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ORIGINAL PAPER

Identification of QTLs Associated with Conversion of Sucrose to Hexose in Mature Fruit of Japanese Pear

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Abstract

Sweetness is the most important trait for fruit breeding and is fundamentally determined by both total and individual sugar contents. We analyzed the contents of sucrose, fructose, glucose, and sorbitol in mature fruit in an F_1 population derived from crossing modern Japanese pear cultivar 'Akizuki' and breeding line '373-55'. A genetic linkage map was constructed using simple sequence repeats (SSRs) and single-nucleotide polymorphisms (SNP). We identified two regions associated with individual sugar contents on linkage group (LG) 1 and LG 7. The percentages of the variance in sucrose, fructose, and glucose explained by the quantitative trait loci (QTLs) were 26.6, 15.9, and 18.5%, respectively, for the region on LG 1, and 22.2, 20.0, and 9.5%, respectively, for the region on LG 7. In both regions, genotypes associated with increases in sucrose were associated with decreases in both fructose and glucose. The 1.5-LOD support intervals of the QTLs on LGs 1 and 7 include SSRs within the regions flanking acid invertase genes *PPAIV3* and *PPAIV1*, respectively. Because acid invertase is a key enzyme in the conversion of sucrose to hexose, these are promising candidates for genes underlying those QTLs and controlling individual sugar contents. We also found one region on LG 11 that explained 21.4% of the variation in total sugar content but was not significantly associated with variation for individual sugars. The information obtained in this study will accelerate research and breeding programs to improve fruit sweetness.

Keywords *Pyrus pyrifolia* · Fruit quality · Sugars · Acid invertase

Introduction

Sweetness is one of the most important factors that determine fruit quality (Kanayama 2017; Ozaki et al. 2009). The major sugars in mature Rosaceae fruits are sucrose, fructose, glucose, and sorbitol. These sugars have different levels of sweetness: if sucrose is rated 1, then fructose is 1.50–1.75, glucose is 0.70–0.80, and sorbitol is 0.55–0.70 (Doty 1976; Pancoast and Junk 1980; Pangborn 1963). In the Rosaceae, sorbitol plays an important role in carbohydrate translocation from sink to source and is converted to fructose and glucose by sorbitol dehydrogenase in fruit (Fig. 1). The sucrose is synthesized from sucrose 6-phosphate by sucrose-phosphate synthase (SPS) and is converted to glucose and fructose by invertase. In addition, sucrose synthase (SUS) catalyzes the reversible conversion of sucrose and uridine diphosphate (UDP) to UDP-glucose and fructose. These enzymes play a critical role in sugar metabolism during fruit development (Kliewer 1966; Moriguchi et al. 1990a, 1990b, 1992; Yamaki 2010; Yamaki and Moriguchi 1989).

Pears (*Pyrus* spp.) belong to the subtribe Pyrinae of the Rosaceae and are one of the most important fruit crops in temperate regions. There are three major species in Asia: *P. pyrifolia* (Burm. f.) Nakai, which is cultivated in Japan, Korea, and China; *P. bretschneideri* Rehder, which is cultivated in China; and *P. ussuriensis* Maxim, a wild species distributed in East Asia. However, these species are thought to be genetically continuous (Iketani et al. 2010, 2012; Katayama et

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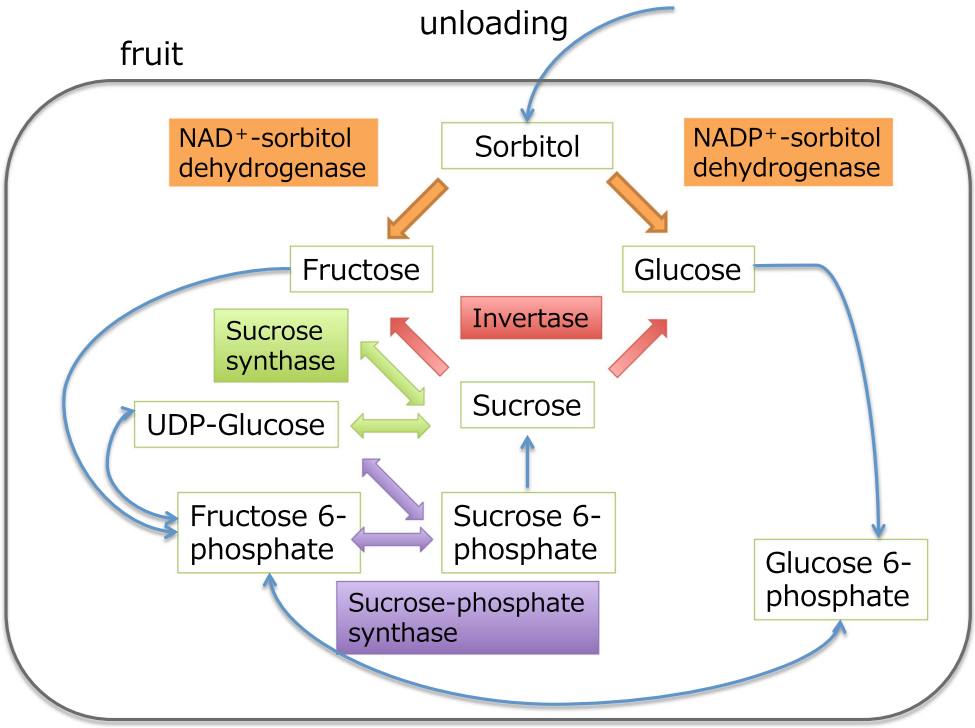

Fig. 1 Sugar metabolism and key enzymes in fruit

al. 2007; Kikuchi 1948). One of the features of this group of species is the large variation in individual sugar contents in mature fruit. The Japanese cultivar 'Nijisseiki' (*P. pyrifolia*), which is a precursor of modern Japanese pear cultivars, and current major cultivars in Japan have sucrose-dominated fruit, whereas Chinese pear cultivars (*P. bretschneideri* and *P. ussuriensis*) and some local cultivars in Japan (*P. pyrifolia*) accumulate very little sucrose (Kajiura et al. 1979; Moriguchi et al. 1992). Recently, the new cultivar 'Kanta', which has high total sugar content (both high sucrose and high fructose) has been released and is promising to become a leading cultivar because of its sweetness. On the other hand, cultivar collections of apple and peach, which are other economically important fruits in the Rosaceae, have less variation in individual sugar contents than in pear. Fructose is dominant in most apple cultivars (Hecke et al. 2006; Wu et al. 2007), while sucrose is dominant in most peach cultivars (Moriguchi et al. 1990a; Byrne et al. 1991). For these reasons, pear is the optimal material with which to clarify the mechanisms and genetic control of individual sugar determination in mature fruit.

Whole-genome sequences have been obtained for several members of the Rosaceae, including apple, peach, strawberry, and pear (Shulaev et al. 2011; Velasco et al. 2010; Verde et al. 2013; Wu et al. 2013). A draft genome sequence of pear was determined using a combination of bacterial artificial chromosome (BAC)-by-BAC and next-generation sequencing (Wu et al. 2013). The assembled genome consists of 2103 scaffolds with an N50 of 540.8 kb, totaling 512.0 Mb with 194× coverage, close to the estimated size of 527.0 Mb. However, this draft sequence is not completely anchored to chromosomes. On the other hand, for apple, a de novo assembly of a 'Golden Delicious' doubled-haploid tree (GDDH13) composed of 280 assembled scaffolds and arranged into 17 pseudo-molecules (Daccord et al. 2017), which makes it possible to predict accurate gene positions. Because of the high collinearity between *Pyrus* and *Malus* genome sequences ($2n = 34$), it is possible to use advanced apple genome information for pear genetic studies.

QTLs for soluble solid concentration (SSC, °Brix) have been mapped in a large number of fruit tree species (e.g., Cirilli et al. 2016). But the QTLs for SSC are complex and fluctuate from year to year (Yamamoto et al. 2014; Zhang et al. 2013). QTLs are sometimes detected on different linkage groups (LGs) in different populations even in the same species (Kenis et al. 2008; Liebhard et al. 2003). Several studies of QTLs for accumulation of individual sugars have been conducted in apple (Guan et al. 2015; Kunihisa et al. 2014), peach (Cirilli et al. 2016; Dirlewanger et al. 1999; Etienne et al. 2002; Quilot et al. 2004; Salazar et al. 2014), and grape (Chen et al. Chen et al. 2015a, Chen et al. 2015b), but candidate genes were not discussed in most of these studies. At present, the position of gene families related to sugar metabolism can be predicted from whole-genome sequence data (Hyun et al. 2011; Li et al. 2012), so it will be important to check the association between QTL peaks and candidate genes.

Several useful DNA markers have been developed and applied to Japanese pear breeding; these include S_4^{sm}-haplotype-specific DNA markers to identify self-compatibility (Ishimizu et al. 1999; Okada et al. 2008), a molecular marker associated with the pear scab resistance gene *Vnk* (Terakami et

al. 2006), one associated with resistance to black spot disease (Terakami et al. 2007), and two associated with fruit ripening day (Iwata et al. 2013; Nishio et al. 2016a; Yamamoto et al. 2014). In addition, QTL analyses, genome-wide association studies (GWAS), and genomic selection (GS) have been carried out to increase the °Brix content in pear fruit (Iwata et al. 2013; Yamamoto et al. 2014; Zhang et al. 2013). So far, however, there are no reliable molecular markers for sugar accumulation that can be applied in pear breeding programs. Kajiura et al. (1979) suggested that the total sugar content fluctuates depending on year but that phenotypic values of individual sugars are more stable than that of total sugar. Thus, QTLs for individual sugars might be found even if QTLs for total sugar content are difficult to detect. The objective of this study was to identify QTLs for individual sugars in pear fruit and to provide useful information for pear breeding programs.

Materials and Methods

Plant Materials and Extraction of Nucleic Acids

A population derived from a cross between modern Japanese pear cultivar 'Akizuki' and breeding line '373-55' (123 F_1 seedlings) was used for constructing genetic maps and performing QTL analyses. 'Akizuki' was released in 1998 and had become one of the leading cultivars in Japan (Kotobuki et al. 2002), while '373-55' was an early-ripening selection from a population derived from 'Chikusui' and 'Tsukuba 43'. Both of these cultivars are derived from local cultivar 'Nijisseiki' and are the product of five generations of crossing in the NIFTS pear breeding program. The seeds were sown in the fall of 2009 and the seedlings were grown with cultural techniques used in commercial production in Japan (Tamura 2006). The trees were trained on horizontal trellises, pruned annually in winter, and treated for pests and diseases. Fruits were thinned to one fruit per three fruit clusters in mid-May and harvested during late July to September according to a color chart that indicates the optimum color for picking Japanese pear (Kajiura et al. 1975). Genomic DNA was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Evaluation of Individual Sugar Contents

Out of the 123 F_1 seedlings, 106 were fruited and evaluated. We analyzed the contents of sucrose (SUC), fructose (FRU), glucose (GLU), sorbitol (SOR), and total sugars (TSC) in the fruit of each F_1 tree. To do this, we extracted and combined the juice from two fruits per sampling date and stored each sample at − 80 °C until analysis. For each genotype, sampling was performed on 2 days in each of 2 years (2014 and 2015). For the measurement of sugar components, we diluted the juice 1:10 with distilled water and added mannitol as an internal standard (at 0.3 mg/mL final concentration). We purified the solution using a Torast Disc (GLCTD-MCE1345; Shimadzu, Kyoto, Japan) and measured SUC, FRU, GLU, and SOR by high-performance liquid chromatography (HPLC) on a Shodex Sugar SP0810 column fitted with an SP-G guard column (Showa Denko, Tokyo, Japan), which was kept at 80 °C and eluted with water at a flow rate of 0.8 ml/min. The average of the two replicates per year averaged over the 2 years was used as the phenotypic data for each trait. TSC was calculated by summing the contents of the four sugars. The Kolmogorov–Smirnov test was used to check the normality of the data distribution for each trait, with $P > 0.05$ indicating a normal distribution. The phenotypic correlation coefficients and their significance were calculated for all trait combinations in SAS JMP v. 9.0.2 software (SAS Institute, Cary, NC, USA).

Simple Sequence Repeat Genotyping

Previously developed simple sequence repeats (SSRs) from apple and pear (Celton et al. 2009; Fernández-Fernández et al. 2006; Guilford et al. 1997; Liebhard et al. 2002; Moriya et al. 2012; Nishitani et al. 2009; Silfverberg-Dilworth et al. 2006; van Dyk et al. 2010; Yamamoto et al. 2002) and three SSRs designed from regions flanking acid invertase (AIV) genes were used for genotyping. In the apple genome, there are presumed to be three *AIV* (also called *VIN*) genes: *MdVIN1* (MDP0000149570), *MdVIN2* (MDP0000377084), and *MdVIN3* (MDP0000124776; Hyun et al. 2011). The scaffolds containing *AIV* genes in the pear genome were obtained from these three gene sequences by BLASTN against the NCBI nucleotide collection (NW_008988257 for *PPAIV1*, NW_008988773 for *PPAIV2*, and NW_008988286 for *PPAIV3*). SSRs located in the regions flanking *AIV* genes in pear were found by using MISA software (http://pgrc.ipk-gatersleben.de/misa/). We designed primer pairs for SSRs near each of the *PpAIVs*. We selected SSRs having good amplification and clear band patterns from among the SSRs near each of the AIVs and named them PPAIV1_near_SSR_sca231, PPAIV2_near_SSR_sca739, and PPAIV3_near_SSR_sca260. The forward (F) and reverse (R) primers used for the *PpAIVs* were as follows: PPAIV1_near_SSR_sca231 (F, CCTCTTGTGGTGCCGACTAT; R, TTGAATCAATCAGC AAGACCAT), PPAIV2_near_SSR_sca739 (F, GAGACATA TCCCGAGGACGA; R, GTCTCCGCAGCATCACATAA), and PPAIV3_near_SSR_sca260 (F, ACCGAAACCCTAAT CAACCC; R, GCCACTGGCATAGAGACCA).

PCR amplification was performed in 10-μL reactions containing 5 μL of 2× Green GoTaq reaction buffer (0.4 mM each dNTP, Taq DNA polymerase, and 3 mM $MgCl_2$, pH 8.5;

Promega, Madison, WI, USA), 20 pmol each of forward primer labeled with a fluorescent chemical (5-FAM or 5-HEX) and unlabeled reverse primer, and 2.5 ng of genomic DNA. Amplification was performed in 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. PCR products were separated and detected in a 3130xl Genetic Analyzer (Life Technologies Co., Carlsbad, CA, USA). The size of each amplified band was determined by comparison with a set of internal standard DNA fragments (400HD-ROX, Life Technologies Co.) in GeneMapper v. 5.0 software (Life Technologies Co.).

Next-Generation Sequencing, Mapping, and SNP Design

We sequenced seven Japanese pear cultivars ('Akizuki', 'Choujuurou', 'Doitsu', 'Hosui', 'Niitaka', 'Nijisseiki', and 'Oushuu') by next-generation sequencing analysis. Genomic DNA of each cultivar was extracted from a young leaf using a DNeasy Plant Mini Kit and used to construct a DNA library. Paired-end sequencing with an insert size of 500 bp was performed in a HiSeq 2000 sequencer (Illumina, Inc., San Diego, CA, USA) using 1 μg of genomic DNA for each sample.

Raw reads were trimmed to 90 bp. The trimmed reads were processed by the following two steps for quality control. First, low-quality bases with a quality score of < 20 were trimmed by self-made script from both ends of the generated reads. Second, the adapter sequences were removed by Cutadapt software (Martin 2011; options: -f fastq -e 0.1 -O 5 -m 20). The processed reads of each cultivar were mapped to the Chinese pear 'Dangshansuli' genome by BWA 0.7.5a (Li and Durbin 2009; option: aln -l 32 -R 30). The mapping results were processed by SAMtools-0.1.19 (Li 2011; option: view -S -b -f 2 -F 12 -q 20 -h) to exclude low-quality mapped reads. Local realignments were performed to correct misalignments by Genome Analysis TK Lite 2.3-9 (McKenna et al. 2010; option: -T IndelRealigner), and PCR duplicates were removed by Picard tools 1.92 (http://broadinstitute.github.io/picard;option: MarkDuplicates.jarREMOVE_DUPLICATE=true).

The SNPs of each cultivar were called by both SAMtools 0.1.19 (option: mpileup -C 50 -DSg -f) and Genome Analysis TK Lite 2.3-9 (option: -T Unified Genotyper -I—genotype_likelihoods_model BOTH), and those with a read depth of 4–150× were used for further analyses.

To design reliable SNP markers, we discarded SNPs meeting any of the following criteria: (1) Other SNPs or indels were detected within the 80-bp flanking regions on either side of the SNP. (2) The 80-bp flanking sequences were aligned to two or more regions in the reference genome of Chinese pear by BLASTN search. (3) Either of the 80-bp flanking regions included a repeat sequence. (4) The read depth was extremely low or high (< 13 or > 56). Among the selected SNPs, those that were unsuitable for probe design because they had a SNP score under 0.7 calculated by the Illumina Assay Design Tool were discarded. The final 1536 SNPs were selected from different scaffolds (Supp. Table 1).

SNP Genotyping

SNPs were genotyped using the Illumina GoldenGate Genotyping Assay (Illumina Inc.). The scanned data were analyzed by the Genotyping module (v. 1.9.4) of Illumina GenomeStudio (v. 2011.1) software to generate genotype data for individuals. Clustering of SNPs was adjusted by eye when necessary. Acceptable SNPs had scores of "GenTrain score" ≥ 0.4, "call freq" ≥ 0.85, "P-P-C errors" ≥ 2, and "minor freq" ≥ 0.01.

Construction of Genetic Linkage Maps

JoinMap v. 4.1 software (Van Ooijen 2006) was used to construct maps of 'Akizuki' and '373-55' with the pseudo-testcross mapping strategy using the BC_1 mode (Grattapaglia and Sederoff 1994). The LOD threshold for mapping was set at 3.0, and the recombination frequency at 0.45. The marker configurations ab × cd, lm × ll, and ef × eg (defined in JoinMap) were used for the maternal map ('Akizuki'), and configurations ab × cd, nn × np, and ef × eg were used for the paternal map ('373-55'). Within each parent, the members of each pair of heterozygous alleles were randomly designated "A" and "H." Since the linkage phase of each marker locus (relative to other loci) is unknown in the pseudo-testcross model, the dataset for each parent was duplicated and the allelic designations were reversed (i.e., those previously designated as A were designated H, and vice versa). After the linkage phase of each marker was inferred and the pairs of linkage groups were deduced, one linkage group from each pair was chosen to represent the linkage group of that parent.

We constructed the integrated map of 'Akizuki' and '373-55' in cross-pollination (CP) mode, using all markers that showed polymorphism in at least one parent. The markers were grouped with a minimum LOD score of 4.0 and a recombination frequency of 0.45. The regression mapping algorithm was used to build the linkage maps, and map distances were calculated according to the Kosambi mapping function (Kosambi 1944). The linkage group names were assigned according to the previously published Japanese pear genetic linkage maps (Yamamoto et al. 2014). The genetic maps were drawn using MapChart ver. 2.2 (Voorrips 2002).

QTL Analyses

MapQTL v. 6.0 software (Van Ooijen 2009) was used for interval mapping. A genome-wide LOD significance threshold was determined for each trait on the basis of a permutation test with 1000 replications, and then QTLs with an LOD that was significant at $P < 0.05$ were identified. We tested individual

scores from 2014 and 2015 and the averages of the 2014 and 2015 scores. The results were almost the same using any of the three scores, so we used the 2014–2015 average values for each individual sugar and TSC to simplify the analyses. QTLs were mapped on each of the two parental maps and the integrated map, and the results were visually compared.

Results

Phenotypic Distribution of Individual Sugars

The contents of SUC, FRU, GLU, SOR, and TSC in 'Akizuki' were 60.2, 39.8, 5.8, 15.8, and 121.6 mg/ml, respectively, while those in '373-55' were 26.9, 41.8, 13.3, 37.3, and 119.3 mg/ml, respectively. In the F_1 population, SUC showed a wide range of phenotypic variation (7.0–80.2 mg/ml, mean 43.0 mg/ml; Supp. Fig. 1). The distributions of FRU, GLU, and SOR were 20.8–61.7 (mean 40.5), 0–34.4 (mean 15.0), and 15.2–42.6 (mean 26.7), respectively. On the other hand, the distribution of TSC was 107.7–141.9 (mean 125.3), a much narrower range than that of SUC. In the fruit from each F_1 tree, the most prevalent sugar was either SUC or FRU. We calculated the phenotypic correlation coefficients and their significances for all trait combinations (Table 1). SUC had large negative correlations with both FRU and GLU ($r = -0.83$ and -0.84, respectively), and FRU had a large positive correlation with GLU ($r = 0.67$). SOR was correlated positively with GLU but negatively with SUC ($r = 0.31$ and -0.29, respectively). TSC was correlated positively with both SUC and SOR ($r = 0.28$ and 0.50, respectively). The distributions of these traits were not significant at $P = 0.05$ by the one-sample Kolmogorov–Smirnov test, indicating that they were normal distributions.

Genetic Linkage Maps

We constructed genetic linkage maps of 'Akizuki' and '373-55' and an integrated map of 'Akizuki' and '373-55' using SSRs and SNPs (Table 2; Supp. Fig. 2). The total numbers of loci were 296, 268, and 725 for the maps of 'Akizuki', '373-55', and the integrated map, respectively. The three maps contain 18, 16, and 18 linkage groups, respectively. LG 7 of the integrated map and LGs 11 and 15 of 'Akizuki' were each divided into two subgroups (CP.7a and CP.7b, Akizuki.11a and Akizuki.11b, and Akizuki.15a and Akizuki.15b, respectively). No markers were mapped on LG 12 of 'Akizuki' or LG 13 of '373-55', so these LGs could not be constructed. The total lengths of the maps of 'Akizuki' and '373-55' (945.0 and 865.1, respectively) were much smaller than that of integrated map (1446.6). Because the inbreeding coefficients of the parents are high (0.15 for 'Akizuki' and 0.21 for '373-55'), each parental map has homozygous regions in which no markers were mapped. The average distance between markers in the integrated map is 2.0 cM, while those of the 'Akizuki' and '373-55' maps are 4.9 and 5.4 cM, respectively.

Table 1 Correlation coefficients calculated for each pair of traits

	SUC	FRU	GLU	SOR	TSC
SUC	1				
FRU	−0.83***	1			
GLU	−0.84***	0.67***	1		
SOR	−0.29**	0.06	0.31**	1	
TSC	0.28**	−0.06	0.05	0.50***	1

SUC sucrose, *FRU* fructose, *GLU* glucose, *SOR* sorbitol, *TSC* total sugar content

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Identification of QTLs for Individual Sugars and TSC

By comparing the locations of QTLs on each of the three maps, we identified two important regions associated with individual sugar content on LG 1 and LG 7 and one region that increases TSC on LG 11. Those regions were named QTL-sugar-metabolism-1, QTL-sugar-metabolism-7, and QTL-sugar-increase-11, respectively (Table 3). Regions with significant effects on SUC, GLU, and SOR were identified at the bottom of LG CP.1, and regions with significant effects on SUC, FRU, GLU, and SOR were identified at the bottom of LG Akizuki.1 (Supp. Fig. 3). Regions with significant effects on SUC and FRU were located at the bottom of LGs CP.7b and Akizuki.7. QTL-sugar-increase-11 had a significant effect on TSC only; however, the regions showing significant LOD values differed between LGs CP.11 and 373-55.11 (top of LG CP.11 vs. bottom of LG 373-55.11).

To compare the QTLs within each of the three regions, we identified the positions where QTLs for SUC and TSC showed the highest LOD values on the integrated map and then summarized the LOD values, allele effects, and percentage of variance explained at these positions (Table 3). For QTL-sugar-metabolism-1, the percentages of variance in SUC, FRU, GLU, and SOR explained by the QTL were all high (26.6, 15.9, 18.5, and 19.5%, respectively; Table 3). Although the LOD score for FRU on the integrated map was not significant, the percentage variance explained by the QTL was relatively high (15.9%), and a significant effect was detected in "Akizuki" (Supp. Fig. 3), so we included FRU in our examination of QTL-sugar-metabolism-1. In this region, genotypes associated with increases in SUC were associated with decreases in FRU, GLU, and SOR. The average value of SUC (mg/ml) was highest for the "ad" genotype (52.2) and lowest for the "bc" genotype (30.3), while those of FRU and GLU (mg/ml) were lowest for "ad" (37.2 and 11.9, respectively) and highest for "bc" (45.8 and 19.0, respectively).

Table 2 Details of the genetic linkage maps of 'Akizuki' and '373-55' and the integrated map of 'Akizuki' and '373-55'

	Linkage group																	
	1	2	3	4	5	6	7[a]	8	9	10	11[b]	12	13	14	15[b]	16	17	Total
No. of mapped loci																		
Akizuki	22	34	43	4	21	5	11	11	14	22	15		21	22	21	18	12	296
373-55	12	7	8	25	3	25	21	17	5	21	15	40		9	29	4	27	268
Integrated map	54	56	65	40	28	39	39	44	23	48	42	50	26	32	64	18	57	725
Length of linkage groups																		
Akizuki	61.4	73.2	77.3	4.1	79.4	35.7	72.4	44.1	60.9	73.0	38.5		82.1	54.8	68.2	47.5	72.6	945.0
373-55	86.4	23.1	32.2	56.2	10.8	68.1	101.8	63.3	23.7	27.5	84.6	74.8		38.1	92.0	2.5	79.8	865.1
Integrated map	83.1	87.2	82.1	98.3	79.5	84.4	105.9	72.8	60.9	75.8	91.1	86.0	82.9	53.5	157.5	47.7	97.9	1446.6

[a] Linkage group 7 was divided into two subgroups (7a and 7b) in the integrated map. The number of mapped loci and length of LG 7 in the integrated map was estimated by combining the data for the two subgroups

[b] Linkage groups 11 and 15 were each divided into two subgroups in the map of 'Akizuki'. For each of these linkage groups, the number of mapped loci and length of linkage groups were estimated by combining the data for the two corresponding subgroups

Also, allele "a" was associated with higher values of SUC than allele "b" (47.0 and 52.2 for "a"-containing genotypes vs. 30.3and 40.8 for "b"-containing genotypes) but with lower values of SOR (24.7 and 24.2 for "a"-containing genotypes vs. 28.7and 30.4 for "b"-containing genotypes). Surprisingly, this region had little effect on TSC.

For QTL-sugar-metabolism-7, the percentages of variance in SUC and FRU explained by the QTL were high (22.2 and 20.0%; Table 3), while those for other individual sugars and TSC were less than 10%. When we compared the average values of each genotype, we found a negative association between SUC and both FRU and GLU, and a positive association between FRU and GLU. The average value of SUC (mg/ml) for genotype "ad" (56.2) was much higher than those for "ac," "bc," and "bd" (41.4, 39.7, and 34.6, respectively), while the average values of FRU and GLU for genotype "ad" (34.9 and

Table 3 Summary of QTLs showing significant LOD scores for SUC and TSC from the integrated map of 'Akizuki' and '373-55'. The LOD and allele effects of these markers on other sugar traits are also listed

Name	Group	Position	Nearest marker	Trait	LOD[a]	μ_ac[b]	μ_ad[b]	μ_bc[b]	μ_bd[b]	% Expl.[c]
QTL-sugar-metabolism-1	LG 1	48.8	TsuGNH250	SUC	7.13*	47.0	52.2	30.3	40.8	26.6
				FRU	3.98	39.1	37.2	45.8	40.9	15.9
				GLU	4.72*	12.8	11.9	19.0	17.0	18.5
				SOR	5.00*	24.7	24.2	28.7	30.4	19.5
				TSC	1.83	123.5	125.5	123.7	129.1	7.7
QTL-sugar-metabolism-7	LG 7	77.2	TsuGNH159	SUC	5.78*	41.4	56.2	39.7	34.6	22.2
				FRU	5.14*	40.8	34.9	41.1	45.9	20.0
				GLU	2.29	16.9	11.2	15.5	16.1	9.5
				SOR	1.38	26.4	24.5	28.1	27.8	5.8
				TSC	0.44	125.5	126.9	124.4	124.3	1.9
QTL-sugar-increase-11	LG 11	23.6	sca114.0_432636	SUC	0.96	42.8	38.1	42.6	51.0	4.1
				FRU	0.44	39.7	42.5	40.8	38.3	1.9
				GLU	0.40	16.4	15.3	15.2	12.9	1.7
				SOR	3.34	26.7	23.8	30.9	26.8	13.5
				TSC	5.55*	125.6	119.8	129.6	128.9	21.4

SUC sucrose, *FRU* fructose, *GLU* glucose, *SOR* sorbitol, *TSC* total sugar content

[a] LOD scores showing significance at $P < 0.05$ are indicated by asterisks

[b] μ_ac, μ_ad, μ_bc, and μ_bd are the estimated means (mg/ml) of the distributions of the quantitative trait associated with the "ac," "ad," "bc," and "bd" genotypes, respectively, as defined in JoinMap v. 4.1. These represent the four possible F1 progeny genotypes from two parents each heterozygous for a different pair of alleles

[c] % Expl. indicates the percentage of variance explained by the QTL

11.2, respectively) were lower than those for "ac," "bc," and "bd" (40.8, 41.1, and 45.9, respectively, for FRU, and 16.9, 15.5, and 16.1, respectively, for GLU). As with QTL-sugar-metabolism-1, QTL-sugar-metabolism-7 had little effect on TSC.

For QTL-sugar-increase-11, the variance in TSC explained was 21.4%, and the average value for "ad" (119.8) was lower than those for "ac," "bc," and "bd" (125.6, 129.6, and 128.9, respectively; Table 3). The LOD value for SOR was not significant, but the percentage of variance explained by the QTL was 13.5%, and there was a positive association between TSC and SOR based on the average values for each genotype. On the other hand, this region had little effect on SUC, FRU, and GLU.

Discussion

We constructed genetic linkage maps using the modern Japanese pear cultivar 'Akizuki' and the breeding line '373-55'. Before our study, several other genetic maps of pear had been constructed (Chen et al. Chen et al. 2015a, Chen et al. 2015b; Dondini et al. 2005; Terakami et al. 2009, 2014; Yamamoto et al. 2007; Zhang et al. 2013). Their total lengths ranged from 799.1 cM (Akiakari; Yamamoto et al. 2014) to 2243.4 cM (integrated map of 'Bayuehong' and 'Dangshansuli'; Wu et al. 2014). The length of our integrated map is 1446.6 cM, which is longer than the maps constructed in previous studies except for those reported by Wu et al. (2014) and Chen et al. (Chen et al. 2015a; Chen et al. 2015b). The average distance between adjacent markers is 2.0 cM in our integrated map, and the marker order on each LG is similar to those reported in previous studies. For these reasons, we judge our map to be reliable, even though the population was derived from modern cultivars and has a narrow genetic base.

The total lengths of the 'Akizuki' and '373-55' maps were smaller than that of the integrated map (945.0, 865.1, and 1446.6 cM, respectively). The inbreeding coefficients are 0.15 for 'Akizuki' and 0.21 for '373-55'. While the molecular markers showing polymorphism in either of the parents could be mapped on the integrated map, it is impossible to map molecular markers within a region that is homozygous in one of the parents onto the corresponding single-parent map. In fact, we found homozygous regions on LGs 4, 6, 8, and 12 in 'Akizuki' and on LGs 2, 3, 4, 5, 9, 10, 13, and 16 in '373-55' (Supp. Fig. 2). In particular, no markers were mapped on LG 12 of 'Akizuki' or LG 13 of '373-55', suggesting that these chromosomes are completely homozygous in the respective parents. It was previously reported that certain genomic regions of LGs 4, 5, and 12 of 'Hosui' and of LGs 4, 5, 6, and 13 of 'Akiakari' were homozygous (Terakami et al. 2009; Yamamoto et al. 2014). Together, these results suggest that there are a number of common homozygous regions in modern Japanese pear cultivars (e.g., on LGs 4, 5, 6, 12, and 13). Since Japanese pear breeding programs have been conducted to improve fruit quality by focusing on flesh texture (Saito 2016), genes controlling fruit quality are likely to be found in these regions.

We identified two regions associated with individual sugar contents (QTL-sugar-metabolism-1 and QTL-sugar-metabolism-7) and one region that increases TSC (QTL-sugar-increase-11). In a previous study, QTLs for SUC, FRU, GLU, and SOR in apple were co-located at the bottom of LG 1 (Guan et al. 2015), almost the same region where we identified QTL-sugar-metabolism-1. As in our population, SUC was negatively correlated with GLU (Guan et al. 2015). These results suggest that the same gene underlies both QTL-sugar-metabolism-1 and the QTL on LG 1 identified by Guan et al. (2015). QTLs associated with sugar metabolism have been reported in peach on LGs 4, 5, and 7 (Cirilli et al. 2016; Dirlewanger et al. 1999; Quilot et al. 2004). However, the effects of these QTLs were not very large, and these regions do not correspond to LG 1 and LG 7 of pear by ancestral genome comparison (Illa et al. 2011; Jung et al. 2012). QTLs associated with TSC or °Brix content have been identified in apple on LGs 2, 3, 6, 8, 9, 10, 14, and 16 (Kenis et al. 2008; Kunihisa et al. 2014; Liebhard et al. 2003) and in pear on LGs 2, 4, 5, 6, 8, 10, and 14 (Wu et al. 2014; Yamamoto et al. 2014; Zhang et al. 2013). The QTL associated with TSC on LG 11 in the present study was not identified in any previous study; thus, QTL-sugar-increase-11 is a newly identified QTL. TSC was correlated positively with SOR in this mapping population, and the percentages of variance in SOR and TSC explained by the QTL were high for this region (13.5 and 21.4%, respectively; Table 3). Thus, a gene controlling this QTL might be related to sorbitol translocation. However, additional information is required before we apply this QTL in pear breeding programs because there is a large gap at this QTL peak on the integrated map and because the LOD peaks on LG 11 were found in different regions of the integrated map and the '373-55' map. Thus, it is necessary to validate this QTL in another population or cultivar collection.

We searched for candidate genes for QTL-sugar-metabolism-1 and QTL-sugar-metabolism-7 using apple genome information. Previous studies of sugar metabolism during fruit development (Moriguchi et al. 1990b, 1992; Yamaki and Moriguchi 1989) indicate that invertase family members, including vacuole invertase (AIV), cell-wall-bound invertase (CIN), and neutral/alkaline invertase (NIN), as well as sucrose synthesis genes SUS and SPS, are involved in sucrose and hexose metabolism. The apple whole-genome sequence has genes for 3 AIVs, 3 CINs, 12 NINs, 5 SUSs, and 6 SPSs (Li et al. 2012). Among them, only *PPAIV3* and *PPAIV1* were located near the QTLs on LGs 1 and 7. We mapped SSRs designed from regions flanking these genes (PPAIV3_near_SSR_sca260 and PPAIV1_near_SSR_sca231) and confirmed that they are located within the 1.5-LOD support intervals of QTL-sugar-metabolism-1 and QTL-sugar-

metabolism-7, respectively. While QTL-sugar-metabolism-1 was significantly associated with both GLU and SOR (Table 3), QTL-sugar-metabolism-7 was not. Thus, other genes related to sugar metabolism or transcription factors might also underlie these QTLs. However, these AIVs are promising candidate genes for these QTLs because of the strong negative association between sucrose and hexose contents when genotypes at each locus were compared.

Moriguchi et al. (1992) suggested that SUS and SPS were key to regulating sucrose accumulation in fruit and that AIV activity was not absolutely required to determine the individual sugar composition of pear cultivars. However, their materials were different from ours. Whereas we used a modern cultivar and a breeding line, their materials included not only leading cultivars but also local Japanese cultivars (*P. pyrifolia*) and Chinese pear cultivars (*P. bretschneideri*). Bayesian structure analysis revealed that modern Japanese cultivars are genetically different from local cultivars and Chinese pear cultivars (Nishio et al. 2016b). It is possible that modern Japanese pear cultivars had been selected for high activity of SPS and SUS and that the sucrose content among modern cultivars may be controlled by AIVs or other enzymes associated with sucrose metabolism.

With respect to the application of QTLs to developing new cultivars with high sweetness, there are two fundamental ways to improve the sweetness of fruit. One is to increase TSC and the other is to convert GLU and SOR (low sweetness) into SUC and FRU (high sweetness). The former approach can be used right now, because there are several QTLs that increase °Brix content and TSC (Wu et al. 2014; Yamamoto et al. 2014; Zhang et al. 2013). The additive effect of each QTL is not very large, probably ranging from 0.5 to 1.0 °Brix, but breeders can introduce and accumulate these QTLs using molecular markers. On the other hand, the latter approach is still difficult with our current knowledge. We identified QTLs associated with conversion of sucrose to hexose, but these QTLs are not effective for increasing sweetness because these reactions convert SUC (high sweetness) to FRU (high sweetness) and GLU (low sweetness). To utilize these QTLs to improve sweetness, we also need to identify other QTLs that control individual sugars (e.g., QTLs for metabolizing GLU or SOR). The newly released cultivar 'Kanta' has high TSC, SUC, and FRU; thus, it is possible that it harbors QTLs associated with increases in both SUC and FRU.

Conclusion

In this study, we constructed the genetic linkage maps using an F_1 population derived from crossing modern Japanese pear cultivar 'Akizuki' and breeding line '373-55'. We identified two regions associated with individual sugar contents on LG 1 and LG 7 and one region associated with total sugar content on LG 11. *PPAIV3* and *PPAIV1* are promising candidates for genes underlying those QTLs on LG 1 and LG 7 and controlling conversion of sucrose to hexose in mature fruit. Sugar metabolism is complex, and more genetic studies using diverse genetic resources are necessary to enable us to control individual sugar levels in fruit. The information obtained in this study will accelerate research and breeding programs to improve the sweetness of fruit.

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