



Article Mechanism by Which High Foliar Calcium Contents Inhibit Sugar Accumulation in Feizixiao Lychee Pulp

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Abstract: The problem of Feizixiao lychee fruit cracking is typically solved by the application of calcium to the leaves. However, lychee trees are sensitive to excessive amounts of calcium, and in practice, it is easy to spray excessive amounts that result in fertilizer burns. This paper intends to explore the effects of excessive calcium fertilizer application on lychee fruit pulp quality and the underlying molecular physiological mechanism. Adult Feizixiao lychee trees were used as test materials; concerning treatment, a 54 µM anhydrous CaCl2 aqueous solution was sprayed onto the leaves, and water was used as a control (CK). The levels of pulp sugar and the activities of key enzymes involved in glucose metabolism were observed, and transcriptome analysis and genetic screening were performed on the pulp. Spraying excessive amounts of calcium onto the leaf surfaces caused the downregulation of trehalase-encoding genes and SUS-encoding genes, thus inhibiting the activities of trehalase, SS-I and SS-II, and further inhibiting the accumulation of glucose, fructose, and sucrose. Moreover, upregulation of VIN gene expression enhanced AI activity and inhibited sucrose accumulation, thus inhibiting upregulation of NI gene expression during fruit growth and expansion; in turn, this inhibited the increase in NI activity in the fruit pulp, which then decreased the glucose and fructose accumulation in the pulp in the high-calcium treatment group compared with the CK group. The downregulation and expression of CHS family genes may lead to a decrease in chalcone accumulation, which may lead to damage caused by active oxygen production in the fruit pulp, thus inhibiting the accumulation of soluble sugars in that tissue.

Keywords: calcium; fertilizer; Feizixiao lychee; sugar accumulation

1. Introduction

Litchi (Litchi chinensis *Sonn*.) is a widely cultivated specialty fruit tree in tropical and subtropical areas of China and has high economic value [1]. The tender, juicy, and delicious fruit of "Concubine Xiao" litchi makes it one of the most popular litchi varieties in China, occupying a large share in the litchi market. Therefore, good appearance and inner quality are fundamental guarantees for "smile of princess" litchi fruit's market competitiveness; however, "smile of princess" litchi fruit experience dehiscent problems during the process of growth and development, which seriously influence yield and quality [2], weaken market competitiveness, and damage growers' economic benefit. Many technical measures have been explored to solve the problem of litchi fruit cracking, among which the simplest one is foliar spraying of Ca fertilizer [3].

Foliar calcium fertilizer has a good effect that prevents the fruit cracking, not only on a variety of fruits, but it also improves the intrinsic quality at the same time. Foliar application of Ca fertilizer effectively reduces grape berry cracking [4], increases the content of sugar in pulp, and promotes the degradation of citric acid in ripe grape pulp [5]. Foliar application of Ca fertilizer reduces cracking [6] and sunburn damage of pomegranate fruits, increases the content of total sugar and chlorophyll, and effectively accelerates the vegetative growth process of the tree [5]. Foliar application of Ca fertilizer can increase



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). the hardness of apple meat by acting on the cell wall [5]. Foliar application of Ca fertilizer improved fruit quality and reduced cracking, increased fruit Ca levels and total phenolic content, and reduced the percentage of dehydrated and rotten berries after storage [5]. Therefore, foliar spraying Ca to prevent fruit cracking has become an effective measure for many fruits.

In actual production, owing to the difficulty in accurate control of operation, the problem of spraying Ca fertilizer solution when the concentration is too high leads to the fruit being damaged by Ca fertilizer, resulting in the deterioration of fruit quality, ion toxicity, and salt stress reaction [7]. At the same time, we found that after spraying 0.6% calcium chloride to the canopy, the branches were unchanged but leaves and only a few fruits were covered with some necrotic spots, and the common abnormal performance was a decline in the soluble sugar content of the fruit. However, the physiological, biochemical, and molecular mechanisms of excessive application of Ca fertilizer to fruit trees are rarely studied. The excessive use of Ca fertilizer in pineapple causes a decrease in glucose, fructose, and β -carotene content [8]. High Ca treatment of grapes results in a decrease in sugar-acid ratio, an increase in tannin content, and a decrease in fruit taste quality [9]. In other plants, excessive calcium also affects the normal growth and development of plants: MICU gene inhibits root growth and reduces mRNA transcription levels in land plants under high Ca conditions [10]. Under high Ca stress, the root volume of wheat plants decreased, and the buds showed greening symptoms and shortened length [11]. Under the influence of high Ca, the germination rate of herbage seeds decreased [12]. High Ca is absorbed by cells and accumulated in mitochondria, which may cause cell death [10].

At present, in litchi production, foliar calcium injection is often used to prevent the fruit from cracking [13], but at the same time, it is also found that the litchi tree body is sensitive to calcin; even a little excessive use causes leaf necrosis and a decline in fruit sugar content, similar to the harm to the fruits and other plants described above. Therefore, it is necessary to explore the fertilizer damage caused by excessive Ca in litchi and its mechanism. This paper intends to start from the mechanism of high leaf calcium inhibiting sugar accumulation in "Feizixiao" litchi pulp, so as to provide a theoretical foundation for the development of comprehensive and effective measures for foliar calcium spraying to prevent litchi fruit from cracking.

2. Materials and Methods

2.1. Experimental Setup and Materials

The lychee orchard at the Yongfa Scientific Research Demonstration Station of the Tropical Fruit Tree Research Institute of Hainan Academy of Agricultural Sciences served as the experimental site (latitude and longitude of 19.9° and 109.8°, respectively). The site is located in the town of Yongfa in northern Chengmai County, Hainan Province. The terrain is a plateau and lies in the tropical monsoon climate area. The annual average temperature is 23.8 °C, the annual average number of sunshine hours is 2059 h, the annual average rainfall is 1786.1 mm, and there is no frost year-round. Rain and heat occur in the same season, and the soil is fertile and lateritic. At the time of this study, the soil organic matter content was 23.37 ± 0.87 g/kg, the content of available K was 125.63 ± 2.38 mg/kg, the content of available Ca was $600.51 \pm 12.35 \text{ mg/kg}$, and the content of available Mg was 150.47 ± 3.86 mg/kg. Ten 20-year-old grafted Feizixiao lychee trees displaying the same growth trend with no signs of poor growth were selected for sampling. Feizixiao lychee plants mature quickly when grown in this area—nearly 3–4 weeks earlier than those grown in the Guangdong production area. The main phenological periods of lychee in this region are as follows: the fruit dropping period is in early April, the fruit expansion period is in late April, maturity and harvest occur in early May, and the autumn shoot growth period is from July to September.

2.2. Field Experimental Design and Sampling Methods

The treatments involved spraying a 54 μ M anhydrous CaCl₂ aqueous solution onto the leaf surfaces (high-Ca treatment hereafter; the CaCl₂ used was chemically pure), and a water treatment served as the control (CK). Five replications of single-tree plots were established. The field experiment was initiated at the beginning of fruit expansion (the aril completely covered the seeds). The foliar fertilizer was sprayed once every 7 d for a total of 3 times. The high-Ca treatment was applied between 8 and 9 a.m. on 10, 18, and 25 April 2019 (corresponding to 35, 43, and 50 d after anthesis). The first sampling occurred on 18 April 2019, and subsequent sampling was conducted every 7 d, i.e., at 43, 50, 57, 64, 71, and 77 d after anthesis. As references, five fruits of moderate size and shape were taken from four sides of the tree around the middle of the crown. Thirty fruits similar to the reference fruits were collected from each tree at each collection time. The fruits were quickly frozen in liquid nitrogen and taken back to the laboratory. Then, they were put into a -80 °C ultralow-temperature freezer for storage.

2.3. Measurement Methods of Observed Indicators

The soluble sugar content was determined by anthrone colorimetry. Then, 0.1 g of the sample was ground in a boiling water bath, centrifuged, and placed in a second boiling water bath. After cooling, the anthrone reacted with free hexose or hexosyl, pentosyl, and hexuronic acid in the polysaccharide. After the reaction, the solution was blue-green and had maximum absorption at 620 nm, and the specific content was obtained by comparison with the standard curve [14]. The contents of sugars such as sucrose, fructose, and glucose were determined by the use of a high-performance liquid chromatograph. Here, 0.5 g of pulp was weighed, and 5 mL of 90% ethanol was added to fully grind the sample. Then, the sample was centrifuged, the supernatant collected, and ethanol used for secondary extraction. The two supernatants were combined, placed in a water bath, evaporated to dryness, and brought up to 10 mL with deionized water. The sample was filtered through a 0.45 μ m filter membrane prior to testing. The sugar content was determined using a Waters 2695 high-performance liquid chromatograph manufactured in the United States and equipped with an evaporative light-scattering detector [15]. The fresh sample was weighed and then dried to an ashed sample. The ashed sample was allowed to cool and 10 drops of deionized water followed by 3–4 mL of nitric acid were added to the sample. Excess nitric acid was evaporated by placing the sample on a hot plate set at 100–120 °C. The sample was returned to the furnace and ashed for an additional 1 h and after being cooled, the ash was dissolved in 10 mL hydrochloric acid and transferred quantitatively to 50 mL volumetric acid. Analysis for calcium was carried out using atomic absorption spectroscopy set at different wavelengths for the optimum working conditions of the minerals.

Extraction of the acid invertase (AI), sucrose phosphate synthase (SPS), sucrose synthase II (SS-II), neutral invertase (NI), and sucrose synthase I (SS-I) enzymes was performed according to Nielsen's method [16], and the enzyme activity was determined according to the avidin biotin peroxidase complex enzyme-linked immunosorbent assay and ELISA reagent test kit (Catalogue No.: KT8013-A, KT50452-A, KT5045-A, KT8107-A, KT5044-A; Meimian, Yancheng, Jiangsu, China). For example: Take 2 g of pulp, add 2 mL of acetic acid buffer, grind into a paste with a mortar in an ice bath, 12 000 r/min for 10 min, and retain the supernatant for enzyme activity determination. Using 2 brace-plug graduated tubes, add 0.8 mL of acetic acid buffer, 0.2 mL of 0.5 mmol/L sucrose solution, and 1 mL of appropriately diluted enzyme solution to each tube, and use the same treatment but no enzyme solution as a blank control, and place it at room temperature for 10 min. Then add 1 mL of Nelson reagent to each tube and place the tubes in a boiling water bath for 20 min. Cool to room temperature, add 1 mL of arsenic–molybdic acid reagent to each tube, and after 5 min, add 7 mL of distilled water to each tube, colorimetric at 510 nm, and determine the optical density (OD) 510nm.

Several key time points were selected: 35 d after anthesis was the starting point and 64 or 71 d after anthesis was the end point. The samples were sent to Wuhan Metwell

Biotechnology Co., Ltd., for transcriptome sequencing. Four unigene databases, namely, NCBI nonredundant (NR) protein, SwissProt, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups of proteins, were used in conjunction with alignment via BLASTX software to obtain protein sequences with high similarity; thus, we obtained the protein functional annotation information corresponding to each unigene. To analyze the differentially expressed genes and their clustering, RSEM software was used for quantitative analysis of gene expression based on fragments per kilobase of exon per million mapped fragment (FPKM) values [17], and DESeq2 was used for screening differentially expressed genes [18] and for analyzing subsequent Gene Ontology (GO) and KEGG functional enrichment. Eight differentially expressed genes were randomly selected from the transcriptome results, and their expression was measured via real-time quantitative PCR (qRT–PCR) [19]; the $2^{-\Delta\Delta CT}$ value was calculated and used to evaluate whether the transcriptome sequencing results were reliable.

2.4. Statistical Analysis

The online version of SAS software was used for statistical analysis of the data. ANOVA was used for variance analysis, and the Duncan method was used for multiple comparison analysis. T tests were used to analyze the significance of the differences between the high-Ca treatment and the CK treatment.

3. Data Analysis and Results

3.1. Effects of High-Ca Treatment on Sugar Content

As shown in Figure 1a,b, the glucose and fructose content in the fruit pulp exhibited the same increasing trend; after 71 d, the contents in the high-Ca treatment group were significantly lower than those in the CK group, so the accumulation of reducing sugars was inhibited by the high-Ca treatment.

As shown in Figure 1c, the dynamic changes in the sucrose content showed a singlepeak curve, but the peak in the high-Ca treatment group occurred 57 d after anthesis, and the peak in the CK occurred 50–64 d after anthesis. There was no significant difference in the sucrose content between the high-Ca treatment and CK before 50 d after anthesis, yet at 57 d after anthesis, the content in the high-Ca treatment group was significantly higher than that in the CK group. Additionally, at 57 d after anthesis, the content in the high-Ca treatment was significantly lower than that in the CK, so the inhibition of sucrose accumulation in response to the high-Ca treatment occurred at this time.







Figure 1. Effects of high-Ca treatment on sugar accumulation and Ca content: (**a**) content of glucose; (**b**) content of fructose; (**c**) content of sucrose; (**d**) content of soluble proportion; (**e**) content of Ca. Note: The symbols with * in the figure indicate significant differences between the CK and high-Ca treatments for the same data type; otherwise, there was no significant difference. The same scheme applies to the figures below.

As shown in Figure 1d, the dynamic change trend of the soluble sugar content of the high-Ca treatment and CK was different, and the phenomenon of "sugar withdrawal" appeared in the flesh at the mature stage. The difference was that there was no significant change from 43 to 57 d after anthesis, and at 64 d after anthesis, the value in the high-Ca treatment was significantly lower than that in the CK. As a result, the accumulation of soluble sugar was significantly inhibited by high-Ca treatment.

3.2. Ca Content

It can be seen from Figure 1e that the Ca content of treatment and CK shows a "W" shaped trend of decreasing, rising, decreasing, and rising. There were two troughs, 57 and 71 d after flowering. Treatment was significantly higher than CK at 57 days after flowering; there was no significant difference between treatment and CK on other dates. It shows that pretreatment can increase the Ca content.

3.3. Effects of High-Ca Treatment on the Activities of Key Enzymes Involved in Glucose Metabolism 3.3.1. AI

The changes in AI activity are shown in Figure 2a. The AI activity of the CK group first increased, then decreased and increased again. Finally, there was no significant difference between the CK group and the high-Ca treatment group. The AI activity of the high-Ca treatment group showed a single-peak curve, and the highest peak occurred at 64 d after

anthesis; this peak was significantly lower and occurred earlier than that of the CK group. There was no significant difference in AI activity between the high-Ca treatment group and CK group at 64 d after anthesis. These results indicated that high-Ca treatment inhibited AI activity in the early stage of sugar accumulation, thus inhibiting the accumulation of reducing sugars.



Figure 2. Effects of high-Ca fertilizer on the activities of key enzymes involved in fructose metabolism: (a) AI; (b) NI; (c) SS-I; (d) SS-II; (e) SPS. (f) The net activity of sucrose invertase in the synthetic direction. Note: The symbols with * in the figure indicate significant differences between the CK and high-Ca treatments for the same data type; otherwise, there was no significant difference.

3.3.2. NI

The changes in NI activity are shown in Figure 2b. The change trend of the enzyme activity in the high-Ca treatment group decreased only at 71 d after anthesis, and the enzyme activity in the CK group experienced two increases and decreases. However, the NI activity in the high-Ca treatment group was significantly higher than that in the CK group at 77 d after anthesis and was significantly lower than that in the CK group overall,

indicating that the high-Ca treatment had an obvious inhibitory effect on the accumulation of reducing sugars in the fruit pulp.

3.3.3. SS-I

The changes in SS-I activity are shown in Figure 2c. The dynamic changes in enzyme activity in the high-Ca treatment group and CK group were different. The values in the high-Ca treatment group were significantly lower than those in the CK group at 71 d after anthesis. There was no significant difference at the other time points. Therefore, combined with the results of the sugar content determination, the results here indicate that high-Ca treatment may inhibit the conversion of sucrose to reducing sugars by reducing SS-I activity and subsequently inhibiting the accumulation of reducing sugars in the fruit pulp.

3.3.4. SS-II

The changes in SS-II activity are shown in Figure 2d. The SS-II activity in the high-Ca treatment group was significantly lower than that in the CK group before 64 d after anthesis and vice versa after 64 d after anthesis, but there was no significant difference at 43 and 77 d after anthesis. These results indicated that high-Ca treatment inhibited SS-II activity before 64 d after anthesis, resulting in the inhibition of sucrose accumulation.

3.3.5. SPS

The changes in SPS activity are shown in Figure 2e. The dynamic changes in enzyme activity in the high-Ca treatment group and CK group showed a single-peak curve; the peak value of the activity in the high-Ca treatment group occurred at 64 d after anthesis, and the peak value in the CK group was at 50 d after anthesis. There was no significant difference in SPS activity between the high-Ca treatment group and the CK group at 64 d after anthesis, but the activity was significantly lower in the high-Ca treatment group than in the CK group at 64-50 d after anthesis. High-Ca treatment inhibited SPS enzyme activity, resulting in the inhibition of sucrose accumulation.

3.3.6. Synthesis Direction and Net Activity of the Sucrose Invertase System

The characteristics of net activity change in the synthesis direction of the sucrose invertase system are shown in Figure 2f. The change trend of the net activity in the high-Ca treatment group was "up, down, and up", while that in the CK showed the opposite trend. At 71 d after anthesis, the net activity of the sucrose invertase system in the high-Ca treatment group was significantly higher than that in the CK treatment group. At this time, a large amount of sucrose in the CK group was transformed into glucose and fructose, while less sucrose in the high-Ca treatment group was converted. Overall, the sucrose in the CK group decomposed into fructose and glucose, while the high-Ca treatment inhibited the conversion of sucrose to reducing sugars, and the accumulation of reducing sugars was inhibited relative to that in the CK group.

3.4. Transcriptome Analysis of Fruit Pulp

3.4.1. Assembly and Analysis of Transcriptome Sequencing Data

The sequencing yield of the lychee pulp transcriptome is shown in Table 1. The GC content of each sample is greater than 44.86%, the percentage of Q30 bases is greater than 91.43%, and the error rate is only 0.02%, which indicates that the quality of the bases is high and that the sequencing results are ideal, which ensures the accuracy and reliability of subsequent analysis.

Figure 3a shows that the number of transcripts and unigenes with a length of more than 200 to 2000 bp exceeds 1000, and the number of transcripts and unigenes with a length ranging from 200 to 300, 300 to 400, and 400 to 500 bp exceeds 10,000. Table 2 shows that a total of 306,396 transcripts with an N50 of 1913 bp, an N90 of 456 bp, and an average length of 1119 bp were assembled. A total of 115,413 unigenes were obtained. The average length of these sequences was 894 bp, the N50 was 1581 bp, and the N90 was 335. Taken together,

these findings indicate that the sequencing results are ideal and that the analysis results are reliable.

Table 1. Statistics of transcriptome sequencing data from the pulp of Feizixiao lychee fruit in the high-Ca treatment and CK treatment groups.

Sample	Raw Reads	Clean Reads	Clean Bases (Gb)	Error Rate (%)	Q30 (%)	GC Content (%)
35d-CK	48,281,265	47,115,740.7	7.0	0.02	92.5	44.9
64d-CK	48,142,673	47,328,952	7.0	0.02	91.4	44.9
64d-Ca	49,977,736	49,345,295.3	7.4	0.02	92.3	44.8
71d-CK	47,102,587	46,452,051.3	6.9	0.02	91.6	44.9
71d-Ca	47,311,827	46,672,307.3	7.0	0.02	91.8	44.8

Notes: 35 d-CK, 64 d-CK, and 71 d-CK represent transcripts in the control Feizixiao lychee fruit pulp at 35, 64, and 71 d after anthesis, respectively; 35d-CK, 64d-Ca, and 71d-Ca represent transcripts of Ca-treated Feizixiao lychee fruit pulp at 35, 64, and 71 d after anthesis, respectively.







(a)

Citrus sinensis 8332 (16.14%) Citrus clementina 4270 (8.27%) Citrus unshiu 2718 (5.27%) Vitis vinifera 2172 (4.21%) Theobroma cacao 1534 (2.97%) Quercus suber 1255 (2.43%) Populus trichocarpa 1136 (2.2%) Hevea brasiliensis 1133 (2.19%) Durio zibethinus 1057 (2.05%) Manihot esculenta 868 (1.68%) Juglans regia 868 (1.68%) Cephalotus follicularis 849 (1.64%) Ricinus communis 824 (1.6%)

Jatropha curcas 715 (1.39%) Gossypium raimondii 684 (1.33%) Herrania umbratica 671 (1.3%) Solanum tuberosum 644 (1.25%) Trema orientalis 627 (1.21%) Dimocarpus longan 603 (1.17%) Ziziphus jujuba 601 (1.16%) Carica papaya 593 (1.15%) Hordeum vulgare subsp. vulgare 566 (1.1%) Cajanus cajan 551 (1.07%) Parasponia andersonii 513 (0.99%) Corchorus olitorius 489 (0.95%) Others 17348 (33.61%)

Figure 3. Cont.



Figure 3. Assembly and annotation of the transcriptome from the pulp of Feizixiao lychee: (**a**) sequence length distribution; (**b**) statistical chart of species with gene homology; (**c**) statistical chart of KOG function classification.

Туре	Number	Mean Length	N50	N90	Total Bases
Transcript	306,396	1119	1913	456	342,893,342
Unigene	115,413	894	1581	335	103,163,097

Table 2. The statistics of assembly results.

3.4.2. Functional Annotation and Analysis of Unigenes

The unigenes were subjected to functional enrichment analysis, and the results are shown in Table 3. By comparing the unigene sequences with sequences in the KEGG, NR, GO, and other databases, we found that 55,352 unigenes were annotated in at least one database, accounting for 47.96% of the total. Among these unigenes, the number of unigene annotations obtained from the TrEMBL database was the largest at 51,397, accounting for 44.53%; this was followed by the annotations obtained from the NR and GO databases. In addition, 36,242 unigenes were annotated in the SwissProt database, and 41,174 and 34,652 unigenes were annotated in the KEGG pathway and Pfam databases, respectively.

Table 3. Unigene annotation results.

Database	Number of Genes	Percentage (%)
KEGG	41,174	35.6
NR	51,132	44.3
SwissProt	36,242	31.4
TrEMBL	51,397	44.5
KOG	32,067	27.7
GO	43,328	37.5
Pfam	34,652	30.0
Annotated in at least one database	55,352	47.9

The results of our evolutionary analysis are shown in Figure 3b. The number of homologous genes is ranked from large to small. The top ten species with homologous genes were *Citrus sinensis*, *Citrus clementina*, *Citrus unshiu*, *Vitis vinifera*, *Theobroma cacao*, *Quercus suber*, *Populus trichocarpa*, *Hevea brasiliensis*, *Durio zibethinus*, and *Manihot regia*.

With 603 homologous genes, Dimocarpus longan, which is closest to lychee, ranks 19th. Based on an in-depth study of citrus fruit growth and development, this study provides a reference method and results.

As shown in Figure 3c, the obtained unigenes were compared with sequences in the EuKaryotic Orthologous Groups (KOG) database to analyze their possible functions. The results showed that these gene sequences were related to 25 biological processes.

Among these biological processes (in terms of general function prediction only), the most abundant terms involved posttranslational modification, protein turnover, chaperones, signal transduction mechanisms, translation, ribosomal structure, and biogenesis. Carbon transport and metabolism; energy production and conversion; and secondary metabolite biosynthesis, transport, and catabolism were the next most abundant terms.

3.4.3. Functional Annotation and Enrichment Analysis of Differentially Expressed Genes

Additionally, we examined the positive and negative regulatory processes related to biological processes and the regulation of enzyme activity related to molecular function. As shown in Figure 4a, there were differences in gene expression during the growth and development of the pulp of the fruit in the high-Ca treatment group and the CK group. With respect to sugar metabolism in the pulp, there were different degrees of gene upregulation and downregulation in the CK group compared with the high-Ca treatment group. In total, 48 genes were upregulated in the high-Ca treatment group compared with the CK group at 50 d after anthesis, and 83 genes were downregulated; at 64 d after anthesis, 92 genes were upregulated, and 19 genes were downregulated. Moreover, at 71 d after anthesis, 49 genes were upregulated the expression of genes during fructose metabolism.

Figure 4b shows that at 64 d after anthesis, in the biological process category, the differentially expressed genes in the high-Ca treatment and CK groups were involved mostly in metabolic processes, cellular processes, and cellular component organization or biogenesis. In the broad cellular components category, the genes in the high-Ca treatment and CK groups were involved mostly in extracellular regions, cells, and cell parts. In the broad molecular function category, the differentially expressed genes in the high-Ca treatment and CK groups were mostly involved in catalytic activity.

Figure 4c shows that there are many subclasses of genes differentially expressed at 71 d after anthesis compared with those at 64 d after anthesis, but the number is similar to that at 64 d after anthesis. At 71 d after anthesis, there were also a greater number of genes involved in response to stimuli and a smaller number involved in cell components, tissues, or biogenesis. In the cellular component category, the number of genes annotated as extracellular regions was relatively small. In the molecular function category, binding was also notably annotated.



(a)

Figure 4. Cont.



(c)

Figure 4. Statistics and functional classification of differentially expressed genes: (**a**) statistical chart of gene difference between treatment and control; (**b**) histogram of GO classification of DEGs in CK and treatment at 64 days after flowering; (**c**) histogram of GO classification of DEGs in CK and treatment at 71 days after flowering.

Pathway enrichment analysis was carried out on the genes differentially expressed between the CK group and the high-Ca treatment group. The pathways that were significantly enriched are shown in Figure 5a,b. The fructose content of the high-Ca treatment and CK groups reached a maximum at 64 d after anthesis, and the fructose content of the high-Ca treatment group was significantly lower than that of the CK group at that time. The pathways most significantly enriched were pentose and glucuronic acid interconversion, flavonoid biosynthesis, and others. At 71 d after anthesis, the sugar withdrawal phenomenon was detected in both the high-Ca treatment group and the CK group, and the enriched pathways causing this difference were mainly involved in plant hormone signal transduction and ABC transporters. This indicates that the inhibitory effect of high-Ca treatment on sugar accumulation is mainly reflected in the effects on the above pathways.



Figure 5. Enrichment statistics of metabolic pathways involving differentially expressed genes: (a) KEGG pathway enrichment of DEGs in CK and treatment at 64 days after flowering; (b) KEGG pathway enrichment of DEGs in CK and treatment at 71days after flowering.

Hierarchical cluster analysis was performed on the expression (FPKM values) after analysis and standardization of the differentially expressed genes, and a cluster heatmap of the groups of genes differentially expressed between the high-Ca treatment group and the CK group at 64 and 71 d after anthesis was constructed, as shown in Figure 6a,b. The results corresponding to the two time points and the CK are grouped into one category. The differentially expressed genes could be divided into two categories: those with high expression and those with low expression.

Statistics of KEGG Enrichment



Figure 6. Hierarchical clustering heatmap of differentially expressed genes: (**a**) hierarchical clustering heatmap of differential genes between 509–CK and 509–T1; (**b**) hierarchical clustering heatmap of differential genes between 516–CK and 516–T1. Note: The abscissa represents the sample name and hierarchical clustering results, and the ordinate represents the differentially expressed gene and hierarchical clustering results shown in Figure 6. Red indicates high expression, and green indicates low expression; 509-CK and 509-T1 correspond to the control and high-Ca treatment groups, respectively, at 64 d after anthesis; 516–CK and 516–T1 correspond to the control and high-Ca treatment groups, respectively, at 71 d after anthesis.

Figure 7a shows the metabolic pathway of starch and sucrose metabolism, which mainly controls carbohydrate metabolism. In sucrose metabolism, there are three pathways labeled 3.2.1.21 involving β -D-glucoside glucose hydrolase, which involves an enzyme that promotes the conversion of other sugars to glucose. Pathway number 5.3.1.9 involves α -D-glucose-6-phosphate aldose ketone isomerase, which is involved in the catalysis of α -D-glucose 6-phosphate and the β -reversible conversion between D-fructose and furanose 6phosphate. The pathway number of the SUS-coding gene is 2.4.1.13; this pathway involves sucrose synthase, which is an enzyme that catalyzes the reversible synthesis of sucrose. Interestingly, the expression level of SUS was downregulated. In starch metabolism, the gene expression in pathway number 3.2.1.28 (involving α -trehalose glucose hydrolase) was downregulated; this enzyme is involved in the catalysis of α , α -trehalose. The hydrolysis of the o-bond of glucoside releases the initial equimolar amount of α - and β -D-glucose, which promotes the synthesis of D-glucose. Since the enzymes encoded by these genes directly affect the conversion and accumulation of sucrose, glucose, and fructose, it is suggested that the difference in the expression of these genes may be related to the inhibition of sugar accumulation detected in the high-Ca treatment group. The enzyme encoded by the sus gene is SS, which indicates that high-Ca treatment leads to downregulation of the SS enzyme-encoding gene. In the analysis of differentially expressed genes involved in glucose metabolism in this paper, no significant change was found in the expression of SPS genes, indicating that there may be other physiological and biochemical mechanisms involved in the regulation of SPS activity in the high-Ca treatment group.

Figure 7b shows the biosynthetic pathway of flavonoids, which compose a main category of plant secondary metabolites. Flavonoids are synthesized from phenylpropanoid derivatives by condensation with malonyl-CoA. The genes in pathway numbers 2.3.1.74 (involving 4- β -D-glucan glucose hydrolase) and 2.3.1.170 (involving malonyl-CoA) were downregulated. Both belong to the chalcone synthase (CHS) superfamily. CHS is the first key structural enzyme in flavonoid synthesis and is responsible for catalyzing the formation of chalcone, which, as a precursor of many flavonoids, participates in the formation of downstream secondary metabolites. The downregulation of these genes inhibits the formation of chalcone and may ultimately inhibit the accumulation of flavonoids.

Figure 7c shows a cluster heatmap of the expression levels of the invertase-related genes CIN and VIN. According to the expression level, it can be concluded that the high-Ca treatment affected the expression of the gene-encoding invertase. Moreover, the expression level of the VIN gene in the CK group significantly decreased during sugar withdrawal, while that in the high-Ca treatment group did not significantly decrease. The expression of the CIN gene was significantly increased, but there was no significant change in the high-Ca treatment group. Since the CIN gene encodes NI and the VIN gene encodes AI, high-Ca treatment suppressed the activity of invertase by downregulating the expression of the invertase-encoding gene, thereby inhibiting the accumulation of glucose and fructose.

FLAVONOID BIOSYNTHESIS Cinnamoyl-CoA Pinocembrin chalcone Pinocembrin Pinobanksin Phenylpropanoid biosynthesis **-**5.5.1.6 1.14.11.9 - D- O-►O Pinobanksin 3-acetate 1.1420.6 1.1420.5 + O Chrysin 1.14.1491 Galangin Pinostrobin Ö Liquiritigenin 1.1.1.219 5.5.1.6 →O 5-Deoxyleucopelargonidin 1.14.11.9 ► Q 🚛 Isoliquiritigenin Garbanzol 1.1420.5 ->(Isoflavonoid biosynthesis 1.14.1482 1.14.19.76 7,4-Dihydroxyflavone 1.14.1482 0 5-Deoxyleucocyanidin Butein 5.5.1.6 1.14.11.9 ___**⊳ò** ₽Ò Butin Prunin →O 24.1.236 →O Naringin Dihydro-4-coumaroyl-CoA Phloretin Phlorizin (Isoflavonoid biosynthesis 2.4.1.185 ►0-<u>2.4.1.35</u>7 ►0 Ы Hesperetin Hesperetin 7-O-glucoside -->O-<u>241185</u>->O Neohesperidin Desmethyl-xanthohumol Xanthohumol Xanthohumol ►O 211338 ►O 211339 ►O 131.117 2.1.1.231 ►O Isosakuranetin Dihydrokaempferol 11.1.219 Leucopelargonidin (-)-Epiafzelechin Naringenin Naringenin chalcone 5.5.1.6 1.14.11.9 1.1420.4 p-Coumaroyl-CoA Pelargonidin 1.17.1.3 21.1.232 →O Sakuranetin Naringenin chalcone 4'-O-glucoside 23.1.133 23.1.133 1.1.1.234 - O Apiforol Kaempferol O (+)-Afzelechin 2.4.1 286 + 0 1.21.3.6 • Aureusidin 6-O-glucoside p-Coumaroyl 8-C-Glucosylnaringenin Vitexin 1.1420.6 **+** • -->(Flavone and flavonol biosynthesis p-Coumaroyl shikimic acid -۲O 2.4.1. 1.1420.5 Apigenin 1.14.1481 1.14.1482 1.141496 1.1413.- 1.141496 Chalcone 2'-O-glucoside 1.21.3.6 1.14.19.76 1.14.1481 1.14.1482 Caffeoyl shikimic acid Caffeoyl quinic acid Flavone and flavonol biosynthesis 1.14.1481 1.14.1482 1.1420.6 • Quercetin 23.1.133 23.1.133 2.4.1.286 • 1.21.3.6 • Bracteatin 6-O-glucoside 1.14.1481 1.14.1482 2',3,4,4',6'-Peptahydroxy -chalcone 4'-O-glucoside Leucocyanidin Cyanidin (-)-Epice (-)-Epicatechin 1.1.1.219 1.14.11.9 4 1.1420.4 2',3,4,4',6'-Pentahydroxychalcone Caffeoyl-CoA Dihydroquercetin Eriodictyol 1.17.1.3 1.1420.5 -0-1 Anthocyanin biosynthesis 8 1.14.19.76 Luteolin (+)-Catechin 2.1.1.104 1.1.1.234 ►O Luteoforol 1.14.1481 1.14.1481 1.14.1481 1.14.1481 -1.1420.6 + Myricetin 1.1420.5 ō ₽Ò

Figure 7. Cont.

Feruloyl-CoA

4,2',4',6'-Tetrahydroxy-3-methoxychalcone Homoeriodictyol

(a)

Dihydrotricetin

Tricetin

1.14.11.9

Leucodelphinidin Delphinidin (-)-Epigallocatechin

1.1.1.219

(+)-Gallocatechin

Dihydromyricetin



Figure 7. Cont.



Figure 7. Statistics and cluster heatmap of differentially expressed genes involved in specific metabolic pathways: (**a**) metabolic pathway of starch and sucrose metabolism; (**b**) flavonoid biosynthesis pathway; (**c**) starch and sucrose metabolism pathway.

3.5. qRT–PCR Validation Analysis

qRT–PCR analysis was performed on eight unigenes. As shown in Figure 8, the linear relationship between the RNA sequencing (RNA-seq) data and qRT–PCR data was significantly positive, indicating that the results of the transcriptome analysis were accurate and reliable.





4. Discussion and Analysis

Soluble sugars are important quality indicators of fruits [20]. In lychee, the main sugars are glucose, fructose, and sucrose [21]. Different kinds of lychees have different types of sugars that accumulate as a result of sugar metabolism, and Feizixiao lychees accumulate reducing sugars [15]. In addition, the sugar withdrawal phenomenon can occur [13], which needs further attention. The results of the present study showed that the soluble sugars in lychee pulp were mainly glucose and fructose, and the sucrose content was significantly lower than that of the two monosaccharides, the results of which were consistent with our previous results. The results also showed that the high-Ca treatment inhibited the accumulation of soluble sugars, which was consistent with data from pineapple treated with high Ca [8].

AI, NI, SS-I, SS-II, and SPS [22–25] play an important role in sugar metabolism. The results showed that the high-Ca treatment obviously inhibited the activities of AI and NI during the sugar accumulation period, inhibited the conversion of sucrose to glucose and fructose, and reduced the accumulation of reducing sugars in the fruit pulp. The high-Ca treatment increased SS-I activity and decreased SS-II activity in the early stage of sugar accumulation, and it also decreased SS-I activity and enhanced SS-II activity after sugar withdrawal; that is, at the early stage of sugar accumulation, SS mainly catalyzed the decomposition of sucrose into fructose, and at the time of sugar withdrawal, SS mainly catalyzed the synthesis of sucrose from fructose. At the same time, SPS activity was inhibited, resulting in a decrease in overall sugar accumulation in the pulp. The Feizixiao lychee fruit pulp industry is mainly controlled by the use of these key enzymes. This result is the same as those in previous reports that sugar metabolism of Feizixiao lychee fruit is mainly regulated by AI and SS [26].

Transcriptome analysis showed that high-Ca treatment did not cause changes in the expression of SPS-encoding genes, indicating that the inhibition of the activities of these two enzymes may occur posttranscriptionally, during translation and posttranslation; nonetheless, the underlying molecular mechanism needs further study.

Among the differentially expressed genes encoding α - and β -D-glucose-6-phosphate aldose ketone isomerase [27], the upregulation of D-glucoside glucohydrolase gene expression [28] suggests that the content of reducing sugars in the fruit pulp under high-Ca treatment remains relatively stable. α -Trehalose and glucose hydrolase can catalyze the

decomposition of trehalose to produce glucose, and the downregulation of related genes directly causes a reduction in glucose synthesis. SUS-encoding genes control SS enzyme synthesis, which catalyzes the NDP- α -D-glucose + D-fructose to ribonucleoside 5'- diphosphate + H⁺ + sucrose (reversible) conversion reaction [29]. The expression of the SUS gene was downregulated in the high-Ca treatment group, which was consistent with the change trend of SS-I and SS-II activities in the high-Ca treatment group, the findings of which were opposite those of the CK group. In the forward direction, SS-II led to a decrease in the synthesis of sucrose in the pulp, and in the reverse direction, SS-I led to a decrease in the amount of sucrose converted to fructose; thus, the accumulation of soluble sugars in the pulp was inhibited.

High-Ca treatment upregulated VIN gene expression and downregulated CIN gene expression. Combined with the measurements of the sugar components, the sugar withdrawal phenomenon occurred after the sugar content peaked. At that time, the VIN expression in the CK group was significantly decreased, while the VIN expression positively regulated AI activity [30], which was consistent with the AI activity trend. Moreover, AI promoted sucrose decomposition to provide a carbon source for growth; that is, by inhibiting the downregulation of VIN expression, high-Ca treatment may cause AI activity to remain at a high level, thus reducing sucrose accumulation. The expression of the CIN gene positively regulated NI activity, but there was no change in CIN activity in the high-Ca treatment group. Among them, the upregulation of the CIN gene promoted the accumulation of reducing sugars [25], while the high-Ca treatment in this experiment inhibited the upregulation of NI expression, thus inhibiting the accumulation of reducing sugars. The results of this paper also confirm that invertase plays an important role in fruit sugar metabolism [31].

By analyzing the transcriptome results at the critical stage of fructose accumulation, we found that the genes of the CHS family involved in the flavonoid biosynthesis pathway were downregulated [32]. CHS is mainly responsible for catalyzing the conversion of coumaroyl-CoA to synthesize naringenin chalcone, which, as a precursor, participates in the formation of flavonoids. According to the results of a test analysis, the downregulation of related genes caused a reduction in chalcone synthesis, which indirectly inhibited the accumulation of flavonoids. High-Ca treatment inhibited the accumulation of glucose, fructose, sucrose, and soluble sugars. It can thus be inferred that the content of flavonoids was reduced [32], and the pulp was damaged by reactive oxygen species, thus inhibiting the accumulation of sugars. However, this needs to be further confirmed.

5. Conclusions

The physiological mechanism that occurs in response to excessive Ca fertilizer being sprayed onto leaves to inhibit sugar accumulation is as follows: First, Ca causes the expression of trehalase-encoding genes and SUS genes to be downregulated and inhibits the activities of trehalase, SS-I, and SS-II, thus inhibiting the accumulation of glucose, fructose, and sucrose. It does not cause the expression of SPS-related genes to change but does inhibit the accumulation of this sugar by inhibiting SPS activity. There may be other mechanisms involved in inhibiting the activity of this kind of enzyme, which needs further study (glucose metabolism). High-Ca fertilizer also causes decreased expression of the VIN gene, thus inhibiting AI activity and inhibiting glucose and fructose accumulation. In turn, these phenomena inhibit the upregulation of CIN gene expression in the fruit during the fruit growth and expansion period, thereby inhibiting the increase in NI activity in the pulp and causing a decrease in glucose and fructose accumulation in the treated pulp relative to that in the fruit in the CK group. Inhibition of CIN expression possibly causes a downregulation of CHS family gene expression, causing a decrease in chalcone accumulation, which may lead to damage caused by active oxygen production in the pulp, thus inhibiting the accumulation of fructose. However, these phenomena need further confirmation.

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