

Genome-wide Association Analysis of Red Flesh Character Based on Resequencing Approach in Peach

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ABSTRACT. Anthocyanins are important molecules that are responsible for fruit color formation and are also beneficial to human health. To date, numerous structural and regulatory genes associated with anthocyanin biosynthesis in peach (*Prunus persica*) have been reported based on linkage analysis. In this study, we sought to identify further genes associated with anthocyanin content in peach by conducting a genome-wide association analysis of 129 peach accessions to detect markers associated with the trait. Significant association signals were detected when anthocyanin content was considered a qualitative character but not when it was considered a quantitative trait. We detected an association region located between 11.7 and 13.1 Mb in chromosome 1, a region in which only 133 of 146 genes have previously been functionally annotated. Gene ontology annotation of the genes in this region showed that membrane-associated genes (including one gene encoding a chloride channel protein and 17 sugar transport/carrier-associated genes) were significantly enriched, and we focused on these in subsequent analyses. Based on *in vitro* induction of anthocyanins in fruit flesh using different exogenously applied sugars and subsequent culture, we found that the expression level of 3 of the 18 membrane-associated genes, *Prupe.1G156300*, *Prupe.1G156900*, and *Prupe.1G157000*, increased during induction treatment. Furthermore, during the fruit development period of a white-fleshed and a red-fleshed peach cultivar, the expression of one gene encoding a transmembrane sugar transport protein was observed to be positively correlated with anthocyanin biosynthesis. These results will facilitate understanding of the molecular mechanism of anthocyanin biosynthesis in peach.

Anthocyanins are important pigments in plants. These compounds are found in stems, leaves, petals, and flesh of fruit (Forkmann and Martens, 2001; Gould et al., 2009), and play important roles in attracting insect pollinators and animal seed dispersers, and in disease and insect resistance, like fungi, herbivores, cold, and excess radiation (Chalker, 2002, 2010; Karageorgou and Manetas, 2006). For example, anthocyanins can absorb visible light without participating in photosynthesis; their presence in leaves should reduce the probability of photon capture by chlorophylls, thereby lowering photosynthesis (Karageorgou and Manetas, 2006). Anthocyanins also are beneficial to human health, and it has been found that foods with high anthocyanin content reduce free radicals, promote blood circulation, prevent cardiovascular and cerebrovascular diseases, and also have antiaging and anticancer properties (Chen et al., 2006; Choi et al., 1997; Xia et al., 2010). Yang et al. (2018) found that anthocyanins from purple wheat

(*Triticum aestivum*) can inhibit the infection capacity and proliferation of hgc-27 and mgc-803 gastric cancer cells. Lila et al. (2016) argued that anthocyanins undergo enterohepatic recycling in the human body, leading to prolonged residence time, and thus enhanced bioavailability; however, levels were found to be very low in routine blood samples taken after ingestion. Single oral administration of anthocyanin has been demonstrated to reduce smoking-induced endothelial dysfunction in young healthy smokers (Akane et al., 2018), whereas cyanidin-3-O-glucoside also can alleviate the Pb-induced decrease in progesterone biosynthesis to reduce the toxicity of heavy metals in rat Leydig cells, and anthocyanin is able to chelate heavy metals and reduce metal ion concentrations, thereby further alleviating heavy metal-induced toxicity (Wen et al., 2018; Zhou et al., 2017).

Peach, a member of the Rosaceae family native to China, is among the four most widely cultivated fruit tree species worldwide (Tuan et al., 2015), and serves as a model for genetic studies owing to its small genome size of 265 Mb (Dirlewanger et al., 2004; Shulaev et al., 2008; Zhu et al., 2012), and the whole-genome sequence of peach has been released (Verde et al., 2013), providing an important foundation for the genome-wide identification of genes in this species (Verde et al., 2017). Typically, peach has one of two types of flesh color: white or yellow. After studying the anthocyanin pigments in peaches, research found that there were two main pigments in peach, cyanidin-3-glucoside and cyanidin-3-rutinoside, with the former content being ≈ 10 times more than the latter. In addition, no clear differences were observed

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The authors declare that they have no competing interests. The RNA-sequencing data of two cultivars (Tianjin Shui Mi and Hakuho peach) have been deposited into the Figshare database (<https://doi.org/10.6084/m9.figshare.5576368.v1>).

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in pigment contents of white and yellow flesh accession (Tomas-Barberan et al., 2001). In recent decades, peaches with red flesh have attracted the attention of a large number of researchers worldwide (Ding, 2017).

To date, candidate genes involved in biosynthesis, regulation, and response to induction by environmental stimuli, such as light, and temperature, and sugar and plant hormones, have been identified in peach (Donoso et al., 2016; Eduardo et al., 2011; Frett et al., 2014; Jaakola, 2013; Jiao et al., 2014; Rahim et al., 2014; Zhou et al., 2017). Among the biosynthetic genes identified, there are a number of structural genes that directly encode enzymes required for the biosynthesis of anthocyanin, including chalcone synthase (CHS), chalcone isomerase, leucoanthocyanidin dioxygenase/anthocyanidin synthase, and UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT) (Tsukaya, 2004). It is currently believed that the process of anthocyanin biosynthesis is mainly regulated by three classes of transcription factors, namely, *v-myb* avian myeloblastosis viral oncogene homolog (MYB), basic helix-loop-helix (bHLH), and tryptophan-aspartic acid 40 (WD40) transcription factors (Xu et al., 2015). Among these, MYB transcription factor genes often are found to be the major determinant of anthocyanin accumulation by acting in conjunction with bHLH and WD40 proteins to activate key anthocyanin biosynthetic genes (Allan et al., 2008; Lin-Wang et al., 2010; Petroni and Tonelli, 2011). The major transcription factor R2R3 MYB10/MYB1/MYBA was shown to be associated with activation of the anthocyanin biosynthesis pathway in the Rosaceae (Allan et al., 2008; Lin-Wang et al., 2010), leading to the development of red flesh pigmentation, and is located within the interval of the major markers associated with blush on linkage group 3 (Frett et al., 2014). However, Zhou et al. (2015) found that the sequence of *PpMYB10.1* did not segregate along with the red flesh phenotype, although their team identified another gene, a NAC transcription factor gene (*PpBL*), that was significantly differentially expressed between the red- and white-fleshed cultivars and confirmed that this gene could affect anthocyanin biosynthesis by regulating *PpMYB10.1* using transient transformation of tobacco (*Nicotiana benthamiana*) and peach (Zhou et al., 2015). Recently, Cao et al. (2018) identified 66 genes were significantly correlated with anthocyanin contents through RNA-sequencing (RNA-seq), 22 of which previously reported as regulatory, biosynthetic, and transporter genes, including *PpMYB* and *PpBL*, are involved in the regulatory network of anthocyanins. Unfortunately, no polymorphic sites have been identified in the coding sequence of the genes in red-fleshed peach. More research is needed to locate key genes involved in red flesh traits in peach.

Genome-wide association study (GWAS) has proved to be a useful tool for dissecting the genetic locus of complex agronomic characters in arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), maize (*Zea mays*), wheat, and barley (*Hordeum vulgare*) (Gyawali et al., 2017; Kim et al., 2016; Revilla et al., 2016; Togninalli et al., 2018; Turuspekov et al., 2017). It has the advantage of not requiring prior establishment of a mapping population and can detect multiple allele loci, thereby saving both time and labor. In peach, Cao et al. (2012) demonstrated GWAS analysis to be an effective complement to traditional quantitative trait locus mapping.

In this study, we performed a GWAS with the aim of locating the region associated with anthocyanin content in peach flesh. Candidate genes were identified based on an in

vitro experiment involving sugar induction of fruit flesh and comparing these genes at different stages of fruit development. The findings of this study provide a more comprehensive understanding of the molecular mechanisms underlying anthocyanin biosynthesis and could be useful in development and application of molecular markers for peach breeding.

Materials and Methods

PLANT MATERIALS. Three sources of plant materials were used in this study, the first of which was 129 peach accessions previously used in GWASs (Cao et al., 2016; Supplemental Table 1). For each of these cultivars, fresh samples of the mesocarp of three ripe fruit were mixed and frozen in liquid nitrogen and then stored at -80°C for anthocyanin evaluation. In addition, total DNA was extracted from the fresh leaves of these accessions for genome re-sequencing.

The second source of material was the flesh of ‘Tianjin Shui Mi’ peach fruit harvested at 15 d before fruit maturity (the key stage of anthocyanin accumulation, 80 d after blooming). The flesh of the harvested fruit was cultured in the presence of exogenous sugar for candidate gene screening. The ripe stage was evaluated according to the grading standards described by Zhang (2008).

The third source of material was two peach accessions with different anthocyanin contents, a white-fleshed peach (‘Hakuho’) and a red-fleshed peach (‘Tianjin Shui Mi’), in which anthocyanin accumulates during the late stage of fruit development (Ding, 2017). The maturation periods of these accessions are all early July in Zhengzhou, China. Three fruit of each cultivar were harvested at three stages, from which three replicate fresh samples were analyzed separately.

ANTHOCYANIN QUANTIFICATION IN FRUIT FLESH. We used a high-performance liquid chromatography system (HPLC; Shimadzu Corp., Kyoto, Japan) coupled with photodiode array detector to analyze flesh anthocyanin contents 1 cm depth from surface of 129 peach accessions at mature time, which are characterized by different flesh colors, and also in two accessions during different fruit development periods, and in sugar-induced fruit with different treated times. Accurately weighed samples (1 g) of powdered fruit flesh were placed into 50-mL flasks containing 25 mL of a methanol/formic acid/water (45:1:4 v/v/v) mixture and incubated overnight (12 h) at 4°C . Following centrifugation at $2000 g_n$, the supernatant was collected and used for HPLC analysis to evaluate cyanidin-3-glucoside and cyanidin-3-rutinoside. The injection volume was 20 μL , the flow rate was $1\text{ mL}\cdot\text{min}^{-1}$, and the detection wavelengths were 280 and 516 nm. A standard curve was prepared using cyanidin-3-O-glucoside as a standard sample. In addition, anthocyanin content was phenotyped in 2014 and 2015 as a qualitative character based on previously published plant genetic resources’ evaluation criteria (Wang and Zhu, 2005). In the book, red pigment can be classified into two types according to some reference cultivars.

GENOME-WIDE ASSOCIATION STUDY. Three models of Tassel v4.0 software (Bradbury et al., 2007) were used to perform GWAS based on the previously identified single-nucleotide polymorphisms (SNPs) of 129 peach accessions (Cao et al., 2016). The first model was general linear model (GLM) without any consideration for principal component analysis (GLM–no PCA); the second was GLM but took PCA results into account as the fixed effect (GLM-PCA); and the third was a mixed linear

Table 1. Details of primers used for quantitative real-time polymerase chain reaction to amplify genes related to sugar metabolism in peach fruit flesh.

Gene accession in reference genome v1.0/2.0 ^z	Forward primer (5'–3')	Reverse primer (5'–3')	Product (base pairs)
<i>ppa004132m/Prupe.1G156300</i>	GCCCTTTGGTGGTGAAAC	TAGGACTGGCGCGTAAAACA	273
<i>ppa004439m/Prupe.1G156900</i>	AGGTTGCCATTCTGGAGGT	AATGCAAGGTCATGAAACGCAG	286
<i>ppa023527m/Prupe.1G157000</i>	CTCCTTCCGTGGTCATCCTG	CAGTGATAGCCTCCAACCCC	222

^zPeach reference genome v1.0 (Verde et al., 2013) and v2.0 (Verde et al., 2017).

model (MLM) using PCA results and the kinship as correction for population structure. In the model, a kinship matrix was calculated using Tassel software and the first three components of PCA were determined using genome-wide complex trait analysis (GCTA) (Yang et al., 2011). Manhattan and quantile-quantile plots were generated using the qqman package in R (Turner, 2014), according to the association results for anthocyanin. We defined a whole-genome significance cutoff from the Bonferroni test threshold (significant genome-wide threshold: $-\log_{10} P = 8.49$, calculated by dividing 0.01 using 3,076,604 SNPs). Gene ontology (GO) annotations of candidate genes were performed using Blast2GO (Conesa and Gotz, 2008) and plotted using WEGO (Web Gene Ontology Annotation Plot) (Ye et al., 2006).

IN VITRO INDUCTION OF ANTHOCYANIN SYNTHESIS BY SUGAR. A red-fleshed peach 'Tianjin Shui Mi' was selected to conduct this experiment. The flesh was cut into small pieces ($2 \times 2 \times 2$ mm) during the stage of development at which the flesh color changes (≈ 15 d before the date of maturity). These samples were incubated in 2-(N-morpholino)ethanesulfonic acid (MES) culture medium (pH = 6.5), containing 100 mM MES (pH 5.5),

5 mM CaCl₂, 10 mM MgCl₂, 1 mM EDTA, 10 mM vitamin C, and 100 mM sugar (glucose, sucrose, fructose, or sorbitol), at room temperature (25 °C). The incubation method was consulted from a previous study (Beruter and Studer Feusi, 1995; Do and Cormier, 1990; Jia, 2013) and adjusted a little. Light was supplied by fluorescent lamps with $62.5 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 24 h. As a control, flesh was incubated in each MES medium containing mannitol to balance the osmotic potential. After treatment for 0, 12, and 24 h, flesh samples were frozen in liquid nitrogen and stored at -80 °C for subsequent RNA extraction and anthocyanin evaluation.

TRANSCRIPTIONAL ANALYSIS OF FRUIT FLESH INDUCED BY SUGAR. Total RNA (2 μg) was extracted of the induced fruit flesh using a Quick RNA Isolation Kit (Waryong, Beijing, China) according to the manufacturer's instructions. Purification of poly (A) messenger RNAs (mRNAs) was performed using oligo-dT attached to magnetic beads. The purified mRNAs were fragmented using ultra-sonication, and then subjected to first- and second-strand complementary DNA (cDNA) synthesis using random hexamer primers. Thereafter, cDNA fragments of ≈ 350 base pairs (bp) in size were gel-purified and used as templates in a polymerase chain reaction (PCR) and sequenced using a high-throughput sequencing platform (BGISEQ-500; GitHub, Shenzhen, China). Finally, the sequencing data were filtered using SOAPnuke (GitHub, 2016) and aligned against the peach reference genome v2.0 (Verde et al., 2017) for better annotation comparison of v1.0. Gene expression was calculated in terms of reads per kilobase per million mapped reads using Cufflinks v2.1.1 (Trapnell et al., 2010).

REAL-TIME QUANTITATIVE PCR.

Fruit of the third source of material was used for extraction of RNA using an RNA extraction kit (EASYspin Plus; Aidlab, Beijing, China), and its purity was evaluated using a spectrophotometer (Nano-Drop 1000; Thermo Fisher Scientific, Waltham, MA). cDNA was synthesized from 2 μg of RNA using a First Strand cDNA Synthesis Kit (ReverTra Ace- α -FSK101; Takara, Dalian, China) according to the manufacturer's protocol. According to gene sequences annotated by Verde et al. (2017), specific

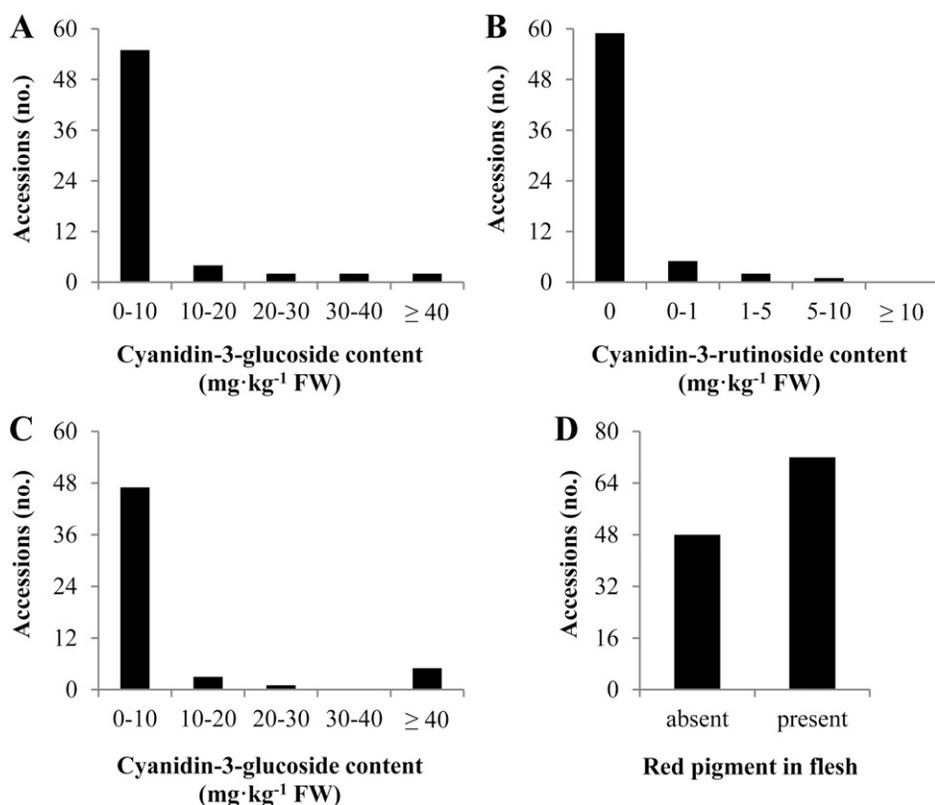


Fig. 1. Anthocyanin quantification in fruit of 129 peach accessions in (A and B) 2014, (C) in 2015, and (D) classification. FW = fresh weight.

primers for three genes (Table 1) were designed using Primer-BLAST software (National Center for Biotechnology Information, Bethesda, MD).

Real-time quantitative PCR reactions were performed using the fluorescent quantitative PCR instrument (LightCycler 480; Roche Diagnostics, Mannheim, Germany) with a 10- μ L reaction mixture containing 10 ng cDNA, 0.5 mM of each primer, 5 μ L Premix Taq solution (Takara), and 3.5 μ L double distilled water. The real-time quantitative PCR reaction program used was as follows: 95 $^{\circ}$ C for 5 min; followed by 35 cycles of 94 $^{\circ}$ C for 30 s, the annealing temperature for 30 s, and 72 $^{\circ}$ C for 30 s; with a final elongation for 10 min at 72 $^{\circ}$ C. Relative expression levels were estimated using the comparative Ct method (Livak and Schmittgen, 2001). The sequences of the primers used in this study are shown in Table 1.

Results

ANTHOCYANIN PHENOTYPING. The anthocyanin contents of 129 peach cultivars in 2014 (Fig. 1A and B) revealed cyanidin-3-glucoside but cyanidin-3-rutinoside as the primary component of anthocyanin, therefore we focused on this molecule in

the subsequent analyses in 2014 and 2015 (Fig. 1A and C). Forty-eight of the assessed cultivars were found to be lacking in all anthocyanin evaluation, whereas among the remainder, cyanidin-3-glucoside showed skewed distribution (Fig. 1A and C). Most of these latter accessions had anthocyanin concentrations ranging between 0 and 10 mg \cdot kg $^{-1}$ of fresh fruit, whereas only \approx 10 cultivars showed higher concentrations. However, we detected no segregation distortion when the anthocyanin concentrations trait was considered as a qualitative character by direct observation (Fig. 1D).

SEQUENCING AND GWAS. In the study, we identified 4,062,178 SNPs in 129 peach cultivars. After filtering the data using a minor allele frequency threshold of \leq 0.05 and absent in more than 20% assessed cultivars, finally, a total of 3,076,604 SNPs remained for GWAS.

Using the MLM, we performed association analysis of cyanidin-3-glucosidase in 2014 and 2015 (Supplemental Fig. 1A and B) because that model was proved to be suitable for the quantitative character (Korte and Farlow, 2013). The result showed that many association signals exceeded the genome-wide significance threshold ($-\log_{10} P > 8.49$) but presented a scattered distribution without any clear association peaks. This abnormal

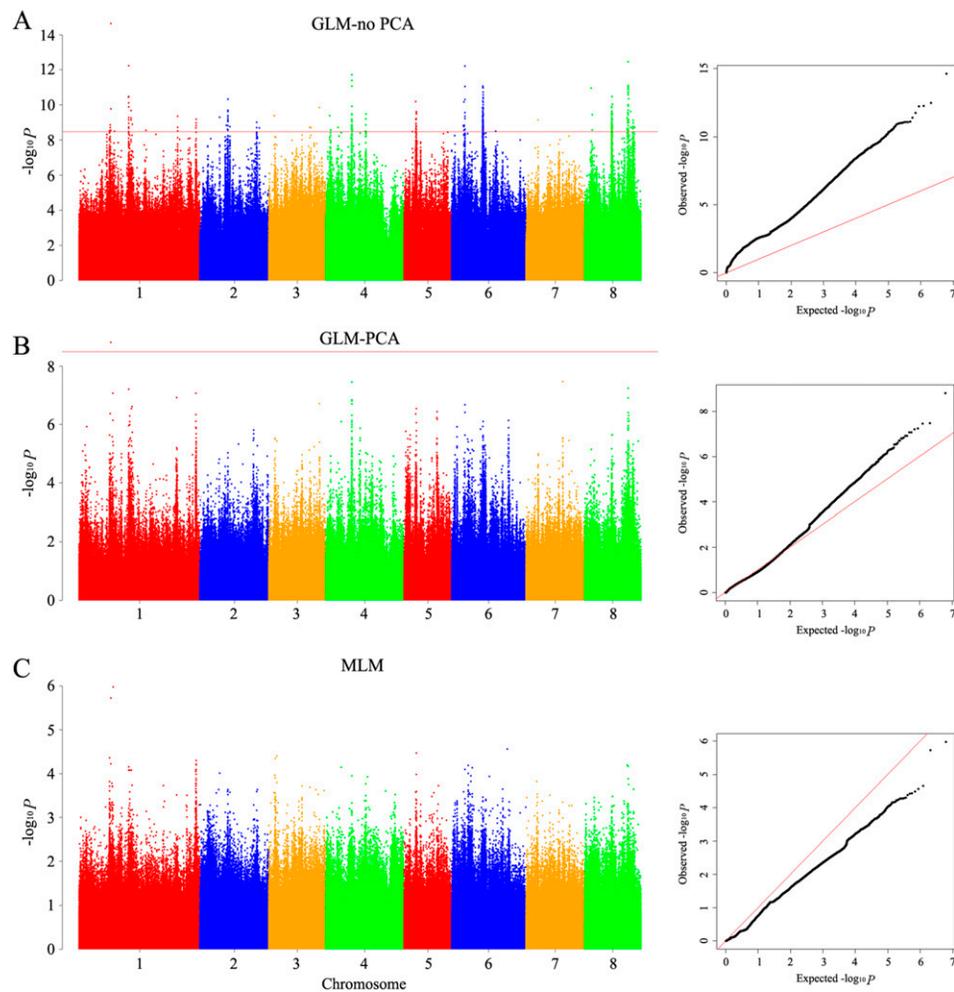


Fig. 2. Manhattan and quantile-quantile plots of estimated $-\log_{10} P$ from association analysis of anthocyanin absent/present in fruit of 129 peach accessions using (A) general linear model (GLM)–no principal components analysis (PCA), (B) GLM-PCA, and (C) mixed linear model (MLM). Negative $\log_{10} P$ values from the genome-wide scan were plotted against single-nucleotide polymorphism position on each of the eight chromosomes. The dotted horizontal line indicates the genome-wide significance threshold ($-\log_{10} P = 8.49$).

association may be related to the substantial bias in separation of the trait (Fig. 1A and C). In 2015, peak signals were found in 37.15, 8.68, 0.04, 19.89, 3.08, 10.17, 3.08, and 5.25 Mb of chromosome (Chr.) 1 to Chr. 8, respectively. And in 2014, peak signals were found in 36.51, 4.94, 3.28, 15.54, 2.01, 19.84, 4.93, and 4.12 Mb of Chr. 1 to 8. The results indicated that there were no year-stable association regions of cyanidin-3-glucosidase contents in peach flesh considering the linkage disequilibrium decay of peach [about 20 kb (Cao et al., 2016)]. Furthermore, we found that significant SNPs were far from the candidate genes involved in anthocyanin biosynthesis. For example, a *CHS* gene, *Prupe.1G002900*, was located in 0.3 Mb in Chr. 1. A *UFGT* gene, *Prupe.2G324700*, was located in 30.0 Mb in Chr. 2. A *MYB* gene, *Prupe.3G163100*, was located in 18.2 Mb in Chr. 3.

When anthocyanin content was considered as a qualitative character (anthocyanin absent/present), three models were used for association analysis (Fig. 2A–C). The QQ-plot result showed that the GWAS of GLM-PCA present a well-defined distribution and the model could obtain clean and obvious associations. The false-positive association signals were avoided by controlling population structure comparing with the GLM–no PCA model (Fig. 2A). However, MLMs present an excessive regulation because no continuous

and obvious association signals were found to be higher than the genome-wide significance level (Fig. 2C). Therefore, the most prominent association signal (Chr. 1: 12,265,694 bp, $-\log_{10} P = 8.81$) being located in the interval between 11.7 and 13.1 Mb on Chr. 1 of GLM-PCA (Fig. 2B) was selected for following analysis. To date, only 133 of 146 genes in this region have been functionally annotated, among which membrane-associated genes are significantly enriched (Fig. 3), including one gene encoding a chloride channel (CLC) protein, and 17 involved in sugar transport and galactosyltransferase activity (Table 2).

CANDIDATE GENE IDENTIFICATION USING FRUIT FLESH CULTURED WITH SUGAR. In the present study, expression of 17 enriched genes associated with sugar identified through GWAS was also evaluated in fruit flesh incubated with different sugars. Our results showed that with an increase in the duration of the culture period, anthocyanin was induced by glucose, sucrose, and fructose, whereas treatment with sorbitol had minor effects comparable to those of the control mannitol (Fig. 4A). In fruit subjected to sugar induction for 12 h, we observed that glucose treatment resulted in the highest content of anthocyanin, followed by sucrose, whereas the levels of anthocyanin in flesh induced by fructose and sorbitol were lower than those induced by the mannitol control. In contrast, after 24 h of induction, the anthocyanin contents of fruit in all four sugar treatments were higher than those induced by mannitol, with the contents induced by glucose and sucrose being slightly higher than those induced by fructose.

On conducting RNA-seq analysis to assess the expression of the aforementioned 17 genes (Fig. 4B), we found that 13 showed no detectable expression. Among the remaining four genes, three (*Prupe.1G56300*, *Prupe.1G56900*, and *Prupe.1G15700*, corresponding to *ppa004132m*, *ppa004439m*, *ppa023527m*, respectively, annotated in reference genome v1.0) showed an expression pattern consistent with anthocyanin

induced by sugar, although the expression levels were higher at 12 h than at 24 h. The expression level of the fourth of these genes (*Prupe.1G166200*) decreased with increasing culture time. Of the three genes with increased expression, *Prupe.1G156300* had higher expression than *Prupe.1G156900* and *Prupe.1G157000*.

CANDIDATE GENE IDENTIFICATION USING FRUIT FLESH DURING FRUIT DEVELOPMENTS. We further analyzed the expression of *Prupe.1G56300*, *Prupe.1G56900*, and *Prupe.1G15700* in fruit flesh during different periods of fruit development (Fig. 5), and accordingly observed the steady expression of *Prupe.1G156300* during the course of fruit development in the white-fleshed peach and induction at the mature stage in the red-fleshed peach, a pattern similar to the accumulation of anthocyanin. *Prupe.1G157000* showed higher expression in the white-fleshed peach at the mature stage, in contrast to the observed anthocyanin levels. We were unable to detect the expression of *Prupe.1G156900* in any of the two examined peach cultivars.

Discussion

GWAS is increasingly being applied in biological studies to facilitate direct correlation between phenotypic traits and the associated genetic mechanisms. The key genes for the red flesh phenotype of peach have previously been identified as *PpMYB10.1* at 12.87 Mb on Chr. 3 and *PpBL* at 4.2 to 4.5 Mb on Chr. 4 (Zhou et al., 2015). A *DBF* gene, known to control flesh color, has been located at the top of linkage group 5 (0.59–0.61 Mb) in a hybrid population of the blood peach cultivar Wu Yue Xian (Shen et al., 2013). Furthermore, *DBF2*, which has been determined to be responsible for red flesh color in almond (*Prunus dulcis*), was found to map to the end of linkage group 1 (corresponding to 35.2 Mb in Chr. 1) in a cross between the almond cultivar Texas and the peach cultivar Earlygold (Donoso et al., 2016). During the study of our laboratory, 66 genes were identified through RNA-seq using different development of fruit of ‘Tianjin Shui Mi’ peach. Among of them, 13 genes were located at Chr. 1 and only *ppa011068m* (corresponding to 10.3 Mb in Chr. 1). In the present study, we conducted a GWAS to identify the marker associated with anthocyanin content using resequencing data. We found an obvious association signal with the GLM-PCA model and the association region to be located between 11.7 and 13.1 Mb on Chr. 1 (Fig. 2C). This locus is close to that located in an interspecific cross (*Prunus davidiana* clone P1908 and *P. persica* cv. Summergrand) (Quilot et al., 2004), which is linked to the marker CFF14 (corresponding to 9.16 Mb in Chr. 1) and gene *ppa011068m* (Cao et al., 2018). The different location from that described previously for the *DBF2* locus may be related to the different backgrounds.

Among the genes previously annotated in the putative association region, one gene encodes a CLC protein, and a further 17 are involved in sugar transport and galactosyltransferase activity. In arabidopsis (Wei et al., 2013) and rice (Diedhiou and Gollmack, 2006), CLCs have been reported to be involved in plant adaptation to salt stress by regulating chloride ion homeostasis. However, sugars not only serve as energy sources and structural materials, but also help osmosis, which has an essential role in plant growth (Archetti et al., 2009; Dong et al., 2013). In addition, sugars also can function as signal molecules in different biological pathways in different plants species (Gazzarrini and Mccort, 2001; Solfanelli et al.,

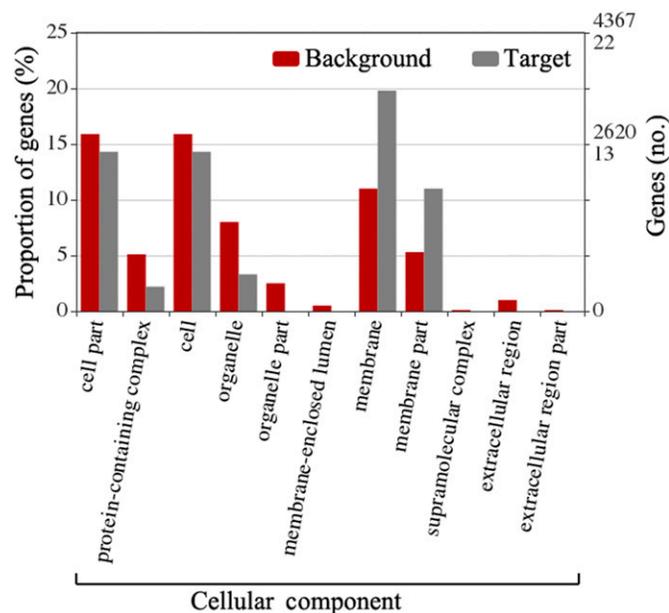


Fig. 3. The cellular component of gene ontology annotation of the 133 target genes and in comparison of all genes annotated in the genome (designated as background genes). The left and right y-axes present the proportion and number of genes among of different categories.

Table 2. Functional annotation of the 18 enriched genes during the quantitative trait loci for red pigments in peach fruit flesh.

Gene accession ^z	Chromosome	Transcript start (base pair)	Transcript stop (base pair)	Description
<i>ppa016440m</i>	1	11780460	11781407	Sugar carrier protein C
<i>ppa020244m</i>	1	11786475	11787125	Sugar carrier protein C
<i>ppa023512m</i>	1	11889517	11896074	Chloride channel protein CLC-d
<i>ppa004132m</i>	1	11903999	11909035	Sugar transport protein 13
<i>ppa004417m</i>	1	11913147	11915173	Sugar transport protein 13
<i>ppa004420m</i>	1	11915952	11917893	Sugar transport protein 13
<i>ppa020009m</i>	1	11932661	11934856	Carbohydrate transmembrane transporter
<i>ppa004423m</i>	1	11973835	11975769	Sugar transport protein 13
<i>ppa004424m</i>	1	11977117	11979137	Sugar transport protein 13
<i>ppa004439m</i>	1	11991191	11994292	Sugar transport protein 13
<i>ppa023527m</i>	1	11999707	12002134	Sugar transport protein 13
<i>ppa008689m</i>	1	12995661	12997002	Xyloglucan galactosyltransferase
<i>ppa018375m</i>	1	12997348	13000030	Xyloglucan galactosyltransferase
<i>ppa007371m</i>	1	13040229	13041816	Xyloglucan galactosyltransferase
<i>ppa004844m</i>	1	13047652	13049409	Xyloglucan galactosyltransferase
<i>ppa005908m</i>	1	13052324	13053908	Xyloglucan galactosyltransferase
<i>ppa025271m</i>	1	13079430	13079987	Xyloglucan galactosyltransferase
<i>ppa006308m</i>	1	13082205	13083502	Xyloglucan galactosyltransferase

^zEach accession name was annotated using peach reference genome v1.0 (Verde et al., 2013).

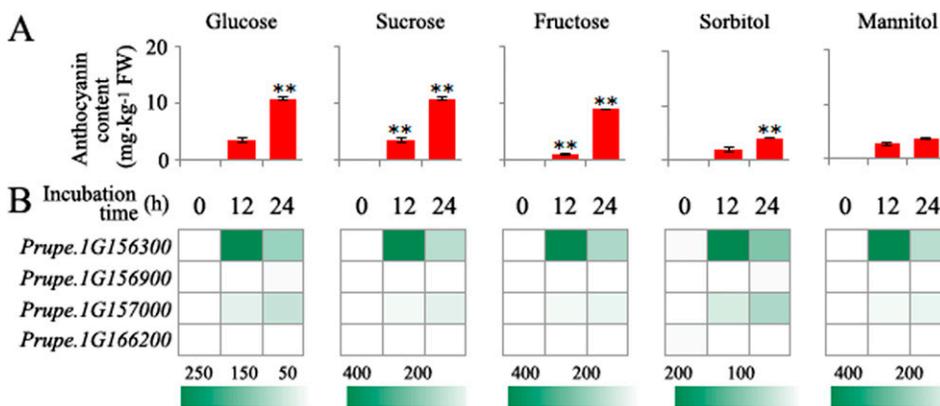


Fig. 4. (A) Anthocyanin content and (B) gene expression profile using RNA-seq in the fruit flesh of a red-fleshed peach ‘Tianjin Shui Mi’ induced by different sugar, including glucose, sucrose, fructose, and sorbitol, at 0, 12, and 24 h, compared with the control (mannitol treatment). The anthocyanin content is expressed as the mean \pm SD of triplicates in each sample. Asterisks indicate significant differences between the sugar and the control in 12- or 24-h treatment (** $P < 0.01$, one-way analysis of variance). FW = fresh weight.

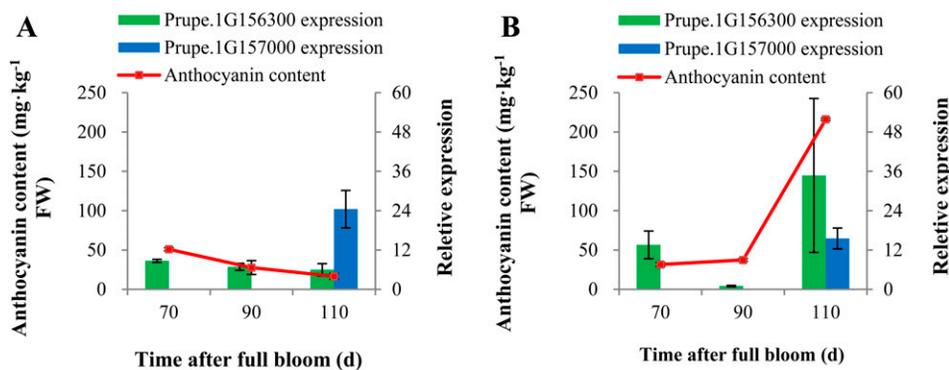


Fig. 5. The relative expression of different genes using quantitative real-time polymerase chain reaction and anthocyanin content in the fruit flesh during the development period of white-flesh [(A) ‘Hakuho’] and red flesh [(B) ‘Tianjin Shui Mi’] peach fruit. FW = fresh weight.

2006), and notably can enhance anthocyanin production in plants (Ai et al., 2016). Sucrose is involved in the expression of numerous genes related to anthocyanin biosynthesis, as well as in the regulation of transcription factors (Cheng and Li, 2010), and can specifically induce the anthocyanin biosynthetic pathway in arabidopsis (Solfanelli et al., 2006). Similarly, exogenous glucose treatment has also been found to enhance anthocyanin content in the petals of *Paeonia suffruticosa* ‘Luoyang Hong’ cut flowers (Zhang et al., 2015). Moreover, these observations have been validated in *Vitis vinifera* cells (Gollop et al., 2001, 2002). Certain genes associated with sugar have been found to be enriched in the markers associated with anthocyanin content in peach (Nikkhah et al., 2007). Therefore, in the present study, we examined the effects of exogenous sugar treatment on fruit flesh to screen the 17 candidate genes. Our RNA-seq results indicated that among the 17 genes induced in fruit flesh; only three genes (*Prupe.1G156300*, *Prupe.1G156900*, and *Prupe.1G15700*) showed an expression pattern similar to that of sugar-induced anthocyanin levels. When we further analyzed the expression of these three genes in fruit flesh during different periods of fruit development, we observed that ex-

pression of *Prupe.1G156300*, a sugar transporter gene, was positively correlated with anthocyanin accumulation in red-fleshed fruit; however, further analysis, including functional studies, are necessary to confirm the role of this candidate gene.

It was noticed that both mannitol and sorbitol increased anthocyanin content of cultured flesh (Fig. 4A), and these findings may be interpreted as the osmotic effects of these sugars on anthocyanin biosynthesis (Do and Cormier, 1990; Zhang et al., 2015).

Conclusion

To identify the candidate gene regulating anthocyanin content in peach, we performed a GWAS and candidate gene screening, and initially detected an association signal in the 11.7 to 13.1 Mb region of Chr. 1. GO annotation of the 146 genes mapping to this region, indicated that 17 may be involved in sugar metabolism, and we accordingly focused on these in our subsequent analyses. Among these 17 genes, the expression of *Prupe.1G156300*, which encodes a sugar transporter, was found to be positively correlated with anthocyanin accumulation in fruit flesh of two red peach cultivars. These findings enabled us to provisionally characterize the genetic basis of anthocyanin biosynthesis in peach fruit.

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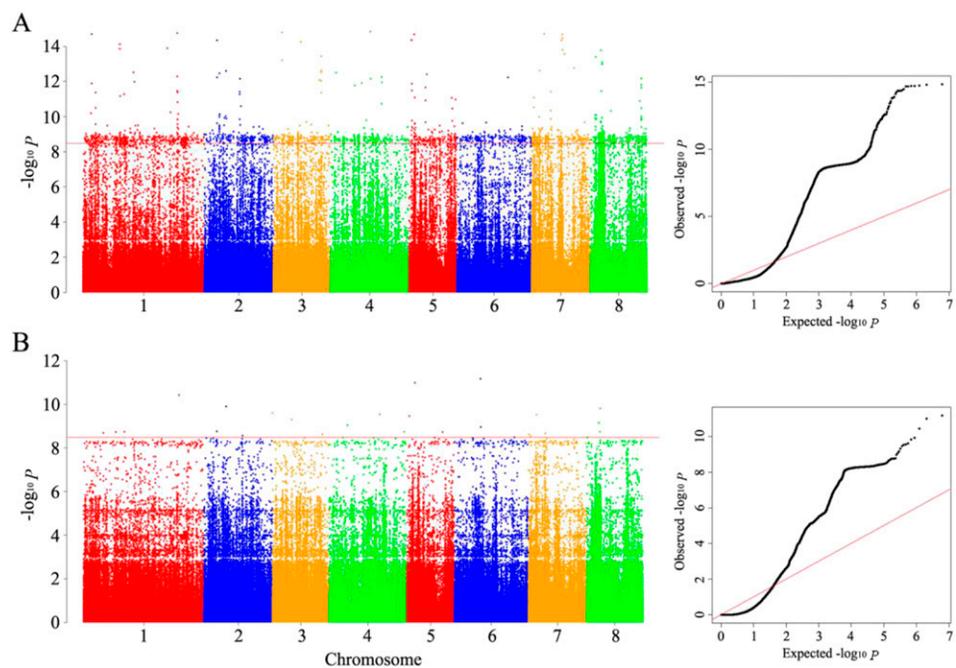
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Supplemental Fig. 1. Manhattan and quantile-quantile plots of estimated $-\log_{10} P$ from association analysis of cyanidin-3-glucoside content in 2014 (**A**) and 2015 (**B**) in fruit of 129 accessions using mixed linear model. Negative $\log_{10} P$ values from the genome-wide scan were plotted against single-nucleotide polymorphism position on each of the eight chromosomes. The dotted line indicates the genome-wide significance threshold ($-\log_{10} P = 8.49$).

Supplemental Table 1. *Prunus* samples used in this study and their key agronomic traits.

Accessions	Species	Population	Fruit shape	Fruit hairiness	Flesh color (white/yellow)	Flesh color (red pigment)
07-4-33#	<i>P. persica</i>	Improved variety	NA	NA	NA	NA
08-X-77#	<i>P. persica</i>	Improved variety	NA	NA	NA	NA
Nonpareil	<i>P. dulcis</i>	Wild specie	Round	Yes	NA	NA
Sa Hong Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Sa Hong Long Zhu	<i>P. persica</i>	Improved variety	Round	Yes	White	No
Chong Ban Xiao Hua Xing	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
09-12west-5	<i>P. persica</i>	Improved variety	Flat	Yes	White	NA
Sa Hua Hong Pan Tao	<i>P. persica</i>	Landrace	Flat	Yes	White	Yes
Wu Yue Xian Bian Gan	<i>P. persica</i>	Landrace	Flat	Yes	White	Yes
You Pan Tao	<i>P. persica</i>	Landrace	Flat	No	White	No
Zhong You 5#	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Hong Li Guang	<i>P. persica</i>	Landrace	Round	No	White	No
Mei Gui Hong	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Hua Guang	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Hong Shan Hu	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Rui Guang 3#	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Zhong You Tao 9#	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Zhong You Tao 9# Hei Xian Bai	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Rou Ya Bian						
07-1-11#	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Zhong You Tao 9# Zao Shu Ya Bian	<i>P. persica</i>	Improved variety	Round	No	White	Yes
Bian Tao	<i>P. persica</i>	Landrace	Flat	Yes	White	No
Rou Pan Tao	<i>P. persica</i>	Landrace	Flat	Yes	White	Yes
Kashi 1#	<i>P. ferganensis</i>	Landrace	Round	Yes	White	No
Ge Gu	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Diao Zhi Bai	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Da Xue Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Hong Ya Zui	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Ping Bei Zi	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Fei Cheng Bai Li 10#	<i>P. persica</i>	Landrace	Round	Yes	White	No
Nanshan Tian Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Wu Yue Xian	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Ying Zui	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Shen Zhou Shui Mi	<i>P. persica</i>	Landrace	Round	Yes	White	No
Suan Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Yu Bai	<i>P. persica</i>	Improved variety	Round	Yes	White	No
Yangzhou 3#	<i>P. persica</i>	Improved variety	Round	Yes	White	No
Fei Cheng Hong Li 6#	<i>P. persica</i>	Landrace	Round	Yes	White	No
Shiwo Shui Mi	<i>P. persica</i>	Landrace	Round	Yes	White	No
Jilin 8903	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Li He Tian Ren	<i>P. persica</i>	Landrace	Round	Yes	White	No
Hua Yu Lu	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Bai Hua	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Jing Yu	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Bai Hua Shan Bi Tao	<i>P. davidiana</i>	Landrace	Round	Yes	White	NA
Hong Hua Bi Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Shandong Si Yue Ban	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Qingzhou Bai Pi Mi Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Qing Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Shi Tou Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Ying Ge Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Li07-1-13	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Zao Shanghai Shui Mi	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Hakuho	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Okubo	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Yan Hong	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Zhong Hua Shou Tao	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes

Continued next page

Supplemental Table 1. Continued.

Accessions	Species	Population	Fruit shape	Fruit hairiness	Flesh color (white/yellow)	Flesh color (red pigment)
07-2-24#	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Hong Hua Shan Tao	<i>P. davidiana</i>	Wild specie	Round	Yes	White	No
Hong Rou Guang He Tao	<i>P. mira</i>	Wild specie	Round	Yes	White	Yes
Guang He Tao (Rikaze)	<i>P. mira</i>	Wild specie	Round	Yes	White	No
Guang He Tao (Aba)	<i>P. mira</i>	Wild specie	Round	Yes	White	No
Xikang Bian Tao	<i>P. tangutica</i>	Wild specie	Round	Yes	White	NA
Chinese Cling	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Bi Nan I	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Shuang Xi Hong	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Shu Guang Hong Xian Ya Bian	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Hei Bu Dai	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Tian Jin Shui Mi	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Wuhan 2#	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Wu Hei Ji Rou Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Da Hong Pao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Da Guo Hei Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Harrow Blood	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes
Huang Jin Pan Tao	<i>P. persica</i>	Landrace	Flat	Yes	Yellow	No
Zhong You Pan 2#	<i>P. persica</i>	Improved variety	Flat	No	Yellow	No
Zhong You Pan 4#	<i>P. persica</i>	Improved variety	Flat	No	Yellow	Yes
Ping Ding You Pan Tao	<i>P. persica</i>	Improved variety	Flat	No	Yellow	No
Rui Guang 2#	<i>P. persica</i>	Improved variety	Round	No	Yellow	No
NJN76	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Okitsu	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Legrand	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
May Fire	<i>P. persica</i>	Improved variety	Round	No	Yellow	No
Zhong You Tao 4#	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Kashi Huang Rou Li Guang	<i>P. persica</i>	Landrace	Round	No	Yellow	Yes
Xia Ye Tao	<i>P. persica</i>	Improved variety	Round	No	Yellow	No
Bai Shu 55# Bian Yi	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Shu Guang	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Zhong You Tao 4# Zao Shu Ya Bian	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Zhong You Tao 9# Huang Rou Ya Bian	<i>P. persica</i>	Improved variety	Round	No	Yellow	NA
Dan Bei Ti	<i>P. persica</i>	Improved variety	Round	No	Yellow	NA
Zao Shu Xia Ye Tao	<i>P. persica</i>	Improved variety	Round	No	Yellow	NA
Zhong You Tao 9# Wan Shu Ya Bian	<i>P. persica</i>	Improved variety	Round	No	Yellow	Yes
Zao Huang Pan Tao	<i>P. persica</i>	Improved variety	Flat	Yes	Yellow	Yes
Mai Huang Pan Tao	<i>P. persica</i>	Improved variety	Flat	Yes	Yellow	Yes
Da Lian 4-35	<i>P. persica</i>	Improved variety	Flat	Yes	Yellow	Yes
Xinjiang Huang Rou	<i>P. ferganensis</i>	Landrace	Round	Yes	Yellow	No
Zhao Shu Huang Gan	<i>P. persica</i>	Landrace	Round	Yes	Yellow	Yes
Elberta	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	Yes
Qing Si Tao	<i>P. persica</i>	Landrace	Round	Yes	Yellow	No
Huo Lian Jin Dan	<i>P. persica</i>	Landrace	Round	Yes	Yellow	Yes
Jin Feng	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	Yes
NJC77	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	No
Maria Serena	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	No
Redhaven	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	Yes
Phillips	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	No
Mao Tao 2-1-55	<i>P. persica</i>	Improved variety	Round	Yes	Yellow	Yes
Xiamiao 1#	<i>P. persica</i>	Landrace	Round	Yes	Yellow	Yes
Long 1-2-4	<i>P. persica</i>	Landrace	Round	Yes	Yellow	Yes
Zhu Fen Chui Zhi	<i>P. persica</i>	Landrace	Round	Yes	White	No
Hong Shou Xing	<i>P. persica</i>	Landrace	Round	Yes	White	No
Bai Gen Gan Su Tao	<i>P. kansuensis</i>	Wild specie	Round	Yes	White	No
Hong Gen Gan Su Tao	<i>P. kansuensis</i>	Wild specie	Round	Yes	White	No

Continued next page

Supplemental Table 1. Continued.

Accessions	Species	Population	Fruit shape	Fruit hairiness	Flesh color (white/yellow)	Flesh color (red pigment)
Xinjiang Pan Tao	<i>P. ferganensis</i>	Landrace	Flat	Yes	White	No
Xinjiang Pan Tao(pollen sterility)	<i>P. ferganensis</i>	Wild specie	Flat	Yes	White	No
Tian Ren Tao	<i>P. ferganensis</i>	Landrace	Round	Yes	White	No
Hong Ye Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Hong Ye Tao Ya Bian	<i>P. persica</i>	Landrace	Round	Yes	White	No
Ju Hua Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Shou Bai	<i>P. persica</i>	Landrace	Round	Yes	White	No
Hun Chun Tao	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Xian Tao	<i>P. persica</i>	Landrace	Round	Yes	White	No
Yuan Yang Chui Zhi	<i>P. persica</i>	Landrace	Round	Yes	White	No
Fen Shou Xing	<i>P. persica</i>	Landrace	Round	Yes	White	Yes
Hong Chui Zhi	<i>P. persica</i>	Landrace	Round	Yes	White	No
Bailey	<i>P. persica</i>	Improved variety	Round	Yes	White	No
Shan Gan Shan Tao	<i>P. davidiana</i> var. potaninii	Wild specie	Round	Yes	White	No
Bai Hua Shan Tao	<i>P. davidiana</i>	Wild specie	Round	Yes	Yellow	No
Zhou Xing Shan Tao	<i>P. davidiana</i>	Wild specie	Round	Yes	Yellow	No
Bai Wu 8#	<i>P. persica</i>	Improved variety	Round	Yes	White	Yes

Note: NA indicates the data were absent.