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Difference of the key softening-regulated factors in relation to cell wall metabolism during fruit ripening of three apple cultivars

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Summary

In this study, the changes of cell wall compositions and related-enzyme activity and gene expression were assessed with fruit softening in three apple cultivars with different softening characteristics. The fruit of 'Golden Delicious' (GD) and 'Gala' apple softened rapidly, and showed a rapid decrease in the content of CSP and hemicellulose and a fast increase in WSP content, which were concomitant with a swift up-trend in cell wall enzyme activity and gene expression. In contrast, 'Fuji' fruit retained the harder flesh with little changes in the content of cell wall component and lower activity and gene expression of cell wall enzymes during the whole storage. Comprehensive pathway analysis indicated that both WSP and β-Gal factors had stronger direct effect and were interacted with each other through stronger indirect effect on 'Gala' fruit softening. The β-Gal factor showed the strongest direct effect and had stronger indirect effect on other factors during fruit softening in 'GD' apple. And the strongest direct role of CSP factor was mainly affected by the indirect role of β-Gal, and the direct role of β-Gal was also indirectly affected by CSP factor during storage of 'Fuji' fruit. Based on above results, the β-Gal may be the most crucial enzyme involved in cell wall degradation, and the changes of WSP, CSP and hemicellulose contents had more correlations with fruit softening with different storage-ability of apple.

Keywords: apple; cell wall metabolic factors; interactive relationship; fruit softening; pathway analysis

Introduction

Apples are widely cultivated around the world and highly favored by people for their nutritional value and flavor. In China, apple production accounts for 50% of the global total production, which occupy a dominant position in the fruit industry (Jia et al., 2016). However, many apple cultivars tend to ripe and soft early, resulting in a loose and fluffy texture after harvest. This makes the fruit susceptible to mechanical damage and increases the risk of pest and disease invasion. The deterioration of flesh texture greatly affects the edibility, market value and storage and transport characteristics of apples, resulting in significant postharvest losses and damaging the enthusiasm of fruit farmers, as well as hindering the development of apple industry (Su et al., 2022). Hence, it is necessary to further explore the softening characteristics of different apple cultivars with distinct fruit storability, as this could provide guidance on suitable measures to delay fruit softening and reduce losses during storage.

Fruit softening is primarily caused by the destruction of the structural integrity of the cell walls (Wakabayashi, 2000; Geitmann et al., 2010). Pectin polymers are orderly embedded in the microfibrils of cellulose and hemicellulose, which form the reticular structure of the cellular skeleton, giving fruit its specific shape and inherent hardness (Brummell, 2006; Jarvis et al., 2011; Wang et al., 2018). Once the modification occurs in the structure and composition of the cell wall, the result is an obvious decrease in flesh firmness and a shortened storage life in many fruits (Goulao et al., 2008). The alterations in cell wall structure and composition are believed to be involved by the action of a series of cell wall hydrolases, such as pectin methylesterase (PME), polygalacturonase (PG), cellulase (Cx), β-galactosidase (β-Gal) and α-arabinofuranosidase (α-Af). These enzymes have been reported to reduce the cell-to-cell adhesion and the molecular size of cell wall polysaccharide polymers by cleaving the backbone or the side-chain residues of the cell wall (Brummell, 2006; Goulao et al., 2007). Cell wall degradation is a complex physiological process in which related factors play different roles in fruit softening, demonstrating the comprehensive and coordinated regulation by cell wall metabolic factors (Goulao, 2008; Gwanpua et al., 2014; Li et al., 2021).

In this study, the fruits of three apple cultivars with different softening attributes were used as test materials to identify the differences in firmness loss cell wall component content dynamics, related hydrolase activity and gene expression during storage. The mutual interactions between firmness and the cell wall metabolic factors were also analysed to further clarify the key softening regulators in different apple cultivars and gain insight into the essence of fruit softening. This provides a theoretical basis for postharvest storage and preservation technology of apples.

Materials and methods

Plant materials and treatments

The apple fruit (*Malus domestica* Borkh.), including 'Fuji' (crisp flesh and good storability), 'Golden Delicious' (GD) (mealy flesh and moderate storability) and 'Gala' (loose-crisp and poor storability), came from an orchard in Changli county, Hebei Province, China. Fuji (about 165-170 days after bloom), GD (about 140-145 days after bloom), and Gala (120-125 days after bloom) were harvested based on their proper commercial maturity. The fruits were selected to be free of mechanical injury, insects and diseases after field heat removal, and were divided into three groups (150 kg of fruit per group) for three replications. The fruits were then packaged in plastic film and held at a room temperature (20 ± 1°C). Samples for analysis were taken at specific intervals according to the different softening rates of the three apple cultivars, as shown in Tab. 1. After measuring the firmness and respiration rate of each sample, the flesh of the apple fruit was cut into pieces, frozen in liquid nitrogen, and stored at −70 °C for further analyses.

Determination of flesh firmness and respiration rate

Firmness was determined in four equatorial regions of the peeled flesh of three apples with ten replications per sampling time by using

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Tab. 1: Sampling points in three apple cultivars

cultivars	sample days during storage (d)					
Fuji	0	12	24	40	56	72
Golden Delicious	0	8	16	24	40	56
Gala	0	4	8	12	16	20

a digital penetrometer (Model GY-4, China) equipped with a flat probe. The respiration rate was measured using the air-stream method with three replications per treatment.

Extraction and assay of cell wall fractions

Cell wall fractions were extracted and measured based on the method described by QI et al. (2015). Fruit flesh (3.0 g) was homogenized in 80% (v/v) ethanol and centrifuged after being stirred 20 min at 80 °C. The precipitate was washed with 80% (v/v) ethanol and pure acetone three times, respectively, and was sequentially immersed in 95% (v/v) dimethyl sulfoxide solution for 12 h and then centrifuged to remove starch. The residue was then oven-dried at 45 °C to obtain the cell wall materials (CWM). The CWM (50 mg) was sequentially extracted by distilled water, 50 mmol L^{-1} trans-1,2-diaminocyclohexane-N,N,N9,N9- tetra acetic acid (CDTA), 50 mmol L^{-1} Na_2CO_3, 4 mmol L^{-1} KOH (containing 0.1% (W/V) $NaBH_4$) to collect the water-soluble pectin (WSP), the ionic-soluble pectin (ISP), the covalent soluble pectin (CSP), and hemicellulose. The contents of WSP, ISP and CSP were determined with the carbazole-ethanol method and the hemicellulose contents were measured by the anthrone colorimetric method.

Enzymes extraction and activities analysis

The extraction of cell wall enzymes was based on the method described by BRUMMELL et al. (2004). Frozen flesh (3.0 g) was powdered and stirred into 6 ml of cold 12% polyethyleneglycol containing 0.2% sodium bisulphite. The homogenate was centrifuged for 10 min at 12000×g at 4 ºC. The centrifugal sediment was washed with 0.2% sodium bisulfite and then immersed in 10 ml of cold extraction buffer [0.1 mol L^{-1} sodium acetate (pH 5.2), 100 mmol L^{-1} NaCl, 2% (V/V) β-mercaptoethanol, and 5% (W/V) polyvinyl- pyrrolidone] for 1.0 hour. Following centrifugation, the supernatant was the crude extract for analyzing the activities of PME, PG, Cx, β-Gal and α-Af. All of above steps were performed at 4 ºC.

For PME activity (QI et al., 2015), a volume of 1 ml of crude extract and 4.0 ml of 0.5% (W/V) citrus pectin (Sigma Chemical Co., St. Louis, MO, USA) was incubated at 37 °C for 1 h, then was titrated with 0.01 mol L^{-1} NaOH to maintain pH 7.4. One unit of activity was calculated as 1 mmol L^{-1} NaOH consumed g^{-1} FW 10 min^{-1}. For PG activity (GROSS, 1982), the enzyme extract (0.5 ml) was mixed with 0.8 ml of 0.5% polygalacturonic acid (Sigma Chemical Co., St. Louis, MO, USA), and incubated at 37 °C for 2 h, then 2 ml of 0.1 mol L^{-1} borate buffer (pH 9.0) and 0.3 ml of cyanoacetamide was added to measure the amount of galacturonic acid released. After boiling for 10 min and then cooling, the absorbency was read at 276 nm. One unit of activity was defined as 1 μg of galacturonic acid released g^{-1} fresh weight (FW) min^{-1}. Cx activity was assayed by 3,5-dinitrosalicylic acid method (ABELES and BILES, 1991). Enzyme extract (1.0 ml) and 2 ml of 1% sodium carboxymethyl cellulose were mixed and incubated at 40 °C for 2 h, and then1.5 ml of 3,5-dinitrosalicylic acid was added in the reaction mixture. After boiling for 5 min and cooling, the absorbance value was measured at 540 nm. One unit of activity was defined as 1 μg of glucose released g^{-1} fresh weight (FW) min^{-1}. Both β-Gal and α-Af activities were assayed as detailed by QI et al. (2015). A mixture containing 0.5 ml of enzyme extract, 0.5 ml of 0.1 mol L^{-1} sodium acetate (pH 5.2), and 0.5 ml of either p-nitrophenyl-β-D-galactopyranoside or p-nitrophenyl-α-D-arabinofuranoside (Sigma Chemical Co., St. Louis, MO, USA) , respectively, was incubated at 37 °C for 30 min, and the reaction was stopped by adding sodium carbonate and the absorbency was read at 400 nm. A calibration curve was obtained by using p-nitrophenol (PNP) (Sigma Chemical Co., St. Louis, MO, USA) as standard. Enzyme activity was expressed as nmol PNP g^{-1} FW min^{-1}. In all assays, the boiled enzyme extract was taken as the control.

RNA extraction and *q*RT-PCR analysis of the related genes

Total RNA was extracted from apple fruit using the modified CTAB method (GASIC et al., 2004). The expression levels of *MdPG, MdPME, MdCx, MdGal* and *MdAf* were determined by *q*RT-PCR. Specific primers for *q*RT-PCR were designed using apple cell wall-associated candidate gene sequences (Tab. 2). The *q*RT-PCR was performed using a PrimeScriptTM RT Reagent Kit (TaKaRa, Japan), with oligo (dT) 20 and random primers for cDNA synthesis according to the manufacturer's protocol. The amplified PCR products were determined on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA), with the SYBR Premix Ex Taq kit (TaKaRa, Japan). The various cDNA samples were standardized with 18S rRNA transcripts. Expression levels were calculated with the $2^{-\Delta\Delta CT}$ by being set at 1.0 for day 0 of storage for each gene. The experiments were repeated three times with three biological samples.

Experimental design and statistical analysis

Experiments were performed based on a completely randomized design. The results were subjected to statistical analysis of variance (ANOVA) by using SPSS 18 software. Least significant differences (LSD) at a significance level of 1% or 5% were detected with Duncan's multiple range tests. The path analysis was carried out through stepwise regression analysis with deleting the insignificant factors.

Results

Flesh firmness and respiration rate

For 'Gala' fruit, flesh firmness was lowest value at harvest, and decreased rapidly just after storage of 8 days, continuing to decline

Tab. 2: Primers of cell wall enzyme-related genes for qRT-PCR in apple fruit

Gene name	Sense primers sequence	Anti-sense primers sequence
MdPG	5' GTAACTGCACCAGAGGACA3'	5' TTCTTCACCACCAAGTTATT 3'
MdPME	5' GATGCCTTGGAGTGGAGA3'	5' TGCTAATGTATTGCGTTC 3'
MdCx	5' TAGGTTGCGATGGTGGTT 3'	5' AGGCTCCGAATGGCTGTA 3'
MdGal	5' AAGAACGGAAAGTCCCCAC3'	5' TCCAATGACCCATACACGG 3'
MdAf	5' AGAAACGCCTATCCTGAC 3'	5' CACGGCATACTCGCTCAC 3'
18S	5' CCATTGGAGGGCAAGTCT 3'	5' GGTTCTCACGCTACACGA 3'

and leading to soft flesh at 20 days of storage. The firmness of 'GD' fruit was at a medium level on the day of harvest and also declined quickly, especially during the early stage of storage, resulting in a mealy flesh after 56 days of storage. However, 'Fuji' fruit not only had the highest firmness value at harvest and maintained a high value even after 72 days of storage, displaying a crisp flesh, more juice and better quality (Fig. 1A).

The rapid decline in firmness was accompanied by a swift increase in respiration rate in both 'Gala' and 'GD' fruits. The respiration rate peaked quickly at 8 days' storage in 'Gala', and 16 days in 'GD'. In contrast, the respiration rate increased only slightly with a minor peak after storage for 40 days in 'Fuji', which was significantly lower than those of both in 'GD' and 'Gala' fruit (Fig. 1B).

In summary, 'Fuji' fruit maintained harder flesh with lower respiration, while firmness lost more rapidly and the respiration rates increased swiftly to the higher peaks in both 'GD' and 'Gala' apples, indicating the different softening characteristics among the three apple cultivars.

Pectin components and hemicellulose contents

During storage, WSP content was highest at harvest and changed little in 'Fuji', whereas it increased rapidly during the early storage period in both 'GD' and 'Gala' fruit (Fig. 2A). Also, the CSP content was the highest and decreased in the slowest rate in 'Fuji', but a rapid decrease rate was found in 'Gala' and 'GD' apple with no obvious difference each other by comparison (Fig. 2B). ISP content increased slightly in both 'GD' and 'Gala' fruit and remained constant in 'Fuji' (Fig. 2C). However, hemicellulose content decreased more rapidly in both 'Gala' and 'GD' fruit, whereas a lower content with little change was observed in 'Fuji' (Fig. 2D).

As shown in Tab. 3, there was a significantly positive correlation between firmness loss and changes in CSP content in the three apple cultivars. However, the significant correlation between firmness loss and changes in WSP and hemicellulose content was only observed in the 'GD' and 'Gala' fruits. This indicates that CSP, WSP and hemicellulose are more closely correlated with changes in fruit texture in apples.

Cell wall-related enzymes activities and genes expressions

PME activity increased slowly in the early stage of storage, followed by a slight decrease in 'Fuji'. A similar trend with middle level was shown in 'GD' fruit, but the fastest increase rate was observed in 'Gala', which quickly exceeded that of 'GD' after 8 days of storage

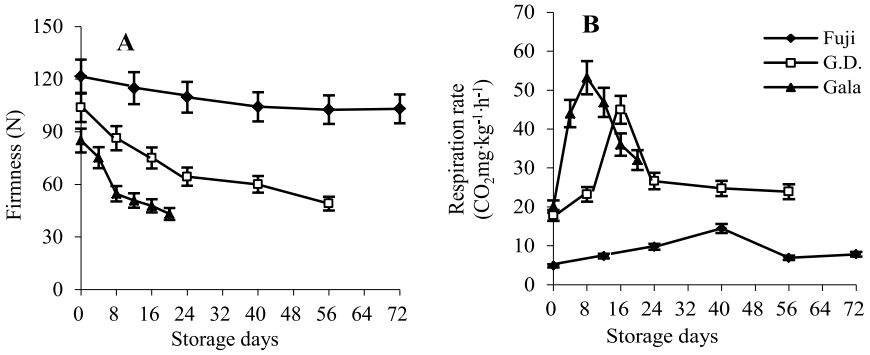

Fig. 1: Changes of firmness and respiration rate during ripening of apple fruit. Firmness values are means of 10 replicates ± S.E. and the values of respiration rate are means of 3 replicates ±S.E.

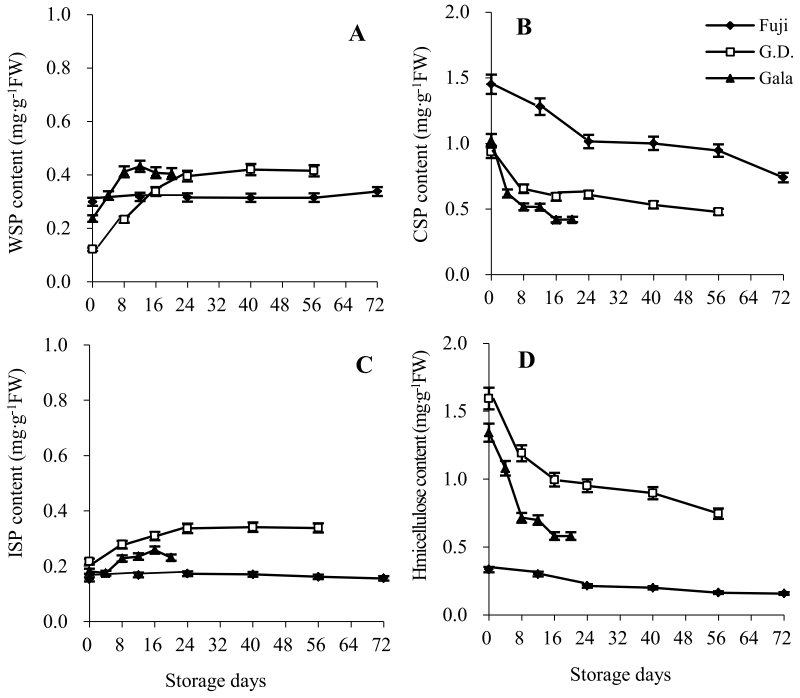

Fig. 2: Changes of the content of cell wall component during ripening of apple fruit. Values are means ± S.E.

Tab. 3: Correlation coefficients of firmness with cell wall compositions and related-enzymes activities

Cultivars	CSP	WSP	ISP	Hemicellulose	β-Gal	α-Af	PME	PG	Cx
Gala	0.900*	-0.967**	-0.680	0.889*	-0.984**	-0.916*	-0.981**	-0.933**	-0.582
Golden Delicious	0.935**	-0.969**	-0.613	0.854*	-0.991**	-0.955**	-0.921**	-0.911*	0.487
Fuji	0.951**	-0.608	-0.462	0.449	-0.868*	-0.810*	-0.147	-0.686	-0.604

Note: * and ** indicate significant linear correlation at $P< 0.05$ and $P< 0.01$ levels, respectively.

(Fig. 3A). The expression of *MdPME* increased fastest in 'Gala', followed by 'GD', with little enhancement in 'Fuji' (Fig. 3D). Similarly, significant differences were found in the activity and gene expression of PG in the three cultivars. PG activity increased faster in 'GD' and Gala than that in 'Fuji' (Fig. 3B). The *MdPG* expression was fastest in 'Gala', followed by 'GD', and the lowest level observed in 'Fuji' (Fig. 3E). Cx activity and gene expression displayed a similar slow increase trend with no significant difference among the three cultivars. The activities were not significantly correlated to firmness loss or the content of each cell wall component (Fig. 3C and Fig. 3F).

A rapid increase was observed in β-Gal activity and gene expression just after harvest in 'Gala'. The lowest activity and constant gene expression was found in 'Fuji' apple, and that of 'GD' fruit was in the intermediate level (Fig. 4A, 4C). The α-Af activity and *MdAf* expression showed the fastest increase rate in 'Gala', followed by the mid-rate in 'GD', and the lowest increase rate was observed in 'Fuji' apple (Fig. 4B, 4D).

Firmness loss was significantly correlated with the activity of cell wall enzymes except of Cx in both 'GD' and 'Gala' fruit. However, only β-Gal and α-Af activity showed a notable correlation with firmness in 'Fuji' apples (Tab. 3). In 'GD' and 'Gala' fruit, β-Gal activity was remarkably correlated with changes of every cell wall component, while PME activity had significant correlation with the content of WSP, CSP and hemicellulose. However, only CSP content had significant correlation with changes of β-Gal, α-Af and PG activity in 'Fuji' fruit (Tab. 4).

Pathway analysis among firmness, cell wall compositions and related enzymes

Pathway analysis was conducted using stepwise regression analysis with deleting the insignificant factors (Tab. 5). For 'Gala' fruit, the factors of WSP, β-Gal and hemicellulose were closely associated with the softening process. Of these, both the WSP and Gal factors exhibited stronger direct negative effects on fruit softening and interacted indirectly with each other through negative interactions. The factor of hemicellulose was also affected by stronger indirect positive roles of WSP and β-Gal factors during fruit softening. Therefore, the WSP and β-Gal factors may be more responsible for the fruit softening in 'Gala'. In the 'GD' apple, although the factors of CSP, hemicellulose, β-Gal and α-Af were also closely linked to fruit softening. However, only the β-Gal factor demonstrated the strongest direct negative effect, while the other factors mainly effected fruit softening through the strong indirect positive effect of the β-Gal factor. This indicates the dominant role of β-Gal involved in the softening process. In 'Fuji' fruit, the CSP, PG, and β-Gal were the primary softening factors. The CSP factor had the strongest directly positive role and was mainly affected by the indirectly positive role of β-Gal. Also, the negative role of β-Gal was primarily influenced by the indirect negative role of the CSP factor. However, the PG factor played the weakest role and had little interactions with CSP and β-Gal factors. Based on the above analysis, β-Gal may be the most crucial enzyme involved in cell wall degradation. Changes in composition of WSP, CSP and hemicellulose were more closely correlated with fruit softening and the storage ability of apple.

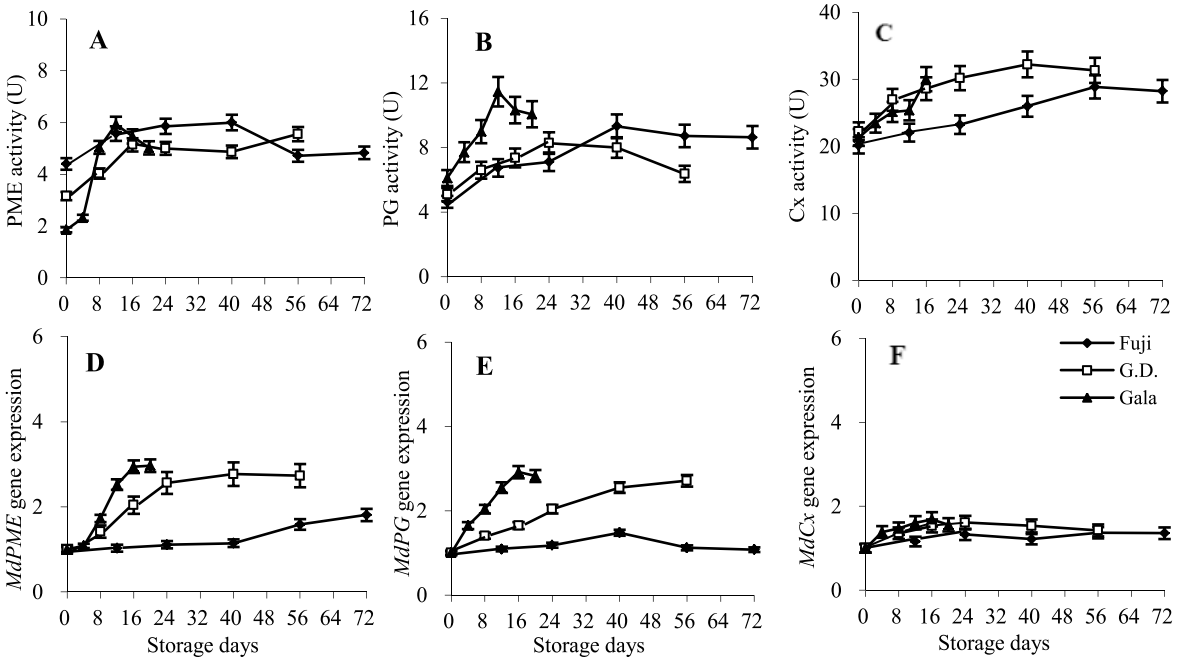

Fig. 3: Changes of PME, PG and Cx activity and gene expression during ripening of apple fruit. Values are means ± S.E.

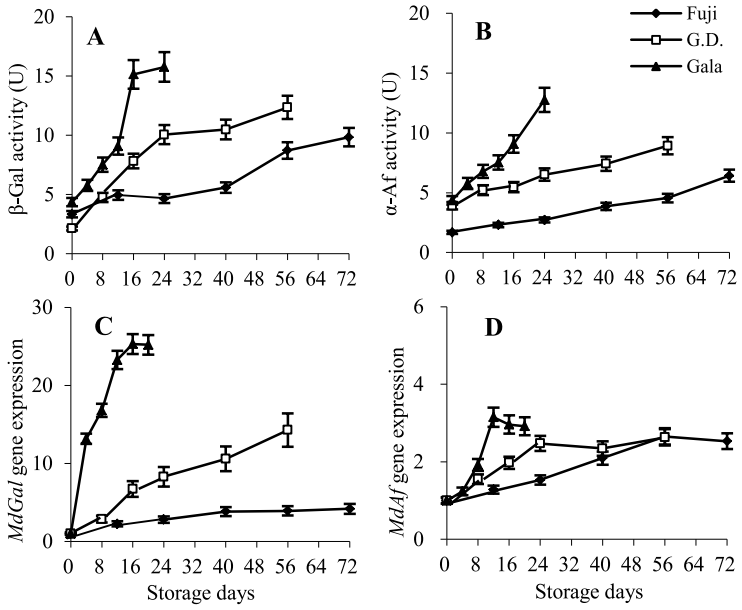

Fig. 4: Changes of β-Gal and α-Af activity and gene expression during ripening of apple fruit. Values are means ± S.E.

Tab. 4: Correlation coefficients between cell wall components and the related enzymes

Apple variety	Cell wall components	β-Gal	α-Af	PME	PG	Cx
Gala	CSP	-0.869*	-0.748	-0.817*	-0.844*	-0.460
	WSP	0.976**	0.675	0.952*	0.936**	0.148
	ISP	0.913*	0.906*	0.637	0.701	-0.299
	Hemicellulose	-0.856*	-0.677	-0.811*	-0.946**	-0.547
Golden Delicious	CSP	-0.912*	-0.871*	-0.966**	-0.607	0.626
	WSP	0.978**	0.876*	0.938**	0.766	-0.340
	ISP	0.966**	0.869*	0.633	0.731	-0.126
	Hemicellulose	-0.948**	-0.888*	-0.984**	-0.642	0.672
Fuji	CSP	-0.863*	-0.947**	0.072	-0.879*	-0.616
	WSP	0.571	0.613	0.020	0.706	0.610
	ISP	-0.149	0.012	0.610	0.158	0.210
	Hemicellulose	-0.180	0.038	-0.689	-0.408	-0.435

Note: * and ** indicate significant linear correlation at $P< 0.05$ and $P< 0.01$ levels, respectively.

Tab. 5: Pathway analysis by stepwise regression among firmness, cell wall compositions and related enzymes in apple fruit

	Factors	**Direct path coefficient**	**Indirect path coefficient**			**Regression equation**
			→WSP	**→Hemicellulose**	**→β-Gal**	
Gala	WSP	-0.6037	—	0.1840	-0.5475	$Y=129.8-122.1X_2-9.7X_4-1.1X_7$,
	Hemicellulose	-0.1944	0.5714	—	0.5118	$R=0.998^{**}$ (X_2, X_4 and X_7 indicate WSP,
	β-Gal	-0.5976	-0.5531	0.1665	—	hemicellulose and β-Gal, resp.)
	Factors	**Direct path coefficient**	**Indirect path coefficient**			**Regression equation**
			→CSP	**→Hemicellulose**	**→β-Gal**	**→α-Af**	
Golden Delicious	CSP	0.2556	—	-0.1404	0.643	0.1766	$Y=105.9+31.6X_1-9.5X_4-3.6X_7-2.3X_8$,
	Hemicellulose	-0.1422	0.2523	—	0.6686	0.1800	$R=0.999^{**}$ (X_1, X_4, X_7 and X_8 indicate
	β-Gal	-0.7052	-0.233	0.1348	—	-0.1915	CSP, hemicellulose, β-Gal and α-Af, resp.)
	α-Af	-0.2027	-0.2227	0.1263	-0.6661	—	
	Factors	**Direct path coefficient**	**Indirect path coefficient**			**Regression equation**
			→CSP	**→PG**	**→β-Gal**	
Fuji	CSP	0.5461	—	-0.0551	0.3196	$Y=173.8+41.2X_1-668.5X_6-1.4X_7$,
	PG	-0.3005	-0.1061	—	0.0551	$R=0.996^{**}$ (X_1, X_6 and X_7 indicate CSP,
	β-Gal	-0.3706	-0.4712	0.0595	—	PG and β-Gal, resp.)

Note: In this table, '—' indicate null number, '→' indicate indirect roles.

Discussions

The modification of cell wall polymers is a major factor involved in fruit softening. The cell wall is composed of pectin, hemicellulose, cellulose and various structural proteins. Pectin polysaccharides bind to cellulose and hemicellulose via hydrogen bonds and covalent bonds, forming the cell wall's network structure (Wakabayashi, 2000; Ng et al., 2013; Cybulska et al., 2015). Covalently bound pectin (CSP) includes the proto-pectin with higher molecular weight and the insoluble pectic acid with high- methoxylation. Water-soluble pectin (WSP) contains low-methoxylated pectic acid with lower molecular weight, which easily detaches from pectin polymers (Brummell, 2006; Vicente et al., 2007; Billy et al., 2008). A higher level of CSP content is associated with the hard flesh firmness and good storage ability, while a rapid increase in WSP content is an important indicator of poor-storage ability in fruit (Li et al., 2020; Su et al., 2022). Hemicellulose is the major component of the cellular skeleton acting and an important contributor to fruit texture (Cheng et al., 2009; Dheilly et al., 2016).

In this study, the relationships between changes in cell wall content and fruit texture characteristics differed among three apple cultivars. Fuji's crisp-texture with good storability was closely correlated to the higher insoluble component (CSP) content and the constant content of cell wall components, while the rapid decrease in the insoluble components (both CSP and hemicellulose) and the rapid increase in the water soluble components (WSP) manifested in the loose texture and the easier softening property in both 'GD' and 'Gala' cultivars. This result is consistent with the previous reports that fruit firmness is negatively correlated with the soluble pectin content, but positively correlated with the proto-pectin content in apple (Ng et al., 2015; Zhang et al., 2024). Similar conclusions have been found in pears (Dong et al., 2017) and blueberries (Chen et al., 2015).

Cell wall disassembly is a key factor in fruit softening that is involved by the co-operation of a multitude of cell wall-localized enzymes, such as pectinase, cellulose, glycosidase (Brummell, 2006; Goulao et al., 2007). Pectin degradation is a crucial physiological process involving PG and PME activities, which are more closely correlated with firmness loss in many fruits (Posé et al., 2013; Chen et al., 2015; Castro et al., 2021). PME has been reported to mainly catalyze pectin de-methylesterification, reducing intercellular connections by cleaving the lateral connection of calcium cross-links in pectin chains and generating suitable substrates for the action of PG on pectin degradation. This indicates their various roles during different softening stages of fruits (Jarvis, 2011; Gwanpua et al., 2014; Lu et al., 2021).

In this study, the activities and the related gene expression of both PG and PME enzymes displayed similar dynamics with fruit softening in three apple cultivars, but only showed significant correlation with firmness in 'GD' and 'Gala' fruit. This indicates that the action of these enzymes on pectin degradation depends on the cultivar. PME activity and gene expression increased faster than that of PG at the early softening stage in 'Gala' fruit. This is consistent with previous reports indicating that PME mainly creates a prerequisite for the action of PG on the degradation of pectin indicating the earlier roles of PME than that of PG on the softening process, and PME activity has been shown higher during the early stage of softening in soft apple fruits (Wei et al., 2010; Gwanpua et al., 2014; Li et al., 2020). The above result is further supported by the report that the *PG* expression is enhanced in later maturity stages and PG enzyme also serves to the later softening stage in apple (Li et al., 2023). However, the activity of Cx showed no obvious difference among three apple cultivars. Although some reports have demonstrated the action of Cx enzyme on texture softening, the exact substrate of Cx enzyme is still not identified until now (Abeles and Biles, 1991; Goulao and Oliveira, 2008) and needs further research.

Glycoside hydrolases cleave glycosidic bonds from side chains of cell wall polymers, promoting cell wall expansion and providing the prerequisite for the other cell wall enzymes to hydrolyze cell wall polysaccharides (Yoshioka et al., 1995; Goulao et al., 2007). The actions of β-Gal and α-Af on fruit softening are in cooperation with pectolytic enzymes in cell wall metabolism and their higher activities prior to PG activity at early softening stage (Wei et al., 2010; Zhao et al., 2019; Su et al., 2022). In our tests, both β-Gal and α-Af activities increased rapidly at early softening stage and had more noticeable correlation with firmness loss. *MdGal* and *MdAf* genes showed a higher expression level in 'Gala' and 'GD' fruit, significantly greater than those in 'Fuji' apple. It can therefore be inferred that the more roles of glycosidase contribute to the early softening of apples with soft texture. Among these, the β-Gal activity showed more significant correlation with the content of every cell wall component than the other enzymes in 'Gala' and 'GD' fruit (Tab. 4). The enzyme of β-Gal may be involved in the softening start-up, contributing more to fruit softening than other cell wall enzymes (Goulao et al., 2007; Qi et al., 2015). The activity of β-Gal and the *MdGal* expression displays significant difference between hard-crisp cultivars and loose-crisp cultivars, which may be responsible for the differences in fruit texture (Li et al., 2023). The higher α-Af activity and gene expression levels are more closely related to firmness loss, which render the cell wall matrix more accessible to be degraded by other enzymes in apples (Nobile et al., 2011).

Finally, based on the pathway analysis (Tab. 5), we identified stronger roles of WSP and β-Gal factors in 'Gala' fruit softening, the dominant role of β-Gal factor in softening process of 'GD' fruit, and the closer correlations of CSP and β-Gal factors with 'Fuji' fruit softening. Moreover, in both 'Gala' and 'GD' fruit, the hemicellulose factor had a strong indirect interaction with β-Gal factor, indicating that the modification of hemicellulose may have more correlation with fruit softening of apple with low storability. Nevertheless, our results cannot adequately explain the complex interactive relationships among the parameters relating to cell wall metabolism, although the pathway analysis provides some reference information for defining key softening regulatory factors in different apple cultivars. However, few reports are in agreement with the conclusions drawn by the pathway analysis in this study, and further research and discussion are needed.

Conclusions

In summary, significant differences were observed in the softening-regulating factors related to cell wall metabolism among the three apple cultivars. In 'GD' and 'Gala' cultivars, a rapid decrease in the content of CSP and hemicellulose and a fast increase in the content of WSP were concomitant with a swift up-trend in cell wall enzymes activities and gene expression, showing closer correlation with firmness loss. In contrast, 'Fuji' apple retained hard flesh with little change in the content of cell wall components and displayed lower activities and gene expression of cell wall-degrading enzymes. The pathway analysis further revealed that both WSP and β-Gal factors were closer associated with 'Gala' fruit softening, and β-Gal enzyme played the dominant roles in the softening of 'GD' apple, and both CSP and β-Gal factors maybe have the closer correlations with the well storability of 'Fuji' fruit.

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Conflicts of interest

The authors declare no conflicts of interest in the submission of this manuscript.

References

Abeles, F.B., Biles, C.L., 1991: Cellulase activity in developing apple fruit. *Scientia Horticulturae* 47 (12), 77-87. DOI: 10.1016/ 0304-4238(91)90029-X

Almeida, D.P.F., Huber, D.J., 2007: Polygalacturonase-mediated dissolution and depolymerization of pectins in solutions mimicking the pH and mineral composition of tomato apoplast. Plant Sci. 172, 1087-1094. DOI: 10.1016/j.plantsci.2007.03.008

Billy, L., Mehinagic, E., Royer, G., Renard, C.M.G.C., Arvisenet, G., Prost, C., Jourjon, F., 2008: Relationship between texture and pectin composition of two apple cultivars during storage. Postharvest Biol. Technol. 47, 315-324. DOI: 10.1016/j.postharvbio.2007.07.011

Brummell, D.A., 2006: Cell wall disassembly in ripening fruit. Funct. Plant Biol. 33, 103-119. DOI: 10.1071/FP05234

Brummell, D.A., Dal Cin, V., Crisosto, C.H., Labavitch, J.M., 2004: Cell wall metabolism during maturation, ripening and senescence of peach fruit. J. Exp Bot. 55 (405), 2029-2039. DOI: 10.1093/jxb/erh227

Castro, R.I., Munoz-Vera, M., Parra-Palma, C., Valenzuela-Riffo, F., Figueroa, C.R., Morales-Quintana, L., 2021: Characterization of cell wall modifycation through thermogravimetric analysis during ripening of Chilean strawberry (*Fragaria chiloensis*) fruit. Cellulose 28, 4611-4623. DOI: 10.1007/s10570-021-03806-w

Chen, H., Cao, S., Fang, X., Mu, H., Yang, H., Wang, X., Xu, Q., Gao, H., 2015: Changes in fruit firmness, cell wall composition and cell wall degrading enzymes in postharvest blueberries during storage. Sci Hortic. 188, 44-48. DOI: 10.1016/j.scienta.2015.03.018

Cheng, G.P., Duan, X.W., Jiang, Y.M., Sun, J., Yang, S.Y., Yang, B., He, S.G., Liang, H., Luo, Y.B., 2009: Modification of hemicellulose polysaccharides during ripening of postharvest banana fruit. Food Chem. 2009, 115, 43-47. DOI: 10.1016/j.foodchem.2008.11.065

Collins, P.P., O'Donoghue, E.M., Rebstock, R., Tiffin, H.R., Sutherland, P.W., Schröder, R., McAtee, P.A., Prakash, R., Ireland, H.S., Johnston J.W., Atkinson, R.G., Schaffer, R.J., Hallett, I.C., Brummell, D.A., 2019: Cell type-specific gene expression underpins remodeling of cell wall pectin in exocarp and cortex during apple fruit development. J. Exp. Bot. 70(21), 6085-6099. DOI: 10.1093/jxb/erz370

Cybulska, J., Zdunek, A., Koziol, A., 2015: The self-assembled network and physiological degradation of pectins in carrot cell walls. Food Hydrocoll. 43, 41-50. DOI: 10.1016/j.foodhyd.2014.04.032

Dheilly, E., Gall, S.L., Guillou, M.C., Renou, J.P., Bonnin, E., Orsel, M., Lahaye, M., 2016: Cell wall dynamics during apple development and storage involves hemicellulose modifications and related expressed genes. BMC Plant Biol. 16, 201. DOI: 10.1186/s12870-016-0887-0

Dong, Y., Zhang, S., Wang, Y., 2018: Compositional changes in cell wall polyuronides and enzyme activities associated with melting/mealy textural property during ripening following long-term storage of 'Comice' and 'd'Anjou' pears. Postharvest Biol. Technol. 135, 131-140. DOI: 10.1016/j.postharvbio.2017.09.010

Gasic, K., Hernandez, A., Schuyler, S., Korban, S.S., 2004: RNA extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol. Biol. Rep. 22, 437a-437g. DOI: 10.1007/BF02772687

Geitmann, A., 2010: Mechanical modeling and structural analysis of the primary plant cell wall. Curr. Op. Plant Biol. 13, 693-699. DOI: 10.1016/j.pbi.2010.09.017

Goulao, L.F., Oliveira, C.M., 2008: Cell wall modifications during fruit ripening: when a fruit is not the fruit. Trends Food Sci. Technol. 19, 4-25. DOI: 10.1016/j.tifs.2007.07.002

Goulao, L.F., Santos, J., Sousa, I., Oliveira, C.M., 2007: Patterns of enzymatic activity of cell wall modifying enzymes during growth and ripening of apples. Postharvest Biol. Technol. 43, 307-318. DOI: 10.1016/j.postharvbio.2006.10.002

Gross, K.C., 1982: A rapid and sensitive spectrophotometric method for assaying polygalacturonase using 2-cyanoacetamide. HortScience 17, 933-934. DOI: 10.21273/hortsci.17.6.933

Gross, K.C., Sams, C.E., 1984: Changes in cell wall neutral sugar composition during fruit ripening: A species survey. Phytochem. 23 (11), 2457-2461. DOI: 10.1016/S0031-9422(00)84075-3

Gwanpua, S.G., Buggenhout, S.V., Verlinden, B.E., Christiaens, S., Shpigelman, A., Vicent, V., Kermani, Z.J., Nicolai, B.M., Hendrickx, M., Geeraerd, A., 2014: Pectin modifications and the role of pectin-degrading enzymes during postharvest softening of Jonagold apples. Food Chem. 158, 283-291. DOI: 10.1016/j.foodchem.2014.02.138

Jarvis, M.C., 2011: Plant cell walls: supramolecular assemblies. Food Hydrocoll. 25, 257-262. DOI: 10.1016/j.foodhyd.2009.09.010

Jia, F., Guo, Y., Liu, D., Yang, X., Deng, H., Meng, Y., 2016: Effect of fermentation on the polysaccharides processing characteristics in apple pomace. Scient. Agri. Sin. 49 (19), 3831-3844. DOI: 10.3864/j.issn.0578-1752.2016.19.014

Li, C., Yuan, S., Xie, Y., Guo, Y., Cheng, Y., Yu, H., Qian, H., Yao, W., 2021: Transformation of fluopyram during enzymatic hydrolysis of apple and its effect on polygalacturonase and apple juice yield. Food Chem. 357, 129842. DOI: 10.1016/j.foodchem.2021.129842

Li, Q., Xu, R., Fang, Q., Yuan, Y., Cao, J., Jiang, W., 2020: Analyses of microstructure and cell wall polysaccharides of flesh tissues provide insights into cultivar difference in mealy patterns developed in apple fruit. Food Chem. 321, 126707. DOI: 10.1016/j.foodchem. 2020.126707

Li, X., Su, Q., Jia, R., Wang, Z., Fu, J., Guo, J., Yang, H., Zhao, Z., 2023: Comparison of cell wall changes of two different types of apple cultivars during fruit development and ripening. J. Integr. Agriculture 22(9), 2705-2718. DOI: 10.1016/j.jia.2023.07.019

Lu, L., Zuo, W., Wang, C., Li, C., Feng, T., Li, X., Wang, C., Yao, Y., Zhang, Z., Chen, X., 2021: Analysis of the postharvest storage characteristics of the new red-fleshed apple cultivar 'Meihong'. Food Chem. 354, 129470. DOI: 10.1016/j.foodchem.2021.129470

Ng, J.K., Schröder, R., Brummell, D.A., Sutherland, P.W., Hallett, I.C., Smith, B.G., Melton, L.D., Johnston, J.W., 2015: Lower cell wall pectin solubilisation and galactose loss during early fruit development in apple (*Malus×domestica*) cultivar 'Scifresh' are associated with slower softening rate. J. Plant Physiol. 176, 129-137. DOI: 10.1016/j.jplph.2014.12.012

Ng, J.K.T., Schröder, R., Sutherland, P.W., Hallett, I.C., Hall, M.I., Prakash, R., Smith, B.G., Melton, L.D., Johnston, J.W., 2013: Cell wall structures leading to cultivar differences in softening rates develop early during apple (*Malus domestica*) fruit growth. BMC Plant Biol. 13, 183-199. DOI: 10.1186/1471-2229-13-183

Nobile, P.M., Wattebled, F., Quecini, V., Girardi, C.L., Lormeau, M., Laurens, F., 2011: Identification of a novel α-L-arabinofuranosidase gene associated with mealiness in apple. J. Exp. Bot. 62, 4309-4321. DOI: 10.1093/jxb/err146

Posé, S., Paniagua, C., Cifuentes, M., Blanco-Portales, R., Quesada, M.A., Mercado, J.A., 2012: Insights into the effects of polygalacturonase *FaPG1* gene silencing on pectin matrix disassembly, enhanced tissue integrity, and firmness in ripe strawberry fruits. J. Exp. Bot. 64(12), 3803-3815. DOI: 10.1093/jxb/ert210

Qi, X., Wei, J., Li, H., Zhao, D., 2015: Cell wall metabolism and related gene expression in *Malus domestica* Borkh. during fruit growth and softening. Fruits 70(3), 1-9. DOI: 10.1051/fruits/2015006

Su, Q., Li, X., Wang, L., Wang, B., Feng, Y., Yang, H., Zhao, Z., 2022: Variation in cell wall metabolism and flesh firmness of four apple cultivars during fruit development. Foods 11, 3518. DOI: 10.3390/foods11213518

Vicente, A.R., Saladie, M., Rose, J.K., Labavitch, J.M., 2007: The linkage between cell wall metabolism and fruit softening: looking to the future. J. Sci. Food Agric. 87, 1435-1448. DOI: 10.1002/jsfa.2837

Wakabayashi, K., 2000: Changes in cell wall polysaccharides during fruit ripening. J. Plant Res. 113, 231-237. DOI: 10.1007/ PL00013932

Wang, D.,Yeats, T.H., Uluisik, S., Rose, J.K.C., Seymour, G.B., 2018: Fruit softening: revisiting the role of pectin. Trends Plant Sci. 23, 302-310. DOI: 10.1016/j.tplants.2018.01.006

Wei, J., Ma, F., Shi, S., Qi, X., Zhu, X., Yuan, J., 2010: Changes and

postharvest regulation of activity and gene expression of enzymes related to cell wall degradation in ripening apple fruit. Postharvest Biol. Technol. 56, 147-154. DOI: 10.1016/j.postharvbio.2009.12.003

YOSHIOKA, H., KASHIMURA, K., KANEKO, K., 1995: β-D-Galactosidase and α-L-arabinofuranosidase activities during the softening of apples. J. Japan. Soc. Hort. Sci. 63, 871-878. DOI: 10.2503/JJSHS.63.871

ZHANG, K., ZHANG, D., WANG, X., XU, X., YU, W., WANG, C., YUAN, Y., YANG, S., CHENG, C., 2024: Integrated analysis of postharvest storage characteristics of seven apple cultivars and transcriptome data identifies *MdBBX25* as a negative regulator of fruit softening during storage in apples. Postharvest Biol. Technol. 207, 112646. DOI: 10.1016/j.postharvbio.2023.112646

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