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Expansin genes are candidate markers for the control of fruit weight in peach

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Abstract Fruit weight is an important target trait in peach breeding and culture. Although many studies have investigated quantitative trait loci (QTL) associated with fruit weight and cell wall development, no causal genes have been identified to date. Expansins (EXPs) are plant cell wall-loosening proteins that modulate the enlargement and softening of fruit, but their role in peach development is unknown. To address this issue, we evaluated the expression profiles of all *EXP* genes in the peach genome. We identified 29 genes that could be classified into six groups according to their temporal expression patterns during fruit development. A genome-wide association analysis showed that six of these genes were located in the confidence intervals of QTL regions of fruit weight. The expression of two of the genes, ppa017982m and ppa010443m, was positively correlated with fruit diameter, suggesting that they control fruit weight by regulating cell enlargement. After screening the single nucleotide polymorphism (SNP) distribution in varieties of peach that bear small or large fruit ($n = 30$ each), an SNP in chromosome 5 (nucleotide 5,026,380) was identified in the promoter region of ppa017982m. This SNP can serve as a molecular marker in breeding programs designed to eliminate small fruit varieties.

Keywords Peach fruit weight · Quantitative trait loci · Expansin · Single nucleotide polymorphism

Introduction

Fruit weight, which is positively correlated with fruit size, is an important commercial trait in fruit crops. Cultivars that consistently produce large fruits are critical for grower profitability. Therefore, knowledge of markers and genes associated with fruit weight in peach can increase the efficiency of producing large-fruited cultivars through breeding programs (Franceschi et al. 2013).

Final fruit weight is determined by cell production and expansion during fruit development (Malladi and Hirst 2010). In apple, these processes occur separately during two stages: early fruit growth involves cell proliferation in the 3–4 weeks after pollination and fertilization, while later stages of growth include cell expansion before 45 days after full blossom (DAB) (Harada et al. 2005). In peach, Growth of peach fruit has been generally divided into three stages. Although a major period of cell division occurs early in fruit development (stage I, from full bloom until 56 DAB)

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and cell enlargement is important during stage III (from 107 DAB to ripe), cell division and cell expansion are not exclusive to either of these growth stages. Rapid growth during stage I is clearly an effect of this division–expansion process (Scorza et al. 1991).

Quantitative trait loci (QTL) have been investigated in order to identify candidate genes that govern fruit weight in crops such as pear (Wu et al. 2014), grape (Doligez et al. 2013), and peach (Dirlewanger et al. 1999; Yamamoto et al. 2001). *FW2.2* was identified by map-based cloning and was found to negatively regulate fruit size through control of cell proliferation during the early development of tomato (Frary et al. 2000; Cong et al. 2002). In addition, plant cell proliferation is regulated via the cell cycle by genes encoding cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors, and CDK-activating kinases (Inze and De Veylder 2006) as well as by endoreduplication, in which DNA replication is not accompanied by mitosis (Chevalier et al. 2011). Auxin-related factor 9 negatively controls cell division during early fruit development in tomato (de Jong et al. 2015). In grapes, candidate genes belonging to the cell number regulator (CNR) family (homologous to FW2.2 in tomato) are located in berry weight QTL in linkage groups (LGs) 1, 8, and 17 (Doligez et al. 2013), while in sweet cherry, two of 23 *FW2.2/CNR*-like genes were found within confidence intervals of major QTL in LGs 2 and 6; one of these genes, *PavCNR12* in LG 2, was associated with fruit size (Franceschi et al. 2013).

Most studies have shown that cell division is more important than cell enlargement in determining final fruit weight (Olmstead et al. 2007; Zhang et al. 2010). Cell size also influences fruit weight; for instance, the final cell diameter in the fruit cortex cells of Grand Gala—a large-sized spontaneous mutant of Gala apples—was 15 % larger than that of Gala, suggesting that the enhanced size of Grand Gala was due to cell expansion during apple fruit development (Malladi and Hirst 2010). Kinesin, a microtubule-based motor protein, regulates fruit weight in cucumber by modulating both cell division and enlargement in early fruit development (Yang et al. 2013).

Members of the expansin (EXP) family of cell wall proteins disrupt hydrogen bonds within the cell wall polymer matrix and are implicated in cell wall polymer disassembly and cell elongation. The physiological functions of EXPs have been studied in different plant species, organs, and developmental stages (Dal Santo et al. 2013); these proteins are involved in root and shoot apical meristem development, seed germination, several types of plant–microbe interaction, and fruit softening. *LeExp1* in tomato contributes to fruit softening by increasing the accessibility of specific cell wall polymers to hydrolases (Rose et al. 1997), whereas *LeExp2* (Caderas et al. 2000) and *LeExp10* (Chen et al. 2001) are highly expressed in the faster growing stem part of the hypocotyls elongation zone and during the period of rapid embryo expansion, respectively. The root-specific *EXP* gene in soybean (*Glycine max*), *GmEXP1*, is involved in root elongation (Lee et al. 2003). In *Arabidopsis*, EXPA7 is required for root hair elongation (Lin et al. 2011). Three *EXP* genes have been cloned in peach; *PpExp2* is abundantly expressed at later stages of development when the fruit expands exponentially before maturation, while *PpExp1* and *PpExp3* are upregulated at the onset of ripening (Hayama et al. 2003). However, it is not known is there any other *EXP* genes are involved in rapid fruit expansion in peach.

To clarify the genetic determinism of fruit weight, we carried out an association analysis using simple sequence repeat (SSR) markers (Cao et al. 2012). QTL for fruit weight were located in LGs 3, 5, 6, and 8. However, the confidence interval of the QTL region was too large—and the density of markers too low—to identify candidate genes. The peach genome released by the International Peach Genome Initiative provides a repertoire of candidate *EXP* genes and accompanying QTL. We carried out a genome wide association analysis (GWAS) of fruit weight using resequenced data instead of SSR markers to obtain a narrow QTL interval, which was screened for *EXP* genes expressed at the fruit expansion stage and that are linked to fruit weight. Our results provide new insight into the genetic control of fruit weight in peach as well as a basis for performing marker-assisted selection of this trait.

Materials and methods

Plant material

Three types of material were used for analyses. Hakuho peaches were picked between April 24 and

July 8, 2014 to determine the expression profiles of all *EXP* genes in the peach genome. In addition, 130 peach accessions (Supplementary Table 1) representing core germplasm resources in China and exhibiting fruit weight between 5 and 350 g were used to identify fruit weight QTL. Finally, two peach varieties with different fruit weights—Robin (about 130 g and designated as a small fruit) and Sunago Wase (about 320 g and designated as a large fruit) were used to evaluate the expression levels of specific genes. These varieties were grafted onto *Prunus davidiana* seedling rootstocks and planted in the same orchard at the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (Zhengzhou, China) with one tree per genotype. All varieties and seedlings were grown under normal conditions of irrigation, fertilization, and pest control.

Measurement of fruit weight and diameter

After the first physiological fruit drop, hand thinning was carried out to reduce fruit to minimize the variation of fruits within a genotype. The mean weight of the 130 varieties was determined by weighing 10 mature fruit which has uniform size per tree in 2007 and 2010 for QTL identification. Hakuho, Robin, and Sunago Wase peaches were weighed at different fruit development periods (FDPs) using an electronic balance (Quintix313-1CN; Sartorius, Göttingen, Germany) in 2014. Fruit polar and cheek diameters were measured using Vernier calipers. The maturity for each tree was determined by several factors, for example, the flesh began to soften, fruit size was no longer grew, and the fruit could be picked easily.

EXP gene expression profile analysis

EXP genes were selected by searching the annotation table of the peach genome on the GDR website (https://www.rosaceae.org/species/prunus_persica/genome_v1.0). Hakuho, Robin, and Sunago Wase peach fruit were picked in field during FDP and brought to the laboratory. Then, the mesocarp was removed immediately and placed in liquid nitrogen. RNA was extracted using the cetyltrimethylammonium bromide (CTAB) method and treated with DNase I (Promega, Madison, Wisconsin, US). cDNA was generated using PrimeScript 1st Strand cDNA Synthesis kit (Takara, Dalian, China) with 1 mg total RNA. Expression profiles of *EXP* genes were determined from unpublished RNA sequencing (RNA-seq) data obtained in our laboratory. The expression of specific genes was determined by quantitative (q)PCR on a Roche LightCycler 480 (Roche, Basel, Switzerland) under the following cycling conditions: 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. Primers were designed using Primer-BLAST (NCBI, Bethesda MD, USA). *Translation elongation factor* 2 was used as a reference gene (Tong et al. 2009).

GWAS of fruit weight

For each accession, DNA was extracted from fresh leaves using the CTAB method as previously described (Murray and Thompson 1980). Then all samples were sequenced by Illumina GA2 with a paired-end read of 100 base pairs (bp) after 500 bp inserted size library constructed. One hundred twenty-one gigabytes of data were generated and the average sequencing depth was 4.21×. Sequenced reads were mapped to the reference genome (The International Peach Genome Initiative 2013) using Burrows-Wheeler Aligner software (Li et al. 2009). SAMtools (Li et al. 2009) was then used to detect single nucleotide polymorphisms (SNPs) in the population. Totally, 4,063,377 high-quality SNPs were identified for following analysis. Association analyses were carried out based on a mixed linear model (MLM) using TASSEL v.4.0 software (http://www.maizegenetics.net). A kinship matrix was generated and the first three components from the principal component analysis were used to correct for population structure, which was incorporated into the MLM association model to calculate P values for the association of each SNP marker with fruit weight of each years.

Results

Analysis of peach *EXP* gene expression profiles

The EXP superfamily comprises four distinct families: EXP A and B and EXP-like (EXL)A and B. Members of the first two families are involved in cell wall modification, whereas the exact functions of EXLA and EXLB remain unclear (Dal Santo et al. 2013). A total of 29 *EXP* gene family members have been identified in the peach genome, with at least one *EXP*

gene detected on each of the eight chromosomes (Supplementary Table 2). Based on the sequence analysis, there were 23 *EXP A*, three *EXP B*, one *EXLA*, and two *EXLB* genes.

RNA-seq data for *EXP* genes collected from April 24 to July 8, 2014 representing 30, 45, 60, 75, 90, and 105 days after full bloom of Hakuho peach revealed stage-specific expression patterns during FDP (Fig. 1), which were used to categorize the genes into six broad groups. In group 1, eight genes showed high expression only at stages 5 and 6 (the final phase of FDP). Two *EXPs* whose expression was upregulated during stages 3 and 4 (the middle phase of FDP) and downregulated thereafter were classified into group 5. Most genes in groups 4 and 6 were expressed only at stage 1. There was only one gene in group 3 (ppa017982m); its expression decreased from stage 2 through 6. Eight of the genes were undetectable during development; these were designated as group 2.

GWAS analysis of fruit weight

GWAS have been used to identify QTL in plants (Begum et al. 2015), including *Arabidopsis* (Atwell et al. 2010), rice (Huang et al. 2010), and foxtail millet (Jia et al. 2013). To determine whether peach *EXP* genes are located within fruit weight QTL, the phenotype of 130 varieties and their SNPs, obtained from resequencing data, were used to carry out a GWAS of the fruit weight trait (Supplementary Table 3). Ten QTL regions of fruit weight in 2007 and 2010 with significant P values were identified, respectively. These QTLs distributed in genome from chromosome (chr.) 1–8. And during different years,

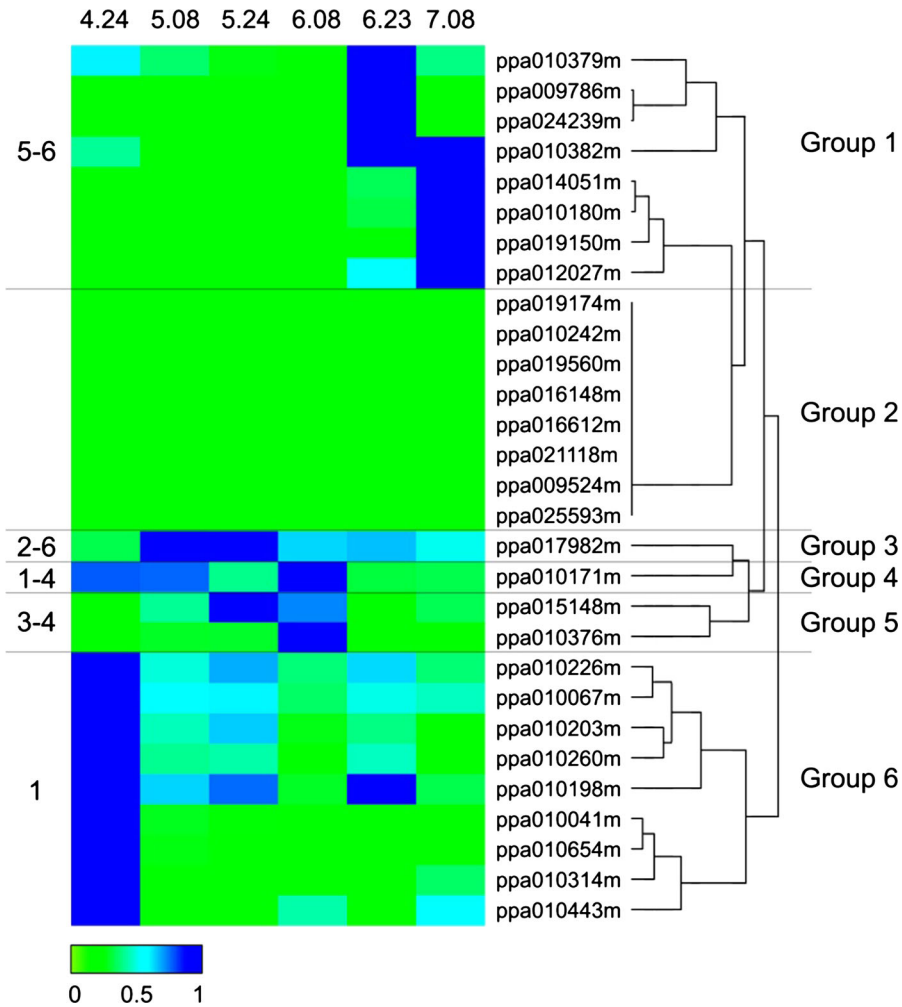

Fig. 1 Expression analysis of *EXP* genes in the peach genome during FDP in Hakuho. *EXP* genes are clustered according to expression patterns over the course of fruit development after normalizing to the maximum expression level of each gene. Six stages of fruit development (1–6 to the left of the figure) are represented. Relative expression levels are represented by different colors from *green* to *blue*. (Color figure online)

the QTLs with most significant P value were all located in chr. 4: 10.9 Mb. At the SNP peak located at about 1 Mb, six *EXP* genes—ppa019150m on chr. 2, ppa015148m on chr. 4, and ppa014051m, ppa017982m, ppa016612m, and ppa010443m on chr. 5—comprised the QTLs for fruit weight. ppa019150m and ppa014051m which had similarly high expression during later development, whereas the other four genes showed distinct expression patterns were classified into group 2, 3, 5, and 6, respectively (Fig. 1).

In addition to a *EXP*, ppa015148 m, descripted previously, 157 genes were predicted to lie within the QTL region around 10.0–11.0 Mb on chr. 4. Among of them, a gene, ppa004744m showed homology to genes encoding E3 ubiquitin-protein ligase. The gene encoding this protein in rice alters the number of cells in the spikelet hull and regulated the rice grain weight (Song et al. 2007), but were not examined further in the present study.

Identification of a candidate fruit weight marker and validation of its expression

We investigated the stage at which fruit weight increases in peach. As for apple (Janssen et al. 2008), peach development can be separated into periods of cell division, cell expansion, maturity, and ripening. We compared the increments in fruit polar and cheek diameters during FDP between two varieties of peaches with different weights, Robin and Sunago Wase (producing small and large fruits, respectively). The increment in fruit polar diameter was high at the first sampling date, reaching 90 % in small fruits and decreasing thereafter (Fig. 2), whereas the increment in large fruits was maximal at the second sampling date—corresponding to the cell expansion stage—and decreased thereafter. A slight increasing was also observed at 5.28–6.05 in varieties with small fruit. Similar trends were observed for fruit cheek diameter.

Although the FDP in Robin and Sunago Wase was shorter than in the Hakuho peach, which was used to study the expression profiles of *EXP* genes, the three varieties flower at almost the same time, such as at April 4th, 6th, and 5th in 2010, respectively. We therefore conjectured that their fruit development curves would be similar, and that a high increment in fruit diameter would also exist in the early developmental stage of Hakuho peach. We then investigated the correlation between the expression levels of peach *EXP* genes and fruit diameter increment. Among the six *EXP* genes, two—ppa017982m and ppa010443m–were highly expressed at stages 1 and 2, suggesting that they are involved in the control of cell expansion and therefore influence final fruit weight. ppa015148m expressed highly during medium term of stages 3 and 4. ppa016612m was not examined during total stages. And two genes, ppa014051m and ppa019150m, showed high expression at stages 5 and 6 (Fig. 1) were also ignored in the following study.

We validated the expression of ppa017982m and ppa010443m by qPCR (Fig. 3), and confirmed that both genes were highly expressed during early stages of FDP and that the level was higher in large than in small fruits. Moreover, the expression of ppa017982m was higher than that of ppa010443m. The finding that these two *EXP* genes are highly expressed during the

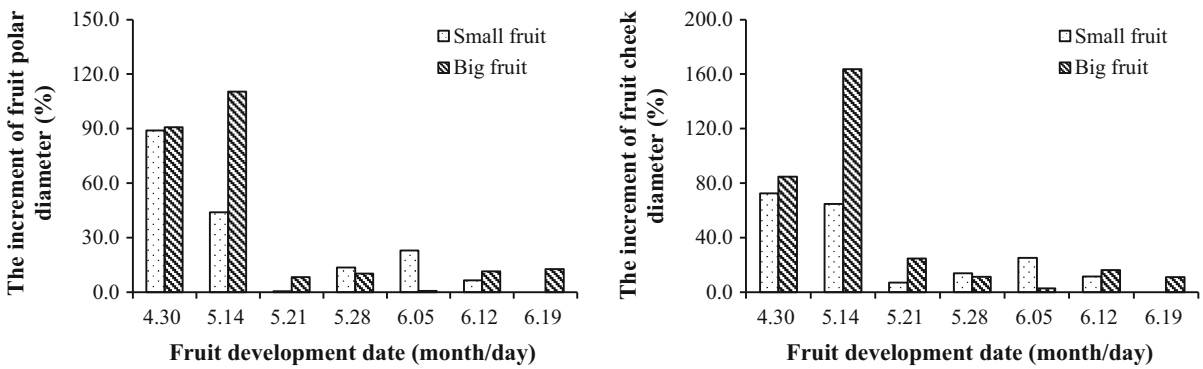

Fig. 2 Increment of fruit polar and cheek diameters during FDP in two peach varieties

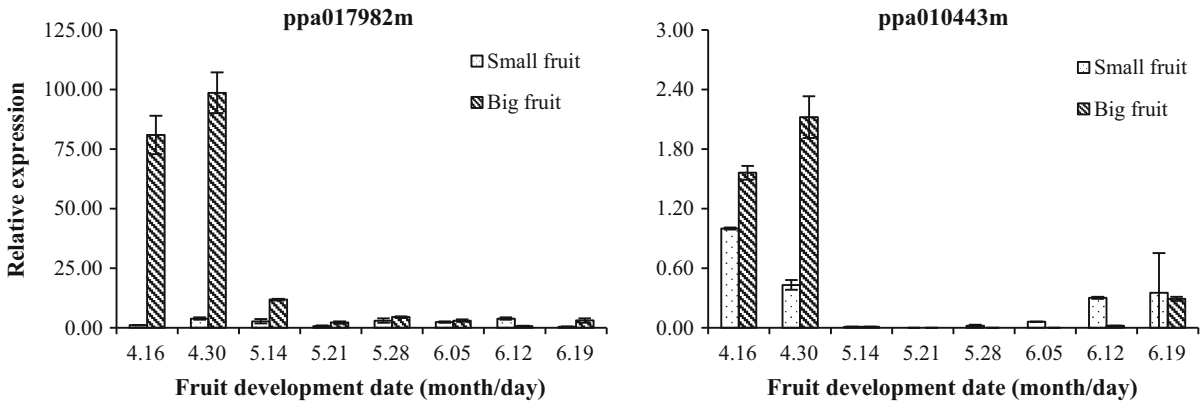

Fig. 3 Expression of two candidate *EXP* genes in different FDPs validated by qPCR. *Bars* indicate standard error of three biological replicates

cell expansion stage suggests that they are likely candidates for fruit weight QTL.

Identification of SNPs associated with fruit weight variation

To identify SNPs associated with fruit weight, the distribution of SNPs in the two candidate genes was analyzed in 30 varieties of peach bearing large fruit ($\geq$150 g) and 30 varieties bearing small fruit ($\leq$70 g) using resequencing data from 130 varieties. A total of 104 SNPs were detected in the ppa017982m promoter (2000 bp upstream of the start codon), exon, and intron sequences at an average density of 33.7 bp between SNPs. For ppa010443m, 58 SNPs at an average density of 64.8 bp between SNPs were identified in the promoter, exonic, and intronic regions. The allelic variation of these genes was evaluated by a χ^2 test for the independence of SNP distribution (Supplementary Tables 4 and 5). The highest χ^2 value was associated with nt 5,026,380 of chr. 5 in ppa017982m ($\chi^2 = 24.6 > \chi^2_{0.05,4} = 9.49$), suggesting that genotype frequencies differ significantly between the two varieties. In the 30 varieties of peach with small fruit, homozygosity of C or G or heterozygosity of C/G at nt 5,026,380 were segregated in a 24: 2:1 ratio, while in the large fruit group, the ratio was 6:18:3. Therefore, we believe that progeny with G/G or C/G genotypes at this locus show the highest fruit weight and that these SNPs can be used to identify varieties that produce larger fruits. We also examined SNPs in peach varieties with moderate fruit weights, and found a ratio of 14:36:13. Another locus was also detected in the same gene at nt 5,025,390 of chr. 5; both loci were located in the promoter region, and may account for the differences in expression levels of this gene between large and small fruit-bearing varieties.

Discussion

Analysis of fruit weight QTL in peach

Studies investigating candidate genes through QTL analysis of fruit weight in peach have identified QTL distributed in LGs 1 (Sosinski et al. 1998; Quilot et al. 2004; Eduardo et al. 2011; Linge et al. 2015), 2 (Quilot et al. 2004; Eduardo et al. 2011; Linge et al. 2015), 3 (Abbott et al. 1997; Yamamoto et al. 2001; Linge et al. 2015), 4 (Quilot et al. 2004; Eduardo et al. 2011; Linge et al. 2015), 5 (Abbott et al. 1997; Quilot et al. 2004; Linge et al. 2015), 6 (Dirlewanger et al. 1999; Yamamoto et al. 2001; Etienne et al. 2002; Eduardo et al. 2011; Linge et al. 2015), and 7 (Quilot et al. 2004; Linge et al. 2015), with none present in LG 8. We found that QTLs for fruit weight were located on total 8 chromosomes in a GWAS based on a panel of resequencing data; some were in regions that had previously been identified as harboring QTL associated with this trait. For example, two QTLs, FW-2007-4 and FW-2010-6, were located approximately 10.8–10.9 Mb away from the top of chr. 4, which is close to the QTL for fruit weight qP-Fw4.1 (10.6 Mb)

that was identified by linkage analysis (Linge et al. 2015). However, some associations were not consistent with the results of other published linkage analyses. One of the stable QTL, FW-2007-2 and FW-2010-2, identified on chr. 2 that was located about 16.6 Mb away from top of the chromosome was far from the linked marker UDP98-406 and SNP_IGA_205001 reported in another two studies (Eduardo et al. 2011; Linge et al. 2015). Other QTL also differed from those previously reported, which may be explained by the different marker systems (Abdurakhmonov et al. 2008) or analytical methods (Thornsberry et al. 2001) that were used. For example, association mapping has several advantages over linkage mapping, including the fact that a potentially large number of alleles per locus—as opposed to only two—can be surveyed simultaneously (Flint-Garcia et al. 2005).

Candidate marker for fruit weight in peach

PpExp3 (ppa010180m) is differentially expressed in the fruit of melting flesh and stony hard flesh cultivars of peach (Hayama et al. 2006). *PpExp1* (ppa010382) and *PpExp2* (ppa010379m) play important roles in peach fruit ripening, but it is unclear whether they regulate changes in fruit firmness during fruit storage (Hayama et al. 2000). In the present study, we also found that ppa010382m, ppa010379m, and ppa010180m had high expression levels during the ripening stage of the melting peach (Hakuho), in agreement with previous studies.

The size potential of fruit is a multi-loci trait in crops (Devoghalaere et al. 2012). Our work indicates that fruit expansion may be partially modulated by two *EXP* genes, ppa017982m and ppa010443m, that are upregulated during the early FDP and colocalize with a QTLs for fruit weight on chr. 5. In addition to these genes, we also investigated genes that control cell number rather than size. A BLAST search of the peach genome using the amino acid sequence of the tomato *FW2.2* gene (XP_004232037) revealed up to 18 gene family members distributed on chr. 1 (10 genes), 2 (three genes), 5 (two genes), 7 (two genes), and 8 (one gene). Importantly, two genes—ppa019431m and ppa010809m—overlapped with fruit weight QTL regions in the GWAS, suggesting a conserved function for *FW2.2* in peach. Therefore, *fw2.2*- or *CNR*-related genes should be more closely examined in future studies.

Major locus for fruit weight and its applications in marker-assisted peach breeding

The QTL in LG 6 is the most important locus in the control of fruit weight in peach, as determined in different populations; it also explains the high incidence of phenotypic variation (Dirlewanger et al. 1999; Yamamoto et al. 2001; Etienne et al. 2002; Eduardo et al. 2011). However, there have been no molecular markers for fruit weight that have been applied to peach breeding, except for some Mendelian trait loci such as flesh texture, fruit hairiness, flesh color, fruit acidity, fruit skin blush, and fruit bacterial spot response (www.rosbreed.org/breeding/dna-tests/peach). We detected an SNP in the promoter of the candidate gene ppa017982m that was associated with fruit weight; the allelic variant with homozygous C showed a high incidence in peach varieties bearing small and moderate-sized fruit. As such, although large fruit varieties cannot be directly selected, accessions with small and moderate-sized fruits can be removed in seedlings.

Conclusions

The *EXP* gene has been implicated in many biological processes associated with fruit growth and development, such as the determination of fruit size through cell expansion and the control of fruit ripening. We report here the expression patterns of different *EXP* genes in the peach genome over the course of fruit development. The SNP distribution in two of the genes—which map to a fruit weight-related QTL—was compared between varieties that differ in terms of fruit weight. In summary, the expression and sequence data suggest that two *EXP* genes, ppa017982m and ppa010443m, are excellent candidate markers for fruit weight QTL on chr. 5 of peach. This finding reveals a genetic basis of fruit weight regulation and provides molecular markers that can be useful for improving peach production.

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