADS-W-KY018-96, and MM-2002O1, and MM-62556O1, respectively) according to the manufacturer's protocols.

RNA isolation, cDNA library construction, transcriptome sequencing, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Undamaged kiwiberries were taken out of the storage box, the skin was carefully removed and the outer pericarp was cut into small pieces as sequencing samples. The sequencing sampling contained three biological replicates. The kiwiberries collected on the 1st, 3rd, 5th, 7th, and 9th day after harvest were frozen in liquid nitrogen and then stored at −80 °C until sequencing. Total RNA was extracted with the TRIzol kit (Invitrogen, Carlsbad, CA, USA). The cDNA library was prepared using the Ion Total RNA-Seq Kit v2 (ThermoFisher Scientific, Waltham, MA, USA). Transcriptome sequencing was performed by Lc-Biotechnology Co., Ltd. (Hangzhou, China) on the Illumina HiSeq 4000 platform following the manufacturer's recommended protocol.

Clean data were aligned to the *A. arguta* reference genome using Hisat2 software and the alignment rate was calculated [27, 28]. The mapped reads of each sample were assembled and all transcripts were merged to reconstruct a comprehensive transcriptome [29]. The expression levels of all transcripts were estimated by calculating the fragments per kilobase of transcript per million mapped reads (FPKM) values using StringTie software [29].

Highly expressed key genes associated with the changes in the hardness of kiwiberry after harvest were selected for RT-qPCR on a 7500 Fast Real-Time PCR system (Thermo Fisher, Singapore) as described by Liu et al. [30]. All reactions were performed with three biological replicates and three technical replicates. Kiwifruit β-actin gene ACTIN (FG454048) was considered as the reference gene [31, 32]. The reference gene and the sequences of the primers used for RT-qPCR are listed in Table S1 of Additional File 1.

Analysis of RNA-seq data

EdgeR software was used to analyse the differential expression of genes in kiwiberries collected at different storage times after harvesting [33]. Differentially expressed genes (DEGs) were determined according to $|\log_2(\text{fold change})| \geq 1$ and $P\text{-value} < 0.05$. Advanced Mfuzz analysis was performed using OmicStudio tools (https://www.omicstudio.cn/tool). To determine gene function, the DEGs were subjected to Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

WGCNA

After filtering out genes with extremely low expression levels and those that were not expressed in all samples, a total of 68,328 genes remained for analysis. The modules of co-expressed genes were identified using the WGCNA package in R software (version 4.0.2) [34]. The eigenvalues of each module were calculated and used to test their correlations with firmness. Transcription factor screening and identification were performed by aligning the gene sequences of *Actinidia arguta* with those of *Arabidopsis*. The firmness-related modules obtained by WGCNA were screened according to a correlation coefficient (r) ≥ 0.7 or ≤ -0.7 and $P \leq 0.01$ as cut-offs. Genes in the meaningful modules were subjected to GO annotation analysis. The expression heatmap of the genes obtained through screening was plotted using TBtools [35].

Regulatory relationship network construction

Pearson correlation analysis was performed for the screened transcription factors and genes related to firmness. Genes significantly correlated ($r \geq 0.9$ and $P \leq 0.01$) with transcription factors were selected to construct the Sankey diagram. The binding motifs of transcription factors were screened using the online tool JASPAR (https://jaspar.elixir.no), and the regulatory relationships of the obtained binding motifs with firmness-related genes were predicted. The regulatory relationship network was constructed using Cytoscape software [36].

Statistical analysis

The statistical analysis of the data obtained in this study was performed using the SPSS version 20.0 software (IBM Inc., New York, NY, USA). Significant differences among means were determined by one-way ANOVA and Duncan's multiple range tests at $P \leq 0.05$.

Results

Morphological and physiological changes in kiwiberry after harvest

The changes in morphological characteristics and physiological indicators of the kiwiberry (Longcheng No. 2) with an increase in storage time under room temperature (24°C) conditions are displayed in Fig. 1. The outer pericarp was green from the 1st to the 5th day after harvest, only turning pale green (RGB: 77,108,47) on the 9th day (Fig. 1A-E). And the colour of the core deepened first and then became lighter with increasing storage time, and presented the deepest yellow (RGB: 196,184,80) on the 5th day after harvest (Fig. 1A-E). In addition, on the 9th day after harvest, the skin of the fruit had rotted and the structure of the inner pericarp was no longer clearly discernible (Fig. 1D).

With respect to the physiological indices, the firmness of the fruit gradually decreased with increasing storage time (Fig. 1F). The statistical analysis results showed that

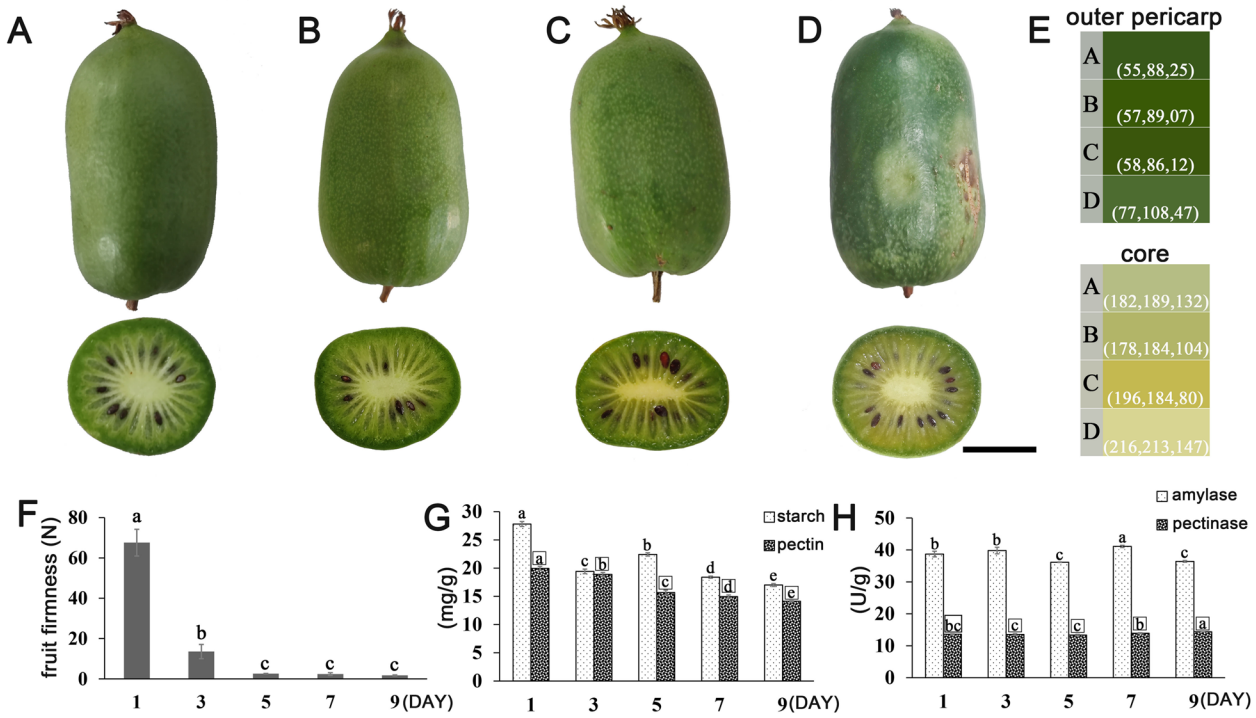

Fig. 1 Morphological characteristics and physiological indices of kiwiberry at different storage times after harvesting.**A**–**D** The morphology of kiwiberry on the 1st (**A**), 3rd (**B**), 5th (**C**), and 9th (**D**) day after harvest (bar = 1 cm). (**E**) RGB colour of the outer pericarp and core. Capital letters A-D correspond to the fruits in panels A-D, respectively. **F** Firmness of kiwiberry at different storage times after harvest. **G** Starch and pectin contents of kiwiberry at different storage times after harvest. **H** Amylase and pectinase activities of kiwiberry at different storage times after harvest. Lowercase letters represent significant differences at the 5% level, while lowercase letters with boxes represent significant differences in pectin and pectinase, respectively

the highest firmness was 67.58 N on the 1st day after harvest and then decreased rapidly over the following two days, reaching only 13.54 N on the 3rd day postharvest. The firmness continued to decrease from the 3rd to the 5th day after harvest, reaching the low value of 2.59 N on the 5th day. Subsequently, the firmness continued to decrease slowly and reached the lowest value of 1.70 N on the 9th day.

The changes in starch and pectin content are important factors affecting fruit softening, so we also studied the starch and pectin content of kiwiberry, as well as the activities of amylase and pectinase. The starch content of the fruit also generally showed a downward trend with the increase of storage time (Fig. 1G). On the 1st day after harvest, the starch content was 27.81 mg/g, reaching 22.43 mg/g on the 5th day and 17.01 mg/g on the 9th day. The pectin content of the fruit also continually decreased after harvest (Fig. 1G), from the highest of 19.95 mg/g on the 1st day to the lowest of 14.13 mg/g on the 9th day. There was no obvious change trend in the activities of amylase and pectinase in the fruit during storage (Fig. 1H). The highest and lowest values of amylase activity were 41.10 U/g and 36.15 U/g, which occurred on the 7th and 5th days after harvest, respectively. The highest and lowest values of pectinase were 14.39 U/g and 13.35 U/g, which occurred on the 9th and 5th days after harvest, respectively.

Through the above research, it was found that the firmness of kiwiberry changed dramatically from the 1st to the 5th day after harvest, and reached a lower firmness value on the 5th day. To further investigate the causes of fruit softening, we conducted transcriptome analysis on kiwiberry stored at different times after harvest.

Transcriptome analysis of different storage times

To further analyse the regulatory mechanism underlying the change of kiwiberry firmness after harvest, postharvest fruits at different storage times were collected for transcriptome sequencing. A total of 75.64 GB of clean data were obtained from kiwiberries at different storage times, with more than 29 million clean reads per sample (Q30 > 94.56%, GC content > 46.76%). The transcripts were mapped to the *Actinidia arguta* genome, with a mapping efficiency ranging from 81.59 to 92.58% (Table S2, Additional File 1), and 148,344 genes were finally obtained (Table S3, Additional File 1).

Principal component analysis and heatmap of the gene expression levels (FPKM values) of postharvest kiwiberry showed that different biological replicates of the samples clustered together, exhibiting high consistency in gene expression (Fig. 2A, S1 in Additional File 2). LC1 is the sample from the 1st day after harvest, which is the initial stage of the fruit after harvesting and the stage with the highest fruit hardness (67.58 N). Using LC1 as a control can identify transcriptome expression differences under any subsequent changes in fruit traits. Therefore, LC1 was chosen as the background for gene differential analysis. Compared with gene expression levels on the 1st day of storage, the total number of DEGs initially increased and then decreased with the increase of storage time, with the largest number of DEGs (40327) identified on the 5th day after harvest (Fig. 2B, Table S4 in Additional File 1). Compared with the 1st day, there were 9389, 15639, 10734, and 10939 up-regulated genes and 20959, 24688, 27475, and 25573 down-regulated genes on the 3rd, 5th, 7th, and 9th day after harvest, respectively. The number of downregulated DEGs in all comparison groups was greater than that of up-regulated DEGs. In addition, there were 17611 DEGs common to the four comparison groups (Fig. 2C), along with unique DEGs identified in each comparison group: 5305 unique DEGs for the 5th day versus 1st day, 4567 unique DEGs for the 9th day versus 1st day, 3902 unique DEGs for the 3rd day versus 1st day, and 3504 unique DEGs for the 7th day versus 1st day.

Functional annotation of genes during storage

To further study the effect of postharvest storage time on the firmness of kiwiberry, the DEGs identified on the 3rd, 5th, 7th, and 9th days after harvest compared with the 1st day were subjected to GO analysis (see Table S5 in Additional File 1). The 20 most significantly enriched GO terms are shown in Fig. 3A–D. There were multiple identical significantly enriched GO terms in the four comparison groups, including "chloroplast," "chloroplast thylakoid membrane," "chloroplast thylakoid," "chloroplast envelope," "integral component of membrane," "thylakoid," "photosynthesis," "chloroplast stroma," "plasma membrane," "plastoglobule," "chlorophyll binding," "response to light stimulus," "membrane," "pigment binding," "photosynthesis, light harvesting in photosystem I," and "oxidation-reduction process." As shown in Figure S2 (Additional File 3), for each GO term, the number of genes included in the 5th day versus 1st day comparison was the largest, while the 3rd day versus 1st day comparison had the lowest number of enriched genes. Among these GO terms, "chloroplast", "integral component of membrane" and "plasma membrane" were the three GO terms with the largest number of genes. In the "integral component of membrane" and "plasma membrane", genes encoding Pectate lyase (PL), Endoglucanase (EGase) and xyloglucan endotransglycosylase/hydrolase (XTH) were identified, which were reported to affect fruit firmness in previous studies (Figure S3 in Additional File 4). Among them, genes Aa10Ag01184, Aa10Bg02446 and Aa10Dg04934 encode EGase, genes Aa15Ag27029, Aa15Bg28556 and Aa15Bg28557 encode PL, and genes Aa11Dg09371, Aa14Bg23550 and Aa15Dg32805 encode XTH. And these genes are relatively highly expressed, which may play the role in the continuous softening process of kiwiberry after harvest over prolonged storage at room temperature.

Through Mfuzz analysis, the expression patterns of DEGs were categorised into 12 clusters (Fig. S4, in Additional File 5), among which clusters 10 and 12, comprising 4234 and 4112 DEGs, respectively, exhibited increasing expression trends. The expression levels of genes in these clusters gradually increased with the increase of postharvest fruit storage time. Clusters 6, 9, and 11, comprising 9383, 4385, and 7230 DEGs, respectively, exhibited decreasing expression trends with increased storage time (Fig. 4A, Table S6 in Additional File 1).

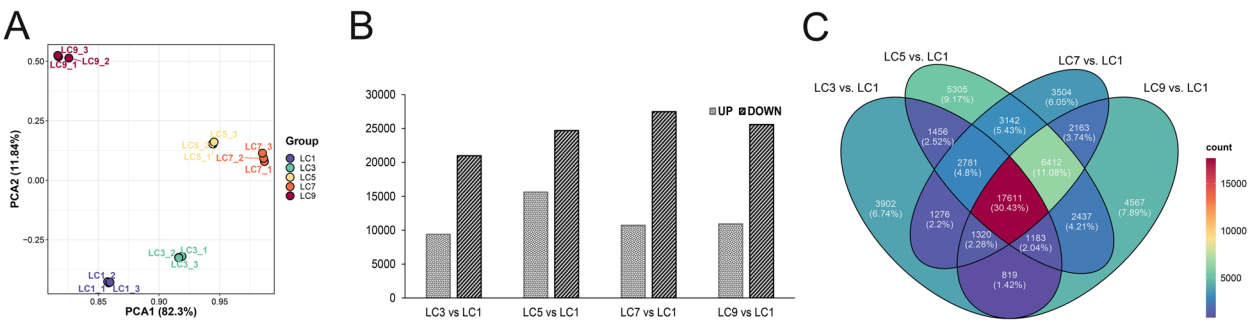

Fig. 2 Gene expression analysis of kiwiberry. **A** Principal component analysis of gene expression in kiwiberry. **B** Differentially expressed genes (DEGs) in the postharvest fruit with increasing storage time. **C** Venn diagram of DEGs in the postharvest fruit with increasing storage time. LC1, day 1 after harvest; LC3, day 3 after harvest; LC5, day 5 after harvest; LC7, day 7 after harvest; LC9, day 9 after harvest

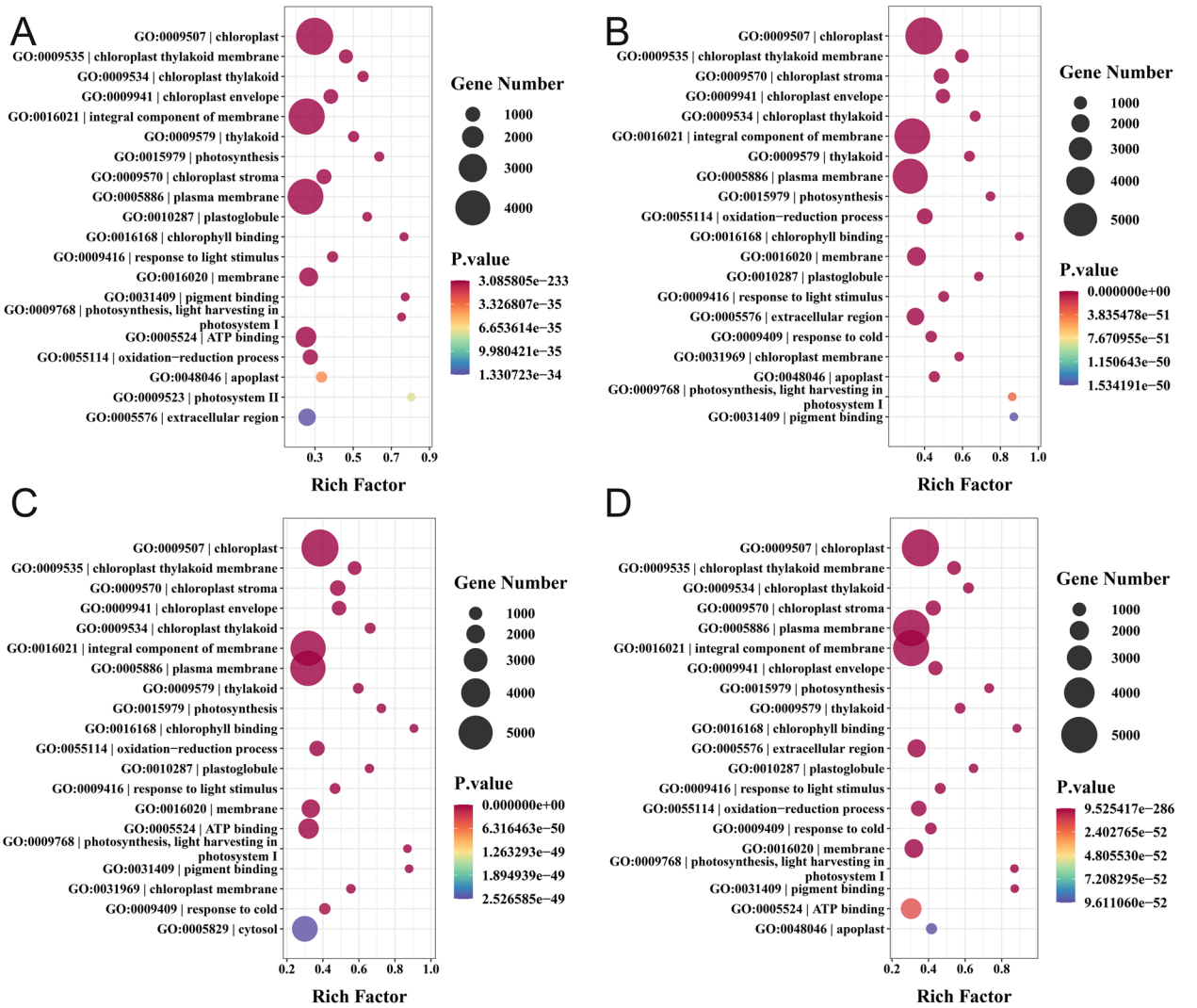

Fig. 3 Gene Ontology (GO) annotation of differentially expressed genes (DEGs) in postharvest kiwiberry fruit with increasing storage time. **A** GO annotation of DEGs in kiwiberry between the 3rd day and 1st day after harvest. **B**–**D** GO annotation of DEGs in kiwiberry for the comparison of the 5th (**B**), 7th (**C**), and 9th (**D**) days and the 1st day after harvest, respectively

A total of 642 and 990 transcription factors were identified among DEGs with up-regulated and down-regulated expression trends, respectively. The 642 transcription factors with up-regulated expression trends during storage time were distributed in 45 transcription factor families, among which the bHLH family and the MYB family had the highest number of transcription factors at 59. The 990 transcription factors with down-regulated expression trends were distributed in 48 transcription factor families, with the highest number found again in the MYB family, including 121 transcription factors. In addition, KEGG pathway analysis showed that the upregulated DEGs were mainly enriched in pathways such as "circadian rhythm – plant," "nicotinate and nicotinamide metabolism," "pantothenate and CoA biosynthesis," "RNA degradation," and "ether lipid metabolism," while the down-regulated DEGs were mainly enriched in pathways such as "ribosome," "photosynthesis," "photosynthesis - antenna proteins," "porphyrin metabolism," and "carbon fixation in photosynthetic organisms" (Fig. 4C).

Key genes and regulatory pathways associated with firmness changes during storage

To identify the important genes that affect the firmness of kiwiberry, WGCNA was performed to identify gene modules related to the changes in firmness at different times after harvest. A total of 12 modules were generated, each of which is represented by a different colour

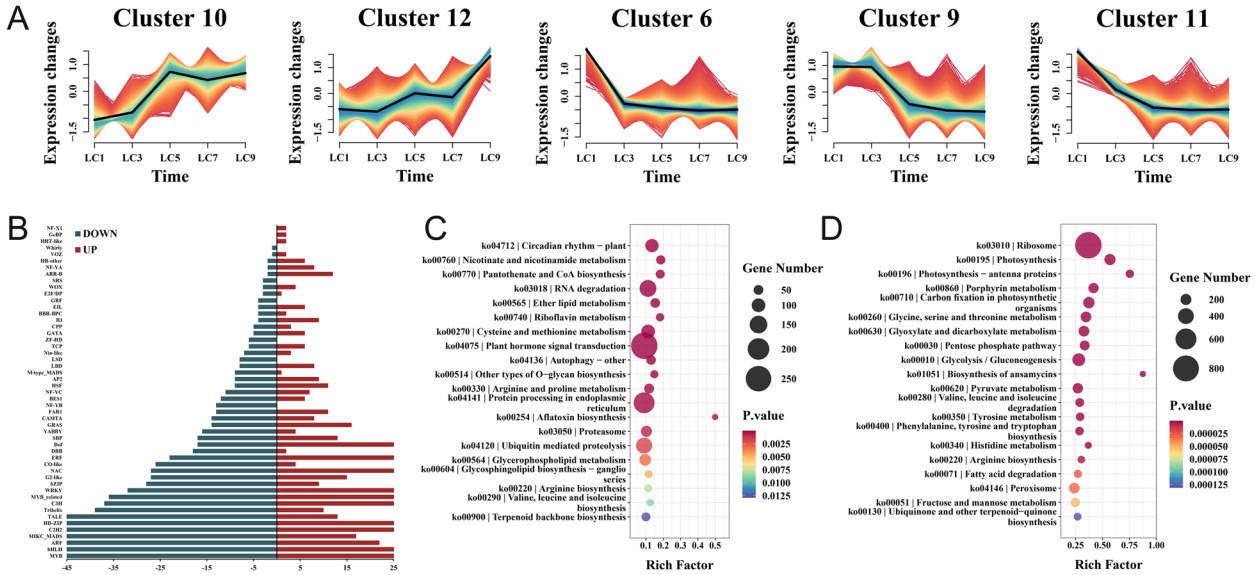

Fig. 4 Dynamic changes of expression and functional analysis of genes in postharvest kiwiberry. **A** Mfuzz clustering of gene expression in kiwiberry at different times after harvest. **B** Transcription factors identified among genes with increasing and decreasing expression trends over storage time. **C** Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of genes with increasing expression trends over storage time. **D** KEGG analysis of genes with decreasing expression trends over storage time. LC1, day 1 after harvest; LC3, day 3 after harvest; LC5, day 5 after harvest; LC7, day 7 after harvest; LC9, day 9 after harvest

in Fig. 5A. The grey modules represent genes that were not classified into other modules and were therefore not further analysed. There were clear differences in the number of genes contained in the different modules, with turquoise being the largest module containing 29791 genes and the green-yellow module being the smallest module containing only 63 genes. The heat map based on the correlation coefficients between the modules and the postharvest firmness of kiwiberry is presented in Fig. 5B. According to a threshold of $r > 0.7$ or $r < -0.7$, the turquoise module was found to be significantly positively correlated with fruit firmness ($r = 0.99$, $P = 2e^{-12}$). The specific genes in this module are listed in Table S7 of Additional File 1. This result indicated that the turquoise module contains genes that play an important role in the postharvest firmness changes of kiwiberry.

Further analysis identified a total of 1458 transcription factors in the turquoise module that are positively correlated with fruit hardness (Fig. 5C). The top three transcription factor families were MYB, bHLH, and C3H, including 193, 100, and 78 transcription factors, respectively.

The results of GO annotation analysis of the genes in the turquoise module are shown in Table S8 of Additional File 1, focusing on the pectin- and starch-related GO terms related to fruit firmness. A total of nine starch-related GO terms were screened in the turquoise module (Fig. 6A). After removing genes that appeared repeatedly in different GO terms, a total of 145 genes were screened, 66 of which were enriched in the term "starch biosynthetic process." The heat map of starch-related genes in the turquoise module is shown in Fig. 6B. In addition, three pectin-related GO terms were screened (Fig. 6A), containing a total of 36 genes, 20 of which were enriched in the term "cell wall pectin biosynthetic process." The heat map of pectin-related genes in the turquoise module is shown in Fig. 6C.

The selected genes in the turquoise module that were positively correlated with kiwiberry firmness after harvesting were further analysed by a Venn diagram with the top 10% module membership (MM) genes, the top 10% genes of significance (GS) to firmness, and the top 100 hub genes. Then 29 key genes related to kiwiberry firmness after harvesting were further screened in the turquoise module (Fig. 7A). Three transcription factors were identified among the top 100 hub genes in the turquoise module, including Aa18Cg49189 (*AGL31*), Aa13Bg17329 (*HAT14*), and Aa23Ag81232 (*ALC*). Correlation analysis between transcription factors and the 29 key genes related to fruit firmness after harvesting revealed that all 29 genes had strong positive correlations ($r > 0.7$) with these three transcription factors (Table S9 in Additional File 1), including 2 pectin metabolism genes, 14 starch biosynthesis genes, 6 starch catabolism genes, and 7 starch metabolism genes (Fig. 7B). To further determine the regulatory relationship between transcription factors

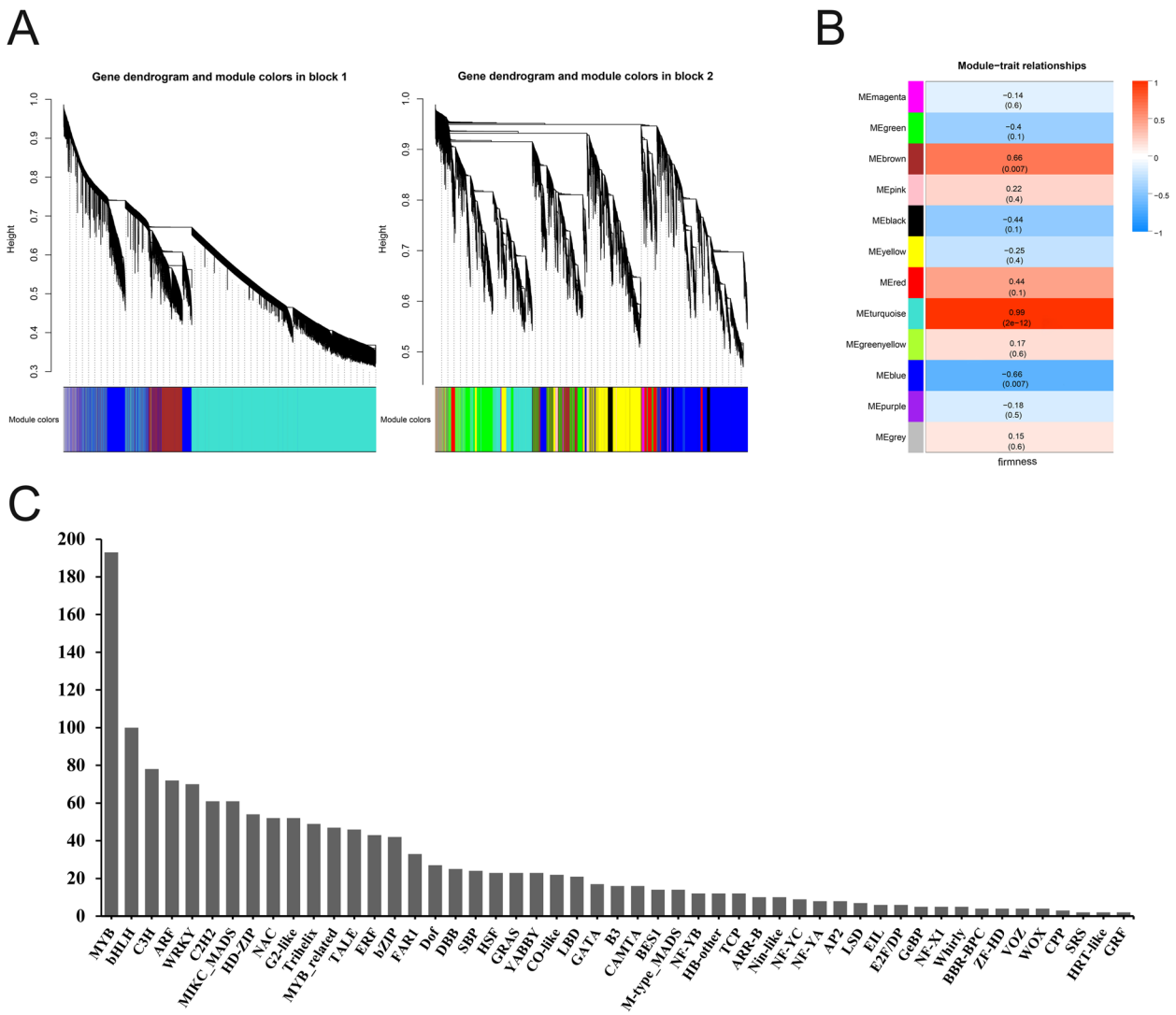

Fig. 5 Weighted gene co-expression network analysis (WGCNA) of genes in postharvest kiwiberry. **A** Hierarchical clustering tree of co-expression modules identified by WGCNA. **B** Association analysis between modules and fruit firmness, with each row representing a colour module. **C** Statistics of transcription factors in the turquoise module

and genes related to fruit firmness after harvest, protein alignment was used to screen the binding motifs of transcription factors *AGL31*, *ALC*, and *HAT14*. Among these three transcription factors, *HAT14* had five binding motifs, including MA1211.1, MA1024.1, MA1406.1, MA1210.1, and MA1198.1 (Fig. 7C). The promoter sequences of the 29 genes that were strongly positively correlated with *HAT14* were extracted and the five binding motifs were used to predict regulatory relationships, ultimately identifying 28 genes regulated by *HAT14* (Fig. 7D, Table S10 in Additional File 1).

The expression trend of the three transcription factors and the 29 key genes related to fruit firmness was further analysed. With respect to temporal changes, the expression levels of the 29 genes were the highest on the 1st day after harvest and then subsequently decreased over storage time. Genes with high expression levels were mainly concentrated in the starch catabolic process category (Fig. 8A).

To verify the reliability of the transcriptome sequencing results, eight genes were selected for RT-qPCR validation. Consistent with the transcriptome results, five genes related to fruit firmness after harvest, including Aa12Bg11957(*DPE2*), Aa12Cg12847(*ADG1*), Aa22Dg79773(*SEX4*), Aa3Cg132087 (*GBSS1*), and Aa4Dg137524(*DUF789*), and three transcription factors, including *AGL31*, *HAT14*, and *ALC*, all had the highest expression levels on the 1st day after harvest. Subsequently, from the 1st to the 5th day after harvest, the

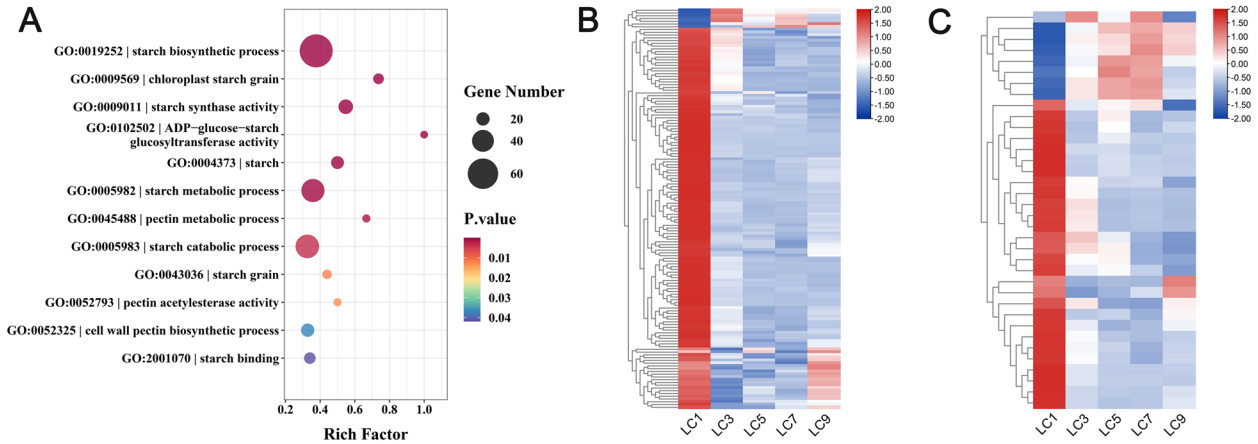

Fig. 6 Analysis of genes in the module related to fruit firmness. **A** Gene Ontology (GO) annotation of genes in the turquoise module. **B** Heat map of starch-related genes in the turquoise module. **C** Heat map of pectin-related genes in the turquoise module

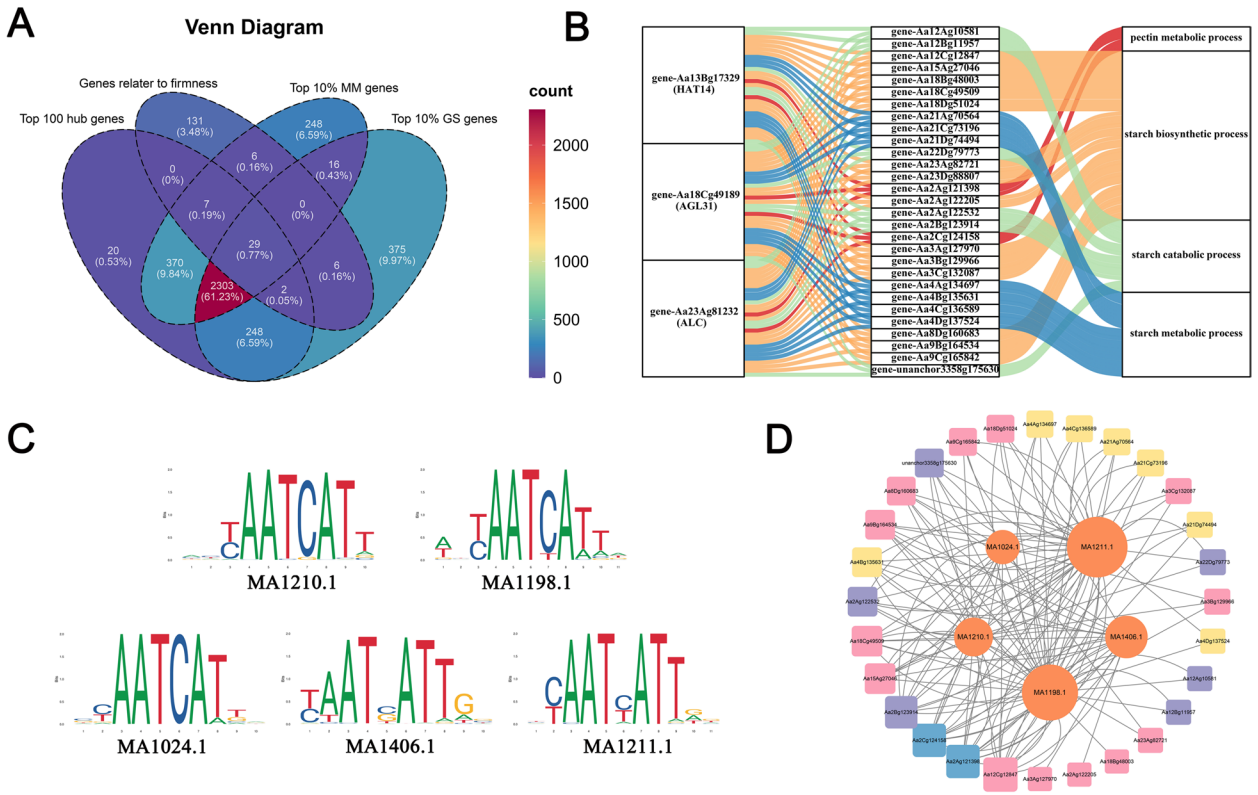

Fig. 7 Identification of key genes related to kiwiberry firmness after harvest. **A** Identification of key genes related to firmness in the turquoise module. **B** Relationship between key transcription factors and genes related to fruit firmness in the turquoise module. **C** Binding motif of transcription factor Aa13Bg17329. **D** Relationship between transcription factor (Aa13Bg17329) binding motif and genes related to firmness. Circles represent binding motifs, squares represent genes related to firmness, pink represents genes involved in starch biosynthetic processes, blue represents genes involved in pectin metabolic processes, purple represents genes involved in starch catabolic processes, and yellow represents genes involved in starch metabolic processes. The size of the circle or square represents the number of relationships

expression levels of these genes gradually decreased and reached the lowest level on the 5th or 7th day after harvest (Fig. 8B–I). In addition, we also conducted RT-qPCR studies on other genes that may affect the changes in fruit firmness, including PG, PL, and BGAL (Fig. S5, in Additional File 6). The results showed that the expression

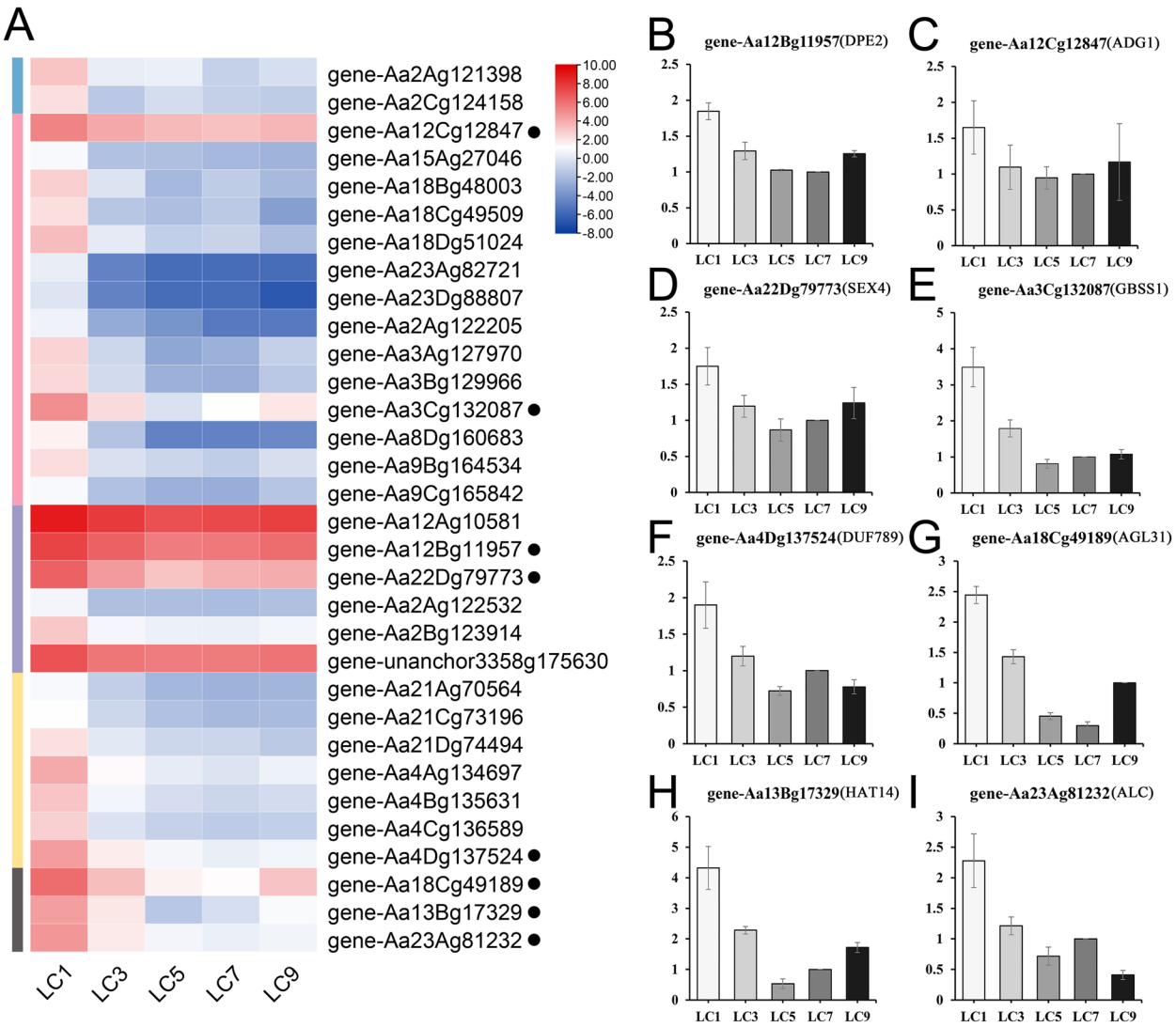

Fig. 8 Expression levels of genes related to kiwiberry firmness after harvest. **A** Expression levels of genes related to fruit firmness after harvest, where pink represents genes involved starch biosynthetic processes, blue represents genes involved in pectin metabolic processes, purple represents genes involved in starch catabolic processes, yellow represents genes involved in starch metabolic processes, and grey represents transcription factors. **B**–**F** RT-qPCR of genes related to fruit firmness, including Aa12Bg11957(*DPE2*) (**B**), Aa12Cg12847(*ADG1*) (**C**), Aa22Dg79773(*SEX4*) (**D**), Aa3Cg132087(*GBSS1*) (**E**), and Aa4Dg137524(*DUF789*) (**F**). **G**–**I** RT-qPCR of transcription factors regulating fruit firmness, including Aa18Cg49189(*AGL31*) (**G**), Aa13Bg17329(*HAT14*) (**H**), and Aa23Ag81232(*ALG*) (**I**)

patterns of these genes were different in the postharvest kiwiberry. The BGAL had the highest expression level on the 1st day after harvest, and then the expression level decreased. The PL and PG had low expression levels on the 1st day after harvest, and then the expression levels increased, reaching the highest expression levels on the 5th and 9th days, respectively.

Discussion

The change of fruit firmness after harvest is an important indicator for determining the storage time [16, 37]. In this study, kiwiberry softened rapidly under room temperature storage and reached a low firmness level on the 5th day of storage, which is considered to be the maximum suitable period for consumption. However, the

fruit began to rot on the 9th day of storage. Compared with Hayward kiwifruit, which retained a firmness of 20 N after one month of room temperature storage [38], kiwiberry softened and rotted more quickly, which indicates that it is relatively less resistant to storage.

The change of fruit firmness is caused by various factors. In this study, as the storage time increased, the pectin and starch contents of kiwiberry showed a downward trend. The cell wall and middle lamella in the fruit are rich in pectin [39, 40]. During the fruit ripening process, the activities of certain enzymes change the structure and composition of the cell wall, resulting in the solubilization and degradation of pectin [41, 42]. The decrease in starch content is due to its conversion into soluble sugars during the fruit ripening process [24, 43]. Thus, the observed decreases in pectin and starch contents represent the modification of the cell wall and the degradation of starch in the fruit, which are the main contributors to the decrease in fruit firmness.

To further explore the transcriptional regulatory mechanism of changes in fruit hardness, transcriptome sequencing was performed on kiwiberries collected at different times after harvest according to the most recently published *A. arguta* genome as reference [27], which is larger than the published *Actinidia deliciosa* genome [27, 44]. We identified 148,344 genes after transcriptome sequencing and genome alignment, which is a higher number of genes than obtained in the sequencing results of *Actinidia deliciosa* [45, 46]. This may be because the sequencing materials and reference genomes used in this study were all tetraploid, and the increase in ploidy leads to an increase in the number of chromosomes and alleles. The total number of DEGs first increased and then decreased during storage, with the largest number of DEGs appearing on the 5th day, indicating that the most drastic physiological activity changes may occur in the kiwiberries on the 5th day after harvest. Venn diagram analysis confirmed that the highest number of uniquely differentially expressed genes was on the 5th day of storage. And the same DEGs among different comparison groups indicated that there were a large number of genes that continued to play a role in the softening process of kiwiberries after harvest.

Previous studies have shown that the reduction in fruit firmness is affected by changes in the expression of genes related to starch and pectin metabolism [17, 47]. In this study, WGCNA was used to screen genes related to starch and pectin, and 29 key genes related to the changes in firmness after harvest in kiwiberry were obtained. Among them, two pectin-related genes named *PME* were annotated as "pectin metabolic process" had been proven to play an important role in the degradation of pectin in tomato [48]. In addition, 27 starch-related genes were annotated as "starch biosynthetic process," "starch catabolic process," and "starch metabolic process." Among these genes, *GBSS1*, *DBE*, *SS* and *SBE* were found to be involved in starch synthesis in potato and banana, but they all showed low expression levels in postharvest kiwiberry [49–51]. However, *DPE* and *SEX* have been shown to play a role in starch degradation in banana and kiwifruit, and were highly expressed in postharvest kiwiberry [19, 52]. This indicates that both changes in starch biosynthesis and degradation processes in kiwiberry have an impact on its postharvest firmness. Compared with pectin-related genes, there were more starch-related genes screened, and genes with high expression levels were concentrated in the "starch catabolic process" term, which may indicate that starch degradation plays a more important role in the changes in firmness during storage of kiwiberry after harvest.

The expression changes of genes related to starch and pectin that affect the postharvest fruit firmness are regulated by transcription factors [53, 54]. A total of 1458 transcription factors were screened in the turquoise module related to kiwiberry firmness, of which *MYB* transcription factors were the largest. *MYB* is a large family of transcription factors. In bananas, *MaMYB16S* is related to the regulation of starch degradation, and its overexpression promotes banana fruit ripening [55], while *MaMYB3* delays banana fruit ripening by directly inhibiting starch degradation-related genes [25]. In addition, *MYB* family transcription factors are known to play a regulatory role in cell wall degradation [56, 57]. In general, *MYB* family members synergistically regulate the ripening and softening process of fruits in various ways. After further analysis, we screened out three key transcription factors in the turquoise module, including *AGL31*, *ALC* and *HAT14*. Among them, previous studies have shown that *AGL31* mainly regulates flower development [58, 59]. *ALC* has been reported to regulate the development and ripening of fruits, and to also play an important regulatory role in cell separation during fruit ripening and cracking [60, 61]. *HAT14* belongs to the *HDZIP* class II transcription factor family, which has been associated with fruit ripening in peach and banana [62, 63]. All three transcription factors were strongly correlated with the 29 key genes screened to affect the changes in kiwiberry firmness after harvest, and the *HAT14* binding motif was predicted to have a regulatory relationship with 28 of these genes. However, a clearer interaction relationship still needs further functional verification.

Changes in fruit hardness greatly affect the time it can be sold on the market, and the characteristics of kiwiberry that it is very easy to soften have limited the market expansion of this fruit [64]. Previous studies

have obtained plants such as tomatoes, strawberries, and apples with delayed changes in fruit hardness after harvest through genetic manipulation, providing reference and confidence for obtaining storage resistant kiwiberry through genetic manipulation [65–67]. In this study, 29 genes and 3 transcription factors were identified through transcriptome sequencing and bioinformatics analysis that may affect the hardness of kiwiberry. These results deepen our understanding of post-harvest fruit softening and provide theoretical support for obtaining kiwiberry varieties with extended shelf life in the future.

Conclusion

In this study, we found that the firmness of kiwiberry reached a low value on the 5th day after harvest and the skin rotted on the 9th day of storage. The starch and pectin contents of kiwiberry decreased with the increase of room temperature storage time. Transcriptome sequencing and WGCNA identified 29 key genes affecting the postharvest firmness changes of kiwiberry, including 2 pectin metabolism genes, 14 starch synthesis genes, 6 starch decomposition metabolism genes, and 7 starch metabolism genes. In addition, three transcription factors, *AGL31*, *HAT14*, and *ALC*, were found to be strongly positively correlated with the 29 genes affecting the postharvest firmness changes of kiwiberry. Further analysis showed that 28 of these 29 key genes were predicted to be regulated by *HAT14*. Overall, our results reveal the changes in morphological characteristics and physiological indicators during the ripening and softening process of kiwiberry under room temperature storage, providing new insight into the transcriptional regulatory mechanism of postharvest kiwiberry.

Abbreviations

WGCNA	Weighted gene co-expression network analysis
FPKM	Fragments per kilobase of transcript per million mapped reads
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
DEGs	Differentially expressed genes
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

Supplementary Material 6.

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Authors' contributions

Z.L. and D.L. conceived and designed the research. Z.L., Y.S., Z.W. and T.L. conducted the experiments. Z.L., Z.L., J.S., and W.Y. collected and analyzed the data. Z.L. and D.L. wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center [68, 69], China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA017428) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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