

Comparative Physiological and Transcriptomic Analysis Reveal *MdWRKY75* Associated With Sucrose Accumulation in Postharvest Apples With Bitter Pits

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Abstract

Background: Calcium (Ca) deficiency can cause apple bitter pits, reduce the quality and shelf life. WRKY Transcription factors play essential role in plant response to multiple diseases. However, the underlying mechanisms causing bitter pits in apple fruit due to Ca deficiency during storage is extremely limited.

Results: In the present study, the nutritional metabolites and reactive oxygen species (ROS) were compared in Ca-deficient and healthy apple fruit (CK) during storage. Results showed that Ca-deficient apples sustained significantly higher production of ROS, PPO activity, flavonoids, total phenol, total soluble solids (TSS), and sucrose contents, but the contents of Ca, H_2O_2 , titratable acids (TA), glucose and fructose were significantly lower than those of CK during storage. Principal component analysis (PCA) showed that TSS, $\cdot O_2^-$, PPO, MDA and Ca were the main factors, and TSS had a positive correlation with sucrose. Furthermore, transcriptome analysis revealed that WRKYs were co-expressed with sucrose metabolism-related enzymes (*SWEETs*, *SS*, *SPS*). RT-qPCR and correlation analysis indicated that *MdWRKY75* were significantly positively correlated with *MdSWEET1*. Moreover, transient overexpression of *MdWRKY75* could significantly increase the sucrose content and promote the expression of *MdSWEET1* in apple fruit.

Conclusions: Calcium deficiency could decrease antioxidant capacity, accelerate nutritional metabolism and up-regulate the expression of WRKYs in apple with bitter pits. Overexpression of *MdWRKY75* significantly increased sucrose accumulation and the expression of *MdSWEET1*. These findings further strengthened knowledge of the basic molecular mechanisms in calcium-deficient apple flesh and contributed to improving the nutritional quality of apple fruit.

Background

Apples with high nutritional value are favored by consumers. However, the physiological obstacles represented by bitter pits result in serious economic losses for postharvest apples [1]. Bitter pit is a common physiological disease caused by calcium deficiency in the tree during ripening and storage of apples, and symptoms frequently appear during or after cold storage; in severe cases, they may appear before harvest [2]. In fruit, approximately 40 % of free calcium is located in the vacuole, and the other 60 % is located in the cell wall. High calcium content is an important factor regulating cell homeostasis. Normal cell metabolism requires the cell membrane to directly contact free calcium to maintain proper cell membrane stability and function [3]. Metabolic changes in Ca^{2+} content in these compartments might be related to Ca^{2+} depletion in apoplastic solution, which can weaken plasma membrane structures, leading to cell death and bitter pit symptoms [4–5].

As essential nutrients for plants, Ca^{2+} play an important role in regulating fruit development and ripening [6]. Calcium pectinate can affect the sensitivity of plants to fungal infection and participate in the regulation of the ripening process of fruit [7]. Reactive oxygen species (ROS), including $\cdot O_2^-$ and H_2O_2 , which are highly reactive and toxic, and can lead to the oxidative destruction of cells, are usually used as

indicators to measure the senescence of fruits. And malondialdehyde (MDA) is an important product of membrane lipid oxidation [8]. It was reported that exogenous calcium ions delayed fruit ripening and maintained nutritional quality and appearance. With an increase in the calcium concentration, papaya fruit ripening and the senescence process were inhibited, slowing down softening and prolonging storage life [9, 10]; firmness and TSS increased and postharvest decay was reduced in strawberries; total phenolics and total antioxidant capacity increased and TA, ascorbic acid and decay decreased in sweet cherry [11]; protein, ascorbic acid, TA and carbohydrates decreased in banana [12]; and calcium sprays decreased ascorbic acid and sugar content and stimulated catalase enzyme activity and pathogen defense genes during storage in grape berries [13, 14]. In apples, low levels of calcium cause faster ripening, and ascorbic acid and firmness are lower [15]. Furthermore, spraying calcium chloride on preharvest apples can effectively reduce the occurrence of bitter pit, and it can significantly improve the firmness and appearance of apple to enhance shelf time [16–18]. Calcium can reduce fruit cracking and promote the healing of mechanical injury [19]. Thus, calcium deficiency can cause many diseases that influence the edible quality and postharvest storage of fruit.

Sugar content is an important determinant of fruit edible quality, and bitter pits increase TSS and sugar contents, such as sucrose content. Sucrose synthesis (SS) can reversibly catalyze sucrose to fructose, glucose and UDP-glucose [20]. SPS is an important enzyme in the irreversible reaction that catalyzes UDPG and 6-phosphate-fructose to sucrose [21]. In peaches, nitric oxide (NO) enhances gene expression and the activities of SPS and SS and leads to an increase in sucrose content [22]. In apple fruit, it was reported that sodium nitroprusside (SNP) treatment delayed loss of quality by enhancing *MdSPS* and *MdSS* expression and then increasing the sucrose content [23], and the sucrose transporter *MdSUT4.1* participates in the regulation of fruit sugar accumulation [24]. SWEETs were identified as sugar transporters responsible for fruit sugar accumulation, and *MdSWEET9b* and *MdSWEET15a* were involved in regulating fruit sugar accumulation in apple [25]. In pear fruit, *PuWRKY31* can accelerate the synthesis of sucrose by binding to *PuSWEET15* [26]. However, whether WRKY TFs regulate the sugar transporter *SWEETs* in calcium-deficient apple fruit remains unclear.

To explore the molecular mechanisms of postharvest quality in apple fruit calcium deficiency caused by bitter pit, the content of nutrients and antioxidant capacity in apple fruit during the storage period were determined. The main factors involved in bitter pits (Ca, TSS, TA and MDA) were screened by PCA and correlation analysis, and the sugar transporter-related enzyme *SWEET1* and *WRKYs* TF were identified by bioinformatics and RT-qPCR analysis. Furthermore, *WRKY75* was transformed and identified by apple transient expression system. These results will provide new insights into candidate genes for sugar accumulation in calcium-deficient apples and the improvement of fruit quality.

Methods

Plant Materials and Treatment

The healthy and calcium-deficient 'Honeycrisp' apple fruit used in this study were provided by Shandong Academy of Agricultural Sciences (Tai'an, Shandong Province). During the ripening period in 2018, 60 calcium deficient apple fruits were harvested from the tree, and 60 healthy fruits without pests or mechanical damage were harvested at the ripening stage as the controls (CK). Every ten healthy fruits/ calcium deficient were randomly divided into one group, and they were respectively placed in six 350 mm glass-vacuum-dryer with the appropriate amount of distilled water to maintain 75~85 % relative humidity at room temperature (25 °C). Ten apple fruits (without seeds and skins) were sampled every seven days until 21 days after storage (DAS). The samples were frozen in liquid N₂ quickly and stored in a -80 °C refrigerator for subsequent experiments.

Determination of Ca content in apple flesh

The Ca content of apple flesh was determined by flame atomic absorption spectrometry (FAAS) according to the methods described by Barea-Álvarez et al. [29]. Apple flesh (1 g, dry weight) was carbonized on a crucible. Then, samples were transferred to a high-temperature muffle furnace, and the temperature gradient was raised to 500 °C (50 °C/30 min) until samples were burnt to white gray. When the samples cooled, they were mixed with 15 mL of a mixture of HNO₃ and HClO₄ (5:1 v/v). The best parameters for determination were $\lambda = 422.7$, current = 10 mA, and spectral resolution = 1.2 nm, and the gases were C₂H₂ and air (C₂H₂ 3.0 L·min⁻¹; atmospheric air 13 L·min⁻¹) (Hitachi Z2000). CaCl₂ solution was used as the standard for calibration.

Determination of flavonoid and total phenol contents and polyphenol oxidase activity

The flavonoid contents were assayed as described by Li et al. [30], and the absorbance was determined at 510 nm. Rutin was used as the standard for calibration.

Total phenols were assayed following the method of Pirie and Mullins [31]. The total phenols were determined by spectrophotometry at 280 nm. Gallic acid was used as the standard to make a calibration curve.

The activity of polyphenol oxidase (PPO) was determined by procedures described by Benjamin and Montgomery [32]. Apple flesh (5.0±0.01 g) was ground in 5 ml of ice-cold extraction buffer [1 mM PEG, 4% polyvinylpyrrolidone (PPVP), 1% Triton X-100, pH 5.5 acetic acid- sodium acetate buffer]. The homogenate was centrifuged, and then the supernatant was collected for protease activity determination. Absorbance was recorded at 540 nm, and the protease activity was quantified as U/g FW.

Determination of malondialdehyde (MDA), hydrogen peroxide (H₂O₂), and superoxide anion (•O₂⁻)

MDA, H₂O₂, and •O₂⁻ were determined according to the methods described by Hu et al. [33]. Absorbance was recorded at 532 nm, and the value for nonspecific absorption at 600 nm was subtracted to obtain the MDA content. Values are expressed as µmol/g.

The H₂O₂ content was obtained by determining the absorbance at 508 nm and is indicated as micromoles per gram.

Superoxide ($\cdot\text{O}_2^-$) production was calculated with an extinction coefficient of $2.16 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Corrections were made for the background absorbance in the presence of 50 units of superoxide dismutase (SOD) and presented as nmol/min/g.

Detection of soluble protein and dry matter contents

For soluble protein, the supernatant was mixed with Coomassie brilliant blue, and the absorbance was determined at 595 nm after 5 min according to the method described by Bradford [34]. The results are expressed as milligrams per gram of FW (fresh weight).

For dry matter, samples ($5.00 \pm 0.05 \text{ g}$) were placed in an oven at 80 °C until the weight at the third weighing remained unchanged. Then, the proportion relative to the initial weight was calculated.

Determination of total soluble solid contents, ascorbic acid and titratable acids

Total soluble solids (TSS) were determined in samples of apple fruit ($5.00 \pm 0.05 \text{ g}$). After grinding and centrifugation (4000 r/min, 10 min), the juice was measured by an Abbe Refractometer (JH-WYA2S, Jiahang Instrument Co., Ltd, Shanghai, China). The titratable acid (TA) content was determined by acid-base neutralization with NaOH [35]. TSS and TA contents were presented as mg/g. The extraction and determination of ascorbic acid in apples followed the method of Nath et al. [36], and the assessment was performed by indophenol titration with minor changes, with values expressed as mg/100 g.

Measurements of the soluble sugar content in apple flesh

The soluble sugars in apple flesh were extracted following the method of Li et al. [26]. Briefly, two grams of the fruit flesh was homogenized, mixed with 5 ml of sterile deionized water, incubated in a water bath at 80 °C for 30 minutes, and then extracted by ultrasonic for 30min at 50W. Finally, the supernatant was collected by centrifugation ($12000 \times g$, 5 min) and filtration through a 0.22 μm membrane. The soluble sugars were measured by HPLC (Agilent Technologies 1260 Series) following the method of Jia et al. [37]. HPLC (Agilent 1260) was performed with a $7.8 \times 300 \text{ mm}$ Carbomix Ca-NP column (Sepax); the mobile phase was ultrapure water with a flow rate of 1 ml min^{-1} ; the column temperature at 80 °C; the refractive index detector temperature at 35 °C, and the injection volume was 20 μl . At each sampling point, at least five fruits were randomly selected and divided into three groups as three biological replicates.

Quantitative reverse transcription PCR analysis

Total RNA from 0.1 g frozen apple fruit samples was extracted by an RNA Extraction Kit (Tiangen, Beijing, China). Then, cDNA was synthesized by a reverse transcription kit (PrimeScript RT Master Mix, Takara, Kyoto, Japan) and further used for quantitative PCR. The specific primers used for qPCR are listed in

Supplementary Table S4. *MdTUB* (*TUB*, accession number G0562615) and *MdUBQ* (*UBQ*, accession number MDU74358) were used as housekeeping genes for the normalization of data. All data are expressed as the means and standard deviations of the values obtained from three biological replicates.

Gene cloning and transient transformation of *MdWRKY75* in apple fruit

The full Coding DNA Sequence (CDS) of *MdWRKY75* (MD13G1122100) in apple was obtained by GDR (<https://www.rosaceae.org/>), and PCR amplification was conducted using Phanta Super-Fidelity DNA Polymerase (P501-d1, Vazyme Biotech Co. Ltd., China) and the primer sequences listed in Table S4. The full CDS fragment of *WRKY75* was inserted into pSAK277 vector under the control of the 35S promoter with *EcoRI* and *XhoI*. The recombinant expression vector *WRKY75*-pSAK277 was transformed into *Agrobacterium tumefaciens* (GV3101), it was cultured at 37°C, and then collected, subsequently resuspended in a solution (included 10 mM MES, 10 mM MgCl₂, and 200 μM acetosyringone) to a final optical density of 0.8~1.0 at OD₆₀₀, and then incubated at room temperature for 3–4 h. The infiltration protocol and culture methods for transient expression were adapted from previously described methods [38,39]. The infected apples were placed at 23°C for 3 days. All fruit samples were frozen in liquid nitrogen upon collection, and stored at –80 °C.

Statistical Analysis

Statistical analysis was performed using a *t*-test in SPSS 22.0. PCA was performed using factor analysis in dimension reduction, and the rotation method was carried out by varimax with Kaiser normalization. Correlation analysis and heatmap analysis were performed by R studio software.

Results

Low calcium content caused bitter pit disease, which shortened the shelf life of apple fruit

In our experiments, calcium-deficient and healthy apple fruit (CK) were analyzed during the storage period. As shown in Fig. 1A, calcium-deficient apple fruit exhibited bitter pit disease 7 days after storage (DAS), while CK did not show disease characteristics during the storage period. The apple peels of bitter pit disease turned dark yellow compared to CK at 21 DAS. Furthermore, the calcium content of apple fruit was determined, and the results showed that the calcium content in calcium-deficient apple fruit was significantly lower than that of the CK fruit during the storage period ($P<0.01$) (Fig. 1B). This showed the reliability of bitter pit disease in calcium-deficient apples.

Comparison of H₂O₂, •O₂[–], MDA and activity of PPO in calcium-deficient and CK apple fruit during the storage period

The results showed that calcium-deficient apple fruit maintained lower levels of H_2O_2 during postharvest storage. Significantly lower levels of H_2O_2 were observed in calcium-deficient fruit than in the CK fruit during the storage period until 14 DAS ($P<0.01$) (Fig. 2A). From 7 to 14 DAS, the control fruit increased as much as 4-fold compared to calcium-deficient apple fruit. Thereafter, the H_2O_2 content in the control fruit decreased gradually due to senescence and rot with prolonged storage time. The production of H_2O_2 in calcium-deficient fruit was accelerated from 0 to 7 DAS and reduced rapidly thereafter.

As shown in Figure 2B, the rate of $\cdot\text{O}_2^-$ production remained high during storage irrespective of disease. Compared with the control fruit, significantly lower $\cdot\text{O}_2^-$ production was observed in calcium-deficient apple fruit during the entire storage period ($P<0.01$).

Figure 2C shows that the MDA content in calcium-deficient fruit was enhanced rapidly during the entire storage period. From 0 to 7 DAS, the levels of MDA in calcium-deficient apple fruit were significantly lower than those in CK. From 7 to 14 DAS, the levels of MDA in calcium-deficient apple fruit were higher than those in the control. Finally, at 21 DAS, the MDA content was approximately the same regardless of disease.

Figure 2D shows the change in polyphenol oxidase (PPO) activity in apple fruit throughout storage. At 0 DAS, the activity of PPO in calcium-deficient apple fruit was nearly 2.5-fold that in CK. Then, the activity of PPO in calcium-deficient apple fruit decreased rapidly and was maintained at a stable state but was always higher than that in control apple fruit during the entire storage time ($P<0.01$). The activity of PPO in control apple fruit rose slowly at first and declined slightly from 14 to 21 DAS.

Comparison of flavonoids and total phenols in calcium-deficient apple fruit with CK fruit during the storage period

To better understand the improved appearance quality in calcium-deficient apple fruit relative to CK, we determined the contents of flavonoids and total phenols. Figure 3A shows that the flavonoid contents in calcium-deficient apple fruit were enhanced at 14 DAS and reduced thereafter. The trend of flavonoids in control apple fruit was similar to that of calcium-deficient apple fruit except at 0 DAS. Until 21 DAS, the flavonoid contents tended to be consistent between calcium-deficient apples and control apple fruit.

In Figure 3B, there was a significant difference in total phenols between the two kinds of apples at 0 DAS. In the next 7 days, the total phenols in calcium-deficient apples increased and then dropped from 14 to 21 DAS. The total phenols of the control apple fruit increased from 7 to 14 DAS and dropped at 21 DAS. Similar to the change trend of flavonoid content, the total phenols in calcium-deficient apple fruit were always lower than those of the control apple fruit (except at 7 DAS).

Analysis of dry matter and soluble protein content in calcium-deficient apple fruit

It was shown that dry matter and soluble protein increased in calcium-deficient apple fruit. During calcium-deficient apple fruit storage for 21 days, the dry matter content increased slightly and was

always higher than that of the control apple fruit ($P<0.01$). However, dry matter in control apple fruit did not obviously change during the entire storage time (Fig. 3C). Similarly, the change trend of soluble protein content was the same trend as dry matter content. During 0 to 7 DAS, the content of soluble protein increased rapidly and then was maintained at a high level (Fig. 3D). In contrast, it was reduced slightly in control apple fruit at the beginning of storage, and then there was a slight increase within 14 to 21 DAS. The content of soluble protein in control apple fruit was always lower than that in calcium-deficient apple fruit ($P<0.01$).

Identification of TA, TSS, ascorbate acid, ratio of TSS/TA and soluble sugars in calcium-deficient apple fruit

TA and ascorbic acid play a sour taste role in apple fruit, while TSS play a sweet taste role. Figure 4 shows the changes in TA, ascorbate, TSS, ratio of TSS/TA and soluble sugars in calcium-deficient apple fruit and control apple fruit. During the whole storage period, TA showed a downward trend in apples regardless of calcium deficiency (Fig. 4A). The TA content in control apple fruit was always higher than that in calcium-deficient apple fruit ($P<0.05$).

The content of ascorbate acid always decreased during the full storage period in the two kinds of apple fruit, and it was the lowest at 21 DAS (Fig. 4B). There was no significant difference in ascorbate acid between the calcium-deficient and control apple fruit mid-storage. However, at the beginning and late stage of storage, ascorbate acid in control apple fruit was higher than that in calcium-deficient apple fruit.

The change of TSS is shown in Figure 4C. During the entire storage time, TSS increased slightly in calcium-deficient apple fruit and was always higher than that in control apple fruit ($P<0.05$ or $P<0.01$). This means that calcium-deficient apple fruit sugar accumulates faster than that in control apple fruit.

The ratio of TSS/TA is an important index for evaluating the flavor of apples. During the storage time, the ratio of TSS/TA in calcium-deficient apples was always higher than that of control apples (Fig. 4D). In particular, the ratio of TSS to TA in calcium-deficient apple fruit was significantly higher than that in control apple fruit (except at 14 DAS) ($P<0.01$).

During the storage time, the sucrose content presented a declining trend in apple fruit, and it was always significantly higher in calcium-deficient apple fruit than in control apple fruit ($P<0.01$) (Fig. 4E).

The glucose contents in calcium-deficient and control fruit shared the same trends. The glucose contents increased from 0 to 7 DAS, decreased from 7 to 14 DAS, and finally increased from 14 to 21 DAS (Fig. 4F). During the whole storage time, the glucose contents of calcium-deficient apple fruit were always significantly lower than those of control apple fruit ($P<0.01$).

During storage, the fructose content was always lower in calcium-deficient apple fruit than in the control fruit (Fig. 4G). At 14 DAS, the fructose contents of calcium-deficient apple fruit and control apple fruit tended to be consistent. However, the fructose content of control apple fruit was significantly higher than that of calcium-deficient apple fruit (except at 14 DAS) ($P<0.01$).

PCA and correlation analysis of the changes in bioactive substances in apple fruit

The PCA results showed that the contribution rates of PC1 and PC2 were 79.8% and 20.2%, respectively. In PC1, Ca and MDA contents were the main factors. In PC2, TA and TSS were the main factors (Fig. 5, Table S1). Correlation analysis indicated that Ca content showed a negative correlation with TSS (*person*: -0.345), sucrose (*person*: -0.4), $\bullet\text{O}_2^-$ (*person*: -0.42) and MDA (*person*: -0.928), and it had a positive correlation with ascorbate (*person*: 0.576), glucose (*person*: 0.405), fructose (*person*: 0.709), H_2O_2 (*person*: 0.386), and TA (*person*: 0.719). In addition, TSS had a positive correlation with sucrose (*person*: 0.713), TSS (*person*: -0.239) and sucrose (*person*: -0.125) and a negative correlation with TA (Fig. S1, Table. S2). The results showed that Ca content was positively correlated with antioxidant capacity and the accumulation of acidic substances and negatively correlated with the accumulation of sweet substances.

Identification Of The Candidate Genes Related Calcium-deficient Metabolism

According to transcriptome data of calcium-deficient “Fuji” apple fruit (T01), calcium-deficient apple healthy flesh (T02) and healthy apple fruit (T03) at fruit ripening period [5], eight expression patterns of all differentially expressed genes (DEGs) were obtained. We selected a total of 42 DEGs from profiles 0, 1, 6 and 7 (Fig. 5B). Among of them, 24 WRKY transcription factors, eleven genes of sugar metabolism (SS, SSL, SWEET and SPS), five genes of apoptosis and two genes of calcium signal have the same or opposite expression trend (Fig. 5C). Furthermore, promoters of genes encoding sucrose synthesis and transport enzymes, specifically *MdSWEET1-like* (MD10G101220), *MdSWEET2a-like* (MD10G1269300), *MdSWEET15* (MD16G1125300), *MdSPS2* (MD04G1013500), *MdSS2* (MD02G1100600), *MdSPS4* (MD05G1006400), *MdSS* (MD15G1223500), *MdSSL* (MD02G1100500) and *MdSWEET1* (MD10G1012200), were predicted by PlantCare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). We found that there were W-box cis-elements in their 2000 bp upstream promoters. This suggested that WRKY TFs may be involved in the regulation of fruit sugar accumulation by binding to genes encoding sucrose synthesis and transport enzymes.

MdWRKY75 was related to MdSWEET1 by RT-qPCR and correlation analysis

In order to further confirm the expression pattern of the above candidate genes in calcium-deficient apple fruit and healthy apple fruit at 0, 14, and 21 DAS, we determined the expression levels of *MdWRKY75* (MD13G1122100), *MdWRKY65* (MD05G1295700), *MdWRKY23* (MD17G1278100), *MdWRKY31* (MD05G1349800), *MdWRKY48* (MD13G1150700), *MdWRKY26* (MD03G1057400), *MdWRKY40* (MD00G1143500), *MdSSL* (MD02G1100500), *MdSS* (MD15G1223500), *MdSWEET1* (MD10G1012200), *MdAmmonium transporter* (MD12G1174700), *MdU-box 21* (MD13G1017300) and *MdU-box 21-like* (MD16G1015400) by RT-qPCR. As shown in Fig. 6, the expression levels of *MdSS*, *MdSSL*, *MdSWEET1*, *MdAmmonium transporter*, *MdU-box 21* and *MdU-box 21-like* were higher in calcium-deficient apple fruit than those of the CK fruit ($P < 0.01$). The expression levels of WRKYs in calcium-deficient apple fruit were

always higher than those in the CK fruit. In particular, the expression patterns of *MdWRKY75* and *MdWRKY31* were similar to those of *MdSWEET1*.

Furthermore, correlation analysis showed that sucrose had positive correlation with *MdWRKY75* (Pearson: 0.815), *MdWRKY23* (Pearson: 0.802). Meanwhile, *MdWRKY75* (Pearson: 0.959) and *MdWRKY31* (Pearson: 0.987) had positive correlations with *MdSWEET1* (Fig. 7, Table. S3). Thus, sucrose accumulation was significantly positively correlated with *MdWRKY75* and *MdWRKY23*, the TFs *MdWRKY75* and *MdWRKY31* were related to *MdSWEET1*. Moreover, *MdWRKY75* had a positive correlation with *MdAmmonium transporter* (Pearson: 0.88), *MdU-box 21* (Pearson: 0.92) and *MdU-box 21-like* (Pearson: 0.95). These results indicated that *MdWRKY75* might regulate the expression of *MdSWEET1* and result in accelerating sucrose accumulation, and might be related to apoptosis in calcium-deficient apple fruit.

Transient transformation of *MdWRKY75* in apple fruit

Because of *MdWRKY75* have a high expression level in calcium deficient apples, and have positive correlation with sucrose content and the expression of apoptosis related genes, we injected *MdWRKY75* into apple fruit and measured the sugar content and the expression level of sugar-, Ca- and apoptosis related genes. As shown in Fig. 8A, the content of sucrose, glucose and fructose in apple fruit were higher than those of the empty vector (pSAK277). Especially, the sucrose content of *MdWRKY75*-oe in apple fruit is 5-fold higher than those of the empty vector. RT-qPCR analysis also showed that the expression levels of *MdWRKY75* and *MdSWEET1* were higher in *MdWRKY75* induced apple fruit than those of the empty vector (Fig. 8B). However, *MdCal1*, *MdCal4*, *MdAmmonium transporter*, *MdU-box 21* and *MdU-box 21-like* don't change obviously (Fig. 8B).

Discussion

Calcium-deficient apples significantly stimulated the activity of ROS and decreased antioxidant capacity

In this study, compared with healthy apples, calcium-deficient apples showed stronger senescence appearance during storage (Fig. 1A), and $\cdot\text{O}_2^-$ and MDA contents and PPO activity were higher than those of healthy apples (Fig. 2B, C, D), while H_2O_2 content was lower in calcium-deficient apples than in healthy apples (Fig. 2A). Thus, the antioxidant capacity of calcium-deficient apples was lower than that of healthy apples. MDA is one of the main products of membrane lipid peroxidation and can be used to reflect the degree of membrane peroxidation. In tomato, MDA content increased sharply in plants with low calcium contents [27]. Moreover, ascorbic acid can deoxidize H_2O_2 to H_2O via its own oxidation. Our results were similar to a report showing that calcium can reduce the degradation of ascorbic acid and enhance the total antioxidant capacity in sweet cherry and 'Royal delicious' [12, 15]. Thus, the antioxidant capacity of 'Honey Crisp' apple with calcium deficiency is lower than that of healthy apple.

The deterioration of calcium-deficient apples was faster than that of healthy apples

Calcium was reported to play a critical role in fruit development and ripening. Calcium deficiency can accelerate the senescence of apples. In our study, there were significant differences in the contents of ascorbic acid, soluble protein, flavonoids, total phenols, TSS and TA between calcium-deficient apples and healthy apples during postharvest storage, which strongly suggested that calcium played a key role in regulating apple fruit senescence. The contents of flavonoids and total phenols in calcium-deficient apples were higher than those in healthy apples (Fig. 3A, B). A previous report suggested a strong correlation between total antioxidant activity and total phenolic content [28]. Soluble protein, as an important component of enzymes in fruit and vegetables, is also one of the important evaluation indexes of fruit and vegetable quality and nutrition. The content of soluble protein in calcium-deficient apples was higher than that in healthy apples (Fig. 3D). Similarly, the TSS content and the ratio of TSS to TA were higher in calcium-deficient apples than in healthy apples (Fig. 4C, D), but the trends of TA and ascorbate content were the opposite (Fig. 4A, B). Thus, it was inferred that the taste of calcium-deficient apples is sweeter than that of healthy apples. The sucrose content of calcium-deficient apple fruit was higher than that of control apple fruit (Fig. 4E), providing direct evidence that calcium deficiency accelerates the accumulation of sweet substances. The TA and carbohydrate contents of banana without exogenous calcium treatment were consistent with our results [12]. Meanwhile, the PCA results showed that Ca was the main factor for bitter pits in postharvest apple fruit (Fig. 5A). The correlation analysis showed that Ca was positively correlated with TA and negatively correlated with TSS accumulation. In addition, TSS was positively correlated with sucrose accumulation (*Pearson*: 0.71) (Table. S2). This result indicated that sugar accumulation is related to calcium deficiency in apples.

MdWRKY75-MdSWEET1 is a potential regulatory model of sucrose transport in calcium-deficient apples

Sugar content was an important criterion for evaluating fruit maturity. The sugar/acid ratio is an index that affects fruit nutritional quality. In this study, we found that the TSS, ratio of TSS to TA and sucrose content of calcium-deficient apples were higher than those of healthy apples (Fig. 4C, D, E). Furthermore, *MdSS*, *MdSSL* and *MdSWEET1* had significantly higher transcript abundance in calcium-deficient apples than healthy apple fruit by transcriptome data mining. This suggested that *MdSS*, *MdSSL*, *MdSWEET1* and twenty-four WRKYs were activated by calcium deficiency. In the other hand, *MdAmmonium transporter*, *MdU-box 21* and *MdU-box 21-like* had similar expression profiles with *MdSWEET1*, *MdSS*, *MdSSL* and WRKYs (Fig. 5C). This suggested calcium deficiency also activated apoptosis. The results of RT-qPCR analysis further showed that the expression patterns of *MdSS*, *MdSSL*, and *MdSWEET1* were consistent with seven WRKY TFs during storage (Fig. 6). Meanwhile, *MdSS* and *MdSWEET1* had a positive correlation with TSS content and a negative correlation with Ca content (Fig. 7, Table S3), and *MdWRKY75* had a strongly positive correlation with *MdSWEET1* by correlation analysis. Recently, it was reported that *PuWRKY31* can bind to the promoter of *PuSWEET15* to regulate sugar accumulation in pear fruit [26]. Thus, we overexpressed *MdWRKY75* in apple fruit by injecting pSAK277-*MdWRKY75*, resulting in sucrose content and expression level of *MdSWEET1* increased (Fig. 8A, B). This suggests that *MdWRKY75* can activate the expression of *MdSWEET1* to increase the accumulation of sucrose in

calcium deficient apple. Furthermore, *MdAmmonium transporter*, *MdU-box 21*, *MdU-box 21-like*, *MdCal1* and *MdCal4* have no obvious change in apple with over-expressing *MdWRKY75* (Fig. 8B). It implied that *MdWRKY75* cannot regulate the expression of calcium signaling and apoptosis related genes in apple, but apoptosis related genes may be involved in the sucrose metabolic pathway of apple fruit and affect the accumulation of sucrose. This study further strengthened the regulatory mechanism of calcium-deficient apple flesh and contributed to improving the appearance quality of apple fruit.

Conclusions

In summary, this study found that the deterioration of calcium-deficient apples, including nutrients and antioxidant capacity, was faster than that of healthy apples. The results also indicated that the TSS and sucrose contents of calcium apples were higher than those of CK during storage. TSS, sucrose, ROS and Ca were identified as the main factors by PCA. In addition, transcriptome data mining, RT-qPCR analysis and transient expression indicated that *MdWRKY75* could activate the expression of sucrose metabolism-related enzyme *MdSWEET1* in Ca-deficient apple fruit. It suggested that *MdWRKY75* could bind to the *MdSWEET1* promoter by W-box cis-elements, and then promotes the contents of sucrose, glucose and fructose in apple fruit. Based on the results of our research, a model is proposed to develop a significant understanding of Ca^{2+} deficiency affecting TSS content through the sucrose metabolic pathway in apples with bitter pits (Fig. 9). Thus, this study provided a deep basis for sugar accumulation in fruit based on sucrose accumulation in calcium-deficient apples and improved fruit quality.

Abbreviations

Calcium, Ca; Days After Storage, DAS; Superoxide Anion, $\cdot\text{O}_2^-$; Malondialdehyde, MDA; Hydrogen Peroxide, H_2O_2 ; Polyphenol Oxidase, PPO; Principal Component Analysis, PCA; Differentially Expressed Gene, DEG; Titratable Acids, TA; Total Soluble Solids, TSS; Quantitative reverse transcription PCR, RT-qPCR; Flame atomic absorption spectrometry, FAAS; Sucrose synthase, SS; Sugars will be eventually exported transporters, SWEET1; Sucrose-phosphate synthase, SPS; reactive oxygen species, ROS.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated and analyzed during this study are included in this published article.

The databases used in this study as follows,

GDR: <https://www.rosaceae.org/> (open)

NCBI database: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA733599> (open)

Competing interests

The authors declare that they have no competing interest.

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Author contributions

C.S., W.W.Z., H.Y.Q., G.F.Y., K.D.H. and H.Z. conceived and designed the experiments; C.S., L.X.L., L.F.Y. and Y.Q.Z. performed the experiments; C.S., L.X.L. and G.F.Y. analyzed the data; C.S., G.F.Y., W.W.Z. and K.D.H. wrote the paper; H.Q.Y., G.F.Y., K.D.H. and H.Z. interpreted the data and revised the manuscript. All authors have read and approved the manuscript.

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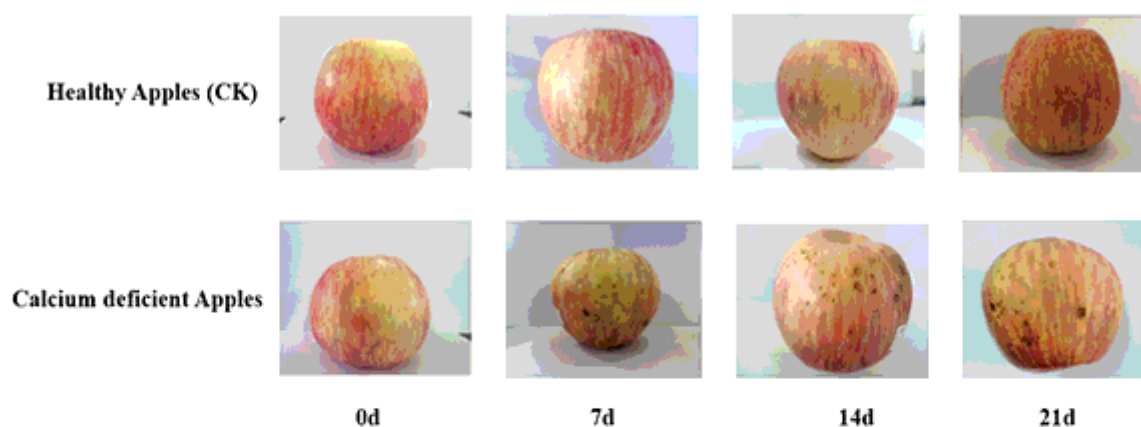
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Figures

A



B

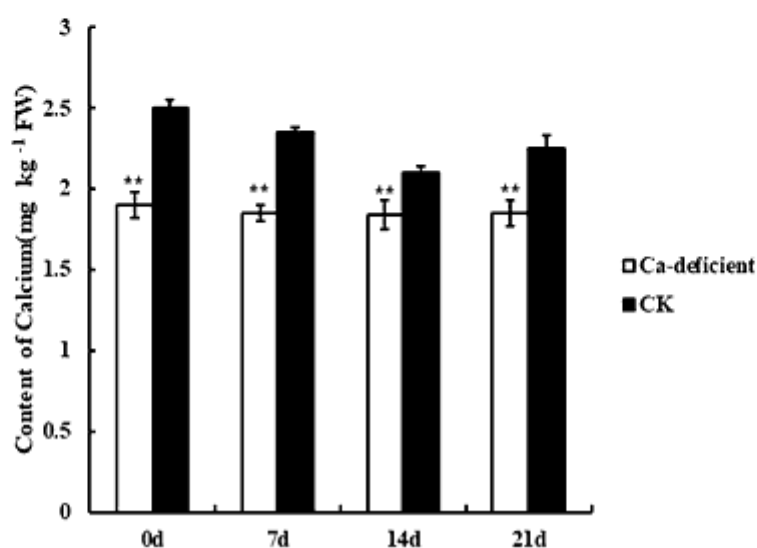


Figure 1

Changes of the phenotype and Ca contents in postharvest calcium-deficient and healthy apples. (A) Changes of the phenotype in the calcium-deficient and healthy apples at 0, 7, 14 and 21days after storage (DAS). (B) Changes of Ca contents in postharvest calcium-deficient and healthy apples at 0, 7, 14 and 21days after storage (DAS). * and ** indicate significance at $P < 0.01$ and $P < 0.05$, respectively.

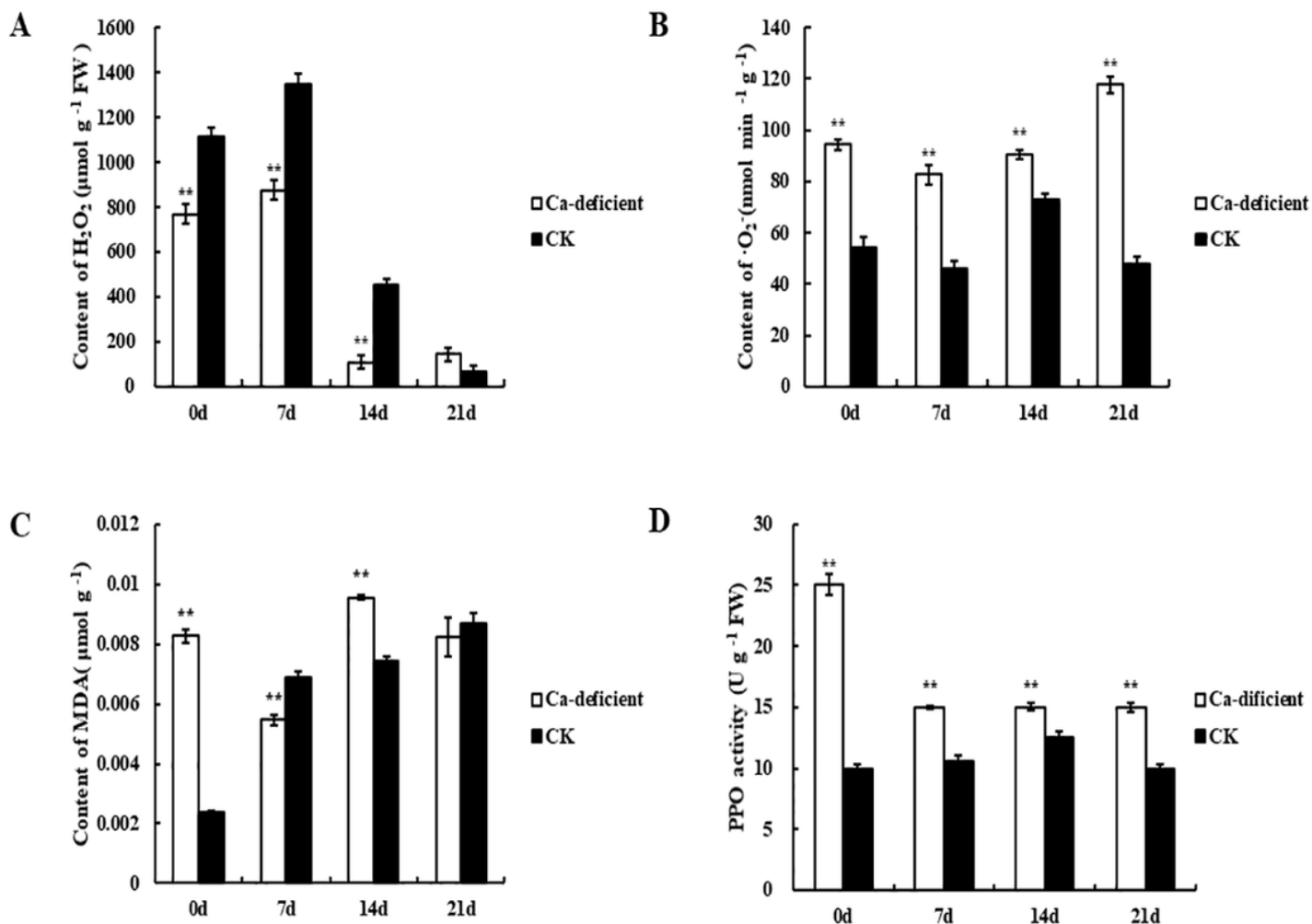


Figure 2

The changes of reactive oxygen species (ROS) in postharvest healthy apples and calcium-deficient apples. (A) hydrogen peroxide, (B) superoxide anion, (C) malondialdehyde and (D) activity of polyphenol oxidase. Data are presented as means \pm SD ($n = 3$). * and ** indicate significance at $P < 0.01$ and $P < 0.05$, respectively.

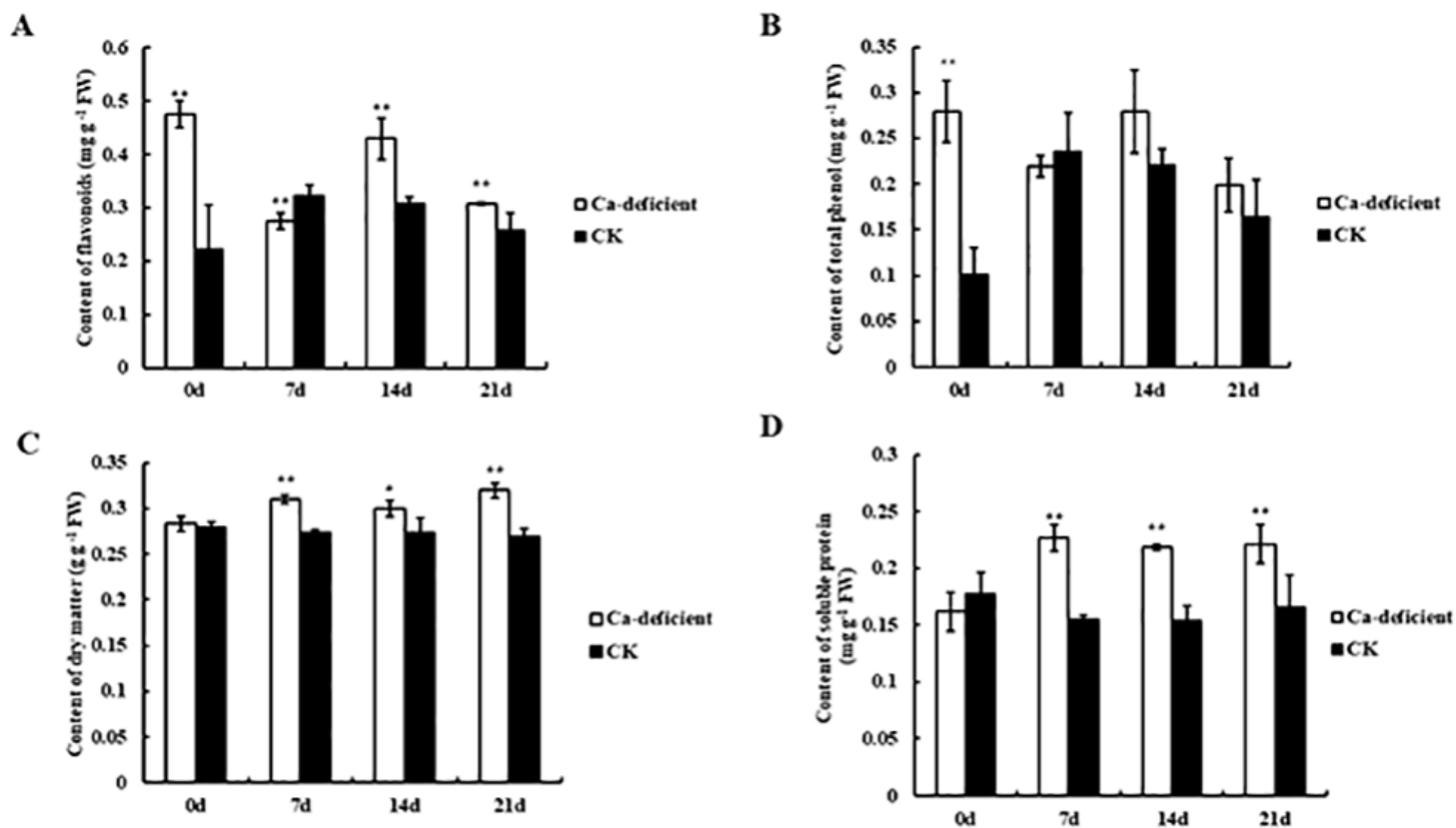


Figure 3

The changes of nutrients in postharvest healthy apples and calcium-deficient apples. (A) flavonoids, (B) total phenols, (C) dry matter and (D) soluble protein. Data are presented as means \pm SD ($n = 3$). * and ** indicate significance at $P < 0.01$ and $P < 0.05$, respectively.

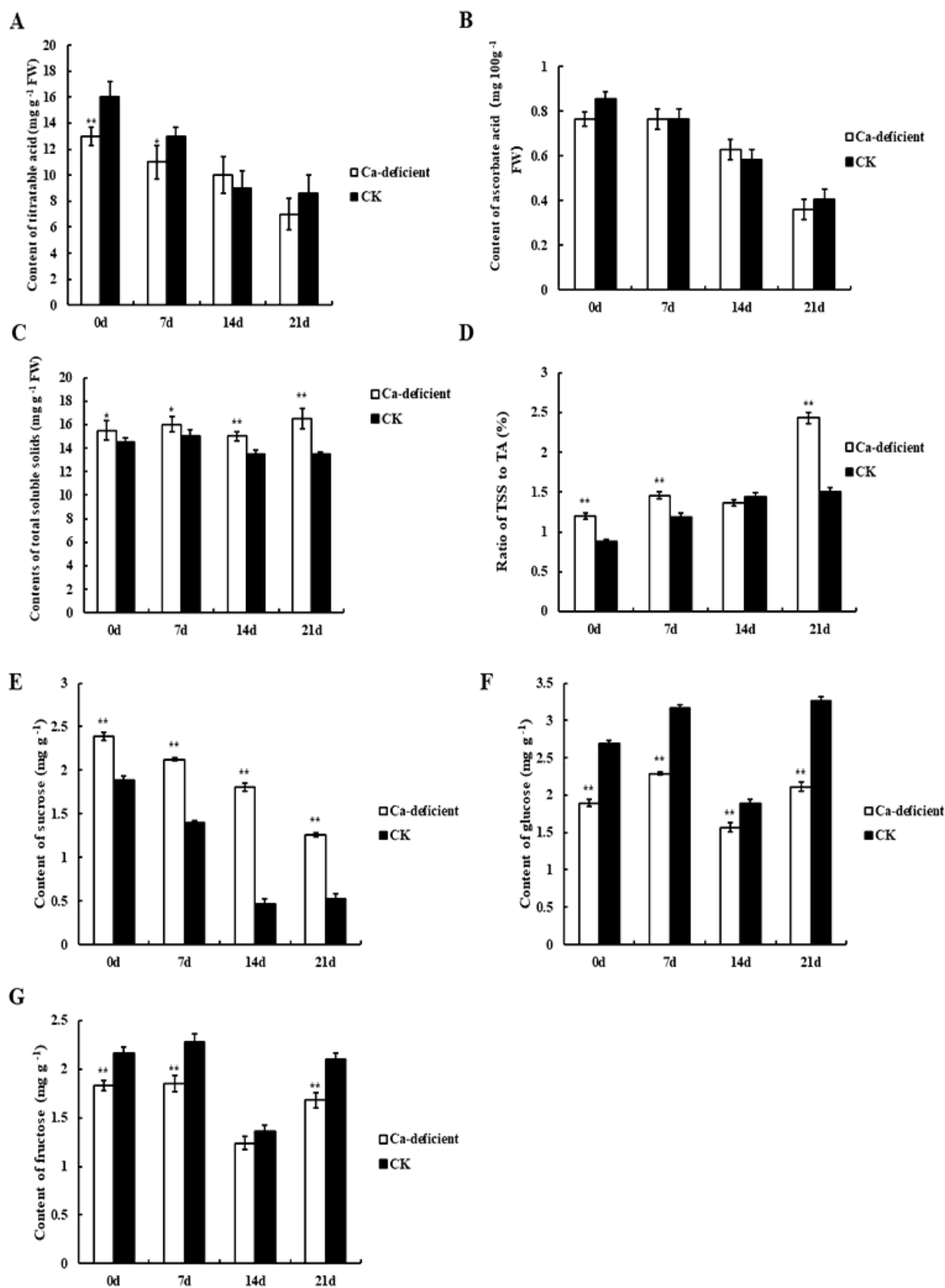
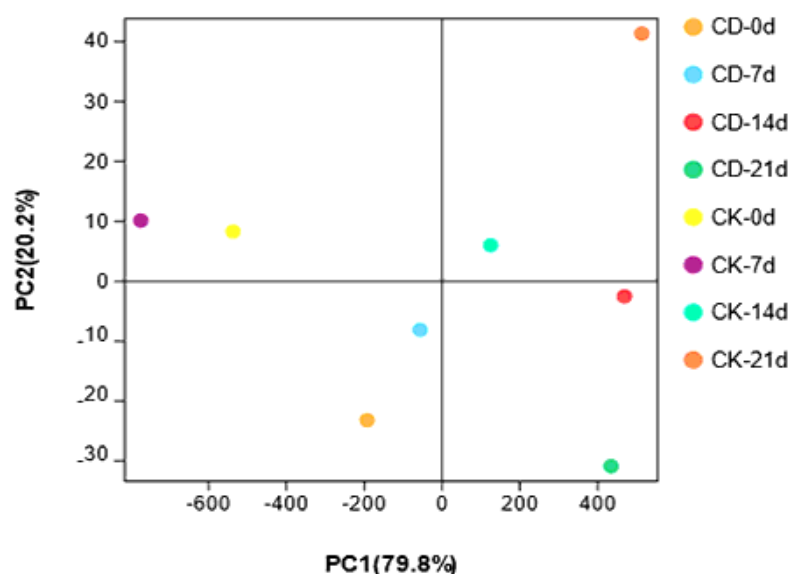


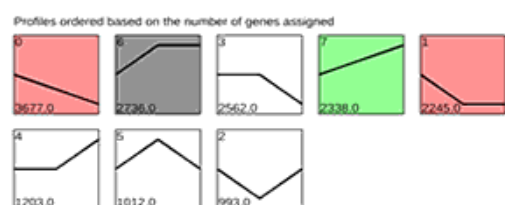
Figure 4

The changes of sugar acid substance in postharvest healthy apples and calcium-deficient apples. (A) titratable acid, (B) ascorbate, (C) total soluble solids, (D) ratio of TSS to TA, (E) sucrose, (F) glucose and (G) fructose. Data are presented as means \pm SD ($n = 3$). * and ** indicate significance at $P < 0.01$ and $P < 0.05$, respectively.

A



B



C

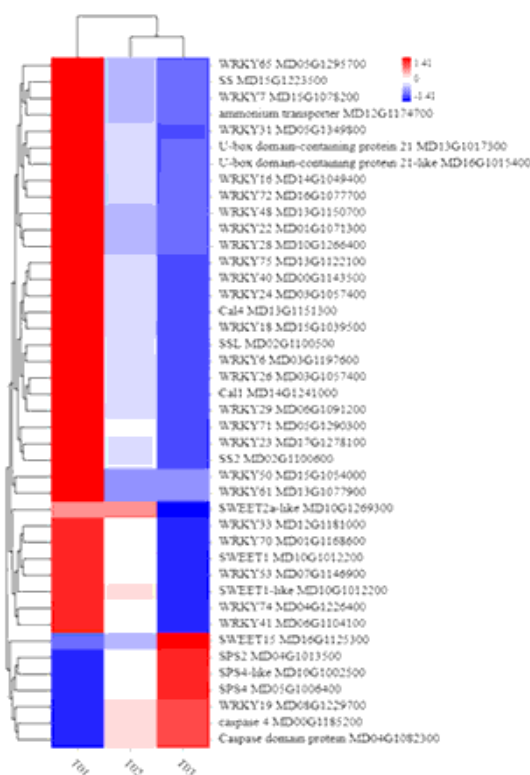


Figure 5

Principal component analysis (PCA) and screening differentially expressed genes (DEGs) from transcriptomic analysis. (A) PCA of postharvest calcium-deficient and CK apple fruit during the storage periods. PC1 and PC2 represent the contribution rates of principal components. (B) The expression pattern of DEGs based on transcriptomic data. (C) Heatmap of the candidate DEGs related to calcium deficient

apple fruit. T01, calcium-deficient apples; T02, healthy flesh of calcium-deficient apples; T03, healthy apple flesh.

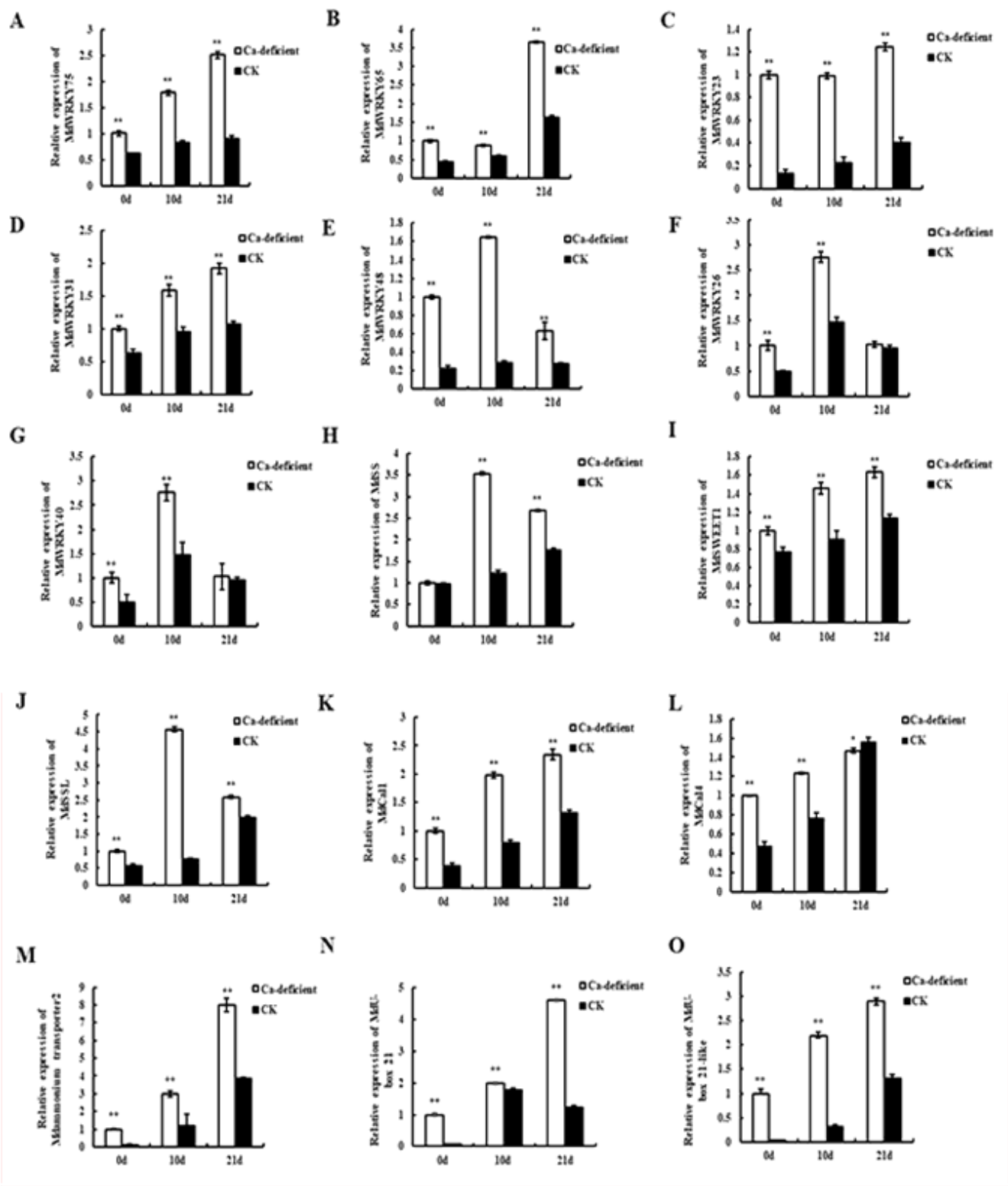


Figure 6

Evaluating the expression levels of candidate genes in postharvest healthy apples and calcium-deficient apples. (A) MdWRKY75, (B) MdWRKY65, (C) MdWRKY23, (D) MdWRKY31, (E) MdWRKY48, (F) MdWRKY26, (G) MdWRKY40, (H) MdSS, (I) MdSWEET1, (J) MdSSL, (K) MdCa1, (L) MdCa4, (M)

MdAmmonium transporter2, (N) MdU-box21 and (O) MdU-box21-like. Data are presented as means \pm SD (n = 3). * and ** indicate significance at $P < 0.01$ and $P < 0.05$, respectively.

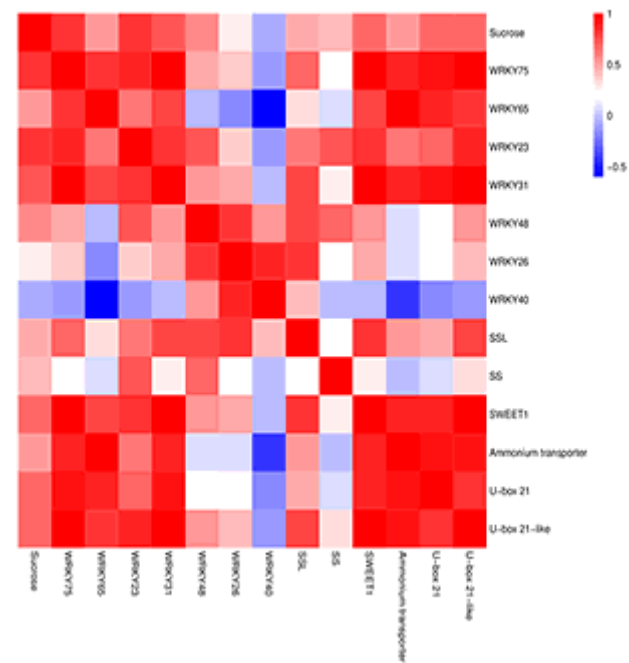


Figure 7

Correlation analysis of the relationship between sucrose content and the expression levels of candidate genes.

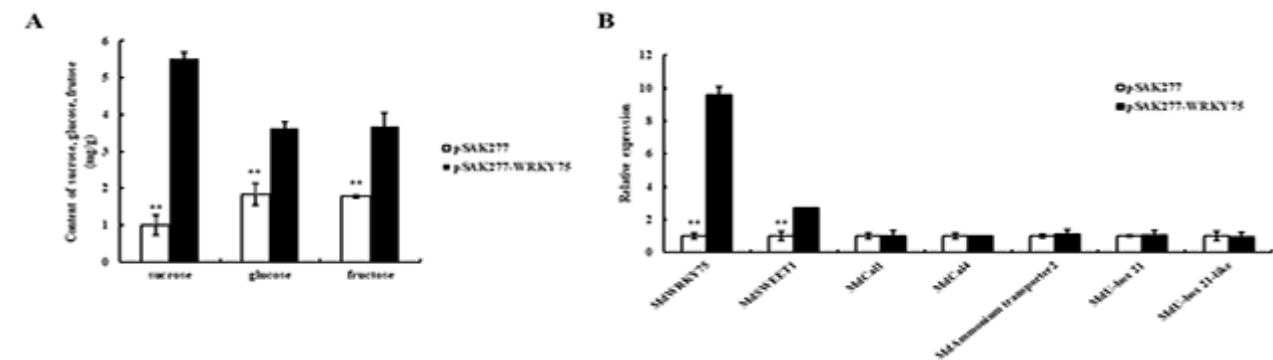


Figure 8

The changes of sucrose, glucose, fructose and related genes expression in apples infected pSAK277-MdWRKY71. (A) Changes in sucrose, glucose and fructose in apple fruit between infected by empty vector and pSAK277-MdWRKY75. (B) Evaluating the expression levels of MdWRKY75, MdSWEET1, MdCal1, MdCal4, MdAmmonium transporter2, MdU-box 21 and MdU-box 21-like in apple fruit between infected by empty vector and pSAK277-MdWRKY75. Data are presented as means \pm SD (n = 3). * and ** indicate significance at $P < 0.01$ and $P < 0.05$, respectively.

Ca²⁺ deficient Apple

Healthy Apple



VS

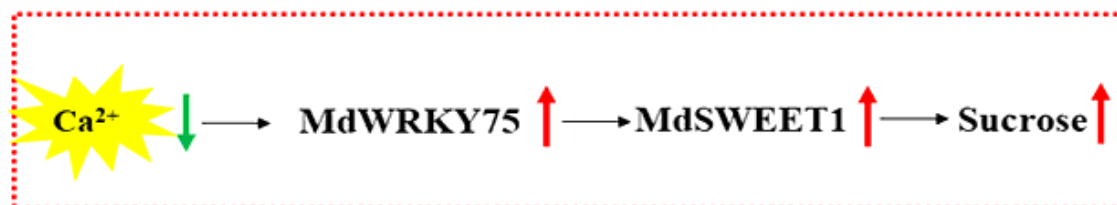
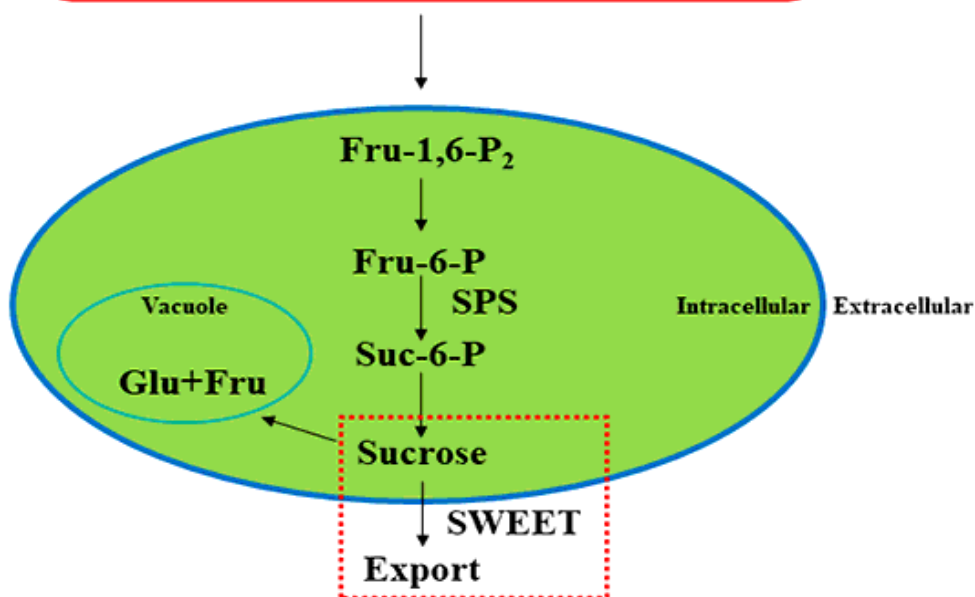
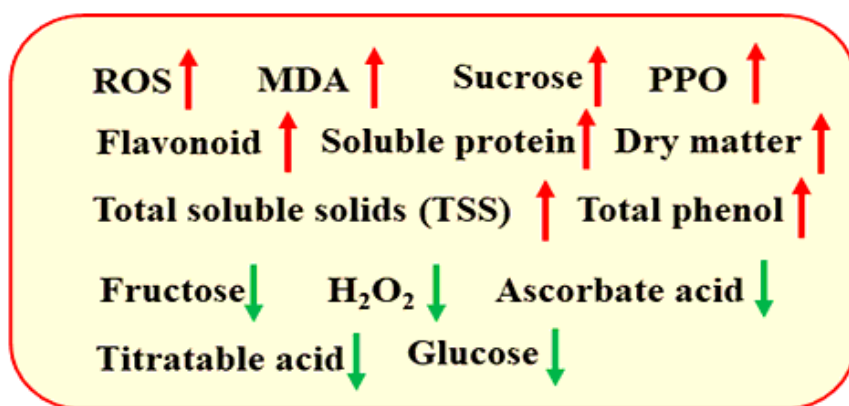


Figure 9

A proposed model of the mechanism of sucrose accumulation, which in apples with Ca deficiency.

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