Original article

Genome-wide identification of MYB family genes potentially related to SCW biogenesis in Korla pear fruit

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Summary

The formation of fruits with a rough-skin is a physiological disorder in Korla pear (*Pyrus sinkiangensis* Yü) that is increasingly common in recent years. Compared with normal fruits, a main characteristic of such rough-skinned fruits is their greater stone cell content. These pear stone cells belong to the sclerenchyma formed by the secondary thickening of parenchyma cell walls, and MYB (myeloblastosis) family transcription factors (TFs) are known to have important functions during secondary cell wall (SCW) biosynthesis. Therefore, this work aimed to investigate MYB family genes in the pear genome and to identify SCW-related MYB family members in Korla pear fruits. A total of 193 *PbMYBs*, which were further divided into 11 clades, were identified in the pear genome. The gene phylogeny and a similarity and structure analysis showed that 33 *PbMYBs* were closely homologous to SCW-associated *MYBs* in *Arabidopsis*. Gene expression analysis revealed that 14 of them were differentially expressed during the experimental time course (prime, late, and stationary stage of stone cell differentiation), being homologs to the SCW-related genes *MYB20*, *MYB42*, *MYB52*, *MYB83*, *MYB85*, and *MYB103* in *Arabidopsis*. Expression pattern clusters indicated that for most of these *MYBs* expression levels peaked in the prime stage of stone cell differentiation, and then were gradually down-regulated in subsequent stages. Furthermore, RT-qPCR results showed that the pear homologs of *AtMYB20*, *AtMYB42*, *AtMYB52*, and *AtMYB85* – downstream TFs of the SCW biosynthesis regulatory network – were significantly upregulated in either or both the peel and pulp of rough-skinned fruits. These results suggest these SCW-associated *PbMYBs* might play fundamental roles during the stone cell differentiation process in pear fruits.

Keywords

Korla pear, rough-skinned fruit, pear genome, MYB, SCW

Introduction

Transcription factors (TFs) are proteins that bind to the specific DNA sequences and can form complexes, either alone or with other proteins, to regulate gene expression by enhancing or interfering the recruitment of specific genes to RNA polymerase (Liu et al., 1999; Riechmann and Ratcliffe, 2000). TFs are located relatively upstream in signaling pathways, and first to respond signaling substances or environ-

Significance of this study

What is already known on this subject?

- The stone cells are formed by secondary thickening of parenchyma cell walls, and MYB family transcription factors (TFs) play important functions during the secondary cell wall (SCW) biosynthesis. Previous studies on pear fruit stone cells mainly focused on transcriptional regulation of some lignin synthetic genes by *MYBs*.

What are the new findings?

- SCWs are mainly composed of cellulose, hemicellulose and lignin. Therefore, the formation of stone cells could not be fully explained merely by lignin deposition. In this work, we identified the homologs of *MYB46/83*, *MYB20*, *MYB42*, *MYB52*, *MYB85* and *MYB103* of *Arabidopsis* that regulate the SCWs synthesis. All of these *MYBs* were abundantly expressed in the critical stages of stone cell differentiation, as well as in the pericarp and pulp of rough-skinned fruits. The genes related to cellulose, hemicellulose, and lignin synthesis are the direct targets of these *MYBs*. This illuminates that the stone cell differentiation requires synergistic regulation of many genes and proteins that are involved in SCW related pathways.

What is the expected impact on horticulture?

- The skin roughening of pear fruits has been adversely affecting the fruit quality of Korla pears recently. Findings in this work may contribute to the understanding of the regulatory mechanism of this phenotype, and would help to take effective measures to reduce or eliminate this physiological disorder during pear production.

mental stimulus, and then regulate the downstream target genes (Riechmann and Ratcliffe, 2000; Zhang et al., 2018). Therefore, in plants many biological processes or cellular activities require involvement of TFs (Dubos et al., 2010; Mmadi et al., 2017).

The MYB family is one of the important TF superfamily in both animal and plant kingdom, R2R3-MYB proteins are the most predominant MYBs in plants (Martin and Paz-Ares, 1997; Dubos et al., 2010). Hitherto, the MYB family genes have been identified at genomic scale in *Citrus sinensis* (Hou et al., 2014), *Solanum lycopersicum* (Zhao et al., 2014), *Pyrus bretschneideri* (Cao et al., 2016a; Li et al., 2016), *Brassica napus* (Hajiebrahimi et al., 2017) and *Prunus persica* (Zhang et

al., 2018). Furthermore, some of the members of this family have been functionally studied in plants. Their results showed that the *MYBs* involved in regulating a wide range of biological processes, including anthocyanin biosynthesis (Lin-Wang et al., 2010; Tuan et al., 2015), the coloration of fruit and flower (Tuan et al., 2015), flavonoid and phenylpropanoid metabolism (Medina-Puche et al., 2014), sugar signaling (Chen et al., 2017), and responses to drought and waterlogging stress (Mmadi et al., 2017). One of the most important functions of *MYBs* is regulating the synthesis of SCWs. The *MYB4*, *MYB7*, *MYB26*, *MYB32*, *MYB46*, *MYB58*, *MYB63*, *MYB83* and *MYB103* in *Arabidopsis*, and their homologs in other plants have been reported to regulate SCW synthesis (Mitsuda et al., 2005; Yang et al., 2007; McCarthy et al., 2009; Zhou et al., 2009; Hussey et al., 2013; Scully et al., 2016; Yang and Wang, 2016; Guo et al., 2017). Most of these TFs are related to the transcriptional activation of genes that regulate the synthesis of secondary wall principal components: cellulose, hemicellulose and lignin (Zhong et al., 2007; Ko et al., 2009; Zhong and Ye, 2012; Kim et al., 2013). Korla pear (*Pyrus sinkiangensis* Yü) is a native cultivar of the Xinjiang Autonomous Region, China. The fruit is known for its unique aroma, juicy flesh and crisp texture. The presence of stone cells is an important characteristic of the pear fruit quality. In regular fruits, the stone cells are unperceivable. However, the high stone cell content adversely affects the external and internal quality of pear fruit. The stone cells in pear fruits belong to the sclerenchyma that are formed by the secondary thickening of parenchyma cell walls (Lu et al., 2014; Cao et al., 2016b; Wang et al., 2017), mainly composed of cellulose, hemicellulose and lignin (Brahem et al., 2017; Mamat et al., 2019). In recent years, the occurrence of rough-skinned fruits is one of the major factors that affect the local pear fruit industry, and the increased stone content is one of the typical characteristics of this phenotype. Therefore, this work attempts to identify the members of *MYB* family

that are associated with the regulation of SCW synthesis in Korla pear fruits. The findings might be helpful to find the upstream signaling molecules or environmental factors that trigger the stone cell differentiation or formation of rough-skinned fruits. Expected results also could provide the theoretical basis for taking effective measures to control the pericarp roughening during pear production.

Materials and methods

Identification of *MYB* family genes in pear and *Arabidopsis*

The genomic information of pear (*Pyrus bretschneideri*) was downloaded from the genome database of Rosaceae (<https://www.rosaceae.org/>), and genome of *Arabidopsis thaliana* was downloaded from the Arabidopsis Information Resource (TAIR) database (<http://www.arabidopsis.org/>). The *PbMYB* (*MYB* family genes in *P. bretschneideri*) and *AtMYB* genes (*MYB* family genes in *A. thaliana*) were searched against Hidden Markov Model (HMM) of *MYB* domain (PF00249) downloaded from Pfam database (<http://pfam.xfam.org/>) with the *E*-value of $1e-10$. The protein sequences identified as containing *MYB* domain were subjected to search against Pfam database and SMART tools (<http://smart.embl-heidelberg.de/>) for further confirmation of presence of *MYB* domain. The confirmed sequences were further searched against Conserved Domains Database (CDD) (<http://www.ncbi.nlm.nih.gov/cdd/>) for checking the completeness of domain structure. At last, iTAK online program (<http://itak.feilab.net/cgi-bin/itak/index.cgi>) was used for further checking whether these TFs could be classified as *MYBs*.

Homologs of SCW biogenesis related *AtMYBs* in pear

OmicBox software (<https://www.biobam.com/omicsbox/>) was used to annotate the *PbMYBs* in different

FIGURE 1. Fruits of Korla pear (*Pyrus sinkiangensis* Yü) at maturity stage. A is the normal fruits and B is the rough-skinned fruits.

biological presses, including SCW biosynthesis. MYB family genes associated with this process were gathered by searching publications that have been functionally verified in *Arabidopsis*. The sequences of *PbMYBs* and *AtMYBs* were aligned with MUSCLE program, and phylogenetic relationships were constructed by using un-rooted neighbor-joining (NJ) method of MEGA-X (Molecular Evolutionary Genetics Analysis) software. The reliability of phylogenetic tree was tested by bootstrapping method of 1,000 replicates. For identification of closely homologous sequences of SCW associated *AtMYBs* in pear, *PbMYBs* and *AtMYBs* were aligned by using BLAST2.6.0+ software with *E*-value < 1e-10.

Gene structure and motif analyses of the SCW associated MYBs

Genomic coordinates of different regions of corresponding *PbMYB* and *AtMYB* genes were uploaded on online tool: Gene Structure Display Server (GSDS) (<http://gsds.cbi.pku.edu.cn/>), and the gene structures were visualized and downloaded. Meanwhile, MEME 4.12.0 online software package (<http://meme-suite.org/>) was used for the prediction of motif structures. The employed parameters of MEME program were as follows: maximum number of motifs to "20", number of repetitions to "any", the width of each motif was between 6 to 50 amino acid residues.

FIGURE 2. Phylogenetic relationships of the MYB family genes in *P. bretschneideri* and *A. thaliana*. Full-length amino acid sequences were aligned with MUSCLE program, the phylogenetic tree was constructed using MEGA-X software package. The phylogenetic trees were derived using the neighbor-joining (NJ) method with bootstrap value of 1,000 replicates. The tree divided the *PbMYBs* into 11 subgroups represented by different colored clades (C1–C11) within the tree. The MYB family genes annotated as associated with SCW biogenesis in pear labeled with blue circles, in *A. thaliana* labeled with red circles on the gene IDs in the figure.

Expression pattern analysis

The fruit samples were collected from 20-year-old Korla pear trees (*P. sinkiangensis* Yü) grown in an orchard in Korla (Xinjiang, China) at three developmental stages (20, 50 and 80 days after flowering, ADF). They are representing prime (named as Day20 samples), late (Day50) and stationary stage (Day80) of stone cell differentiation in pear fruits, respectively (Mamat et al., 2020). Each sampling stage contains three biological replicates. Each biological replicate was the mixed sample of ten fruits collected from three pear trees. The fruit skins were peeled and pulps were sampled, then flash frozen in liquid nitrogen, ground into powder, and stored at -80°C prior to transcriptome sequencing (Novogene Bioinformatics Technology Co. Ltd., Beijing, China).

The sequenced data (raw reads) was in fastq format. After quality control, the clean reads were aligned back to the pear reference genome with Hisat2 v.2.0.5. The read counts of genes in each sample were estimated, the expression abundance of genes were normalized and calculated by uniquely mapped reads with FPKM (fragments per kilobase of exon per million fragments mapped) method. Heatmap.2 function in Gplots package was used to analyze the gene expression pattern in fruit samples from different developmental stages.

Quantification of selected MYBs in rough-skinned fruits

The rough-skin is phenotypically invisible in the early stage of fruit development, and appears gradually in the later stage of fruit development from late July to mid-August (Figure 1). Hence, the skin and pulp of normal and rough-skinned fruits at 120 DAF (mature stage) were collected. Each group contains three biological replicates. Each biological replicate was the mixed sample of ten fruits collected from three pear trees. The fruit skin and pulp were sampled separately. Fruit skin were evenly peeled with a peeler, while cylindrical samples were obtained from the pulp 15 mm in diameter, then frozen in then liquid nitrogen, ground into mixed powder and stored at -80°C until further usage.

14 MYBs, that were homologous to SCW associated MYBs in *Arabidopsis*, were selected for PCR quantification of their expressions in rough-skinned fruits from Korla pear. The primers were designed with Oligo 7.0 software (Supplemental Table S1). The specificity of primers was tested by running the amplification products on agarose gel. 1 µg of total RNA was used to synthesize the cDNA by using reverse transcriptase RR037A (Takara, U.S.A.). The Real-Time PCR was carried out in 20 µL system that contains 1 µL of forward and reverse primers of each, SYBR Green Real-Time PCR Master Mixes (Invitrogen, U.S.A.) and 1 µL of cDNA templates on STEP ONE PLUS™ Real-Time PCR System (Applied Biosystems, U.S.A.). The PCR cycling conditions were: 95°C for 15 min followed by 40 cycles of 95°C for 10 s, 58°C for 20 s, and 72°C for 20 s. Each sample had three replicates and the expression level of each target gene was normalized to internal reference gene of *Tubulin*. Relative expression levels were calculated by the method of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Results

MYB family genes in pear and *Arabidopsis*

All protein sequences encoded from pear and *Arabidopsis* genome were searched against MYB family domain HMM model, and found that 424 transcripts (encoded by 333 genes) and 435 transcripts (encoded by 243 genes) contain MYB protein domain, respectively. Based on *E*-value, the transcripts that were aligned to the domain model with

the lowest value among those transcripts that transcribed by the same gene, was chosen as a representative MYB encoded by that particular gene. The selected transcripts were also searched against SAMRT and Pfam database for further confirming the presence of the domain in these sequences. Furthermore, the confirmed transcripts were aligned to CDD database and found 193 transcripts in pear and 142 transcripts in *Arabidopsis* that contain MYB complete domain structure (Supplemental Tables S2 and S3).

MYB family genes related to SCW biogenesis in pear fruit

By annotating *PbMYB* genes against GO database at level eight, eight genes (namely *LOC103945877*, *LOC103935652*, *LOC103963897*, *LOC103959908*, *LOC103959431*, *LOC103954864*, *LOC103965544* and *LOC103935651*) were enriched into the biological process of SCW biogenesis. In *Arabidopsis*, three master switches (*MYB26*, *MYB46* and *MYB83*) and 13 downstream TFs (*MYB4*, *MYB7*, *MYB20*, *MYB32*, *MYB42*, *MYB43*, *MYB52*, *MYB54*, *MYB58*, *MYB63*, *MYB69*, *MYB85* and *MYB103*) were reported to be associated with this process (Mitsuda et al., 2005; Yang et al., 2007; McCarthy et al., 2009; Zhou et al., 2009; Hussey et al., 2013; Scully et al., 2016; Guo et al., 2017). By multiple sequence alignment and phylogenetic tree construction, the homologous relation between the sequences of *MYB* genes of pear and *Arabidopsis* was primarily established (Figure 2). The SCW associated *MYBs* in *Arabidopsis* and their homolog genes in pear were clustered into the clade (C) of C3, C7, C8 and C10. The homolog sequences of SCW associated *AtMYBs* in pear genome were listed in Table 1. Among them, five genes were homologous to *Arabidopsis* SCW regulatory master switch *MYB26*, and six of them were homologous to *MYB83*, which has high sequence similarity to master switch *MYB46*. Besides, 22 *PbMYBs* were homologues to *Arabidopsis* SCW biogenesis regulatory network downstream TFs, includes *AtMYB4* (three *PbMYBs* homologous to *AtMYB4*), *AtMYB22* (two *PbMYBs*), *AtMYB42* (one *PbMYB*), *AtMYB43* (two *PbMYBs*), *AtMYB52* (four *PbMYBs*), *AtMYB58* (one *PbMYB*), *AtMYB63* (one *PbMYB*), *AtMYB85* (four *PbMYBs*) and *AtMYB103* (four *PbMYBs*), respectively.

Gene structure and motif distribution of SCW biogenesis related MYB homologs

All sequences assigned to the clade C3, C7, C8 and C10 from the phylogenetic tree (Figure 2) were extracted, as they are including all the SCW related genes in pear and *Arabidopsis*. And their genetic relation, gene structure and sequence motif distribution were further visualized (Figure 3). By multiple sequence alignment and reconstructing the phylogenetic relation, these genes were divided into eight subgroups. In subgroup I, the pear gene *LOC103959431*, *LOC103959908*, *LOC103945688* and *LOC103945691* were closely located to the gene *MYB43* and *MYB20* of *Arabidopsis*. The blast result showed that the gene *LOC103959431* and *LOC103959908* have the highest sequence similarity to *MYB20* (Table 1). Pear gene *LOC103963418*, *LOC103959844* and *LOC103961970* were clustered together with *MYB42*. Meanwhile, gene *LOC103952062* and *LOC103933770* were clustered with and blasted with the highest identity to *MYB58* and *MYB63* of *Arabidopsis*, respectively. In subgroup III, the pear gene *LOC103965608* and *LOC103965615* clustered with the SCW associated gene *MYB4*, *MYB7* and *MYB32* in *Arabidopsis*. The blast result indicated that they were highly identical to the sequence of *MYB4* of *Arabidopsis*. In

subgroup V, pear gene *LOC103936957* and *LOC103953387* clustered together and have the highest identity to *MYB103* in *Arabidopsis*. All three SCW master switches of *Arabidopsis* and their homologs in pear were clustered into subgroup VI, gene *LOC103935652*, *LOC103963897*, *LOC103935651* and *LOC103965544* clustered with master switch *MYB46*, and gene *LOC103954864* and *LOC103945877* clustered with and have the highest identity to *AtMYB83*. Pear gene *LOC103932780*, *LOC103953418*, *LOC103953419* and *LOC103946534* were clustered with the master switch *AtMYB26*, among them, last three genes blasted with the highest similarity. Last but not least, the pear gene *LOC103933820*, *LOC103936882*, *LOC103957898* and *LOC103951125* were clustered together with *Arabidopsis* SCW biogenesis network downstream regulator *MYB52*, *MYB54* and *MYB69* in subgroup VIII. It is interesting to note that all those aforementioned homologs tend to have similar gene structures and motifs distributions except for *LOC103932780*, which has the rather low sequence identity to *MYB26*, with the short of motif 4 in its amino acid sequence compared with the neighbor sequences in the phylogenetic tree.

The expression clustering of MYBs in pear fruits

After the quality control of sequenced data, the clean reads were mapped back to the reference genome for gene expression analysis. The result showed that, among the 193 MYB members identified in pear genome in this study, 142 of them had expression at different developmental stages of Korla pear fruits. Genes with similar patterns of mRNA expression frequently have similar functions (Eisen et al., 1998; Brown et al., 2000; Wu et al., 2002). Thus, we have used Heatmap.2 function of Gplots package, clustered the expression value (FPKM) of these genes. When the dendrogram was cut with the height of 1.5 (Figure 4A), the gene expressions clustered into 10 different patterns among these genes during the fruit development (Figure 4B). In cluster I, thirteen genes showed relatively higher expressions in the samples from group Day20 and Day50 compared with group Day80. In cluster III, there were 21 genes that had higher expression in the fruits from 50 DAF in contrast with the samples of group Day20 and Day80. In cluster V, ten genes were highly expressed in Day80. In cluster VII, 43 MYB members were clustered together as they were highly expressed in Day20

TABLE 1. Homologous sequences of SCW related *AtMYBs* in pear.

No.	<i>P. bretschneideri</i> gene ID	<i>A. thaliana</i>		Identity	E-value	Score
		Gene name	Gene ID			
1	LOC103953418	MYB26	AT3G13890	88.19%	1.59E-77	236
2	LOC103953419	MYB26	AT3G13890	88.19%	1.59E-77	236
3	LOC103946534	MYB26	AT3G13890	88.19%	1.71E-77	236
4	LOC103953353	MYB26	AT3G13890	75.59%	9.86E-67	207
5	LOC103958062	MYB26	AT3G13890	56.06%	1.04E-66	207
6	LOC103954864	MYB83	AT3G08500	47.49%	3.66E-83	249
7	LOC103945877	MYB83	AT3G08500	46.07%	1.53E-82	247
8	LOC103935651	MYB83	AT3G08500	43.57%	1.57E-76	232
9	LOC103935652	MYB83	AT3G08500	43.57%	1.57E-76	232
10	LOC103963897	MYB83	AT3G08500	43.31%	1.87E-75	229
11	LOC103965544	MYB83	AT3G08500	42.33%	2.75E-74	226
12	LOC103965608	MYB4	AT4G38620	62.63%	2.4E-117	330
13	LOC103965615	MYB4	AT4G38620	62.63%	2.4E-117	330
14	LOC103952575	MYB4	AT4G38620	61.06%	2.31E-91	266
15	LOC103959908	MYB20	AT1G66230	61.91%	2.3E-101	291
16	LOC103959431	MYB20	AT1G66230	58.55%	4.23E-97	280
17	LOC103963418	MYB42	AT4G12350	53.07%	7.49E-89	258
18	LOC103960944	MYB43	AT5G16600	61.41%	1.55E-81	242
19	LOC103959683	MYB43	AT5G16600	60.87%	7.74E-81	240
20	LOC103946882	MYB52	AT1G17950	55.36%	2.68E-90	261
21	LOC103933820	MYB52	AT1G17950	52.02%	4.38E-81	238
22	LOC103957898	MYB52	AT1G17950	83.64%	1.01E-67	207
23	LOC103951125	MYB52	AT1G17950	82.73%	2.31E-67	206
24	LOC103952062	MYB58	AT1G16490	44.88%	1.34E-78	236
25	LOC103933770	MYB63	AT1G79180	57.52%	1.33E-59	189
26	LOC103945688	MYB85	AT4G22680	55.99%	2.6E-102	293
27	LOC103945691	MYB85	AT4G22680	55.99%	2.6E-102	293
28	LOC103959844	MYB85	AT4G22680	51.70%	1.26E-89	260
29	LOC103961970	MYB85	AT4G22680	52.20%	1.43E-89	260
30	LOC103936957	MYB103	AT1G63910	51.77%	2.6E-106	309
31	LOC103953387	MYB103	AT1G63910	50.81%	4E-104	304
32	LOC103953426	MYB103	AT1G63910	84.03%	5.74E-76	231
33	LOC103936966	MYB103	AT1G63910	83.19%	7.61E-73	229

compared with samples from two later stages in question. In cluster VIII, eighteen genes also showed higher expression in Day20, but some of them have little or no expression in some replicated of group Day20. The rest of the MYB members clustered to cluster II, IV, VI, IX and X showed no constant expression in all biological replicates of fruit samples at any developmental stages.

Identification of SCW biogenesis related *PbMYBs* in pear

We have selected MYB family members from the expression pattern cluster I, VII and VIII, as they were highly expressed in pear fruits 20 DAF and/or 50 DAF compared with

the samples that were collected at stone cell differentiation stationary period. The differential expression of these genes was analyzed (Table 2). The results showed that six of them were significantly upregulated (P_{adj} -value <0.05) in group Day20 and Day50 samples in cluster I. In cluster VII, 26 pear *MYBs* were differentially upregulated (P_{adj} -value <0.05) in Day20 samples and most of them also showed upregulation (P_{adj} -value <0.05) in Day50 samples. It is interesting to note that 13 of these genes were highly homologous to those of SCW master switches, such as *MYB83*, and regulatory network downstream transcription factor *MYB20*, *MYB42*, *MYB52*, *MYB85* and *MYB103* in *Arabidopsis*. In cluster VIII,

FIGURE 3. Phylogenetic relationships, gene structures, and architecture of conserved protein motifs in 99 *MYB* genes in *P. bretschneideri* and *A. thaliana*. A is the phylogenetic tree that constructed by MEGA-X using the NJ method. Eight subgroups on the phylogenetic tree were shown by different background colors. B is the structures of *MYB* genes, green boxes indicate UTR regions, yellow boxes indicate exons and black lines indicate introns. C is the motifs distribution of *MYB* proteins. Different motifs were indicated by different colors for motif 1 to 20. The sequence information for each motif is provided in Supplemental Table S4.

pear gene *LOC103945688* was upregulated (P_{adj} -value < 0.05) in the fruits from Day20 and Day50 samples, which is a homolog of *MYB85* from *Arabidopsis*.

Quantification of SCW related MYBs in the rough-skinned fruits

The fourteen homologs of SCW associated *AtMYBs* in pear, which were differentially upregulated at the key stages of stone cell differentiation in pear fruits, were chosen for the quantification of their expression in rough-skinned fruits from ripening stage. During the experiment, the expression levels of gene *LOC103936957*, *LOC103953387*,

LOC103945877 and *LOC103963897* could not be quantified, this may be because they had a very low expression or not expressed at the mature stage in pear fruits. Among the rest of 10 *PbMYBs*, seven genes were highly expressed in either or both the peel and pulp of rough-skinned fruits (Figure 5). Pear gene *LOC103963418* and *LOC103945688* were upregulated more than 2.58 folds in both the peel and pulp of rough-skinned fruits compared with normal fruits, and they are homologous to *MYB42* and *MYB85* of *Arabidopsis*, respectively. Gene *LOC103959908* and *LOC103959431* were upregulated in the peel of rough-skinned fruits around 2.26 folds compared with normal fruits, which are the homologs

FIGURE 4. Expression clustering of MYB family genes in transcriptomes of Korla pear fruits at different developmental stages. A is the dendrogram of FPKM value clusters of MYB family genes; the vertical axis represents the height of the dendrogram, the horizontal axis represents the genes in different clusters. B is the gene expression heat map; vertical axis represents 10 branches after cutting the dendrogram, the horizontal axis represents different samples that collected from different fruit developmental stages; red and green colors in the heat map represent the high and low levels of expression of *PbMYBs*, respectively.

FIGURE 5. Quantification of SCW-related *PbMYBs* expression in rough-skinned fruits. Blue column represents the gene expression level in the fruit skin and pink column represents the gene expression level in the fruit pulp. The bar height represents the mean expression values of three replicates $\pm$ standard deviation (SD).

TABLE 2. Potential SCW biogenesis related MYB family genes in pear.

Gene ID	Day20 vs. Day80		Day50 vs. Day80		Cluster
	LogFC	P_{adj} -value	LogFC	P_{adj} -value	
LOC103952719	5.71	1.1E-78	5.40	1.6E-85	I
LOC103942517	2.23	9.3E-07	1.90	3.9E-04	I
LOC103945316	0.91	6.8E-06	1.27	1.9E-11	I
LOC103950641	1.50	3.4E-04	1.83	4.5E-05	I
LOC103935943	1.62	8.5E-04	2.43	1.6E-09	I
LOC103947724	0.91	2.7E-02	1.52	2.2E-06	I
LOC103936957●	29.78	1.6E-86	24.02	3.3E-02	VII
LOC103953387●	29.48	7.2E-75	24.13	5.4E-02	VII
LOC103963897●	29.01	3.1E-63	24.80	5.7E-04	VII
LOC103959908●	4.97	3.6E-54	2.75	4.0E-19	VII
LOC103959844●	7.42	1.8E-51	3.47	5.6E-06	VII
LOC103961970●	6.90	5.7E-33	3.19	2.6E-05	VII
LOC103946882●	8.83	1.2E-31	4.85	7.4E-02	VII
LOC103954864●	7.84	1.4E-30	3.23	4.1E-01	VII
LOC103940825	2.91	1.2E-26	1.61	1.0E-05	VII
LOC103945877●	6.38	2.3E-25	1.00	9.5E-01	VII
LOC103963418●	4.29	5.3E-25	2.86	2.1E-16	VII
LOC103957479	2.98	7.7E-23	0.63	3.7E-01	VII
LOC103937520	5.44	8.5E-22	3.62	5.1E-06	VII
LOC103933079	2.01	9.4E-22	0.58	7.2E-02	VII
LOC103957898●	6.13	3.2E-20	2.50	1.8E-01	VII
LOC103933794	1.58	1.5E-15	0.63	1.6E-02	VII
LOC103960282	3.30	5.4E-15	1.82	6.4E-04	VII
LOC103967156	3.65	1.8E-14	2.19	4.5E-03	VII
LOC103952966	7.51	3.8E-13	2.88	8.3E-02	VII
LOC103959431●	6.48	5.4E-12	4.49	1.8E-02	VII
LOC103928619	4.19	6.3E-11	2.45	1.4E-01	VII
LOC103938545	1.22	2.2E-09	-0.14	5.1E-01	VII
LOC103927194	3.61	2.2E-07	2.51	2.5E-02	VII
LOC103958466	5.43	2.4E-06	2.93	2.2E-01	VII
LOC103950808	1.67	4.6E-04	0.19	1.0E+00	VII
LOC103933820●	3.67	1.3E-03	-2.59	8.3E-01	VII
LOC103945688●	27.86	1.7E-12	24.27	6.3E-02	VII

● indicates the *PbMYBs* that are highly homologous with SCW associated *MYBs* in *Arabidopsis*.

of *AtMYB20*. The gene *LOC103959844*, *LOC103961970* and *LOC103933820* were upregulated more than 2.57 folds in the pulp of rough-skinned fruits. Among them, the former two genes are the homologs of *MYB85* and the last one is homologous to *MYB52* of *Arabidopsis*.

Discussion

Currently, formation of rough-skinned fruits is one of the major factors that adversely affects the fruit quality in Korla pear (*Pyrus sinkiangensis* Yü). Increased stone cell content is one of the typical characteristics of rough-skinned fruits. Stone cells are formed by secondary thickening of parenchyma cell walls, which consist of a large amount of lignin, cellulose and hemicellulose (Nie et al., 2009; Brahem et al., 2017; Mamat et al., 2019). Being a type of sclerenchyma, as fibers, stone cell differentiation must be carried out by cell autonomous processes (Smith et al., 2013). Being the most prevalent TFs in plants, the MYBs regulate the coordinated activation of secondary wall biosynthetic genes (Mitsuda et al., 2005; Zhong et al., 2010; Yang and Wang, 2016). In this work, we identified 14 DEGs encoding different MYB mem-

bers in pear that are highly homologous to a battery of SCW associated MYBs in *Arabidopsis*, including *MYB46/83*, *MYB20*, *MYB42*, *MYB52*, *MYB85* and *MYB103*, all of which specifically expressed in secondary wall-forming cells (Zhong et al., 2008; McCarthy et al., 2009). Among these DEGs, the gene *LOC103954864*, *LOC103945877* and *LOC103963897* were homologs of *AtMYB46/AtMYB83*, as the second-level master switches, regulates a series of downstream MYBs and some direct target genes that involve in the biosynthesis of secondary cell walls (Zhong et al., 2007; McCarthy et al., 2009). All of these three genes were abundantly expressed at the prime stage of stone cell differentiation, with higher fold changes in expression between prime and stationary stages of stone cell differentiation, indicating that these two TFs may play critical roles in the process of secondary thickening of cell walls in pear stone cells. Supporting this, both *AtMYB46* and *AtMYB83* genes and their close homologs in other plants have been reported to express in secondary wall-forming cells, induce ectopic secondary wall deposition when over-expressed, and mutation or repression of these genes causes a defective secondary wall phenotype (Zhong et al., 2008;

McCarthy et al., 2009; Zhou et al., 2009; Zhong et al., 2011; Zhong and Ye, 2015). In addition, secondary wall-associated cellulose synthases genes (*CESA4*, *CESA7* and *CESA8*), xylan biosynthesis genes (*IRX8*, *IRX9*, *IRX14*, and *IRX15-L*) and some lignin synthetic genes have also been reported to be upregulated by MYB46 through directly binding the promoter region of these genes (Kim et al., 2012, 2013, 2014; Zhong and Ye, 2012). All these genes showed upregulation during the prime stage of stone cell differentiation, and the stone cell components (cellulose, hemicellulose and lignin) also peaked during this period in pear fruits (Mamat et al., 2020), suggesting that pear homologs of *AtMYB46* and *AtMYB83* might play important regulatory functions during stone cell differentiation in Korla pear fruits.

After being induced, *AtMYB83* and *AtMYB46* trigger the expression of downstream *MYBs* (Zhong et al., 2007; McCarthy et al., 2009). In this work, we found 11 DEGs that are homologous to *MYB20*, *MYB42*, *MYB52*, *MYB85* and *MYB103* in *A. thaliana*. All of these genes showed peak expression at the prime stage of stone cell differentiation, then decreased rapidly in later stages. It was consistent with the tendency of changes in stone cells and lignin in Korla pear fruits (Mamat et al., 2019, 2020). *MYB85* is recognized as lignin-specific transcriptional activator, which positively regulates various lignin synthesis genes (Zhong et al., 2007, 2008; McCarthy et al., 2009; Zhou et al., 2009). Dominant repression or overexpression of *MYB85* results in a specific repression or activation of lignin biosynthesis and concomitant ectopic deposition in both vessel and fiber cells (Zhong et al., 2008; Zhou et al., 2009). It is reported that *MYB20* and *MYB42* regulate lignin biosynthesis by direct activation of phenylalanine and lignin synthetic genes. Similar to *MYB85*, disruption of these genes caused lignin defect phenotype in *Arabidopsis* (Zhong et al., 2008; Zhou et al., 2009; Geng et al., 2020). Accordingly, synchronizing with the lignin accumulation period (Mamat et al., 2019), all homologs of *AtMYB85*, *AtMYB20* and *AtMYB42* in pear showed peak expression in the prime stage of stone cell differentiation, and reached the lowest level in the stationary stage in pear fruits, suggesting that the homologs of *MYB20* and *MYB42* together with *MYB85* may play crucial regulatory functions during lignin accumulation in Korla pear fruits.

MYB52 and *MYB103* are also reported to be the secondary wall-associated *MYBs*. In our transcriptome data, three homologs of the *AtMYB103* were differentially expressed during the different stages of pear fruit development, with the highest expression shown in prime stage of stone cell differentiation. In *Arabidopsis*, the overexpression of *MYB103* results in increased, but otherwise (repression or mutation) loss of SCW in fibers (Zhong et al., 2008). Similar phenotype was observed in plants with dominant repression of *MYB52*, but the overexpression of *MYB52* was unable to induce the ectopic secondary wall deposition, just slightly induce *CESA8*, *IRX9* and *4CL1* (Zhong et al., 2008; Hao and Mohnen, 2014). It is also reported that *MYB103* regulates the expression of *CesA8*, indicating that transcription factor *MYB103* may affect the SCW thickening of cell walls by the regulation of cellulose biosynthesis.

Stone cells are commonly present in all pear varieties, and appear at cell division period during normal fruit development. At mature stage, they are nearly invisible in fruits of all cultivated pear varieties under normal conditions (Nie et al., 2009; Jin et al., 2013; Yan et al., 2014; Mamat et al., 2019). As for the rough-skinned fruits, also known as hard-end fruits in 'Whangkeumbae' pear (*Pyrus pyrifolia*), it is a physi-

ological disorder that has become increasingly common in recent years, resulting in considerable economic loss to the pear growers. And the increased stone cell content is a main cause of hard-end disorder in pear fruits (Lu et al., 2014; Li et al., 2017, 2019). All the SCW associated *MYBs* that differentially expressed in this study were abundantly expressed at the prime stage of stone cell differentiation, then expression levels started to decrease gradually. This trend is similar with the changing trends of cellulose, hemicellulose and lignin content during the fruit development. Further, the homologs of *AtMYB20*, *AtMYB42*, *AtMYB52* and *AtMYB85* were upregulated in either or both the skin and pulp of rough-skinned fruits. These *MYBs* are the downstream TFs that regulate the biosynthesis of SCW components in *Arabidopsis*. Hence, the upregulation of these *PbMYBs* in rough-skinned fruits indicate that they may play crucial roles in the process of stone cell differentiation or fruit skin roughening on Korla pear fruits.

Conclusions

In this work, 193 MYB family genes were identified in pear genome. Among them, thirty-three were closely homologous to SCW associated *MYBs* in *Arabidopsis*. During the stone cell differentiation in pear fruits, fourteen *PbMYBs* which are homologs of SCW related *AtMYBs* (namely *MYB20*, *MYB42*, *MYB52*, *MYB83*, *MYB85* and *MYB103*), showed peak expression in the prime stage of stone cell differentiation, and down regulated gradually in later stages. Furthermore, pear homologs of *AtMYB20*, *AtMYB42*, *AtMYB52* and *AtMYB85* were significantly upregulated in either or both of the skin and pulp of rough-skinned fruits from ripening stage. These results indicated that these SCW associated *PbMYBs* play fundamental roles during the stone cell differentiation and rough-skin formation in pear fruits.

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Data availability statement

The transcriptome sequencing (RNA-seq) data supporting the results of this work can be accessed in the Short Read Archive (SRA) of National Center for Biotechnology Information (NCBI) with the accession number SRX7122321 – SRX7122329.

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