Transcriptome analysis reveals candidate genes for dietary fiber metabolism in *Rosa roxburghii* fruit grown under different light intensities

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Abstract

The fruit of the perennial rosebush *Rosa roxburghii* were valued for their high levels of ascorbic acid (AsA), superoxide dismutase activity, and cancer preventing effects. The high cellulose and low pectin content of *Rosa roxburghii* fruit results in an undesirable fibrous texture and hence needs to be addressed. However, little is known about the molecular mechanisms underlying dietary fiber metabolism in this fruit. Here, we report that the contents of cellulose, pectin, and lignin were increased by shading treatments at the maturation stage of fruit development. Under 50% shading, the soluble pectin content increased by 16.39%, which may improve the fruit palatability. However, deeper shading of 100% caused the lignin content to increase by 28.86%, which conversely may lower fruit quality. Based on transcriptome analysis, we identified candidate genes involved in dietary fiber metabolism, including *cellulose synthase (CesA) 1, 2, 3,* and *5*, *â-1,4-xylosyltransferase (IRX)*, *arabinosyltransferase (ARAD) 1* and *2*, *galacturonosyltransferase (GAUT)*, *cellulolytic enzyme (Cx)*, and *pectin methylesterase (PME)*, in which *CesA1, CesA2, CesA3, IRX, ARAD2*, and *GAUT3* significantly responded to shading and positively correlated with the content of their corresponding component. Furthermore, *cinnamyl alcohol dehydrogenase (CAD)* was significantly regulated by shading treatment and positively correlated with increasing lignin concentration. These results may facilitate a better understanding of the molecular mechanisms of dietary fiber metabolism in *R. roxburghii* fruit under low light conditions and provide a framework for future crop improvement.

Keywords: Dietary fiber · Gene expression· Molecular mechanisms · *Rosa roxburghii* fruit · Shading treatment

1 Introduction

Rosa roxburghii Tratt is a perennial rosebush native to China that is becoming more commonly cultivated, especially in Guizhou Province, due to the nutritional and health-promoting properties of its fruit. While direct consumption of the fruit remains low, due in part to its fibrous texture, an increasing number of health care products, cosmetics, and functional foods containing *R. roxburghii* fruit extracts are available (Xu et al. 2019). To date, the cultivation area of this species in China stands at over 130,000 hectares. The mature fruit of *R. roxburghii* contains large quantities of total dietary fiber (23.80%), cellulose (8.70%), hemicellulose (5.10%), and lignin (1.40%), but relatively low levels of total pectin (3.40%), of which insoluble pectin is 2.8% and soluble pectin only 0.6% (Liu et al. 2015a). The high ratio between the

content of dietary fiber and pectin is one of the principal causes of undesirable organoleptic properties in fruit of *R. roxburghii*, and hence an important target for future breeding programs.

Cellulose, hemicellulose, lignin, and pectin are the four major components of *R. roxburghii* fruit dietary fiber. The quantity of these cell wall polymers in the developing and ripening fruit depends on their synthesis, remodeling, and degradation (Galanakis 2011). Our previous research suggested that the lignin-related activities of peroxidase (POD), cinnamoyl-CoA reductase (CCR), cinnamoyl-CoA reductase (4CL), and shikimate O-hydroxycinnamoyltransferase (HCT) play a crucial role in lignin biosynthesis (Lu et al. 2020).

Cellulose is a linear polysaccharide of (1 → 4)-linked β -D-glucosyl residues, whose synthesis is catalyzed by the *CesA* genes, which encode the probable catalytic subunits of the plant cellulose synthase enzyme complex, visible in the plasma membrane as rosettes (Schneider et al. 2016). At least 10 *CesA* isoforms exist in *Arabidopsis thaliana*, which exert distinct role/s in the cellulose synthesis process (Takata & Taniguchi 2015). In apple (*Malus domestica*), seven *CesA* genes were found to be downregulated during the transition from tight cluster flowers to anthesis (Guerriero et al. 2014). It has been suggested that *hydrolase-cellulase* (*Cx*) may also be implicated in the metabolism of (hemi-) cellulose in citrus (Dong et al. 2009).

Hemicelluloses are a diverse group of heterogeneous polysaccharides whose function is to cross-link cellulose microfibrils and encompass the heteromannans, xyloglucan, heteroxylans, and mixed-linkage glucan (Pauly et al. 2013). Polysaccharide synthesis is mediated mostly by glycosyltransferases (GT). The synthesis of the backbone of xyloglucan is catalyzed by members of the GT2-CSL superfamily. Group CSL-C catalyzes the xyloglucan backbone, CSL-A catalyzes the mannan and glucomannan backbone, and CSL-F catalyzes the mixed-linkage glucan backbone. The xylan backbone is thought to be synthesized by GT43 proteins with beta-1,4-xylosyltransferase activity (Lee et al. 2012). On the other hand, the synthesis of heteromannans and mixed-linkage glucan is catalyzed by *cellulose synthase-like* (*CSL*), which shares several common features with *CesA* genes. Evidence exists that all *CSL* gene products are also integral membrane proteins and contain the D, DxD, D, and QxxRW motifs (Saxena et al. 1995). Additionally, xyloglucan endo-trans-glycosylase/hydrolase (Miedes & Lorences 2009), β-xylosidase (Figueroa et al. 2010; Takizawa et al. 2014), and α-L-arabinofuranosidase (Figueroa et al. 2010) are enzymes associated with hemicellulose degradation.

Pectic polysaccharides, a group of complex polysaccharides, are commonly referred to as pectin, which comprise mainly homogalacturonan and rhamnogalacturonan I and II (Smith 2013; Salima et al. 2018). Homogalacturonan is the most common form among them, which consists of residues of

(1-4)-α-D-galacturonic acid arranged linearly and catalyzed by *galacturonosyltransferase* (*GAUT*) (Sterling et al. 2006). Enzymes involved in pectin degradation, such as polygalacturonase (PG) and pectin methylesterase (PME), have been extracted from fruits. The activity of PG and PME was found to gradually increase during the development of orange (*Citrus* × *sinensis*) fruit (Zeng et al. 2006). However, the PG activity decreased gradually during the development of strawberry (*Fragaria* × *ananassa*) fruit (Figueroa et al. 2010). In tomato (*Solanum lycopersicum*), *PME* genes make up a small gene family including at least four genes (Mutsumi et al. 2015).

Light is an important environmental factor affecting fruit growth and development (Zhang et al. 2019). Studies have shown that light affects the synthesis and accumulation rate of dietary fiber components mainly by two aspects. One is to promote the synthesis of sugars, phenylalanine, and other organic compounds by affecting photosynthesis and the other is to directly promote the synthesis of dietary fiber components by regulating related enzyme activities in the biosynthetic pathway. The accumulation of dietary fiber components in fruit cell walls was closely related to low light (Zhang et al. 2019). Furthermore, low light intensity reduced the cellulose content in cotton (*Gossypium hirsutum*) (Chen et al. 2014) and decreased the lignin content in tea (*Camellia sinensis*) (Wang et al. 2012). Under low light conditions, photorespiration is increased, and therefore, the ethylene/sugar ratio also increases, and the abscission rate of reproductive structures is higher（Millenaar et al. 2010). This can, for instance, result in reduced yield and fiber quality in cotton (Echer et al. 2019). In melon (*Cucumis melo*), low light intensity decreased the pectin content (Toshiyuki *et al*., 2006) and reduced the hemicellulose content (Suparjo et al. 1990). Shading treatment also inhibits the activity of phenylalanine ammonium-lyase (PAL), cinnamyl alcohol dehydrogenase (CAD), 4CL, and POD, which had significant effects on lignin accumulation (Liu et al. 2019). A comparison of rice (*Oryza sativa*) plants with low light-resistant and low light-susceptible genotypes showed that shade-tolerant plants had a higher lignin content (Wang *et al*., 2015). The enzymatic activities of PAL, CAD, 4CL, and POD were also higher in shade-resistant plants compared to shade-susceptible plants (Hussain et al. 2020). In shade-grown japonica rice, the expression of genes involved in secondary cell wall synthesis, namely *PAL*, *caffeic acid O-methyltransferase* (*COMT*), *caffeoyl-CoA O-methyltransferase* (*CCoAOMT*), *CCR*, and *CAD2,* and primary cell wall synthesis genes *CesA1*, *CesA3*, and *CesA8* was significantly downregulated (Wu et al. 2017).

The high cellulose and low pectin content of *R. roxburghii* fruit causes an undesirable fibrous texture. Although the genes involved in dietary fiber metabolism have been extensively studied in many plant species, dietary fiber accumulation processes and their underlying molecular mechanisms remain largely unexplored in *R. roxburghii.* In this study, we treated *R. roxburghii* fruit with two different shading intensities during fruit development. We then screened the genes involved in the synthesis and

accumulation of dietary fiber components from fruit transcriptome sequences and analyzed the correlation between gene expression and the content of the corresponding dietary fiber component. This study may facilitate a better understanding of the molecular mechanisms of dietary fiber metabolism in *R. roxburghii* fruit under low light conditions and provide a framework for future crop improvement.

2 Materials and methods

2.1 Plant material

Samples were collected from 8-year-old plants of *Rosa roxburghii*‘Guinong 5’(Fan et al. 2011), which were grown in the fruit germplasm repository of Guizhou University, Guizhou, China, in 2019 (26°42.408'N, 106°67.353'E). To study the effect of shading on the accumulation of dietary fiber, developing fruits were covered at 15 days after anthesis (DAA) with a translucent white bag (light transmittance measured at 50%) or an opaque yellow bag (light transmittance measured at 0%), or left uncovered as a control, thereby giving shade levels of 0, 50, and 100%. Then, 80, 60, and 40 fruit were collected at three different developmental stages at 30, 60, and 90 DAA, which represented the young fruit stage, fruit development stage, and mature stage, respectively (Fig. 1). After collection, samples were immediately frozen in liquid nitrogen and stored at -80°C for further use.

2.2 Determination of cellulose content

Cellulose content was determined through anthrone colorimetry as per Chen et al. (2010), with minor modifications. Air-dried fruit tissue (0.2 g) was digested in 60 mL of 60% H_2SO_4 for 30 min in a cold-water bath at 4°C. The digested cellulose solution was transferred to a volumetric flask which was then filled to 100 mL with 60% H_2SO_4. The mixture was shaken well and filtered through a Brinell funnel. Next, l.5 mL of filtrate was added to a 100 mL volumetric flask, diluted with distilled water in a cold-water bath, shaken well, and then 2 mL was taken into a tube with a plug. Then, 0.5 ml of 2% anthrone reagent was added, and 5 mL of H_2SO_4 was added along the wall of the tube, shaken well, and allowed to stand for 12 min. Absorbance was measured at 620 nm.

2.3 Determination of hemicellulose and lignin content

Kiln-dried tissue (0.2 g) was weighed into a 100 mL beaker and 10 mL of 60% Ca $(NO_3)_2$ was added and the mixture was heated for 10 min before filtration. The residue was washed with distilled water 3 times before oven drying at 70℃. The residue was transferred to a 250 mL conical flask to which 10 mL of 2 M HCl was added, and the flask was submerged in a water bath at 100℃ for 50 min. After cooling and

filtering, the residue was washed with distilled water three times and the volume of the filtrate was measured. Then, 0.5 mL of filtrate was added to 1.5 mL of DNS solution, and again incubated in a water bath at 100℃ for 10 min. After cooling, the volume was adjusted to 25 mL and the absorbance was measured at 540 nm according to Jin et al. (2017). The lignin content was measured using a lignin content determination kit (Solarbio, Beijing, China), according to the manufacturer's instructions.

2.4 Determination of pectin content

Carbazole colorimetry was used as per Einhorn-Stoll et al. (2018), with minor modifications. Fresh fruit tissue (2 g) was weighed, ground in a mortar, and washed into a conical flask with 50 mL of 95% ethanol, then extracted with 95% ethanol at 70℃ 3 times. Filtered, discarded the filtrate and washed the residue with 95% ethanol until there was no soluble sugar in the filtrate. The residue was washed into a conical flask with 40 mL of ddH_2O and incubated in a 50 ℃ water bath for 1 h before filtering. The residue and filter paper were washed with a small amount of distilled water. The filtrate was transferred into a 50 mL volumetric flask and used for the determination of soluble pectin (WSP). The residue was washed into a triangular flask with 80 mL of 0.5 M H_2SO_4, heated in a water bath (100℃) for 1 h, cooled and filtered, then transferred to a 100 mL volumetric flask, and used for the determination of insoluble pectin.

2.5 Differentially expressed genes and functional enrichment

Transcriptome data of *R. roxburghii* fruit were analyzed as per our previous study (Lu et al. 2020). Differential expression analysis of two conditions/groups was performed using the DESeq R package (1.10.1). DESeq was used to determine the differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *P*-value < 0.05 found by DESeq were assigned as differentially expressed. To annotate the unigene sequences of *R. roxburghii*, a BLASTx search ($E < 10^{-5}$) was used to search against Kyoto Encyclopedia of Genes and Genomes (KEGG) databases based on sequence similarity.

2.6 Real-Time Quantitative PCR Analysis

Candidate differentially expressed genes (DEGs) involved in dietary fiber metabolism were selected for validation by real time quantitative PCR (qRT-PCR). Total RNA was extracted through a TaKaRa MiniBEST Plant RNA Extraction Kit (TaKaRa, Inc., Dalian, China). RNA quality was evaluated by agarose gel electrophoresis and the NanoDrop system (Implen, Los Angeles, CA, USA). cDNA was synthesized with the PrimeScrip RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Inc.,

Dalian, China). The primer sequences used for qRT-PCR are listed in Table S1. qRT-PCR was performed on an ABI ViiA 7 DX system (Applied Biosystems) using SYBR Premix Ex Taq II (TaKaRa) with the ubiquitin gene as an endogenous control. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method. Values for mean expression and standard deviation (SD) were calculated from the results of three independent experiments.

2.7 Statistical analysis

All experiments were conducted in at least triplicate, and data were expressed in means ± standard deviations. Statistical analysis was performed using SPSS 20.0 software (SPSS 20.0, IBM, Armonk, NY, USA), and the differences among mean values were tested by one-way ANOVA (SPSS 20.0, IBM, Armonk, NY, USA), taking a level of $p < 0.05$ as significant to Duncan's multiple range test. The original 8.0 software was used to draw the graph. TBtools software was used to draw the heat map of gene expression (Chen et al. 2020).

3 Results

3.1 Effect of shading on fruit coloring of *R. roxburghii*

The effect of shading treatment on the color change of *R. roxburghii* fruit during fruit development is shown in Fig. 1. The young fruit (30 DAA) had a green coloration in the control sample, which was slightly paler in the 50% shade treatment, turning to a pale yellow-white color in the 100% shade treatment. The developing fruit (60 DAA) displayed a yellow-green color in the control group, which was much paler in the 50% shade treatment and had more pink-red hues in the 100% shade group. The mature fruit (90 DAA) had a pale-yellow color in the control group, which darkened to a more vivid yellow in the 50% shade group, and further to a rich yellow with hints of orange in the 100% shade group. Shade treatments, especially 100% shade, caused a significant reduction in fruit weight.

3.2 Content of dietary fiber components in *R. roxburghii* fruit and its response to shading

The changes in the content of dietary fiber components of the *R. roxburghii* fruit during development under different shade treatments are presented in Fig. 2. During fruit development, cellulose content showed a linear downward trend in the control group, but a more uniform concentration during development in the shaded samples. In the young fruit stage (30 DAA), shading treatment significantly inhibited the accumulation of cellulose in the fruit, while in the later stage of fruit development, it significantly promoted the accumulation of cellulose, and the effect became more obvious with the increase of shading; the content of cellulose increased by 12.26% and 18.25% under 50% and 100%

shading, respectively (Fig. 2a). The accumulation of hemicellulose in *R. roxburghii* fruit showed a downward trend in the whole development process, but shading had no obvious effect on the accumulation of hemicellulose (Fig. 2b). The lignin content increased slowly first and then decreased rapidly in the control group, but shading effected lignin accumulation. Before 60 DAA, shading inhibited the accumulation of lignin, but at 90 DAA, under the 50% and 100% shading treatments, shade promoted the accumulation of lignin by 13.07% and 28.86%, respectively (Fig. 2c). Pectin content first decreased at 60 DAA and then slightly increased or slightly decreased further at 90 DAA depending on the treatment. At the mature stage, both the 50% and 100% shading treatments promoted the accumulation of soluble pectin by 16.39% and 18.78%, respectively. However, only 100% shading promoted the accumulation of total pectin and insoluble pectin by 27% and 28.6%, respectively (Fig. 2d-f).

3.3 Clustering and Kyoto Encyclopedia of Genes and Genomes pathway enrichment of the differentially expressed genes

To study the transcriptional regulation of the genes involved in fruit development and maturation, 17,470 differentially expressed unigenes were classified into 18 types of clusters based on the modulation of expression patterns (Fig. 3). The gene expression patterns of cluster 1 (548 DEGs), cluster 2 (1,208 DEGs), cluster 3 (231 DEGs), cluster 12 (732 DEGs), cluster 15 (1,747 DEGs), and cluster 17 (207 DEGs) exhibited similar changes during fruit development. The gene expression levels showed a rapid increase/decrease from 30 to 60 DAA, but insignificant changes were observed from 60 to 90 DAA. These genes may exert important functions in the young fruit. The gene expression patterns of cluster 6 (88 DEGs), cluster 8 (179 DEGs), and cluster 9 (299 DEGs) were similar. The gene expression levels showed a rapid increase/decrease from 30 to 60 DAA, followed by a rapid decrease/increase from 60 to 90 DAA. The genes in this group may function in the middle stages of fruit development. The gene expression patterns of cluster 4 (3,824 DEGs), cluster 5 (1,108 DEGs), cluster 7 (1,521 DEGs), cluster 14 (331 DEGs), cluster 16 (87 DEGs), and cluster 18 (377 DEGs) exhibited similar changes during fruit development. The gene expression levels remained stable from 30 DAA to 60 DAA, and then a rapid decrease/increase from 60 to 90 DAA was observed. Hence, the genes in this group may exert their functions during the maturation stage of fruit development. Additionally, similar gene expression patterns were found in cluster 10 (457 DEGs) and cluster 11 (1021 DEGs), which showed a trend of continuous increase/decrease from 30 to 90 DAA. The genes in this group may function during all stages of fruit development.

3.4 Genes mediating dietary fiber metabolism

A total of 139 DEGs were found to be associated with dietary fiber metabolism. After removing the partially assembled transcripts and obvious discrepancies, 33 unigenes encoding enzymes were identified, including *CesA* (12), *CSL* (1), *IRX* (4), *ARAD* (2), *GAUT* (8), *Cx* (1), *PG* (2), and *PME* (2) as illustrated in Fig. 4. Of these 33 unigenes, 18 were significantly positively correlated with the content of total dietary fiber, cellulose, hemicellulose, and total pectin. They belonged to five different gene expression clusters (Table S2): *PME* (2-3k.c45874/2/1923) belonged to cluster 4; *CesA2* (3-6k.c17146/1/4436 and 2-3k.c13506/4/2290), *CesA3* (3-6k.c2764/2/3959), *CesA5* (1-2k.c16341/1/1287), *IRX* (1-2k.c22164/1/1427), and *GAUT*(1-2k.c54278/1/1356) to cluster 5; *CesA1* (3-6k.c21066/11/3801) to cluster 7; *Cx* (2-3k.c56230/7/2136) to cluster 11; and *CesA3* (2-3k.c53599/4/2184 and 3-6k.c6339/1/3496), *ARAD* (1-2k.c25067/1/1220 and 2-3k.c54088/9/2163), and *GAUT* (1-2k.c21559/1/1082, 2-3k.c26947/2/2489, 2-3k.c2467/1/2261, 2-3k.c50854/1/2501, and 2-3k.c28445/1/4160) to cluster 13. As shown in Fig. S1, there was a strong correlation between RNA-seq data and qPCR data for most of the genes. These results confirmed the accuracy of our transcriptome profiling. In addition, lignin biosynthesis-related genes in *R. roxburghii* fruit were identified, including *4CL*, *HCT*, *coumaroylquinate (coumaroylshikimate) 3′-monooxygenase* (*C3 'H*), *CcoAMT*, *CCR*, *CAD, COMT*, and *POD* (Lu et al. 2020).

3.5 Effect of shading on dietary fiber metabolism-related gene expression

The expression of genes encoding intermediates in dietary fiber biosynthetic pathways across fruit development are shown in Fig. 5. During the development of fruit of *R. roxburghii*, the expression levels of *CesA1*, *CesA2,* and *CesA5* showed a downward trend, while those of *Cx* and *CesA3* showed a downward trend first and then an upward trend. This indicated that *CesA1*, *CesA2*, *CesA5*, and *Cx* are strongly correlated with the rate of cellulose accumulation. Shading significantly affected the expression of cellulose-related genes during fruit development. Shading of 50% inhibited the accumulation of cellulose and up-regulated the expression of *CesA1*, *Cx*, and *CesA3* before 60 DAA. However, at the mature stage (90 DAA), cellulose accumulation was promoted, and the expression of *CesA1*, *CesA3*, *CesA5*, and *Cx* was down-regulated. Shading of 100% decreased the content of cellulose at the young fruit stage (30 DAA), and increased the expression of *CesA5*, while it significantly increased the content of cellulose at the mature stage (90 DAA), indicating that *CesA5* may play an important role in the accumulation of cellulose under shading treatment (Fig. 5).

Of the genes involved in the hemicellulose biosynthetic pathway, *ARAD1* expression first increased and then decreased, while that of *ARAD2* and *IRX* had a downward trend across fruit development, which correlated with the trend of hemicellulose content. Shading had no obvious effect on the accumulation of hemicellulose; however, the relative gene expression identified that the trend of hemicellulose content

correlated strongly with the expression of *IRX* under 50% shading, suggesting that *IRX* may influence hemicellulose accumulation (Fig. 5).

cDNA sequences of lignin synthesis pathway genes were identified from previous transcriptome data and further explored by qRT-PCR to analyze the expression level through fruit development and their response to shading. These included phenylpropanoid pathway genes（*4CL1, 4CL2*, and *4CL3*), special pathway genes（*HCT, CCR1, CCR2, CCR3, CCR4, C3'H, CAD, COMT1, COMT2*, and *CcoAOMT*), and those involved in lignin monomer polymerization (*POD1, POD2, POD3, POD4, POD5*, and *POD6*). The expression levels of *4CL1, 4CL2, 4CL3, HCT, CCR3, CCR4, C3'H, COMT1, POD1, POD2, POD3, POD4*, and *POD5* showed a downward trend throughout development, while *CCR2, COMT2*, *CcoAOMT,* and *CAD* increased in expression throughout development. *CCR1* and *POD6* expression first increased and then decreased towards the latter stages of development, which was consistent with the trend of lignin content, suggesting that *CCR1* and *POD6* may influence lignin accumulation. Shading treatment (50%) down-regulated the expression of *POD3, POD5*, and *HCT* before 60 DAA. However, at 90 DAA, the expression of *HCT, POD3*, and *CCR2* was up-regulated. This suggests that *HCT* and *POD3* expression promoted lignin accumulation. Likewise, the 100% shading treatment down-regulated the expression of *POD2, POD4, POD5, CcoAOMT, CCR1, CCR2, CCR4, HCT, COMT1*, and *4CL1* before 60 DAA. However, at 90 DAA, the expression of *CCR1, CCR3*, and *CAD* was up-regulated. This suggests that *CCR1, CCR3*, and *CAD* may regulate lignin accumulation under shading treatment (Fig. 5).

The mechanism of pectin synthesis in *R. roxburghii* fruit was similarly studied. The expression of *GAUT1*, *GAUT5*, and *GAUT2* showed a trend of first increasing and then decreasing, whereas *PME* and *GAUT4* expression declined, and *GAUT3* expression initially decreased before increasing, which was consistent with the pectin contents, suggesting that *GAUT3* may influence pectin accumulation throughout fruit development. However, the effect of shading on the expression of pectin-related genes varied across fruit developmental stages. 50% shading increased the expression of *PME*, *GAUT1*, and *GAUT5*, and down-regulated the expression of *GAUT3* and *GAUT4* at 30 DAA. However, at 60 DAA, the expression of *PME* and *GAUT4* was up-regulated, and the expression of *GAUT1*, *GAUT2*, and *GAUT5* was down-regulated. Furthermore, at 90 DAA, the expression of *PME* and *GAUT4* was up-regulated, while the expression of *GAUT3* was down-regulated, which overall suggests that *PME* and *GAUT1* may regulate pectin accumulation in fruit grown under 50% shading. On the other hand, 100% shading up-regulated the expression of only *GAUT1* and *GAUT5*, and down-regulated the expression of *GAUT2* and *GAUT4* at 30 DAA. At 60 DAA, the expression of *GAUT4* was up-regulated, while the expression of *GAUT1*, *GAUT2*, and *GAUT3* was down-regulated. At 90 DAA, the expression of *PME*, *GAUT3*, and

GAUT4 was down-regulated. Here, *GAUT3* expression positively correlated with pectin accumulation under 100% shading (Fig. 5).

3.6 Correlation analysis of dietary fiber components content and gene expression

Further correlation analysis was conducted between the dietary fiber components content and the expression levels of related genes, and indicated that there was a significant correlation between the cellulose content of fruit and *CesA1*, *CesA2*, and *CesA3* expression. Hemicellulose content was significantly correlated with *IRX* and *ARAD2* expression. The expression levels of *CAD* genes were significantly positively correlated with lignin content. The expression levels of *POD1, POD2, POD3, POD4, POD5, CcoAOMT, 4CL1, 4CL2*, and *4CL3* were significantly negatively correlated with lignin content. The relative expression level of *GAUT3* in fruit was significantly positively correlated with the total pectin and insoluble pectin content (Table 1).

4 Discussion

Cellulose, hemicellulose, and pectin are plant cell wall polysaccharides, usually studied as cellular structural substances (Guillon et al. 2017; Xu et al. 2016). They are also predominant components of fibers, but their metabolic patterns have not received much attention, especially in fruit organs. Dong et al. (2009) researched the dietary fiber metabolism in orange but focused on genes of the decomposition process. However, the accumulation of dietary fiber is the result of synthesis and decomposition, which is a dynamic process. Here, we show that from 30 to 90 DAA, the content of cellulose, hemicellulose, and total pectin decreased steadily. In addition, gene expression clusters 5, 10, 13, and 14 seemed to behave similarly. After conducting correlation analysis, 18 related genes were identified that significantly correlated with fiber content, mainly in clusters 5 and 13. Nine unigenes were present in cluster 5, 6 and 13, all of which were involved in cellulose, hemicellulose, and pectin biosynthesis, including *CesA*, *IRX*, *ARAD*, and *GAUT*. The CSL proteins are regarded as intrinsic for hemicellulose biosynthesis in Arabidopsis (Richmond & Somerville 2000, 2001), rice (Hazen et al. 2002), and other plants. However, here we identified only one differentially expressed unigene annotated as CSL. Furthermore, no significant correlation was detected between its expression abundance and the content of total dietary fiber, cellulose, hemicellulose, and total pectin, suggesting its limited role in *R. roxburghii* fruit maturation-related processes. The same findings and supposition are valid for the enzymes related to the degradation of hemicellulose. *Cx* and *PME* were placed in cluster 11 and 4, respectively, which displayed a negative correlation to the content of total dietary fiber, cellulose, hemicellulose, and total pectin, and

are responsible for cellulose and pectin degradation. These results suggest that fiber accumulation is dependent on both biosynthesis and degradation in a dynamic process.

The expression levels of *RrCesA1*, *RrCesA2*, *RrCesA3*, and *RrCesA5* were significantly correlated with the contents of cellulose, hemicellulose, and pectin in fruit of *R. roxburghii*. In Arabidopsis, *CesA1* and *CesA3* are necessary for the synthesis of primary wall cellulose, while *CesA2* and *CesA5* have some functional redundancy to *CesA6* (Song et al. 2018). In apple fruit, there are no *CesA2* or *CesA5* genes, but only 3 *CesA6* genes (Guerriero et al. 2014). Contrastingly, here we did not find any *CesA6*s in the fruit of *R. roxburghii*, but we found *CesA2* and *CesA5.* This might be because *R. roxburghii* is a high-fiber fruit compared to apple, and the high content of cellulose requires more *CesA*s to work synergistically. In this study, the cellulose content decreased through maturation, which is consistent with research on apple (Guerriero et al. 2014). Therefore, *CesA1*, *CesA2*, *CesA5*, and *Cx* may play an important role in cellulose accumulation. In Arabidopsis, soybean (*Glycine max*), and other dicotyledonous plants and citrus fruits, the expression of *CesA* positively correlated with cellulose content (Nawaz et al. 2019; Li et al. 2016). Furthermore, previous studies have shown that shading reduced the cellulose content in cotton (Chen et al. 2014), but this is inconsistent with our results. This difference may be linked to the abundance of trichomes on the surface of *R. roxburghii*, which are not present in Cotton (Wang et al. 2019). In this study, *CesA1*, *CesA2,* and *CesA3* appear to play an important role in the accumulation of cellulose under shading treatment. Research by Joshi (2003) identified all the conserved features of typical plant CesA proteins, namely a zinc-binding domain, eight transmembrane domains, two hypervariable regions, and processive glycosyltransferases' signature motif D-D-D-QXXRW. Furthermore, the first of these two hypervariable regions, HVR1, of *CesA5* was shown to have low homology with *CesA1* and *CesA*2 (Kalluri et al. 2003). Therefore, we infer that these differences may have led to the apparent responsiveness of *CesA1, CesA2,* and *CesA3* expression to shading.

Hemicellulose content decreased in fruit throughout maturation, and the expression of *IRX* and *ARAD2* strongly correlated with this change. In Arabidopsis, *IRX* is involved in the synthesis of the xylan backbone and is largely expressed during the synthesis of secondary cell walls (Ren et al. 2014). Studies in tobacco (*Nicotiana tabacum*) and other plants also show that *IRX* is involved in the synthesis of the xylan backbone (Lee et al. 2012; Pauly et al. 2010). Therefore, it can be inferred that xylan is the main component of hemicellulose in *R. roxburghii*, which is consistent with the results of studies in Arabidopsis and tobacco. The effect of shading on hemicellulose accumulation has rarely been reported. Here, shading had no obvious effect on the accumulation of hemicellulose, which is consistent with our previous study (Zhang et al. 2020). However, our expression analysis has further demonstrated that *IRX* likely plays an important role in hemicellulose accumulation under shading.

Lignin can bind with cellulose to increase the rigidity and strength of plant cell walls. Changes in its content can change the physical properties of plant tissues and affect the development of tissue structure, for instance, to confer lodging or disease resistance to crops (Ralph et al. 2004). Moreover, previous studies have shown that there is a high lignin content in *R. roxburghii* fruit, especially in the epidermal prickles, which are modified trichomes (Lu et al. 2020). Lignin mainly accumulates in the prickly skin part of the *R. roxburghii* fruit (Liu et al. 2015a). In our study, during the development of *R. roxburghii* fruit, lignin content first increased and then decreased, which is consistent with the change in leaves of tobacco, corn (*Zea mays*), and other plants during maturation (Yu et al. 2013). Correlation analysis showed that *4CL1*, *4CL2*, *HCT*, *C3'H*, *CCoAOMT*, *COMT1*, *CAD2*, and *CCR1* play important regulatory roles in lignin synthesis (Tomotaka et al. 2016), and their expression levels are closely related to the lignin content (Lu et al. 2020). POD is one of the key enzymes of enzymatic browning (Oliveira et al. 2016), which can reduce H_2O_2 in cells to H_2O and remove free radicals in fruits. Therefore, a decline in POD activity accelerates the senescence of fruits (Han et al. 2017), which is consistent with our findings. 4CL is a key enzyme in the biosynthesis of phenylpropane derivatives, such as lignin and flavonoids. Different species of plants contain varying numbers of lignin biosynthetic gene families, and there is also a degree of variation in their structure and function (Meng et al. 2017). Some studies have shown that the evolutionary relationship of 4CL is generally divided into class I and class II, involved in the synthesis of lignin and flavonoids, respectively (Yuan et al. 2014). In *japonica* rice, under shade conditions, *OsPAL*, *OsCOMT*, *OsCcoAMT*, *OsCCR*, and *OsCAD2* expression decreased significantly (Wu et al. 2017). Here, the expression of *CCR1*, *POD2*, *POD3*, *POD4*, *POD5*, *HCT*, *4CL1*, *4CL2*, and *4CL3* also decreased significantly; the possible reason may be that there is a functional element ERF related to stress or GT-1 motif, ASF-1 motif, GATA-box, or I-box elements associated with light regulation in the promoter sequence (Hu et al. 2020).

Pectin is produced in the cell wall in a highly methylesterified arrangement and *PME* genes subsequently de-esterify it (Zega et al. 2016). PMEs may also influence the extent to which demethylated polygalacturonans are available by PGs for degradation, releasing galacturonic acid or oligogalacturonate, and the availability of carboxylic groups of homogalacturonan for calcium (Ca^{2+}) binding, leading to the formation of supramolecular assemblies and gels. These gels are believed to affect the mechanical characteristics of the cell wall, increasing firmness (Wang et al. 2018). In most fruits, *PME* is expressed before ripening and has a minor role in fruit softening, but it does affect the integrity of tissues (Kalia et al. 2015). *PME* expression increases during fruit development, which is consistent with the findings in pomelo (*Citrus maxima* (Burm.) Merr) (Liu et al. 2015b). Galacturontransferase genes *GAUT1*, *GAUT2*, and *GAUT5* initially increased in expression before decreasing towards the end of fruit maturation,

indicating that these genes were highly expressed during the fruit expansion process, in which it was speculated to affect fiber length. *GAUT3* and *GAUT4* decreased in expression throughout fruit development, and these genes may play a role in the initial stage of fiber development. In melon, low light intensity decreased the pectin content (Toshiyuki et al. 2006). Wei (2015) researched shading treatment in jujube (*Ziziphus jujube*) cv. Jun zao, and found that shading increased the pectin content and improved the taste of fruit. Here, at the mature fruit stage, under 50% and 100% shading the soluble pectin content increased by 16.39% and 18.79%, respectively, while only 100% shading promoted the accumulation of total pectin and insoluble pectin. Analysis of the expression of related genes showed that *GAUT3* correlates with pectin accumulation under shading, suggesting that the *GAUT3* promoter sequence has a GT-1 motif, ASF-1 motif, GATA-box, or I-box elements associated with light regulation (Gangappa et al. 2013). In this study, the increase in soluble pectin content was much greater than that of cellulose or lignin under 50% shading. The high expression of *PME* may transform large amounts of insoluble pectin into soluble pectin, thereby improving the taste of the fruit. Although soluble pectin levels also increased under 100% shade, it was accompanied by a marked increase in the content of lignin, cellulose, and insoluble pectin, and as such, would not improve the taste of the fruit.

Light intensity significantly affects fruit color. Reduced light intensity promotes the degradation of chlorophyll, thus expediting the color transformation of fruit (Bárcena et al. 2020). Some studies, however, have shown that shading can deleteriously affect the color of the fruit by altering the ratio of carotenoids through changing gene expression (Chen et al. 2017). Here, the fruit color became paler in the earlier stages of fruit development, then developed orange and yellow hues in the middle and late stages of fruit development. The pale color early in development is likely attributed to reduced chlorophyll levels, and it is hypothesized that in the later stages, shading treatment may promote the accumulation of carotenoids and flavonoids, resulting in the orange color of the fruit. Studies have shown that under shade conditions, the low far-red ratio inhibited the synthesis of plant pigments and increased the activity of gibberellin (Gommers et al. 2013). While increased gibberellin activity may have been expected to promote heavier fruits, in our study, shade treatments reduced fruit weight, which may have been a result of reduced photosynthetic capacity. Future work to determine the content of flavonoids and carotenoids in fruit after shading treatment would further elucidate the causes of fruit color change in response to shading.

5 Conclusion

Short-read Illumina and long-read SMRT sequencing were used in conjunction to construct a transcriptome of *R. roxburghii* during fruit development. Using functional enrichment and KEGG

analysis of DEGs, we identified candidate genes involved in dietary fiber metabolism, including *CesA1*, *CesA2*, *CesA3*, *CesA5*, *IRX*, *ARAD2*, *GAUT*, *Cx*, and *PME*. To our knowledge, this is the first time that *CesA2* and *CesA5* have been identified from the edible fruit. The accumulation of cellulose, lignin, and pectin was increased by shading treatment at the maturation stage of fruit development. Analysis of the expression of related genes showed that *CesA1, CesA2*, *CesA3, IRX*, *ARAD2, GAUT3*, and *CAD* play an important role in dietary fiber metabolism with shading treatment. Light is an important environmental factor affecting the accumulation of dietary fiber in *R. roxburghii* fruit, with 50% shading offering a potential to redress the imbalance between soluble pectin and lignin and thus improve fruit quality. These results provide a basis for understanding the molecular mechanisms of dietary fiber metabolism in *R. roxburghii* fruit and provide a framework for future crop improvement.

Table S1. Primer sequences for qRT-PCR analysis

Gene ID	Gene name	Primer sequence 5'-3'	
3-6k.c21066/11/3801	*CesA1*	TTGCCTGTAATGAGTGTGCCTTCC	TTGCGGACAAGCCTGGTTGC
3-6k.c17146/1/4436	*CesA2*	TGGTGAAGCACGAAGGAGGAATTG	AATAGGCCAAGAACCGCAAGACG
2-3k.c53599/4/2184	*CesA3*	AGTGGTGTTGGAATTGACGAGTGG	CCTTGGAGGTGACAGTGAAGTTGG
1-2k.c16341/1/1287	*CesA5*	GTCTGCCTGCCATCTGTCTTCTG	TGCGATGAAGCACCTCCAATTACC
2-3k.c56230/7/2136	*Cx*	TGGTCGGTAGCAGAAGGATGAGG	TTGGTCTTACGTCTCACTGTTGGC
1-2k.c25067/1/1220	*ARAD1*	GAACACTGAGGCTTGGAAGAGGTC	TCTGACATGCCACATTGCGACTG
2-3k.c54088/9/2163	*ARAD2*	ACAAGAGGTTGCAGGACAAGTTGG	GCAAGCACGAACATGGCAGAAC
1-2k.c22164/1/1427	*IRX*	TGCTGCCTTGGTTGTGGAGATTG	CCAAGACATCACCAGCACTACCTG
2-3k.c28853/1/2146	*CCR1*	TCCGCCTCTTCCTCTTCCAGTTC	GCCTCGTTCTAAGCAGCAAGACTC
1-2k.c5667/2/1269	*CCR2*	CATCGATCAGCGACCCACAG	AACTGCCGCCATGGAAGATG
1-2k.c12160/5/1395	*CCR3*	TCTGTCGCAAGTTCAAGCTATGGC	GCACGCCGTCTTCATACATCTCC
1-2k.c54650/6/1411	*CCR4*	TGACTCGGAAGAGAAGCTCGTCTG	CGGAGGAGGAGGCGGTTGAC
3-6k.c9867/1/4805.2	*HCT*	ATCCTGATGACGCTGCTGAAGTTC	CAGAGCCGTAGCAACAGCCTTAG
1-2k.c17541/21/1413	*COMT1*	GCTGACCACTCCACCATTACCATG	GTGCCGCCTCCGACATCAAC
1-2k.c10057/3/1300	*COMT2*	GCCATAGAACTTGGTGTGCTCGAC	GCAGACAGAAGGCGAAGCATACG
1-2k.c44996/1/1611	*CAD*	TCGTTGGTGGAAGCGACATTGG	AGCCAAGACTACTGAGACGAGGAG
1-2k.c9880/6/1245	*POD1*	CTCTCCTTCGCCTTCACTTCCATG	GACCTGCTGTCTTCTCACCAGTG
1-2k.c15601/2/1502	*POD2*	GAGCAATGAGGAAGAGCCAGGTC	TGGACAGCACAGTCATGGAAGATG
1-2k.c54417/2/1276	*POD3*	TCTGCTCAGCTTAGGACGGACTTC	GAAGAGTCGAAGAGTGGCTGGAAC
1-2k.c51022/1/1295	*POD4*	TTCTTGGCGTGCTTCTACTGTTGG	GCCTGCTGCTAAGGTTGGATCAG
1-2k.c54627/3/1218	*POD5*	GCTTCATGTCCTGGTGTGGTCTC	AGGTTGGTGCTGGTAATTGTCTGG
1-2k.c32151/2/1389	*POD6*	AGCAGGTTCGGTAGTCGGATCG	TTCGGACACTGTTGTTGCAGCTC
1-2k.c25768/1/1004	*CcoAOMT*	ATCTTCGTTGACGCAGACAAGGAC	GTGCCACCACAGAGCCGTTC
1-2k.c47100/1/1669	*C3'H*	AAGTTCAGCAAGGACGGTCAAGAC	TGGCGGTGACCTCGTCTTCC
2-3k.c26005/8/1941	*4CL1*	CGAGCGTGTCTCAGCAGGTTG	CCACATAGCAAGACCGAGTTCAGC
2-3k.c2539/43/2037	*4CL2*	GCCACGTCATCCTCCTCCTCTC	CCGAGTTGTGAAGGCGAGAACG
2-3k.c12242/4/1959	*4CL3*	TATGTTCACGCTGCCGCTGTTC	CGACTTGGTCAGAGCCACAATCAG
2-3k.c45874/2/1923	*PME*	TCTCAGCCGAATCCGTAACAATGC	CAGGCGACGCTCAAGGAAGTTC
1-2k.c21559/1/1082	*GAUT1*	CATGGCTGGAGTTGGCGATGAC	AGGAGCAGGTGAACGAGACAGAG

2-3k.c26947/2/2489	*GAUT2*	TGATGGTGAGGAATGTGGTGATGC	AAGAGGAGGAGGAGGAGGAGGAG
1-2k.c54278/1/1356	*GAUT3*	CAGGTGTGATGGTGATGGACTTGG	AGCCTCTACATCTCCACCGAAGAC
2-3k.c50854/1/2501	*GAUT4*	CTGCCGCTGCCTGTTCTTCC	TCCTGGACAAGCTGGTTGAATGAC
2-3k.c28445/1/4160	*GAUT5*	TCCTGGACAAGCTGGTTGAATGAC	GGAGTGACGCATCAGTTCTCAGAG
	UBQ	ATGCAGATTTTGTGAAGAC	ACCACCACGRAGACGGAG

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855 **Table S2.** Differentially expressed genes possibly related to dietary fiber metabolism

gene ID	Annotation	correlation coefficient				cluster
		Total dietary fiber	Cellulose	hemicellulose	Total pectin	
3-6k.c21066/11/3801	*CesA1*	0.99993	0.98690	0.99960	0.96962	7
3-6k.c17146/1/4436	*CesA2*	0.98556	0.94913	0.99158	0.99606	5
2-3k.c13506/4/2290	*CesA2*	0.96868	0.92063	0.97790	0.99997	5
2-3k.c53599/4/2184	*CesA3*	0.99845	0.97890	0.99988	0.97939	13
3-6k.c2764/2/3959	*CesA3*	0.96941	0.92178	0.97851	0.99994	5
3-6k.c6339/1/3496	*CesA3*	0.93802	0.87561	0.95121	0.99550	13
1-2k.c16341/1/1287	*CesA5*	0.98114	0.94118	0.98813	0.99792	5
1-2k.c22164/1/1427	*IRX*	0.97192	0.92578	0.98061	0.99977	5
1-2k.c25067/1/1220	*ARAD*	0.93746	0.87485	0.95072	0.99534	13
2-3k.c54088/9/2163	*ARAD*	0.90395	0.82981	0.92044	0.98332	13
1-2k.c21559/1/1082	*GAUT*	0.96161	0.90974	0.97189	0.99982	13
2-3k.c26947/2/2489	*GAUT*	0.93202	0.86732	0.94586	0.99375	13
1-2k.c54278/1/1356	*GAUT*	0.92691	0.86034	0.94128	0.99211	5
2-3k.c2467/1/2261	*GAUT*	0.91507	0.84444	0.93057	0.98784	13
2-3k.c50854/1/2501	*GAUT*	0.89836	0.82254	0.91533	0.98090	13
2-3k.c28445/1/4160	*GAUT*	0.86279	0.77745	0.88245	0.96349	13
2-3k.c56230/7/2136	*Cx*	-0.99605	-0.97155	-0.99882	-0.98558	11
2-3k.c45874/2/1923	*PME*	-0.87641	-0.79451	-0.89509	-0.97051	4

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857 preparations were carried out by X.Z. X.Z., M.L., and W.M. performed the laboratory experiments and
858 analyses. Z.X. and M.L. drafted the manuscript. M.L., H.A., and R.L. revised the manuscript. All authors
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Table 1. The correlations between cellulose, hemicellulose, lignin, and pectin content and relative gene expression levels in fruit during fruit development in *R. roxburghii.*

	Cellulose	Hemicellulose	Total Pectin	insoluble pectin	Soluble pectin	Lignin
CesA1	0.602**					
CesA2	0.534**					
CesA3	0.427*					
CesA5	0.194					
C_X	-0.345					
IRX		0.758**				
ARAD1		-0.026				
ARAD2		0.641**				
PME			-0266	-0.304	-0.091	
GAUT1			0.085	0.058	0.182	
GAUT2			0.061	0.064	0.041	
GAUT3			0.379*	0.388*	0.310	
GAUT4			0.115	0.125	0.068	
GAUT5			-0.015	-0.005	-0.053	
CCR1						-0.041
CCR2						0.225
CCR3						-0.085
CCR4						-0.204
HCT						-0.152
COMT1						-0.202
COMT2						-0.186
POD1						-0.349*
POD2						-0.354*
POD3						-0.406*
POD4						-0.209*
POD5						-0.338*
POD6						0.088
CcoAOMT						-0.332*
C3'H						-0.147
4CL1						-0.415
4CL2						-0.419*
4CL3						-0.499*
CAD						0.608**

** Significant correlation at the 0.01 level (bilateral), * Significant correlation at the 0.05 level (bilateral). *CesA: cellulose synthase; Cx: hydrolase-cellulase; IRX: â-1,4-xylosyltransferase; ARAD: arabinosyltransferase; PME: pectin methylesterase; GAUT: galacturonosyltransferase; CCR: cinnamoyl-CoA reductase; HCT: O-hydroxycinnamoyltransferase; COMT: caffeic acid Omethyltransferase; POD: peroxidase; CcoAOMT: caffeoyl-CoA O-methyltransferase; C3'H: coumaroylquinate (coumaroylshikimate) 3'-monooxygenase; 4CL: 4-coumaroyl/CoAligase.*

FIG LEGENDS:

Fig 1. Effect of shading on the appearance and morphology of fruit of *R. roxburghii* at different developmental periods. White bar = 2 cm.

Fig 2. Effects of shading on the accumulation of dietary fiber components in *R. roxburghii* fruit. All experiments were conducted in triplicate. Values represent mean ± standard deviation, and the error bars are standard deviations. Different letters (a-i) in the same column indicate significant differences at $p < 0.05$ determined by ANOVA. In a, b, and c, % represents the proportion of each component content in dry weight, while in d, e, and f, % represents the proportion of each component content in fresh weight.

Fig 3. Cluster analysis of the differentially expressed genes (DEGs) in each comparison. DEGs were categorized into 18 clusters depending on their expression during fruit growth and maturation.

Fig 4. Heat map depicting the expression profile of dietary fiber metabolism-related genes in *R. roxburghii* during fruit development. The values used for heat map construction were the mean from the transcriptome data of triplicate experiments. The gene name and corresponding candidate gene are presented on the right side of the heat map. The expression level is represented by a color scale ranging from saturated green for RPKM = 0 to saturated red for RPKM = 23.67.

Fig 5. Heatmap of the 33 identified differentially expressed genes in the 50% shading (B) and 100% shading treatments (C) compared to the control (A) in *R. roxburghii* during fruit development.

Fig S1. Expression analysis of 9 differentially expressed genes related to dietary fiber metabolism in *R. roxburghii* during fruit development. *UBQ* was used as the internal control. The error bars represent the standard error of three biological replicates. The numbers above the graphics correspond to values obtained with the Pearson correlation. Pearson correlation between the RNA-seq data and qRT-PCR data was calculated using the value of FPKM and the relative expression level.