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Transcriptome and metabolite profiling reveal that spraying calcium fertilizer reduces grape berry cracking by modulating the flavonoid biosynthetic metabolic pathway

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ABSTRACT

Grape (*Vitis vinifera* L.) is one of the most widely cultivated fruit crops globally. Fruit cracking during fruit growth and development severely affects yield and quality, resulting in significant economic losses. Currently, calcium fertilizer application is used to prevent berry cracking. However, the mechanisms by which calcium fertilizer treatment reduces berry cracking are poorly understood. To explore this, transcriptomics and metabolomics were used to identify the differentially expressed genes (DEGs) and differentially abundant metabolites in *V. vinifera* '90-1'. We found that secondary metabolic pathways were enriched during the veraison and maturity stages, including the flavonoid biosynthesis pathway. Enrichment analysis indicated that most of the DEGs were enriched in the functional category of flavonoid biosynthesis. As secondary metabolites are largely antioxidants, the spraying of calcium fertilizers may improve the antioxidant capacity of the berries by regulating genes related to the flavonoid metabolism pathway, thus reducing the occurrence of berry cracking.

1. Introduction

Fruit cracking is a phenomenon that occurs during the growth and development of horticultural crop fruits (Khadivi-Khub, 2015). Fruit cracking influences fruit appearance, and fungal infections may occur in the cracked sites, thus reducing the commercial value and ultimately resulting in significant economic losses (Khadivi-Khub, 2015).

The current research on fruit cracking has mainly focused on the factors affecting fruit cracking occurrence, such as fruit phenotypic characteristics, cell anatomical structure and mechanical properties, external environmental factors, and cultivation management (Correia, Schouten, Silva, & Gonçalves, 2018). In contrast to studies on the physiological mechanisms that influence fruit cracking, studies on the molecular biological mechanisms underlying fruit cracking are limited (Balbontín, Ayala, Rubilar, Cote, & Figueroa, 2014; Li, Wu, Zhang, Shi, Liu, Shu, & Wei, 2014; Li, Fu et al., 2019; Li, Li et al., 2019).

The main purpose of understanding mechanism of fruit cracking is to prevent and control its occurrence. Current measures include spraying mineral elements, plant growth regulators, and other substances (Garcı´a-Luis, Duarte, Kanduser, & Guardiola, 2001; Kafle, Khot, Zhou, Bahlol, & Si, 2016). Growth regulators are beneficial for reducing the incidence of fruit cracking (Garcı´a-Luis et al., 2001), and their physiological effects include increasing the plasticity of the cell wall, which, in turn, affects hydrolytic activity and cell growth. In terms of the prevention and control of fruit cracking, the direct application of Ca in mineral elements has been extensively studied (Kafle, Khot, Zhou, Bahlol, & Si, 2016). In field trials, reports of the use of Ca salts alone or in combination with other nutrients have yielded positive results and can reduce the rate of fruit cracking to some extent (Kafle, Khot, Zhou, Bahlol, & Si, 2016).

Researchers have attempted to elucidate how Ca application prevents fruit cracking. Ca participates in the overall development of the fruits, especially changes in the metabolism of cell walls (Pham, T, Singh, & Behboudian, 2012). Ca^{2+} has the function of interacting with cell wall components and has important physiological functions such as maintaining the stability of the cell wall structure and regulating cell wall metabolic enzyme activity (White & Broadley, 2003). Ca^{2+} in the intracellular space interacts with the cell wall to synthesize Ca^{2+}-glycan crosslinked polymers, which help promote extrusion between pectin polymers, form a cell wall network, enhance mechanical strength, and limit cell wall hydrolase entry (White & Broadley, 2003). Ca treatment can significantly increase Ca^{2+} content in the cell

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wall of the skin; in particular, it can significantly increase the content of bound calcium in the cell wall of the skin. In addition, it can inhibit the degradation of pectin, cellulose, and hemicellulose, reduce arabinose and galactose contents, and increase water-soluble pectin in the cell wall of the skin (Blanco, Fernández, & Val, 2010). However, studies on the details of the associated mechanisms are limited.

Grape is one of the most widely grown fruit crops (Massonnet et al., 2017). Grapevine berries are commercially grown for fresh fruits, juices, and raisins, but are mainly used for fermentation into wine (Massonnet et al., 2017). Berry development is a complex process that involves profound physiological and metabolic changes. Grapevine berries undergo two different growth stages: the green ripening stage from flowering to 60 days and the ripening stage (Coombe, 1992). During the green ripening period, the berries grow rapidly through cell division, and then the cells swell (Keller, 2010). The end of the green ripening period is characterized by slower berry growth, wherein malic acid accumulates in the pulp, and the total organic acid concentration reaches the highest level. The process of transitioning to maturity is called veraison, which occurs when the seeds mature. At the ripening stage, further metabolic changes make the fruit edible and attractive, which promotes the spread of seeds and includes changes in peel color, cell swelling and influx of water, berry softening, accumulation of sugar in the pulp, loss of organic acids, and synthesis of tannins and volatile aromatic compounds (Keller, 2010).

Field observations have shown that grape berry cracking starts during the veraison period and is the most severe when ripe (Zhang et al., 2020). Several studies have revealed that spraying calcium-containing solutions can reduce the occurrence of fruit cracking (Kafle, Khot, Zhou, Bahlol, & Si, 2016); however, studies on the molecular mechanisms of fruit cracking prevention are limited. Here, a preliminary study on the molecular mechanism of spraying calcium fertilizers to prevent and control berry cracking was conducted using transcriptome and metabolome analyses.

2. Methods

2.1. Plant materials

In the present study, the grape variety '90–1' (red table grapes) was obtained from the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (113°39′E, 34°43′N), and was selected from the early-ripening grape variety 'Zana' through the bud mutation line. The grape variety '90–1' was introduced from the Luoyang Advanced College for Professional Training, Henan Province, China. '90-1' possesses various excellent traits such as good yield and very early maturity (the growth period is 70 days, which is 30 days less than 'Zana'), but it is prone to cracking, which seriously affects its large scale promotion.

The vines were pruned into two branches with one or two clusters for each branch, and the branches were pruned vertically to about 1.5 m. "Groquiter ® Calcium" (water-solubility; total calcium ≥ 10.0%; chelated calcium ≥ 9.0%; Ca^{2+} ≥ 1.0%; Badische Anilin-und-Soda-Fabrik, Ludwigshafen, Germany) was sprayed onto the clusters during the berry green growth stage from when the berry was pea sized to the onset of veraison. The same amount of distilled water was sprayed onto the plants as the control. For conventional pest management, no growth regulators were used during growth. Unless otherwise specified, berries were sampled between 08:00 and 10:00 in the morning. Berries at the same maturity stage (45 days after full bloom) and with no defects on the surface were collected.

2.2. Statistics of the berry cracking rate at 80 days after full bloom (during berry maturity)

Three berry clusters treated with calcium fertilizer (treatment) or distilled water (control) were randomly selected, and the total number of berries and the number of berries with cracks were counted. Berry-cracking rate = number of cracked berries/total number of berries.

2.3. RNA preparation and RNA sequencing

The total RNA of the material was extracted using the TRIzol (Sigma-Aldrich, St. Louis, Missouri, USA) method, and the samples were treated with DNase I (Beijing Solarbio Science & technology co., Ltd., Tongzhou District, Beijing, P.R.China) to eliminate DNA contamination. A Nanodrop 1000 (Thermo Scientific, NanoDrop Technologies, Inc., Wilmington, DE, USA) was used to detect the purity and concentration of the RNA, and 1% agarose gels (Sigma-Aldrich, St. Louis, Missouri, USA) were used to detect the integrity of the RNA. The mRNA was enriched with oligo (dT) beads and randomly broken into smaller fragments. The small fragments were then used as templates for reverse transcription with random primers and for the synthesis of double-stranded cDNA. Following end-repair of the double-stranded cDNA, a single nucleotide A and a connector were added. The modified cDNA was used as a template for PCR amplification for the *de novo* RNA-seq cDNA library.

2.3.1. Sequence assembly and annotation

Trim Galore software (Stanford University, Palo Alto, California, USA) was used to dynamically remove linker sequence fragments and low-quality fragments from the 3′ end of the Illumina sequencing data. FastQC software (Bioinformatics, Babraham Institute, Cambridge, UK) was used for quality control analysis of the preprocessed data. Trinity software (Trinity Technologies & software Solutions Pvt Ltd., Hubli, Karnataka, India) was used to *de novo* assemble high-quality sequences, and the transdecoder program in this software was used to predict the potential protein-coding region of the transcript based on the Markov model principle. The transcript or longest sequence where the longest protein is selected constitutes the Uni-Gene. Finally, the BLAST2GO program (https://www.blast2go.co–m), the Universal Protein Resource (UniProt) database (http://www.uniprot.org), Non-redund-ant (Nr) database (http://www.ncbi.nlm.nih.gov), Gene Ontology (GO) database (http://gene-ontology.org), Kyoto Encyclopedia of Genes and Genomes (KEGG pathway) database (http://www.genome.jp/kegg), and Interpro database (http://www.ebi.ac.uk/interpro) were used to perform functional annotation on the above-mentioned UniGenes, and the E-value was set to 1×10^{-3}.

2.3.2. Gene expression pattern analysis

The Fragments Per Kilobase per Million (FPKM) calculation method was used to estimate the expression level of each transcript, and the differentially expressed genes (DEGs) were screened according to the criterion that the difference in expression level between the groups was > 2 times and the *P*-value was < 0.05. The DEGs were used for GO enrichment and KEGG analysis.

2.4. Metabolome analysis

2.4.1. Metabolite extraction and derivatization

Metabolites were extracted in 400 μL of 75% methanol (TCI Shanghai, Fengxian District, Shanghai, P.R.China), and 20 μL of ribitol (Sigma-Aldrich, St. Louis, Missouri, USA) was used as an internal standard. Steel balls (Sunan Weijie Steel Balls Co., Ltd, Changzhou, Jiangsu, P.R.China) were added to the sample extract, which was ground at 40 Hz for 4 min and then sonicated in an ice bath for 5 min. The supernatant was centrifuged at 4 °C and 12000 rpm for 15 min. After the extract had been dried by a vacuum concentrator (Beijing JM Technology Co., Ltd, Zhongguancun, Beijing, P.R.China), it was derivatized. First 60 μL methoxyamine salt reagent (20 mg/mL pyridine dissolved, Sigma-Aldrich, St. Louis, Missouri, USA) was added to the extract and treated at 80 °C for 30 min, following which 80 μL BSTFA (containing 1% TMCS, v/v, Shanghai Aladdin Biochem-

ical Technology Co., Ltd, Fengxian District, Shanghai, P.R.China) was quickly added, and the solution was incubated at 70 °C for 2 h in preparation for gas chromatography time-of-flight mass spectrometry (GC -TOF-MS, LECO Corporation, St. Joseph, Michigan, USA) on-board detection.

2.4.2. GC-TOF-MS metabolite detection and data processing

An Agilent 7890 gas chromatography-PEGASUS HT TOF mass spectrometer (Agilent Technologies, Palo Alto, California, USA) was used for the GC-TOF-MS analysis. The system used a DB-5MS capillary column (Agilent Technologies Inc., Palo Alto, California, USA) with a size of 30 m × 250 μm × 0.25 μm. Helium was used as the carrier gas, and 1 μL of the sample was loaded in splitless mode. Chroma TOF4.3X software (LECO Corporation, St. Joseph, Michigan, USA) was used to extract the original peaks, filter and correct the data baseline, and then identify the peaks by comparing peaks and analyzing and deconvoluting them. After that, the retention index (RI) and the LECO-Fiehn Rtx5 database were used to annotate the metabolites. The RI tolerance was 5000.

Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were performed on the metabolites using SIMCA V14.1 software (Umetrics AB, Umea, Scania, Sweden). The screening principle for the differential metabolites was a Variable Importance in the Projection (VIP score > 1 and a p-value < 0.05. OmicsShare software (Guangzhou Genedenovo Biological Technology Co., Ltd., Guangzhou, Guangdong, P.R.China) was used to perform cluster analysis and produce a heatmap. The parameter settings were as follows: the metabolite data were first z-transformed (centered on the mean and divided by the standard deviation of the metabolite), and then distance measurements were performed using Pearson's correlation coefficient and average clustering algorithms.

2.4.3. KEGG annotation and enrichment analysis

The identified metabolites using the KEGG compound database (http://www.kegg.jp/ke-gg/compound/), and then the annotated metabolites were mapped to the KEGG pathway database (http://www.kegg.jp/kegg/pathway.html). The metabolite regulatory pathway was then input into MSEA (metabolism group enrichment analysis), the importance of which was determined by the *P*-value of the hypergeometric test.

2.5. Statistical analysis

Data analyses were performed using SPSS version 16.0 (IBM, Armonk, NY, USA) and R (https://www.r-project.org).

3. Results

3.1. Rate statistics of berry cracking in treated and control mature grapevine berries

The rate statistics of berry cracking in the treated and control mature grapevine berries is shown in Table 1. The rate of berry cracking after spraying with calcium fertilizer was significantly lower than that without (35.5% *vs*. 85.05%). The berry cracking rate decreased by 58.26%.

Table 1
Berry-cracking rate of treatment and control mature grapes.

Group name	Berry-cracking rate/%
Control	85.05 ± 2.69**
Treatment	35.05 ± 3.91

Notes: ** represents an extremely significant difference at the P = 0.01 level based on Student's *t*-test.

3.2. Metabolomics analysis of the mechanism whereby calcium fertilizer reduces the berry cracking rate

3.2.1. Analysis of detected metabolites

After qualitative and quantitative analysis of the detected metabolites, the fold-change of the quantitative information of the metabolites in each group could be compared. Figs. S1 and S2 show the differences in multiple log2 values in the group in Turn CK *vs*. Turn T and Ripe CK *vs*. Ripe T, respectively. The metabolite results based on ranked changes are shown. 'CK' refers to control samples (not treated), 'T' refers to treated samples. 'Turn' refers to the veraison stage, and 'Ripe' refers to the mature stage.

3.2.2. OPLS-DA of the different groupings

The results of the OPLS-DA based on the different grouping is shown in Figs. S3 and S4. The model Q2 score of Turn CK *vs*. Turn T was 0.98, and that of Ripe CK *vs*. Ripe T was 0.958; both were excellent models, indicating that the models were stable and reliable.

3.2.3. Screening of differential metabolites

Differential metabolites were screened based on the *P*-value of the *t*-test and the VIP values of the OPLS-DA model in three biological replicates. The screening criteria were as follows: FC > 2, *P*-value < 0.05, and VIP > 1. The detailed screening results of Turn CK *vs*. Turn T and Ripe CK *vs*. Ripe T are shown in Tables S1 and S2, respectively. The number of differential metabolites in each group is shown in Table S3. From Turn CK *vs*. Turn T, 44 significantly different metabolites were screened out, of which 27 were upregulated and 17 were downregulated, Ripe CK *vs*. Ripe T screened 26 significantly different metabolites, of which 12 were upregulated and 14 were downregulated. Based on the Venn diagram (Fig. S5), the relationships between the differential metabolites between groups were compared and analyzed. Turn CK *vs*. Turn T and Ripe CK *vs*. Ripe T had 11 identical metabolites that differed significantly. A volcano plot allowed for the visualization of the differences in the expression levels of metabolites in the two groups, as well as the statistical significance of the differences. The differentially expressed volcano map of Turn CK *vs*. Turn T and Ripe CK *vs*. Ripe T is shown in Fig. S6.

3.2.4. Functional annotation and enrichment analysis of differential metabolites using KEGG

The KEGG database was used to annotate differential metabolites (Tables S4 and S5). The number of KEGG annotations of differential metabolites in each group was calculated (Table S6). The number of differential metabolites in Turn CK *vs*. Turn T and Ripe CK *vs*. Ripe T was 44 and 26, respectively, and the number of differential metabolites annotated to KEGG was seven and nine, respectively.

3.2.5. Comparison of differential metabolites during veraison and the maturity period

The analysis showed that seven different metabolites could be annotated during the color transition (veraison) period, and nine different metabolites were annotated at maturity. The color transition period and mature period were annotated to the metabolites catechin and L-epicatechin. A comparison of the differences in metabolite contents between the treatments and controls during the veraison and maturity periods is shown in Fig. 1 and Table S7. Further analysis revealed that compared with the stages of veraison and maturity and the control berries, (−)-epigallocatechin, L-epicatechin, galactinol, D-(+)-sucrose, guanine, and cynaroside exhibited a positive correlation with metabolite content, and (+)-gallocatechin, catechin, rutin, choline alfoscerate, xanthosine, quercitrin, L-(−)-cystine, and matairesinol were inversely correlated with the metabolite content. Com-

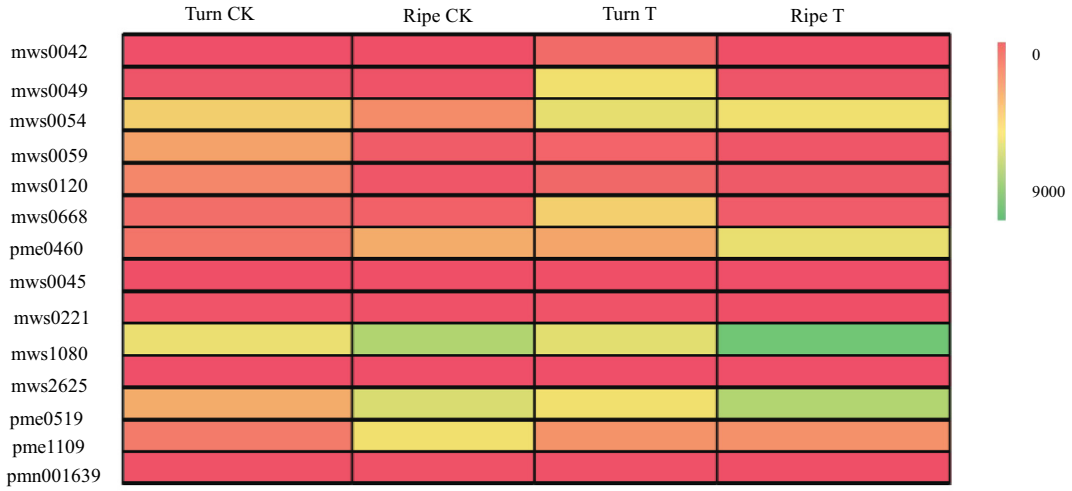

Fig. 1. Heat map of differential metabolite content annotated by KEGG. Notes: A heatmap showing the differential metabolite contents annotated by KEGG. Mws0042: (−)-epigallocatechin; mws0049: (+)-gallocatechin; mws0054: catechin; mws0059: rutin; mws0120: choline alfoscerate; mws0668: xanthosine; pme0460: L-epicatechin; mws0045: quercitrin; mws0221: L-(−)-cystine; mws1080: galactinol; mws2625: matairesinol; pme0519: D-(+)-sucrose; pme1109: guanine; pmn001639: cynaroside. The red font indicates the differential metabolites annotated during the veraison period; the green font indicates the differential metabolites annotated during the maturity period; the purple font indicates the differential metabolites annotated in both the veraison and maturity periods. 'CK' refers to control samples (not treated), 'T' refers to treated samples. 'Turn' refers to the veraison stage, and 'Ripe' refers to the mature stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pared with the veraison and maturity stages, L-epicatechin and D-(+)-sucrose were positively correlated to metabolite content, whereas the content of the other metabolites was negatively correlated. At the berry maturity stage, the metabolites nws1080 (galactinol) and pme0519 [D-(+)-sucrose] showed the highest content between the treatment and control groups. Galactinol and D-(+)-sucrose in the treated berries were 2.12- and 2.26-fold higher than in the control, respectively.

3.2.6. KEGG functional enrichment analysis of differential metabolites

Cluster profiler is an R package specifically used for enrichment analysis of GO and KEGG pathways. Cluster profiler was used to enrich and analyze the annotation results of the differential metabolite KEGG pathway analysis by hypergeometric testing, and three types of diagrams were drawn, including a classification diagram (Fig. S7), point diagram (Fig. S8) and network diagram (Fig. 2). Fig. S8 shows that Turn CK *vs*. Turn T possessed significantly different metabolites enriched in six pathways (flavonoid biosynthesis, ether lipid metabolism, caffeine metabolism, flavone and flavonol biosynthesis, glycerophospholipid metabolism, and purine metabolism), particularly the flavonoid biosynthesis pathway. Significantly different metabolites in Ripe CK *vs.* Ripe T were enriched in seven pathways (flavone and flavonol biosynthesis, galactose metabolism, flavonoid biosynthesis, starch and sucrose metabolism, cysteine and methionine metabolism, ABC transport, and purine metabolism), and the highest enrichment was observed in the categories of flavone and flavonol biosynthesis, galactose metabolism, flavonoid biosynthesis, and ABC transport. The KEGG enrichment dot map of different metabolites (Fig. S8) shows that Turn CK *vs*. Turn T and Ripe CK *vs*. Ripe T were enriched in the flavonoid metabolism pathway, and two metabolites, nws0054 and pme0460, were enriched in the network diagram (Fig. 2).

3.3. Transcriptome analysis of the mechanism by which calcium fertilizer reduces the occurrence of berry cracking

To study the molecular changes that occur in grapevine berries sprayed with calcium fertilizer and to assess their correlation to metabolic profiles, we compared the transcriptomes of the control and treatment groups at veraison and ripening.

3.3.1. Statistics of the number of the DEGs

The number of DEGs is shown in Table S8 and indicates that there were 1066 DEGs during the veraison period, of which 530 were upregulated and 536 were downregulated, and there were 816 DEGs at maturity, of which 255 were upregulated and 561 were downregulat-ed. A Venn diagram was drawn for each group of differential genes (Fig. S9). The Venn diagram shows the number of differential genes unique to each comparison group and the number of common differential genes between the comparison groups. Among these, there were 847 unique DEGs during the veraison period and 597 unique DEGs during the maturity phase. There were 219 DEGs during the veraison period and mature phases.

3.3.2. KEGG pathway enrichment analysis of the DEGs

We performed pathway enrichment analysis of the DEGs, which applies the hypergeometric test to identify pathways that are significantly enriched with DEGs compared with the entire genome background. The results show the top 20 pathways with the smallest significant Q-values (Fig. S10).

Fig. S10 shows that the DEGs during veraison and ripening were enriched in flavonoid biosynthesis (ko00941), phenylalanine metabolism (ko00360), plant circadian rhythm (ko04712), and phenylpropanoid biosynthesis (ko00940), whereas the DEGs during the veraison stage enriched the tyrosine metabolism (ko00350), photosynthesis-antenna proteins (ko00196), tropane, piperidine and pyridine alkaloid (ko00960), starch and sucrose metabolism (ko00500), carotenoid biosynthesis, ko00906, isoquinoline alkaloid biosynthesis (ko00950), β-alanine metabolism (ko00410), and plant hormone signal transduction (ko04075) pathways. The DEGs of the ripening stage were enriched the functional categories of stilbenoid, diarylheptanoid and gingerol biosynthesis (ko00945), nitrogen metabolism (ko00910), and cutin, suberine, and wax biosynthesis (ko00073). In addition, these Q-values were small, indicating that these pathways possessed the highest enrichment of DEGs. The results show that the differential expression of related genes in the aforemen-

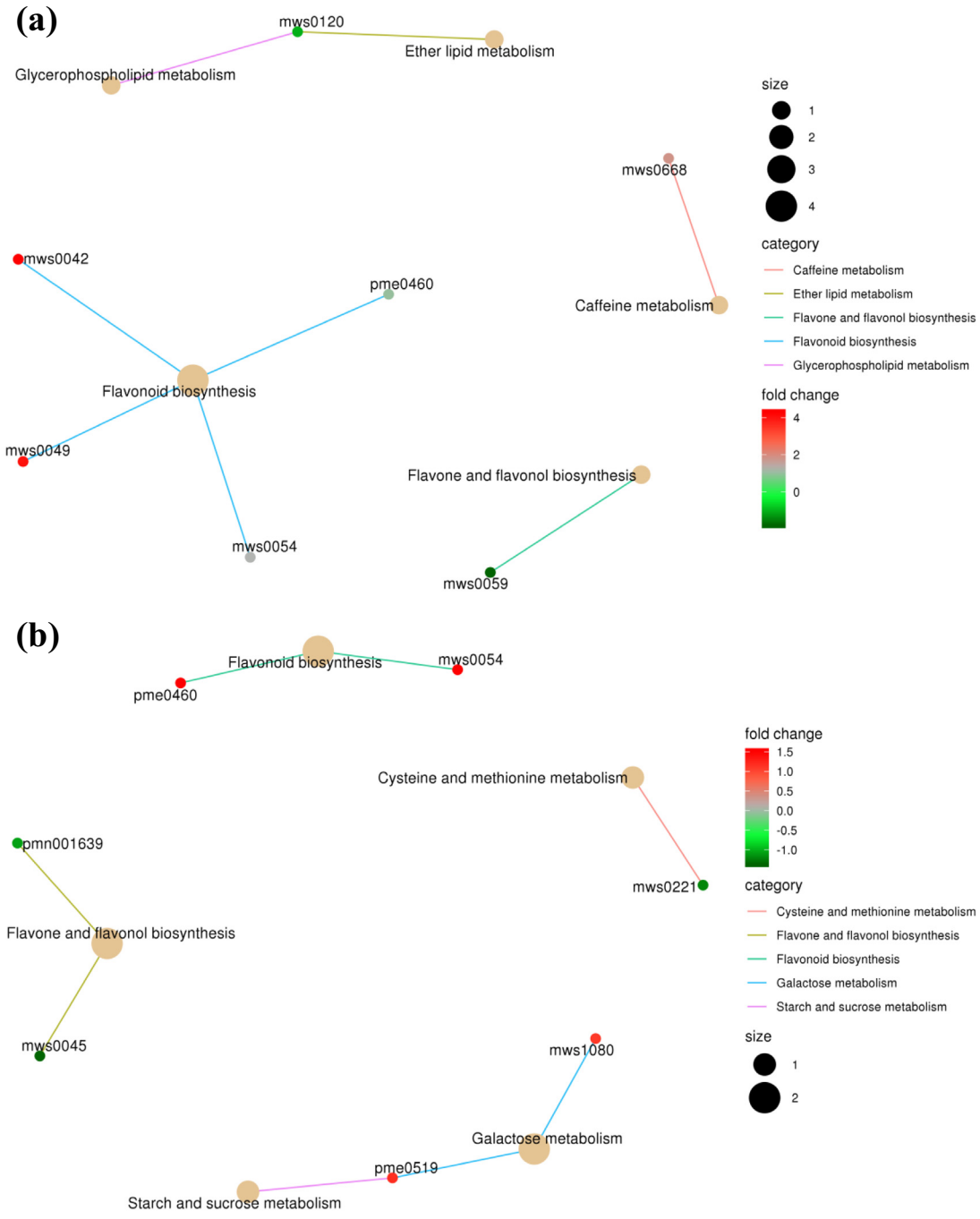

Fig. 2. KEGG enrichment network of differential metabolites. Notes: KEGG enrichment network of differential metabolites in the treatment and control groups during **a,** the veraison stage and **b,** the ripe stage. The light-yellow nodes in the figure are pathways, and the small nodes connected to these are specific metabolites annotated to the pathway. The figure shows a maximum of five pathways. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tioned metabolic pathways was most active during the veraison and maturity stages.

3.3.3. Effect of calcium fertilizer application on the flavonoid biosynthetic pathway

Fig. 3 shows that calcium fertilizer application regulates the expression of most genes that encode structural enzymes involved in the flavonoid pathway (Savoi et al., 2016). Fig. 4 shows the effect of calcium fertilizer application on the relative expression levels of genes related to the flavonoid metabolism pathway. At the berry veraison and ripening stages, three of these DEGs were annotated as phenylalanine ammonia lyase (*VviPALs*) genes with different expression levels.

Among these, *VIT_06s0004g02620* (*VviPAL*) was initially upregulated and then downer-gulated; *VIT_08s0040g01710* (*VviPAL*) was continuously downregulated; and *VIT_13s0019-g04460* (*VviPAL2*) was continuously upregulated. One *trans*-cinnamate 4-monooxygenase (*VviC4H*; *VIT_06s0004g08150*) was continuously upregulated, whereas the other *trans*-cinn-amate 4-monooxygenase (*VviC4H*, *VIT_11s0065g00350*) was continuously downregulated. Three 4-coumarate-CoA ligases (*Vvi4CL*; *VIT_02s0025g03660*, *VIT_11s0052g01090*, *VIT_1-8s0001g00290*) were continuously down-

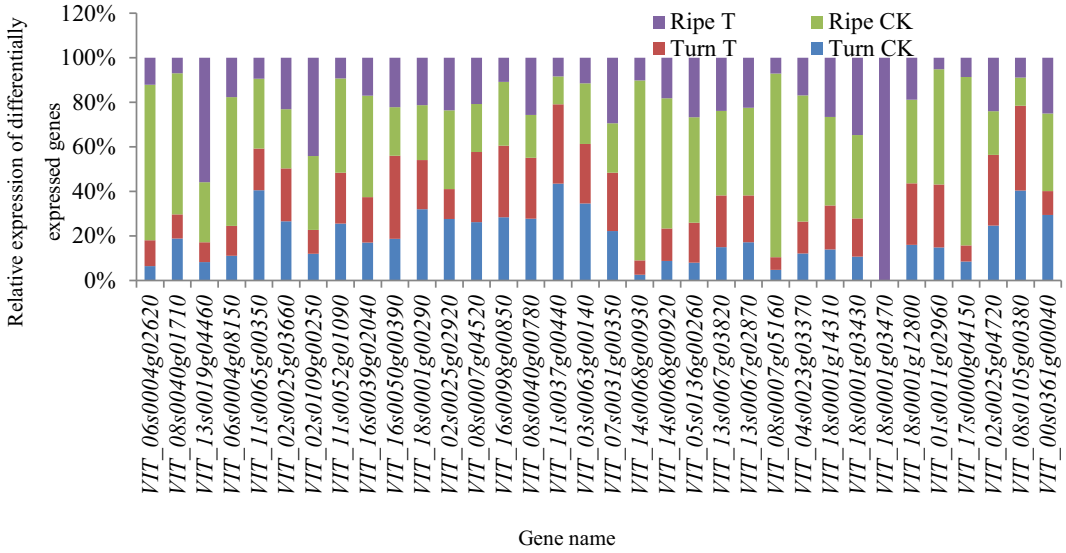

Fig. 3. Effect of calcium application on relative expression of differentially expressed genes in the flavonoid biosynthesis metabolism pathway. Notes: Purple font indicates the relative gene expression in the treatment of at the ripening stage; light green font indicates the relative gene expression in the control treatment at the ripening stage; red font indicates the relative gene expression of the treatment at the veraison stage; blue font indicates the relative gene expression of the control at the veraison stage. 'CK' refers to control samples (not treated), 'T' refers to treated samples, 'Turn' refers to veraison stage, and 'Ripe' refers to the mature stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regulated; one 4-coumarate-CoA ligase (*Vvi4CL*; *VIT_16s0050g00390*) was continuously upregulated; one 4-coumarate-CoA ligase (*Vvi4CL*; *VIT_02s0109g00250*) was initially downregulated and then upregulated; and one 4-coumarate-CoA ligase (*Vvi4CL*; *VIT_16s0039g02040*) was first upregulated and then downregulated. One p-coumaroyl shikimate 3′-hydroxylase (*VviC3H*) was first downregulated and then upregulated; and one hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransfera-se (*VviHCT*) was continuously downregulated. Two caffeic acid 3-O-metyltransferases (*VviCOMT*; *VIT_08s0007g04520*, *VIT_16s0098g00850*) were first downregulated and then upregulated; and the other caffeic acid 3-O-metyltransferase (*VviCOMT*; *VIT_02s0025g02920*) was continuously downregulated. Finally, one caffeoyl-CoA 3-O-methyltransferase (*VviCCo-AMT*; *VIT_03s0063g00140*) was continuously downregulated, and the other *VviCCoAMT* (*VIT_07s0031g00350*) was continuously upregulated.

At the same time, calcium fertilizer application regulated the expression of most of the structural flavonoid genes, particularly three chalcone synthases (*VviCHSs*), two chalcone isomerases (*VviCHIs*), one flavonoid-3′5′-hydroxylase (*VviF3′5′H*), two flavanone-3-hydrox-ylases (*VviF3Hs*), one dihydroflavonol reductase (*VviDFR*), two leucoanthocyanidin dioxygenases (*VviLDOX*), two flavonol synthases (V*viFLSs*), two leucoanthocyanidin reductase (*VviLARs*), and one anthocyanidin reductases (*VviANR*). Among these genes, two chalcone synthases (*VviCHSs*; *VIT_14s0068g00930*, *VIT_05s0136g00260*), two chalcone isomerases (*VviCHls*; *VIT_13s0067g03820*, *VIT_13s0067g02870*), one flavonoid-3′5′-hydro-xylase (VviF3Hs; *VIT_04s0023g03370*, *VIT_18s0001g14310*), one flavonol synthase (*VviF-LS1*, *VIT_18s0001g03430*), one dihydroflavonol reductase (*VviDFR*; *VIT_18s0001g12800*), and one leucoanthocyanidin reductase (*VviLAR1*; *VIT_01s0011g02960*) were initially downregulated and then upregulated; one chalcone synthase (*VviCHS2*; *VIT_14s0068g00920*) and one leucoanthocyanidin dioxygenase (*VviLDOX*; *VIT_02s0025g04720*) were continuousl-y upregulated; and one leucoanthocyanidin reductase (*VviLAR2*; *VIT_17s0000g014150*), one leucoanthocyanidin dioxygenases (*VviLDOX*; *VIT_08s0105g00380*), and one anthocyanidin reductase (*VviANR*; *VIT_00s0361g00040*) were continuously downregulated. In addition, one flavonol synthase (*VviFLS*; *VIT_18s0001g03470*) was not expressed during the color changing phase, but the expression was upregulated during the maturation phase. leucoanthocyanidin reductase (*VviLAR*) and anthocyanidin reductase (*VviANR*) are key regulators of the biosynthesis of flavan-3-ol and proanthocyanidin. Calcium fertilizer *application* always downregulated the expression of one leucoanthocyanidin reductase (*VviLAR2*; *VIT_17s0000g04150*) and one anthocyanidin reductase (*VviANR*; *VIT_00s0361g00040*). Moreover, leucoanthocyanidin reductase (*VviLAR1*; *VIT_17s0000g04150*) was initially upregulated and subsequently downregulated.

3.3.4. Effect of calcium fertilizer application on flavonoid and flavonol biosynthetic pathways

Fig. S11 shows the effects of calcium fertilizer application on the relative expression levels of genes related to the flavonoid and flavonol metabolism pathways. Calcium fertilizer application regulated the expression of genes encoding the flavonoid and flavonol metabolic pathways (Fig. S12). At the fruit maturity stage, four DEGs were screened out, and three genes were upregulated, including flavonoid 3′, 5′-hydroxylase 2 (*VIT_08s0007g05160*), flavonoid 3′hydroxylase (*VVF-3′H2, VIT_17s0000g07210),* and caffeic acid *3-O*-methyltransferase *(VIT_16s0098g00850),* whereas the anthocyanin *O-methyltransferase (FAOMT, VIT_01s0010g03510)* was downregulated. However, there were no differences in the expression of these four genes between the treatment and control groups during the color changing stage.

3.4. Molecular mechanistic model of reducing berry cracking by calcium fertilizer

The molecular mechanistic model of the control of reducing berry cracking by calcium fertilizer application is shown in Fig. 5. It can be seen that the application of calcium fertilizer at the grape berry ripening stage regulated the pathway of flavone and flavonol biosynthesis and flavonoid metabolism pathways. Among these, the flavonoid metabolism pathway as most highly enriched in DEGs, reaching 38, which consisted of 29 upregulated genes and nine downregulated genes. There were four DEGs enriched in flavone

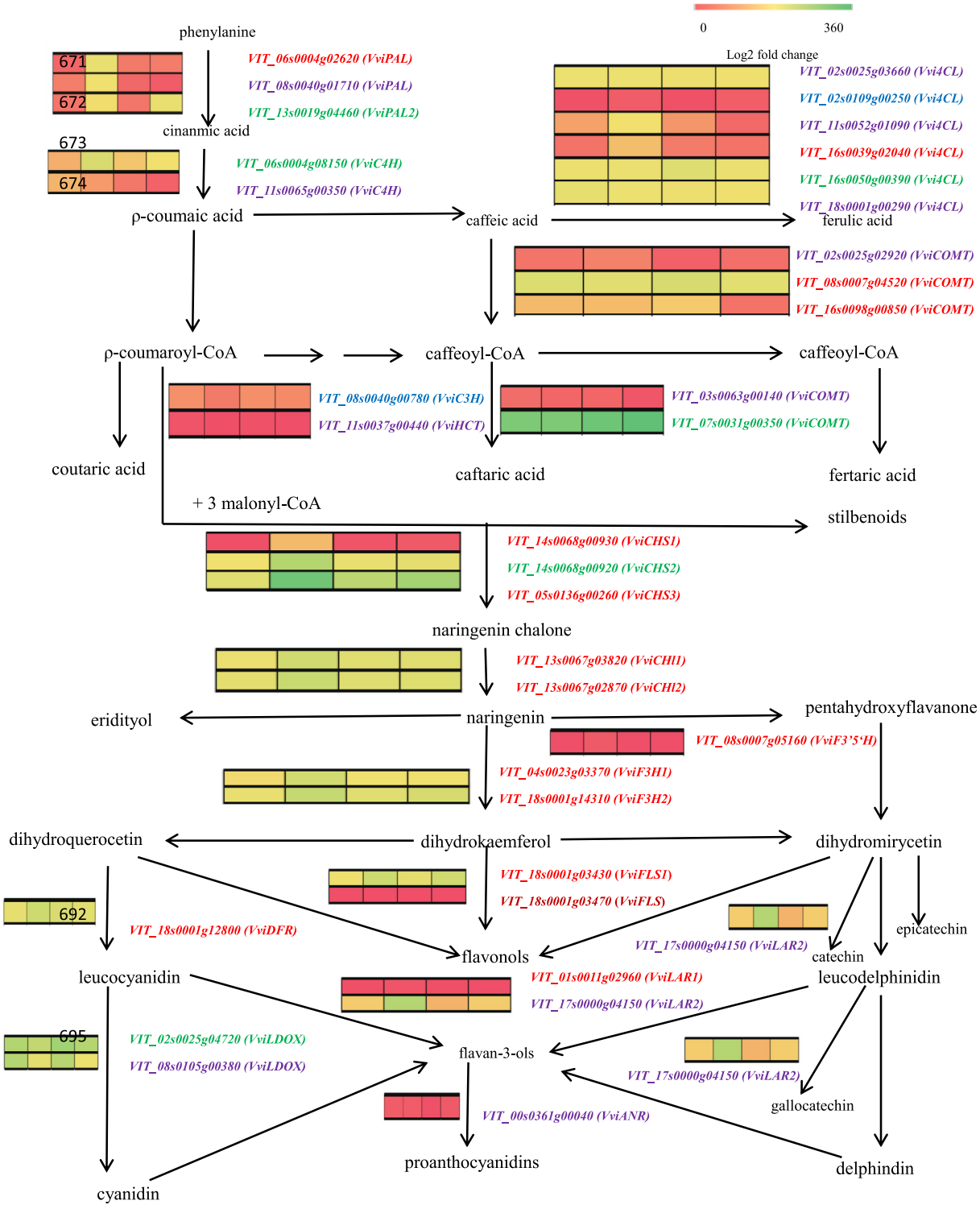

Fig. 4. Modulation of the flavonoid pathway under calcium fertilizer application. Log_2FC (D/C) levels of DEGs are presented at Turn-CK, Ripe-CK, Turn-T, and Ripe-T, respectively, from left to right. Notes: The bold red font in the gene name indicates genes that were upregulated during the veraison period and downregulated during maturity; the bold purple font are genes that were downregulated during both veraison period and maturity periods; the bold green font are genes that were upregulated during both veraison period and maturity periods; genes that are in bold blue were downregulated during the veraison period and genes that were upregulated during maturity; those that are marked in bold dark red are genes that were expressed unchanged during the veraison period and upregulated during maturity. 'CK' refers to control samples (not treated), 'T' refers to treated samples, 'Turn' refers to veraison stage, and 'Ripe' refers to the mature stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and flavonol biosynthesis, consisting of one upregulated gene and three were downregulated genes. Under the influence of these metabolic pathway-related genes, it is possible to reduce the occurrence of grape berry cracking by increasing the contents of catechin and L-epicatechin, and downregulating the contents of quercitrin and cymaroside.

4. Discussion

Fruit cracking is a disorder that commonly occurs in fleshy fruits and is associated with agricultural economic losses (Correia, Schouten, Silva, & Gonçalves, 2018; Khadivi-Khub, 2015). Currently, only a few varieties can fully withstand cracking (Correia, Schouten, Silva, & Gonçalves, 2018). Numerous theories on the mechanisms underlying fruit cracking have been proposed, such as fruit anatomy and cuticle mechanical properties, water potential, osmotic potential and turgor pressure, and water uptake pathways (Yang et al., 2016; Correia, Schouten, Silva, & Gonçalves, 2018).

Research on the mechanism of fruit cracking has informed appropriate management strategies for preventing and controlling fruit cracking, which mainly include rain cover protection (Overbeck, Schmitz, & Blanke, 2017), mineral spraying (Kafle, Khot, Zhou, Bahlol, & Si, 2016; Knoche, Grimm, & Schlegel, 2014), and the application of growth regulators (Suran, Vavra, & Zeleny, 2016). Among these management strategies, the application of calcium solution has been extensively studied (Kafle, Khot, Zhou, Bahlol, & Si, 2016; Knoche, Grimm, & Schlegel, 2014). However, studies on the detailed mechanisms whereby fruit cracking is reduced under calcium application are limited.

With the rapid development of molecular biology, a growing number of genes related to fruit cracking have been identified. Genomic and transcriptome sequences are useful tools for revealing signal transduction pathways as well as new genes (Li et al., 2014; Li, Fu et al., 2019; Li, Li et al., 2019). The research objects of fruit cracking have primarily been apple (Li, Zhang, Zhang, Ji, Zhang, Liu, & Chen, 2013), sweet cherry (Balbontín et al., 2014), lychee (Li et al., 2014), jujube (Li, Fu et al., 2019; Li, Li et al., 2019), and atemoya (Li, Fu et al., 2019; Li, Li et al., 2019). The genes related to cracking that have been identified at the transcription level are mainly related to carbohydrate metabolism (Chen, Duan, Hu, Li, Sun, Hu, & Xie, 2019), cell wall and cuticle (Balbontín et al., 2014; Li et al., 2014; Li, Fu et al., 2019; Li, Li et al., 2019), plant hormones (Li et al., 2014; Li, Fu et al., 2019; Li, Li et al., 2019), and calcium transport-related proteins (Li et al., 2014).

In the present study, we evaluated the mechanism by which calcium fertilizer application prevents grape berry cracking via transcriptomic and metabolomic analysis. Differential metabolites in the color changing and maturity periods were annotated, which indicated that secondary metabolites accounted for 85.71% and 55.56% of the annotated differential metabolites, respectively. Calcium fertilizers control the occurrence of grape berry cracking mainly by regulating secondary metabolic pathways.

Our analysis found that the secondary metabolites (−)-epigallocatechin, (+)-gallocatec-hin, catechin, xanthosine, and L-epicatechin were upregulated, and rutin content was downregulated during the color changing period. The upregulation range was 100.38% –1849.52%. The (−)-epigallocatechin content was most significantly upregulated, while L-epi-catechin was the least upregulated, and the rutin content decreased by 71.00%. At maturity, the secondary metabolites catechin and L-epicatechin were upregulated by 185.15% and 185.62%, respectively, and quercitirin, matairesinol and cynaroside were downregulated by 52.77% – 99.72%. Among these, catechin and L-epicatechin, were annotated together during the color changing and maturity stages. The two metabolite annotation pathways (catechin and L-epicatechin) were included flavonoid biosynthesis (ko00941) and biosynthesis of secondary metabolites (ko01110). This shows that flavonoid biosynthesis regulates the content of catechin and L-epicatechin.

Studies on reducing the occurrence of cracked fruit by calcium fertilizer application to affect the secondary metabolite biosynthetic pathways are limited. The present study demonstrated that secondary metabolites responded to calcium fertilizer application at the transcription and metabolite levels, and determined that secondary metabolite concentrations generally increased. Studies have shown that calcium has an effect early on in the secondary metabolic pathways (Martins, Garcia, Costa, Sottomayor, & Gerós, 2018). Calcium influx is necessary to stimulate secondary metabolite accumulation (Hu, Li, Chen, & Yang, 2009). This study discovered that catechin and L-epicatechin in the flavonoid biosynthetic pathway were annotated at the berry color changing and ripening stages and were upregualted by 100.38% – 135.25% and 185.15% – 185.62%, respectively. At the mature stage, quercitrin and cynaroside, the differential metabolites of the flavonoid and flavonol biosynthetic pathways, were downregulated by 61.22% and 52.77%, respectively.

Flavonoids are a large class of (poly)phenolic compounds that are widely distributed in the plant kingdom. They are structurally divided into flavonoids, flavonols, anthocyanins, proanthocyanidins, and isoflavones. Flavonoids are secondary metabolites with diverse structures in plants and have multiple functions, which include regulating plant development, pigmentation, and UV protection, as well as a series of roles in defense and signaling between plants and microorganisms (Mathesius, 2008). An important aspect of the biological activity of flavones is their structural diversity, which enables flavones to interact with a variety of other molecules, thereby determining their function in lipid oxidation, as well as DNA and protein binding. These interactions are the basis for their multiple effects on human health, and their functions in controlling redox status, enzyme function, lipid peroxidation, and the cell cycle are well established (Mathesius, 2008). Flavonoids are considered antioxidants due to their ability to scavenge free radicals, inhibit lipid oxidation or chelate metal ions (Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007).

There are few studies on the regulation of flavonoid metabolism by calcium fertilizer application to reduce fruit cracking. However, due to the high antioxidant enzyme activity of flavonoids (Mathesius, 2008; Tripoli et al., 2007), the imbalance in oxidative metabolism during fruit ripening is closely related to the occurrence of fruit cracking (Giné-Bordonaba et al., 2017). After spraying calcium fertilizer, the increased content of flavonoids may reduce fruit cracking. It is widely accepted that increased H_2O_2 levels during fruit ripening produce a variety of oxidative stresses leading to physiological damage. Studies have noted that the oxidation process related to H_2O_2 accumulation may play an important role in determining the susceptibility of cultivars to cracking (Giné-Bordonaba et al., 2017). The increase in H_2O_2 levels is in equilibrium with the increase in malondialdehyde, especially in the cracking-susceptible sweet cherry variety ‘Prime Giant’, and the results show that this variety is subject to higher oxidative damage and membrane lipid peroxidation (Giné-Bordonaba et al., 2017). The higher sensitivity to cracking appears to be related to the increased peroxidative damage caused by the reduced ability to convert H_2O_2 (superoxide dismutase [SOD] activity) from destructive reactive oxygen species (ROS) to H_2O (polyphenol oxidase [POX] activity) (Giné-Bordonaba et al., 2017). SOD can eliminate active oxygen generated during the aging of cells, tissues or organs, protect cell membranes, and maintain cellular metabolic balance (Tosun, Yağcı, & Erdurmuş, 2014). One study found that the SOD content of the cracking-susceptible tomato variety ‘LA1698’ was lower than that of the cracking-resistant variety ‘LA2683’ (Yang, Wu, Zhang, Hu, Zhou, & Jiang, 2016). The above studies show that higher antioxidant capacity can reduce the occurrence of fruit cracking. The present study found that the contents of catechin and L-epicatechin increased after spraying with calcium fertilizer. This may increase of the antioxidant capacity of the berry, which can reduce the occurrence of grape berry cracking. However, further direct evidence is required to support this.

The transcriptome results of the present study revealed that calcium fertilizer application regulated the gene expression of components of the flavonoid biosynthetic pathway. There were five different trends in these genes during color changing and maturity

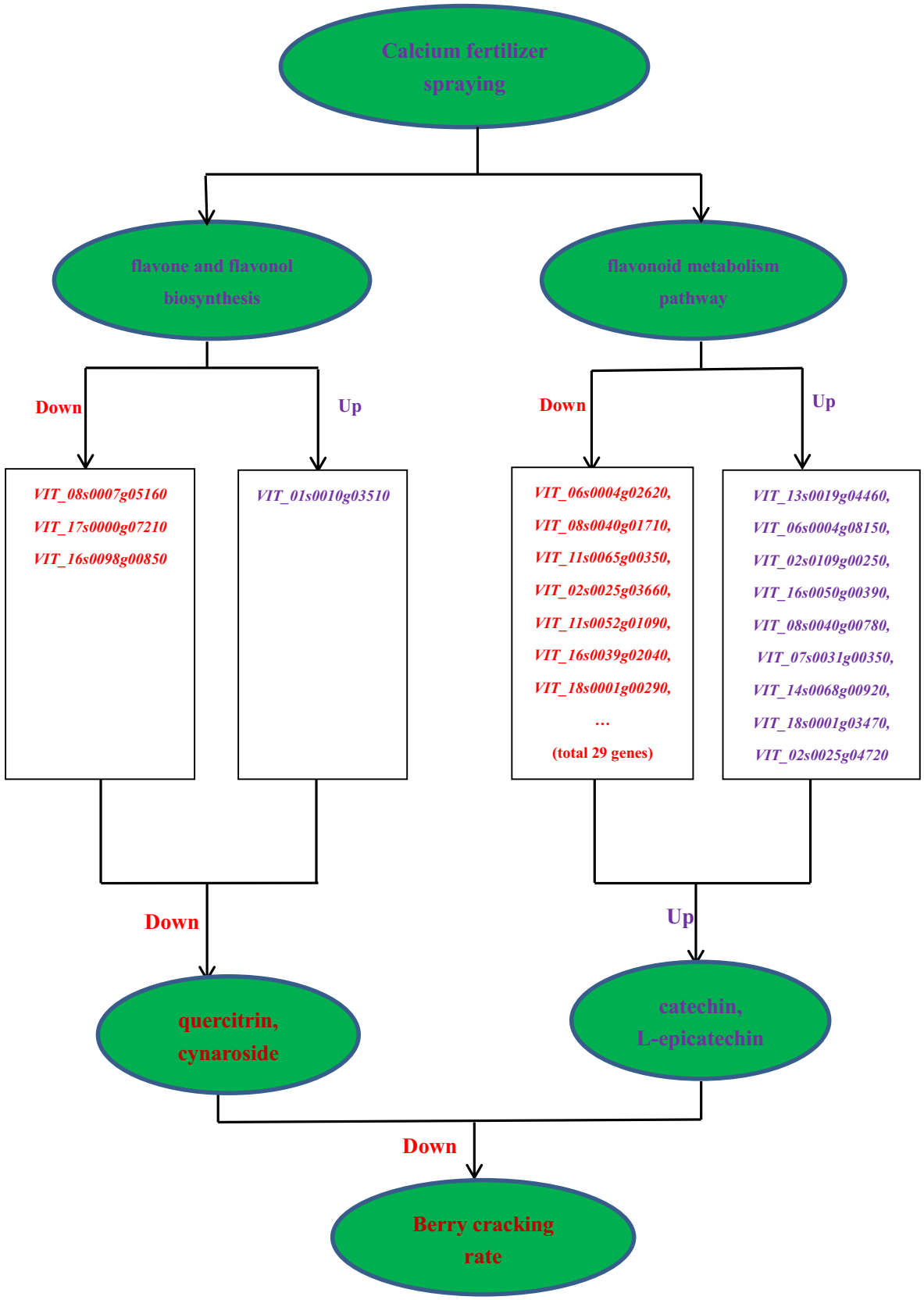

Fig. 5. Mechanism of reducing grape cracking under calcium fertilizer application. Notes: Red bold font indicates downregulated genes or metabolites; purple bold font indicates upregulated genes or metabolites. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(upregulation of the color changing and downregulation of maturity stages, including downregulation during color changing and upregulation during maturity; downregulation during both color changing and maturity; upregulation during both color changing and ripe maturity; and maintenance of the expression level during the color changing stage and upregulation at the mature stage. The numbers of related genes ranged from 1 to 14. Among these, the number of genes that were upregulated and downregulated during the maturity stage was the largest and mainly included phenylalanine ammonia lyases (*VviPAL*; *VIT_06s0004g02620*), 4-coumarateCoA ligase (*Vvi4-CL*; *VIT_16s0039g02040*), caffeic acid 3-O-metyltransferase (*VviCOMT*; *VIT_08s0007g045-20*, *VIT_16s0098g00850*), chalcone synthases (*VviCHS1*, *VIT_14s0068g00930*; *VviCHS3*, *VIT_05s0136g00260*), chalcone isomerases (*VviCHl1*, *VIT_13s-0067g03820*; *VviCHl2*, *VIT_13s0067g02870*), flavonoid-3′5′-hydroxylase (*VviF3′5′H*; *VIT_08s0007g05160*), flavanone-3-hydroxylases (*VviF3H1*, *VIT_04s0023g03370*; *VviF3H2*, *VIT_18s0001g14310*), flavonol synthase (*VviFLS1*; *VIT_18s0001g03430*), dihydroflavonol reductase (*VviDFR*; *VIT_18s0001g12800*), and leucoanthocyanidin reductase (*VviLAR1*; *VIT_01s0011g02960*). The expression level was maintained during the color changing period, and only one gene was upregulated during maturation, namely, flavanol synthase (*VviFLS*; *VIT_18s0001g03470*). In the flavonoid and flavonol biosynthetic pathways, transcriptome analysis revealed that spraying calcium fertilizer during the fruit ripening period only regulated the differential expression of four genes. The upregulated genes were flavonoid 3′, 5′-hydroxylase 2 (*VIT_08s0007g05160*), flavonoid 3′ hydroxylase (*VVF3′H2, VIT_17s0000g07210*), and caffeic acid 3-O-methyltransferase (*VIT_16s0098g00850*), and the downregulated gene was anthocyanin O-methyltransferase (*FAOMT*, *VIT_01s0010g03510*).

Calcium fertilizer application regulated the expression of genes in the flavonoid pathway, particularly catechin and L-epicatechin, is higher, which resulted in increased antioxidant capacity (Colon & Nerin, 2012), which can reduce the occurrence of grape berry cracking to a certain extent. Fruit cracking often occurs during maturity (Zhang et al., 2020). Fruit maturation is a complex process including cell wall disintegration, compositional changes, and cell aging, which ultimately leads to organ death (Almeida & Huber, 2001). A recent study suggested that oxidative damage and membrane lipid peroxidation may play an important role in the occurrence of fruit cracking (Giné-Bordonaba et al., 2017). However, it was noted that after the application of calcium fertilizer, flavonoids and metabolites in the flavonol biosynthetic pathways (quercitrin and cynaroside) during the fruit ripening period were downregulated by more than 50% of all differential metabolites. The specific role of these two substances in the process of reducing fruit cracking requires further investigation.

5. Conclusion

This study used a combination of transcriptome and metabolome analyses to elucidate the molecular basis whereby calcium fertilizer application reduces the severity of grape berry cracking. We found that the application of calcium fertilizer mainly modulated the occurrence of grape berry cracking by regulating the secondary metabolite contents of genes related to the flavonoid biosynthesis pathway. In addition, genes related to other metabolic pathways may also be involved in the regulation of grape berry cracking. The obtained information will provide novel perspectives on the mechanisms underlying berry cracking susceptibility.

Author contributions

ZC and FJG provided the experimental ideas and designed the research; ZC specifically implemented the research and performed data analysis; DTY and ZPA made preliminary revisions to the paper; CLW and ZC made the final decision on the paper. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2021.100025.

References

Almeida, D. P. F., & Huber, D. J. (2001). Transient increase in locular pressure and occlusion of endocarpic apertures in ripening tomato fruit. *Journal of Plant Physiology, 158*(2), 199–203. https://doi.org/10.1078/0176-1617-00130.

Balbontín, C., Ayala, H., Rubilar, J., Cote, J., & Figueroa, C. R. (2014). Transcriptional analysis of cell wall and cuticle related genes during fruit development of two sweet cherry cultivars with contrasting levels of cracking tolerance. *Chilean Journal of Agricultural Research, 74*(2), 162–169. https://doi.org/10.4067/S0718-58392014000200006.

Blanco, A., Fernández, V., & Val, J. (2010). Improving the performance of calcium-containing spray formulations to limit the incidence of bitter pit in apple (*Malus* × *domestica* Borkh.). *Scientia Horticulturae, 127*(1), 23–28. https://doi.org/10.1016/j.scienta.2010.09.005.

Chen, J., Duan, Y., Hu, Y., Li, W., Sun, D., Hu, H., & Xie, J. (2019). Transcriptome analysis of atemoya pericarp elucidates the role of polysaccharide metabolism in fruit ripening and cracking after harvest. *BMC Plant Biology, 19*, 219. https://doi.org/10.1186/s12870-019-1756-4.

Colon, M., & Nerin, C. (2012). Role of catechins in the antioxidant capacity of an active film containing green tea, green coffee, and grapefruit extracts. *Journal of Agricultural & Food Chemistry, 60*(39), 9842–9849. https://doi.org/10.1021/jf302477y.

Coombe, B. G. (1992). Research on the development and ripening of the grape berry. Americal Journal Enology and Viticulture, 43, 101–110. doi:https://10.1016/S0065-2164 (08)70256-9.

Correia, S., Schouten, R., Silva, A. P., & Gonçalves, B. (2018). Sweet cherry fruit cracking mechanisms and prevention strategies: A review. *Scientia Horticulturae, 240*, 369–377. https://doi.org/10.1016/j.scienta.2018.06.042.

Garcı´a-Luis, A., Duarte, A. M. M., Kanduser, M., & Guardiola, J. L. (2001). The anatomy of the fruit in relation to the propensity of citrus species to split. *Scientia Horticulturae, 87*(1-2), 33–52. https://doi.org/10.1016/S0304-4238(00)00158-8.

Giné-Bordonaba, J., Echeverria, G., Ubach, D., Aguiló-Aguayo, I., López, M. L., & Larrigaudiè, C. (2017). Biochemical and physiological changes during fruit development and ripening of two sweet cherry varieties with different levels of cracking tolerance. *Plant Physiology and Biochemistry, 111*, 216–225. https://doi.org/10.1016/j.plaphy.2016.12.002.

Hu, X., Li, W., Chen, Q., & Yang, Y. (2009). Early signals transduction linking the synthesis of jasmonic acid in plant. *Plant Signaling & Behavior, 4*(8), 696–697. https://doi.org/10.4161/psb.4.8.9181.

Kafle, G. K., Khot, L. R., Zhou, J., Bahlol, H. Y., & Si, Y. (2016). Towards precision spray applications to prevent rain-induced sweet cherry cracking: Understanding calcium washout due to rain and fruit cracking susceptibility. *Scientia Horticulturae, 203*, 152–157. https://doi.org/10.1016/j.scienta.2016.03.027.

Keller, M. (2010). The science of grapevines: anatomy and physiology || phenology and growth cycle. Academic Press, Burlington, Massachusetts. 49–83. doi:https://10.1016/B978-0-12-374881-2.00002-7.

Khadivi-Khub, A. (2015). Physiological and genetic factors influencing fruit cracking. *Acta Physiologiae Plantarum, 37*, 1718. https://doi.org/10.1007/s11738-014-1718-2.

Knoche, M., Grimm, E., & Schlegel, H. J. (2014). Mature sweet cherries have low turgor. *Journal of the American Society for Horticultural Science, 139*(1), 3–12. https://doi.org/10.1080/01448765.2013.849208.

Li, M., Zhang, Y., Zhang, Z., Ji, X., Zhang, R., Liu, D., & Chen, X. (2013). Hypersensitive ethylene signaling and ZMdPG1 expression lead to fruit softening and dehiscence. *Plos One, 8*(3). https://doi.org/10.1371/journal.pone.0058745 e58745.

Li, W. C., Wu, J. Y., Zhang, H. N., Shi, S. Y., Liu, L. Q., Shu, B., & Wei, Y. Z. (2014). De novo assembly and characterization of pericarp transcriptome and identification of candidate genes mediating fruit cracking in Litchi chinensis Sonn. *International Journal of Molecular Sciences, 15*(10), 17667–17685. https://doi.org/10.3390/ijmsl51017667.

Li, N., Fu, L., Song, Y., Li, J., Xue, X., Li, S., & Li, L. (2019). Water entry in jujube fruit and its relationship with cracking. *Acta Physiologiae Plantarum, 41*, 162. https://doi.org/10.1007/s11738-019-2954-2.

Li, H., Li, W., Zhang, T., Zhong, J., Liu, J., Yuan, C., & Liu, K. (2019). Comparative transcriptomic analysis of split and non-split atemoya (*Annona cherimola* Mill. × *Annona squamosa* L.) fruit to identify potential genes involved in the fruit splitting process. *Scientia Horticulturae, 248*, 216–224. https://doi.org/10.1016/j.scienta.2019.01.017.

Martins, V., Garcia, A., Costa, C., Sottomayor, M., & Gerós, H. (2018). Calcium-and hormone-driven regulation of secondary metabolism and cell wall enzymes in grape berry cells. *Journal of Plant Physiology, 231*, 57–67. https://doi.org/10.1016/j.jplph.2018.08.011.

Massonnet, M., Fasoli, M., Tornielli, G. B., Altieri, M., Sandri, M., Zuccolotto, P., ... Pezzotti, M. (2017). Ripening transcriptomic program in red and white grapevine varieties correlates with berry skin anthocyanin accumulation. *Plant Physiology, 174*(4), 2376–2396. https://doi.org/10.1104/pp.17.00311.

Mathesius, U. (2008). Flavonoid functions in plants and their interactions with other organisms. *Plants, 7*(2), 30. https://doi.org/10.3390/plants7020030.

Overbeck, V., Schmitz, M., & Blanke, M. (2017). Targeted forcing improves quality, nutritional and health value of sweet cherry fruit. *Journal of the Science of Food and Agriculture, 97*(11), 3649–3655. https://doi.org/10.1002/jsfa.8224.

Pham, T, T, M., Singh, Z., & Behboudian, M. H. (2012). Different surfactants improve calcium uptake into leaf and fruit of "Washington navel" sweet orange and reduce albedo breakdown. *Journal of Plant Nutrition, 35*(6), 889–904. https://doi.org/10.1080/01904167.2012.663442.

Savoi, S., Wong, D. C. J., Arapitsas, P., Miculan, M., Bucchetti, B., Peterlunger, E., ... Castellarin, S. D. (2016). Transcriptome and metabolite profiling reveals that prolonged drought modulates the phenylpropanoid and terpenoid pathway in white grapes (*Vitis vinifera* L.). *BMC Plant Biology, 16*(1). https://doi.org/10.1186/s12870-016-0760-1.

Suran, P., Vavra, R., & Zeleny, L. (2016). Effectiveness of potential products to reduce rain cracking of cherry fruit. *Acta Horticulturae, 1137*, 183–186. https://doi.org/10.17660/ActaHortic.2016.1137.26.

Tosun, M., Yağcı, R., & Erdurmuş, M. (2014). *Glaucoma and antioxidant status. Handbook of Nutrition, Diet and the Eye*. Academic Press. https://doi.org/10.1016/B978-0-12-401717-7.00009-5.

Tripoli, E., Guardia, M. L., Giammanco, S., Majo, D. D., & Giammanco, M. (2007). Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry, 104*(2), 466–479. https://doi.org/10.1016/j.foodchem.2006.11.054.

White, P. J., & Broadley, M. R. (2003). Calcium in plants. *Annals of Botany, 92*(4), 487–511. https://doi.org/10.1093/aob/mcg164.

Yang, Z., Wu, Z., Zhang, C., Hu, E., Zhou, R., & Jiang, F. (2016). The composition of pericarp, cell aging, and changes in water absorption in two tomato genotypes, mechanism, factors, and potential role in fruit cracking. *Acta Physiologiae Plantarum, 38*, 215. https://doi.org/10.1007/s11738-016-2228-1.

Zhang, C., Guan, L., Fan, X., Zheng, T., Dong, T., Liu, C., & Fang, J. (2020). Anatomical characteristics associated with different degrees of berry cracking in grapes. *Scientia Horticulturae, 261*, 108992. https://doi.org/10.1016/j.scienta.2019.108992.