Postharvest Properties of Ultra-Late Maturing Peach Cultivars and Their Attributions to Melting Flesh (M) Locus: Re-evaluation of M Locus in Association With Flesh Texture

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The postharvest properties of two ultra-late maturing peach cultivars, “Tobihaku” (TH) and “Daijumitsuto” (DJ), were investigated. Fruit were harvested at commercial maturity and held at 25°C. TH exhibited the characteristics of normal melting flesh (MF) peach, including rapid fruit softening associated with appropriate level of endogenous ethylene production. In contrast, DJ did not soften at all during 3 weeks experimental period even though considerable ethylene production was observed. Fruit of TH and DJ were treated with 5,000 ppm of propylene, an ethylene analog, continuously for 7 days. TH softened rapidly whereas DJ maintained high flesh firmness in spite of an increase in endogenous ethylene production, suggesting that DJ but not TH lacked the ability to be softened in response to endogenous and exogenous ethylene/propylene. DNA-seq analysis showed that tandem endo-polygalacturonase (endoPG) genes located at melting flesh (M) locus, Pp-endoPGM (PGM) and Pp-endoPGF (PGF), were deleted in DJ. The endoPG genes at M locus are known to control flesh texture of peach fruit, and it was suggested that the non-softening property of DJ is due to the lack of endoPG genes. On the other hand, TH possessed an unidentified M haplotype that is involved in determination of MF phenotype. Structural identification of the unknown M haplotype, designated as M0, through comparison with previously reported M haplotypes revealed distinct differences between PGM on M0 haplotype (PGM-M0) and PGM on other haplotypes (PGM-M1). Peach M haplotypes were classified into four main haplotypes: M0 with PGM-M0; M1 with both PGM-M1 and PGF; M2 with PGM-M1; and M3 lacking both PGM and PGF. Re-evaluation of M locus in association with MF/non-melting flesh (NMF) phenotypes in more than 400 accessions by using whole genome shotgun sequencing data on database and/or by PCR genotyping demonstrated that M0 haplotype was the common haplotype in MF accessions, and M3 and M1 haplotypes.
INTRODUCTION

Fruit firmness is an important quality that influences consumer preference, damage during distribution, and shelf life. Studies associated with the decrease in fruit firmness after harvest have been conducted with an eye toward reducing distribution loss and prolonging shelf life and thus, supplying high-quality fruit to consumers (Nimmakayala et al., 2016; Moggia et al., 2017; Tucker et al., 2017; Fernandez et al., 2018; Liu et al., 2018; Carrasco-Valenzuela et al., 2019). Fruit can be classified as climacteric or non-climacteric depending on their respiration and ethylene production patterns during ripening (Biale and Young, 1981). In climacteric fruit, ethylene is acknowledged to play an important role in controlling ripening- and senescence-related phenomena including fruit softening, due to the fact that massive ethylene production commences at the onset of ripening; exogenously applied ethylene and/or ethylene analog, propylene, induces ripening and senescence; ethylene inhibitors retard the progress of fruit ripening and senescence; and mutants and transgenic lines defective in ethylene production ability exhibit suppressed fruit ripening, especially softening (Gapper et al., 2013; Minas et al., 2015; Tucker et al., 2017).

Peach [Prunus persica (L.) Batsch] is generally known to belong to the climacteric type and to exhibit dramatic increases in respiration and ethylene production during ripening (Tonutti et al., 1991). In melting flesh (MF) peaches, the increased ethylene stimulates fruit softening principally through cell wall modification (Brummell et al., 2004; Hayama et al., 2006, 2008; Liu et al., 2018). MF peaches are highly perishable, softening rapidly after harvest. The increasing interest in improving peach shelf life has sparked investigations and resulted in findings of peach strains with long shelf lives. Those studies have demonstrated phenotypic variability associated with fruit softening and identified the possible causal genes for peach shelf life, as described below.

Intensively studied peaches that have long shelf lives are the stony-hard (SH) and slow-ripening (SR) peaches (Brecht and Kader, 1984; Haji et al., 2005; Bassi and Monet, 2008). The SH is determined by Hdhd gene and fruit with SH flesh bear the hdhd genotype (Haji et al., 2005). SH peaches are characterized by the absence of ethylene production and high firmness during postharvest storage, which are caused by the reduced expression of ethylene biosynthesis related gene PpACS1 encoding 1-aminocyclopropane-1-carboxylic acid synthase (Tatsuki et al., 2006). YUCCA flavin mono-oxygenase gene PpYUC11, which is involved in the auxin biosynthesis pathway, has been proposed as a candidate for causal gene for this phenotype (Pan et al., 2015; Tatsuki et al., 2018). SR peaches are known to show delayed maturation on the tree, thereby resulting in late harvest. In SR peaches harvested earlier than the optimum harvest date, flesh firmness decreased slowly (Brecht and Kader, 1984). Its genetic base was characterized and a deletion mutation in a gene encoding the NAC transcription factor was reported to be responsible for the SR phenotype (Eduardo et al., 2015; Nuñez-Lillo et al., 2015; Meneses et al., 2016).

Another peach strain that shows high flesh firmness during postharvest ripening is non-melting flesh (NMF) peaches (Fishman et al., 1993; Yoshioka et al., 2011). Whereas MF peaches soften dramatically and bear melting texture during the final stage of ripening called “melting phase,” NMF fruit appear to lack this “melting phase” of softening and remain relatively firm during ripening not only on the tree and but also after harvest (Fishman et al., 1993; Yoshioka et al., 2011). The MF/NMF phenotypes segregate as a single locus (M) that is linked tightly to the stone adhesion locus (Bailey and French, 1949; Monet, 1989). MF is dominant over NMF and the recessive allele determines the NMF character (Bailey and French, 1949; Monet, 1989). In NMF peaches, solubilization of cell wall pectin and enzymatic activity and protein accumulation of endo-polygalacturonase (endoPG), a pectin hydrolase, are markedly reduced compared with MF peaches (Pressey and Avants, 1978; Fishman et al., 1993; Lester et al., 1996; Yoshioka et al., 2011). Studies aimed at demonstrating endoPG(s) as a candidate gene for M locus have shown suppressed or undetectable expression of endoPG(s) in NMF peaches and polymorphisms in endoPG genes coinciding with MF/NMF phenotypes (Lester et al., 1994, 1996; Callahan et al., 2004; Peace et al., 2005, 2007; Morgutti et al., 2006, 2017; Gu et al., 2016). M locus is located at 3.5 cM interval on the bottom of linkage group 4 of the peach map, the position within which a genomic region with clusters of endoPG genes exists (Cao et al., 2016; Gu et al., 2016). Two tandem endoPG genes in that region, Pp-endPGM (PGM) and Pp-endPGF (PGF), corresponding to sequences Prupe.4G262200 in v2.0 of peach genome (ppa006857m in v1.0) and Prupe.4G261900 (ppa006839m in v1.0), respectively, were found to be responsible for determining the MF/NMF phenotypes (Gu et al., 2016). Gu et al. (2016) proposed a scenario where M locus has three allelic copy number variants of endoPG genes designated by H1 (possessing PGF and PGM), H2 (only PGM), and H3 (null). Accessions harboring either H1 and/or H2 haplotype (H1H1, H1H2, H1H3, H2H2, and H2H3) exhibit MF phenotype whereas those harboring homozygous recessive H3 (H3H3) show NMF

Keywords: fruit, softening, ethylene, Prunus persica, melting flesh locus, endoPG, postharvest

Abbreviations: M locus, Melting flesh locus; MF, melting flesh; NMF, non-melting flesh; SH, stony-hard; SR, slow-ripening; SSC, soluble solids content; endo-PG, endo-polygalacturonase; NADH, nicotinamide adenine dinucleotide dehydrogenase; SRA, sequence read archive; WGS, whole genome shotgun sequencing; SNP, single nucleotide polymorphism; indel, insertion/deletion.
phenotype (Gu et al., 2016). It was also speculated that H2 is the ancestral haplotype whereas H1 and H3 haplotypes are two variants due to the duplication and deletion of PGM, respectively, (Gu et al., 2016). However, research on different NMF peach germplasms suggested that mutations in endoPG gene(s) could be of more than one type arising from more than one source (Lester et al., 1996; Callahan et al., 2004; Peace et al., 2005; Morgutti et al., 2006, 2017) and some NMF accessions seemed to be incompatible with the model proposed by Gu et al. (2016). Much more comprehensive evaluation of M locus in association with flesh textural traits is required. Pursuing M locus evolution with much broader genetic resources covering Prunus species is also necessary for precise judgment.

Peach is known to have high diversity with regard to not only flesh texture but also fruit maturation date (Elsadr et al., 2019). In Japan, MF peaches reaching maturation stage in early July through September are mainly produced. Recently, because of the increasing demand for fresh peach in late autumn, ultra-late maturing cultivars whose optimum harvest dates are October and November are gathering the attention of growers. However, the postharvest properties of some of these rare cultivars have not yet been characterized.

In this study, first, two postharvest properties, namely, ethylene production and fruit softening, of two extremely late harvest cultivars, “Tobihaku” (TH) and “Daijumitsuto” (DJ), were investigated. It was revealed that TH showed normal MF peach ripening properties, whereas DJ possessed unique properties in that the fruit did not soften at all in spite of significant endogenous ethylene production and exogenous propylene treatment. Second, DNA-seq analysis of these cultivars demonstrated that DJ was a homozygote of an allele lacking PGM at M locus, whereas TH possessed a previously structurally unidentified haplotype that contained one endoPG. Third, accessing database sequences at M locus in more than 400 peach accessions and Prunus species indicated that the newly identified haplotype was an important allele that distributed widely within MF accessions, determined MF phenotype, and seemed to have been diverse from the other haplotypes before the speciation of P. persica. The scenario is discussed in which not three but four allelic variants at M locus are associated with the flesh texture and the newly identified haplotype is one of the two dominant determinants of MF texture.

MATERIALS AND METHODS

Plant Materials

Ultra-late maturing peach (Prunus persica) cultivars “Tobihaku” (TH) and “Daijumitsuto” (DJ), whose genetic backgrounds are unknown, were examined. TH fruit were harvested on November 7, 2018, the commercial harvest date, from two trees grown in a commercial orchard in Okayama Prefecture located in southwestern Japan. DJ fruit were harvested on October 12, 2018, the commercial harvest date, from two trees grown in the Research Farm of Okayama University. For each cultivar, 26 fruit without any disease and injuries were selected and used for investigation described below. As regards DJ, 16 fruit from a different production area, namely, Fukushima Prefecture, which is located in northeastern Japan, were harvested on October 22, 2018, the commercial harvest date, and 12 fruit without any disease and injuries after transfer to Okayama were used to investigate the effects of growing conditions and harvest maturity. Climate conditions in the peach production areas, which were obtained from the website of the Japan Meteorological Agency, are listed in Supplementary Table S1.

Regardless of cultivar or growing area, fruit were grown under suitable climate conditions for peach production by skilled growers using conventional growing techniques in Japan, including fruit thinning and bagging by the end of June. The fruit were harvested on the basis of skilled growers’ visual evaluation using de-greening of fruit ground color as harvest index. It is equivalent to color chip No. 3 (color meter score of L: 76.8, a*: −8.16, b*: 29.7) of de-greening harvest index reported in Yamazaki and Suzuki (1980). After harvest, 16 out of the 26 fruit of TH and DJ harvested from Okayama Prefecture were ripened at 25°C for 3 weeks and ethylene production rate, flesh firmness, soluble solids content (SSC), and juice pH were measured on days 0, 7, 14, and 21 at 25°C. DJ fruit harvested from Fukushima Prefecture were packed carefully in a corrugated cardboard box and transported by vehicle at ambient temperature to Okayama University 2 days after harvest, where 12 fruit without any disease and injuries were ripened at 25°C.

Propylene Treatment

Ten out of 26 fruit of TH and DJ harvested from Okayama Prefecture were used for experiment with continuous propylene treatment. Six fruits per each cultivar, excluding those used for measurement of values at day 0, were treated with 5,000 ppm of propylene, an ethylene analog, continuously for 7 days at 25°C, as described previously (McMurchie et al., 1972; Hiwasa et al., 2004). Ethylene production rates and flesh firmness were measured on treatment days 0, 3, and 7.

Measurement of Ethylene Production Rate, Flesh Firmness, SSC, and Juice pH

Ethylene production rate, flesh firmness, SSC, and juice pH were measured as described previously (Kawai et al., 2018; Nakano et al., 2018). For the measurement of ethylene production rate, individual fruit were incubated in a 1.3 L plastic container at room temperature for 30 min. Headspace gas withdrawn from the container was injected into a gas chromatograph (GC8 CMPF; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (set at 200°C) and an activated alumina column (ϕ 4 mm × 1 m) set at 80°C. For flesh firmness, the cheek parts of each fruit were cut and peeled, and flesh penetration force was measured using a rheometer (FUDOH RTC Rheometer; RHEOTECH, Tokyo, Japan) with a 3-mm-diameter cylindrical plunger and expressed in Newton per plunger area (N/mm²). The relationships between the flesh penetration force measured by this system and fruit maturity indexes are listed in Supplementary Table S2. SSC and juice pH in the cheek parts of each fruit were measured with a

1http://www.jma.go.jp/jma/index.html

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refractometer (PR-1; Atago, Tokyo, Japan) and a pH meter (B-712; HORIBA, Kyoto, Japan), respectively.

Statistical Analysis
Three to four fruit were used as biological replicates at each measurement point. In order to evaluate postharvest changing patterns in flesh firmness and ethylene production in different cultivars or production areas under the condition with and without propylene, Tukey's multiple comparison test was conducted after one-way ANOVA. The different letters shown in each figure indicate significant differences among measurement days by Tukey's test ($p < 0.05$).

Mapping WGS Data and Variant Calling
Genomic DNA was isolated from the leaves of DJ, TH, and “Benihakuto” (BH) by Nucleon PhytoPure (Cytiva). DNA-seq analysis was performed by Novogen and 9G data of PE150 reads, corresponding to approx. 30 times coverage, were obtained. We further searched SRA (Sequence Read Archive) database to obtain whole genome shotgun sequencing (WGS) data for doubled haploid “Lovell” (dhLL), “Dr. Davis” (DD), and “Big Top” (BT). Illumina WGS reads were mapped to peach reference genome (ver. 2.0) (Verde et al., 2017) by CLC Genomics Workbench or minimap2 (Li, 2018). SNP, indel, and structural variants were called by CLC Genomics Workbench.

De novo Assembly and Structural Comparison
DNA-seq analysis of BH was further performed by Macrogen to obtain 46G data of PE150 reads, which correspond to approx. 170 times coverage of $P. persica$ genome. Illumina reads were assembled by ABysS 2.0 (Jackman et al., 2017). Contigs encompassing $M$ locus were detected by Blastn analysis using PG1, PG2, PGM/F, NADH, and F-box genes, which were located in the $M$ locus region, as query. $M^0$ and $M^1$ haplotype sequences (see section “Results”) were compared by numerc (Kurtz et al., 2004) and their relationship was drawn by Circos\(^2\). We generated new reference sequence set “PpREF20 + M0,” in which the $M^0$ haplotype sequence was added to $P. persica$ reference genome. Illumina short reads of DJ, TH, BH, dhLL, DD, and BT were mapped to PpREF20 + M0 by minimap2. Coverage was analyzed from BAM file by samtools and drawn by Circos.

Genotyping by PCR
Genomic DNA was extracted from leaves of peach accessions. Genotyping by PCR was conducted with six sets of primers described in Supplementary Table S3. PCR was performed with BIOTAQ DNA Polymerase (Bioline, United Kingdom) using the following program: 30–35 cycles at 95°C for 20 s, annealing for 15 s, and extension at 72°C an initial denaturation at 95°C for 3 min, and a final extension at 72°C for 7 min. Annealing temperature and extension time are described under “PCR conditions” in Supplementary Table S3. PGM/F products were separated on a 15% acrylamide gel and others were separated on an agarose gel. PCR products were stained with UltraPower DNA Safedye (Gellex International Co., Ltd., Japan).

RESULTS
Fruit Ripening Characteristics of Two Ultra-Late Maturing Cultivars
Postharvest changes in ethylene production rate, flesh firmness, SSC, and juice pH were investigated in two ultra-late maturing peach cultivars, TH and DJ. In TH, as much as 22 nl·g$^{-1}$·h$^{-1}$ of ethylene was produced at harvest and the amount increased gradually during storage at 25°C (Figure 1A). Flesh firmness was 0.7 N/mm$^2$ at harvest but decreased dramatically to less than 0.2 N/mm$^2$ by day 7 at 25°C (Figure 1D). Thereafter, the decrease became slight until the last day of experiment (day 21). SSC and juice pH did not change remarkably during storage (Supplementary Figure S1). In DJ harvested from the Research Farm of Okayama University on October 12, ethylene production rate was almost negligible at harvest. Thereafter, ethylene production rate increased and peaked on day 7, reaching more than 10 nl·g$^{-1}$·h$^{-1}$ (Figure 1B). Flesh firmness was around 0.85 N/mm$^2$ at harvest (Figure 1E). In spite of considerable ethylene production, flesh firmness showed no significant decrease during storage at 25°C and was almost unchanged until the last day of experiment (day 21). SSC slightly increased during storage, reaching a peak of 18 °Brix on day 14, and juice pH was maintained at around pH 4.0 during storage (Supplementary Figure S1).

To confirm that the unique characteristics of DJ were not due to climatic effects and/or misestimated harvest maturity, DJ fruit harvested from a different production area were investigated. In the case of DJ harvested from a commercial orchard in Fukushima Prefecture on October 22, fruit delivered to Okayama University two days after harvest had an ethylene production rate of as high as 5.2 nl·g$^{-1}$·h$^{-1}$ and flesh firmness of 0.6 N/mm$^2$ (Figures 1C,F). Substantially high level of ethylene production was maintained during storage at 25°C, scoring 16 nl·g$^{-1}$·h$^{-1}$ on day 21. On the other hand, flesh firmness did not change significantly during storage; flesh firmness on day 21 was almost the same as that at harvest. SSC and juice pH did not change remarkably during storage (Supplementary Figure S1). Figure 2 shows DJ fruit on day 14. The external appearance showed no significant deterioration; however, the longitudinal sections showed flesh browning and slight flesh breakdown around the stones.

Different Responses to Exogenous Propylene Treatment of TH and DJ
Although ethylene production rate during storage was higher in TH than DJ, generally speaking, the amount of ethylene produced in DJ is sufficient to induce physiological effects on fruit ripening including flesh softening (Mathooko et al., 2001; Hiyasa et al., 2004; Nishiyama et al., 2007; Hayama et al., 2008; Liu et al., 2018). In order to confirm that the non-softening characteristic of DJ is not due to the low ethylene production rate of this

\(^2\)http://circos.ca
cultivar, TH and DJ were treated with 5,000 ppm of propylene, an ethylene analog, continuously for 7 days. This concentration of propylene is equivalent to 50 ppm of ethylene and is sufficient to induce ethylene response in climacteric fruits (Burg and Burg, 1967; McMurchie et al., 1972). Ethylene production rates of TH and DJ were increased rapidly by the propylene treatment, reaching 45 and 22 nl·g⁻¹·h⁻¹ on day 3 of treatment, respectively (Supplementary Figure S2). Flesh firmness of TH decreased rapidly whereas that of DJ decreased slightly, and high firmness was maintained in DJ even after 7 days of continuous propylene treatment (Figure 3). These results suggest that regardless of the differences in the ethylene production rate, DJ lacked the ability to be softened in response to ethylene.

Structural Features of M Locus in DJ and TH and an Unidentified Haplotype in TH

M locus is involved in the regulation of peach flesh texture (Bailey and French, 1949; Monet, 1989; Bassi and Monet, 2008). Four polygalacturonase genes, PG1, PG2, PGM, and PGF; three NADHs; and one F-box gene were annotated in the M locus region of peach reference genome (Figure 4A). Gu et al. (2016) walked the chromosome of three accessions by using PCR based on sequence information of reference genome, and proposed three haplotypes, H1, H2, and H3. The sequence of H1 haplotype was identical to reference genome, in which tandem duplication of endoPG genes, PGM, and PGF, was observed. H2 haplotype possessed only PGM and lacked PGF. The 70 kbp
also mapped to the entire reads were mapped uniformly to homozygote, was used for peach genome sequencing and the two contigs covering assembly with PE150 reads of BH was conducted and sequence polymorphism and mapping pattern indicated that the sequence and structure of the unidentified haplotype of TH, BT and BH was highly diverged from those of the reference sequence (M1). We decided to obtain the unidentified haplotype sequence by de novo assembly. However, polymorphism shown in the M locus of TH had a negative effect on de novo assembly. As we found that BH was a homozygote of the unknown haplotype, de novo assembly with PE150 reads of BH was conducted and two contigs covering M locus were obtained. PG1, PG2, PGM, and NADH were located in one of the two contigs. The F-box gene was found in another contig. The haplotype composed of the two contigs was defined as M0 according to the locus name. Haplotypes H1, H2, and H3 in Gu et al. (2016) were renamed M1, M2, and M3, respectively, based on haplotype features, to avoid confusion and define the haplotypes precisely.

When the genome structure of M0 was compared with M1 sequence, the region corresponding to PFG was not found in M0 and only one NADH was located on M0 (Figure 5). The sequences were conserved among the two haplotypes but M1 specific sequences were found in the region from PG2 to PGM and the downstream region of NADH3. Many non-specific reads were mapped to the region spanning from NADH3 to NADH2 of M1 haplotype, the region corresponding to M1

TH and BT showed similar mapping patterns and thus were expected to possess the same haplotypes (Figure 4). The DJ mapping results indicated that the genotype of DJ was not identical to those of TH and BT, but the three accessions were expected to share one haplotype. Mapping the pair reads of DJ to peach reference genome revealed many broken pairs around 19,026,186 and 19,096,680 bp (Figures 4A,B,G). Only a few reads were mapped between them, except for the many non-specific reads from other chromosomal regions that were mapped between NADH3 and NADH2, the region named M1 insertion in this study (see below). Structural mutation analysis by CLC Genomics Workbench indicated that the region from 19,026,186 to 19,096,680 bp was deleted in DJ, suggesting that DJ is homozygote for the deleted haplotype identical to that reported as H3 by Gu et al. (2016). Many broken pairs were found at the same position as DJ when mapped with TH and BT pair reads, indicating that one of the two haplotypes was H3 (Figures 4A,B,G). The other haplotype was presumed to be an unidentified novel haplotype that cannot be predicted directly by mapping to reference genome. In TH and BT, SNPs were heterozygous in the outer region of H3 deletion, whereas they were homologous in the inner region of H3 deletion (Figures 4B,G and Supplementary Table S4). All SNPs in this region were homoyzogous in BH, a Japanese MF accession. In contrast, no SNP was detected in either dhLL or DD. In the region of PGM and PGF, the coverage of TH, BT, and BH was low and many SNPs and broken pair reads were observed (Figures 4C,E). Throughout M locus and its surrounding region, SNPs were hardly observed in dhLL, DD, and DJ, whereas many mutations were detected in TH, BT, and BH (Figure 4 and Supplementary Table S4), suggesting the existence of an unidentified haplotype in TH, BT, and BH.

**Identification of M0 Haplotype**

Sequence polymorphism and mapping pattern indicated that the sequence and structure of the unidentified haplotype of TH, BT and BH was highly diverged from those of the reference sequence (M1). We decided to obtain the unidentified haplotype sequence by de novo assembly. However, polymorphism shown in the M locus of TH had a negative effect on de novo assembly. As we found that BH was a homozygote of the unknown haplotype, de novo assembly with PE150 reads of BH was conducted and two contigs covering M locus were obtained. PG1, PG2, PGM, and NADH were located in one of the two contigs. The F-box gene was found in another contig. The haplotype composed of the two contigs was defined as M0 according to the locus name. Haplotypes H1, H2, and H3 in Gu et al. (2016) were renamed M1, M2, and M3, respectively, based on haplotype features, to avoid confusion and define the haplotypes precisely.

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**FIGURE 3** | Effect of propylene treatment on postharvest fruit softening in (A) TH and (B) DJ. Harvested fruit were treated with 5,000 ppm of propylene continuously for 7 days. Flesh firmness was measured on days 0, 3, and 7. For DJ, fruit harvested on October 12 from the Research Farm of Okayama University were used. Each point on day 0, 3 and 7 represents the mean value SE (n = 3–4). Statistical analysis was conducted by Tukey’s multiple comparison test after one-way ANOVA. Different letters indicate significant differences among measurement days by Tukey’s test (p < 0.05).
FIGURE 4 | Mapping DNA-seq reads to M haplotype. Illumina short reads of DJ, TH, “Benihakuto” (BH), “Big Top” (BT), “Dr. Davis” (DD), and doubled haploid “Lovell” (dhLL) were mapped to reference genome of P. persica (ver 2.0) (Verde et al., 2017) by CLC Genomics Workbench. Properly paired reads that are in the correct orientation and distance are shown in blue and light blue. Green and red reads are broken pairs. Non-specific matches are shown in yellow.

(A) Overview of mapping graph in the M locus region. PG1 to F-box above graph are genes named according to Gu et al. (2016). (B,G) show the region around the junction of M3 deletion. (D,F) are those of M2 deletion. (C,E) are PGM and PGF, respectively.

The insertion described above (Figures 4, 5). The M1 insertion was assumed to be translocated from another chromosomal region and inserted into NADH to disrupt it, because both NADH3 and NADH2 were partial and their structures appeared to be generated from one NADH gene divided at the third intron by the M1 insertion (Supplementary Figures S3, S4 and NADH3/2 of Supplementary Figure S5).

The CDS sequence of PGM of M0 (PGM-M0) showed 99% similarity to those of PGM of M1 (PGM-M1) and PGF of M1. One amino acid substitution between PGM-M0 and PGM-M1 was found at residue 49, where Ser was substituted to Phe in PGM-M1 (Supplementary Figure S6). The Ser at residue 49 of PGM-M0 was conserved in other Prunus species. Amino acid sequence comparison between PGM-M0 and PGF showed substitution of Ser for Thr at residue 269 in PGF, although it was not considered to have a significant effect on the function of the PG protein. The S49F substitution between f and fl alleles was reported by Peace et al. (2005) and Morgutti et al. (2017). However, because they did not perform resequencing analysis, it was not possible to understand the entire structure of the f haplotype and its origin. It was probable that f haplotype was the same as M0 in this study. We further demonstrated that this M0 haplotype is not a specific haplotype but a widely found haplotype in various peach accessions, and plays an important role in MF phenotype determination.

DNA-seq reads of BH, TH, DJ, dhLL, DD, and BT were mapped to the reference “PpREF20 + M0” to identify M genotype. We finally predicted M genotypes of 412 accessions from the WGS mapping pattern and/or by PCR genotyping.

Structural Variety of M Haplotype Identified by WGS Mapping Patterns and PCR Genotyping

It was demonstrated that WGS data were useful for the precise genotyping of M locus. Many WGS data of peach accessions were registered in the SRA database and mapped to “PpREF20 + M0” to identify M genotype. We finally predicted M genotypes of 412 accessions from the WGS mapping pattern and/or by PCR genotyping.
genotyping. $M^0$ and $M^1$ haplotypes were the most popular in the peach accessions analyzed (Figure 6 and Supplementary Table S5). On the other hand, the frequency of $M^2$ haplotype was very low. In addition to the four main haplotypes, $M^0$, $M^1$, $M^2$, and $M^3$, we found variant-type haplotypes and chimeric haplotypes. The former haplotypes exhibited deletion in the region different from those found in $M^2$ and $M^3$, whereas the latter haplotypes appeared to be generated by the recombination between $M^0$ and $M^1$ (Figure 6). In total, 11 haplotypes were structurally identified at $M$ locus (Figure 7). They were first classified into $M^0$ to $M^3$ on the basis of the existence of $PGM-M^0$, $PGM-M^1$, and $PGF$; the haplotype containing only $PGM-M^0$ was $M^0$; the haplotype containing $PGM-M^1$ and $PGF$ was $M^1$; the haplotype containing only $PGM-M^1$ was $M^2$; and the haplotype containing neither $PGM$ nor $PGF$ was $M^3$. Furthermore, variants and recombinant types of haplotypes were identified from structural variations and such characters as b, c... or r1, r2... were added to their names, respectively.

Although no significant structural change in gene composition was detected, the sequence variation was found in the accessions with $M^0$ haplotype, when analyzed on the basis of the mapping patterns of DNA-seq reads of TH. The insertion, whose length was unknown, was found in the 1.8 kbp upstream region of $PGM-M^0$, and SNPs were also detected in $PGM-M^0$, one of which was located in CDS and led to a synonymous substitution (Supplementary Figure S7). It was expected that $M^0$ was also diversified. However, we regarded both types of $M^0$ as $M^0$ in this study because no changes in gene composition (structural feature as haplotype) and no amino acid substitutions were found.

$M^0$ Haplotype Was Widely Spread Among MF Accessions

We designed primers for PCR genotyping on the basis of the sequence variations at the third intron of $PGM$ and $PGF$ and the $M$ haplotype structural differences (Figure 8, Supplementary Figure S8, and Supplementary Table S5). The structural
variations and the PGM and PGF compositions of M haplotypes identified in this study indicated that the four main haplotypes could be classified using three primer sets, PGM/F, M2D, and M3D. PGM/F primer set detected the indel at the third intron of PGM and PGF, and three different fragment sizes were amplified (Figure 8). The upper fragment was derived from PGM-M1, the lower one was from PGF, and the middle one was from PGM-M0. When the middle fragment was amplified, the accession possessed M0 haplotype. From M1 haplotype, both the upper and the lower fragments should be amplified. When the lower fragment was not amplified and only the upper one was amplified, this meant that only PGM-M1 was amplified, showing that the accession had M0 haplotype. The genotyping results obtained with the PGM/F primer set could be confirmed by amplification with the M2D primer set that detects the deletion on M1. Because no fragment was amplified from M3 with the PGM/F primer set, the M3D primer set, which amplifies the junction of M3 deletion, should be useful to identify M2 haplotype.

The three primer sets were used to genotype 14 accessions. The M genotypes of six accessions, “Okubo” (OB), DJ, TH, BH, LL, and “Myojo” (MJ), were predicted from the mapping patterns of WGS data, which were re-confirmed by PCR genotyping. In the other eight accessions, we found inconsistent amplification patterns in “Mochizuki” (MZ) and “Okayama-3” (O3). These two accessions were expected to have M2 haplotype judging from the result that the PGM/F primer set amplified PGM-M1 but not PGF. However, no amplification was observed in M2D. These results suggested that M2 haplotype of MZ and O3 was a variant type of M2. Indeed, an additional primer set showed that they have M2b (Supplementary Figure S9).

“Hakuto” (HT), a progeny of “Chinese Cling” (CC), was mainly used as germplasm for MF peach breeding in Japan.
(Supplementary Figure S10A; Yamamoto et al., 2003a,b). PCR genotyping showed that HT was a homozygote of \( M^6 \), indicating the possibility that \( M^6 \) haplotype had been spread among Japanese peaches. All the major Japanese cultivars tested in this study were found to have \( M^6 \) haplotype (Supplementary Figure S10B). All cultivars except OB and “ShimizuHakuto” (SM) were homozygotes of \( M^3 \). SM was a typical MF cultivar in Japan (Yamamoto et al., 2003b), and its genotype was \( M^3M^3 \), the same as that of TH (Figure 8).

**M\(^2\)** and **M\(^3\) Could Confer NMF Phenotype**

DJ was an \( M^3 \) homozygote and did not have PGM and PGF. Thirty-seven \( M^3 \) homozygotes were identified in this study. Flesh textural phenotypes of 16 out of 37 accessions were reported, and 12 accessions were reported as NMF except those whose flesh texture was considered NMF (Yoshida, 1981). We suspected that the reads of EG registered in SRA were confused with those of the other accessions. This is because “Nishiki” (NK) (\( M^2M^2 \)), an NMF accession, was the parent of EG (Yoshida, 1981) and one of EG haplotypes was supposed to be \( M^2 \).

**M Locus Structure in Prunus Species**

The structure of M locus was identified from reference genome sequences of other Prunus species, including *P. mira*, *P. kansuensis*, almond (*P. dulcis*), apricot (*P. armeniaca*), Japanese apricot (*P. mume*), sweet cherry (*P. avium*), and Yoshino cherry (*P. x yedoensis*; called “Sakura” in Japan), and compared with the four main haplotypes of peach structurally identified in this study (Figure 9 and Supplementary Figure S11). PGM and PGF were found in the “Lauranne” genome (PdLN) of almond, which belongs to subgenus *Amygdalus* together with peach, indicating that the PdLN M haplotype was similar to peach \( M^1 \) haplotype. As shown in \( M^1 \) of peach, NADH located downstream of PGM in PdLN was broken by an insertion.
TABLE 1 | Flesh texture of \( M^2 \) or \( M^3 \) homozygotes and heterozygotes.

| Cultivar          | Haplotype     | \( PGM-M^0 \) | \( PGM-M^1 \) | \( PGF \) | Flesh texture | References                                                                 |
|-------------------|---------------|---------------|---------------|----------|--------------|-----------------------------------------------------------------------------|
| **M2 homozygote** |               |               |               |          |              |                                                                             |
| Dr. Davis         | M2M2          | No            | Yes           | No       | Non-melting  | Peace et al., 2005                                                         |
| Phillips          | M2M2          | No            | Yes           | No       | Non-melting  | Cao et al., 2016 Old canning peach cultivar (Davis, 1937), Some progenies were non-melting (Wang and Lu, 1992; Font i Forcada et al., 2013) |
| Oro A             | M2M2          | No            | Yes           | No       | Non-melting  | Morgutti et al., 2017                                                     |
| Carson            | M2M2          | No            | Yes           | No       | Non-melting  | Infante et al., 2011; Font i Forcada et al., 2013                           |
| NJC105            | M2M2          | No            | Yes           | No       | Non-melting  | Pan et al., 2015                                                           |
| Myoyo             | M2M2          | No            | Yes           | No       | Non-melting  | Gu et al., 2016                                                            |
| Nishiki           | M2M2          | No            | Yes           | No       | Non-melting  | Haji et al., 2005. Parent of Early Gold (Yoshida, 1981)                    |
| Loadel            | M2M2          | No            | Yes           | No       | Non-melting  | Font i Forcada et al., 2013                                                |
| G. Klamt          | M2M2          | No            | Yes           | No       | Non-melting  | Font i Forcada et al., 2013                                                |
| Everts            | M2M2          | No            | Yes           | No       | Non-melting  | Font i Forcada et al., 2013                                                |
| Golden Queen      | M2bM2b        | No            | Yes           | No       | Non-melting  | Font i Forcada et al., 2013                                                |
| **M3 homozygote** |               |               |               |          |              |                                                                             |
| Daijumitsuto      | M3M3          | No            | No            | No       | Non-melting  | This study                                                                 |
| Dawangzhuang Huang Tao | M3M3      | No            | No            | No       | Non-melting  | Cao et al., 2016                                                           |
| Maria Serena      | M3M3          | No            | No            | No       | Non-melting  | Font i Forcada et al., 2013                                                |
| Long 1-2-4        | M3M3          | No            | No            | No       | Non-melting  | Yoon et al., 2006                                                          |
| NJC77             | M3M3          | No            | No            | No       | Non-melting  | Pan et al., 2015                                                           |
| NJC47             | M3M3          | No            | No            | No       | Non-melting  | Pan et al., 2015                                                           |
| Meiko             | M3M3          | No            | No            | No       | Non-melting  | Personal communication                                                     |
| Ying Zui Tao      | M3M3          | No            | No            | No       | Non-melting  | Cao et al., 2016                                                           |
| Xi Jiao 1         | M3M3          | No            | No            | No       | Non-melting  | Cao et al., 2016                                                           |
| Yu Bai            | M3M3          | No            | No            | No       | Non-melting  | Zhang et al., 2019                                                         |
| Rou Pan Tao       | M3M3          | No            | No            | No       | Non-melting  | Yoon et al., 2006                                                          |
| Zhang Huang 9     | M3M3          | No            | No            | No       | Non-melting  | Cao et al., 2016                                                           |
| Fen Ling Chong    | M3M3          | No            | No            | No       | Melting      | Cao et al., 2016                                                           |
| Mai Huang Pan Tao | M3M3          | No            | No            | No       | Melting      | Cao et al., 2016                                                           |
| Tsukuba 85#       | M3M3          | No            | No            | No       | Melting      | Cao et al., 2016                                                           |
| Zhong You Pan Tao 2 | M3M3      | No            | No            | No       | Melting      | Cao et al., 2016                                                          |
| **Heterozygote**  |               |               |               |          |              |                                                                             |
| Mochizuki         | M2bM3         | No            | Yes           | No       | Non-melting  | Yoshioka et al., 2011                                                     |
| NJF16             | M2M3          | No            | Yes           | No       | Non-melting  | Clark and Finn, 2010                                                       |
| Okayama-3         | M0M2b         | Yes           | Yes           | No       | Melting      | Cao et al., 2016. A germplasm for canning cultivar breeding (Yoshida, 1981) |
| Tsukuba 86        | M0M2          | Yes           | Yes           | No       | Melting      | Cao et al., 2016                                                           |
| Big Top           | M0M3          | Yes           | No            | No       | Melting      | Bassi and Monet, 2008. Flesh softening was very slow                       |
| Tobihaku          | M0M3          | Yes           | No            | No       | Melting      | This study                                                                 |
| Chinese Cling     | M0M3          | Yes           | No            | No       | Melting      | Pan et al., 2015                                                           |
| Shimizu Hakuto    | M0M3          | Yes           | No            | No       | Melting      | Gu et al., 2016                                                            |
| Lovell*           | M1M2          | Yes           | No            | Yes      | Melting      | Some progenies were non-melting (Font i Forcada et al., 2013)               |
| Georgia Bell      | M1M3          | No            | Yes           | Yes      | Melting      | Some progenies** were non-melting (Peace et al., 2005)                      |

Twenty-two M2 homozygotes, including variant and recombinant types, and 37 M3 ones were found in this study. Accessions whose flesh texture was reported are listed in this table. *This “Lovell” was not doubled haploid used for genome sequencing. **Includes offspring from self-pollination and cross of “Dr. Davis” and “Georgia Bell.” The observed sequence, although this insertion sequence was not similar to the M1 insertion, \( F-box \) and \( NADH \) were pseudogenes, probably due to multiple genome rearrangements occurring in almond “Texas” genome (PdTX). One \( \text{endoPG} \) was found in addition to \( PG1 \) and \( PG2 \), and it was expected to be \( PGF \), because \( PGFs \) in PdLN and PdTX shared amino acid substitutions specific to them and genomic sequence similarity was found not only in the gene region, but also in the intergenic region.
flanking them. A frameshift mutation was found in *PdLN-PGF*, indicating that *PdLN-PGF* did not function. The absence of this mutation in *PdTX* suggests that the mutation in *PdLN-PGF* occurred after the haplotype diverged. No tandem duplications of *PGM*, *PGF*, and M1 insertions were observed in *P. mira* or *P. kansuensis*. NADH appeared to be a fragmented structure in *P. kansuensis*, but because disrupted exons were located at different contigs in the draft genome, their actual relationship was unclear. *PGM*, *PGF*, and NADH were not present in *P. mira* genome.

In subgenus *Amygdalus*, the M1 insertion was found only in peach. However, a sequence similar to the M1 insertion was found in apricot of subgenus *Prunus*. The insertion also disrupted NADH, which was located downstream of *PGM*. These findings implied that the two insertions originated from the same event (haplotype), and that this insertion event occurred before the divergence of subgenera *Amygdalus* and *Prunus*. A structure similar to M⁰ was found in Japanese apricot (*P. mume*) of subgenus *Prunus*. PG2, *PGM*, intact NADH, and F-box were clustered in *P. mume* haplotype, but PG1 was translocated to the downstream region to M locus. Sweet cherry and Yoshino cherry belong to subgenus *Cerasus*, which is far from subgenus *Amygdalus* (Chin et al., 2014). Their M haplotype structure and gene composition were similar to those of peach M⁰ haplotype.

**DISCUSSION**

**Different Postharvest Properties of TH and DJ**

In this study, first, the postharvest properties of ultra-late maturing peach cultivars TH and DJ were investigated. Generally, peach is considered to be a climacteric fruit in which ripening-related phenomena, such as accelerated endogenous ethylene biosynthesis and fruit softening, were reported to be controlled by ethylene (Liguori et al., 2004; Hayama et al., 2006; Liu et al., 2018). TH exhibited the characteristics of normal MF peach fruit, including rapid fruit softening associated with appropriate level of endogenous ethylene production. In contrast, DJ did not soften at all even though significant ethylene production was observed. From their sugar contents and pH-values at day 0, it was confirmed that DJ fruit used in this study were not harvested at too early maturity...
stage to be ripen normally (Supplementary Figure S1). It seemed that DJ possessed the ability to produce ripening-related ethylene but lacked the ability to be softened in response to the ethylene. The lack of softening ability in response to ethylene in DJ was supported by the continuous propylene treatment. Propylene, instead of ethylene, treatment has been used to monitor endogenous ethylene production in parallel to other ripening-related changes (McMurchie et al., 1972). As much as 5,000 ppm of propylene was used for treatment in this study. According to previous reports, this concentration of propylene is equivalent to 50 ppm of ethylene and is sufficient to induce ethylene response in climacteric fruits (Burg and Burg, 1967; McMurchie et al., 1972). Indeed, in peach fruit treated with 500–5,000 ppm of propylene, induction of autocatalytic ethylene production and dramatic fruit softening were reported (Yoshioka et al., 2010; Liu et al., 2018). In this study, DJ exhibited only a slight decrease in flesh firmness and maintained almost similar firmness to that at harvest even after 7 days of continuous propylene treatment. As TH showed dramatic softening by the propylene treatment, it is suggested that the propylene treatment in this study is capable of inducing ethylene response in peach and that DJ has a non-softening characteristic in response to both endogenous and exogenous ethylene/propylene.

**Involvement of M Locus in Determining Different Postharvest Properties of DJ and TH**

The genetic background of DJ and TH and its relationships with other cultivars having long storability and/or shelf lives are unknown. One of the peach strains reported to have a long shelf life is SH peach (Haji et al., 2005). SH peaches, however, are characterized by the absence of ethylene production (Tatsuki et al., 2006). It is reported that ethylene sensing is normal in SH peaches and the fruit soften rapidly with exogenous ethylene or propylene treatment (Haji et al., 2003; Yoshioka et al., 2010), in contrast to DJ. A similar ripening characteristic observed in DJ has been reported in early harvest fruit of SR peaches (Brecht and Kader, 1984). In SR peaches harvested at an earlier date than the optimum harvest date, autocatalytic ethylene production was induced with or without propylene treatment whereas flesh firmness decreased quite slowly. However, DJ is phenotypically different from SR peaches in that it bears large fruit with red coloration, as shown in Figure 2, whereas SR fruit do not show normal ripening in terms of fruit size and coloration (Giné-Bordonaba et al., 2020). In agreement with these phenotypical differences between DJ and SH and/or SR peaches, the genomic sequences of DJ and TH did not exhibit any significant differences and/or mutations in the candidate causal genes for these specific strains, YUCCA flavin mono-oxygenase (Pan et al., 2015; Tatsuki et al., 2018) and NAC transcription factor (Eduardo et al., 2015; Nuñez-Lillo et al., 2015; Meneses et al., 2016) genes (data not shown).

On the other hand, significant differences between DJ and TH were found with regard to genomic sequences at M locus, which has been reported to control MF and NMF textures (Bassi and Monet, 2008; Gu et al., 2016). M locus is composed of two tandem endoPG genes, PGM and PGF (Gu et al., 2016). Resequencing analysis revealed that DJ is a homozygote of the haplotype that lacks both PGM and PGF, designated as M0 in this study, whereas TH is a heterozygote of M0 and a structurally uncharacterized haplotype that possesses PGM (PGM-M0) but not PGF, designated as M0 in this study (Figures 4, 5). Many reports have demonstrated that high level of enzymatic activity, protein accumulation, and gene expression of endoPG are observed only in MF, and supported the involvement of endoPG in the determination of the flesh texture (reviewed in Bassi and Monet, 2008). Thus, it is considered that DJ is a member of NMF peaches. NMF peaches are known to be firm at maturity and to soften slowly during ripening without melting. Different from DJ, it was reported that softening progressed steadily during postharvest ripening in NMF peaches (Fishman et al., 1993; Yoshioka et al., 2011) and thus, the possible involvement of a number of factors in the non-softening property of DJ other than the lack of endoPG genes cannot be excluded. Nevertheless, it was indicated that the non-softening postharvest property of DJ is attributed to the lack of endoPG genes at M locus. The rapid softening property of TH is due to the presence of newly characterized M0 haplotype (PGM-M0) gene in TH genome. This was further confirmed by the re-evaluation of M haplotype in relation to flesh textural phenotypes in 412 accessions, as described below.

**Re-evaluation of M Locus in Relation to Flesh Textural Phenotypes**

In this study, we revealed that M0 was not only a unique M haplotype as shown in TH, but also a widely spread haplotype in MF accessions, particularly popular peach cultivars grown in Japan, and was responsible for the MF phenotype in these accessions (Figure 6, Supplementary Figure S10B, and Supplementary Table S5). Based on the results obtained from the re-evaluation of M locus in 412 accessions in relation to flesh textural traits, we proposed the scenario in which four M alleles/haplotypes, M0 to M3, were involved in the determination of flesh texture, with M0 and M1 dominantly controlling MF texture over M2 and M3.

Various alleles/haplotypes have been proposed for M locus. The correspondence of M alleles/haplotypes in previous studies to those in this study is summarized in Supplementary Tables S7, S8. Peace et al. (2005) classified endoPGs at M locus into four alleles, F, f1, f, and null, on the basis of the results obtained from germplasm derived from peach cultivars GB and DD with f allele being hypothesized to be segregated via outcross from an unknown origin. Morgutti et al. (2017) assumed the same four haplotypes F, f1, f, and fnull by referring to Peace’s classification and further found two variations, PGSM0 and PG5BT, in haplotype. The haplotype structures of H1, H2, and H3 reported by Gu et al. (2016) corresponded to those of F, f1, and fnull alleles, respectively, although the haplotype corresponding to f was not reported by Gu et al. (2016). In this study, we structurally identified four main haplotypes M0 to M3. Judging from S49F substitution and indel at the third intron detected between PGM-M0 and PGM-M3, M0 appeared to be identical to f haplotype. However, the derivation of
f(M^0) haplotype assumed in this study was different from that in Morgutti et al. (2017), in which f(M^0) was expected to be derived from F(M^1) via f(M^2). We detected sequence diversifications and large structural differences between M^0 and M^1/M^2 and assumed that M^0 was not derived from M^1/M^2 directly. This assumption was supported by a structural comparison of M loci of other Prunus species (Figure 9). A similar specific structure to peach M^1, such as M1 insertion and/or tandem duplication of endoPG, was found in M haplotypes of almond and apricot. On the other hand, there was no insertion to disrupt NADHD in peach M^0 or M haplotypes of the Japanese apricot, sweet cherry, and P. x yedoensis. These findings suggested that the ancestral haplotypes of M^0 and M^1 diverged relatively early, before subgenus divergence, and evolved independently of each other. On the other hand, we could not find any SNP-level variation among M^1, M^2, and M^3 (Supplementary Table S4), suggesting that the divergence of M^1 into M^2 and M^3 occurred relatively recently after P. persica speciation. It was also suggested that M^2 did not lead to M^1 but rather M^2 was derived from M^1. This might be supported by the fact that the frequencies of M^2 and M^3 were much lower than that of M^1 at least in the accessions investigated in this study (Figure 6). In this study, we showed 11 haplotypes in total (Figure 7), but more haplotypes are expected to exist. We only examined reference genomes in Prunus species other than P. persica. Considering the divergence of M haplotype before speciation, it would not be surprising to find other species harboring haplotypes similar to both M^0 and M^1 haplotypes.

Gu et al. (2016) did not consider the presence of PGM-M^0 (f allele) and defined H2 as the sole haplotype harboring PGM but not PGF because they attempted to distinguish each haplotype on the basis of copy number of endoPG genes quantified by qPCR. Therefore, not only M^2 but also M^0 was genotyped as H2 in Gu et al. (2016). This misgenotyping of M^0 as H2 produced results that included incongruity between genotype and phenotype as M^0 (PGM-M^0) and M^2 (PGM-M^1) were likely to have different effects on flesh texture. For example, the genotyping in Gu et al. (2016) identified that SM and “Hakuho” (HH), both of which are popular MF cultivars in Japan, were H3H2 and H3H2, respectively. Supposing PGM-M^1 on H2 is not functional, as assumed in this study, SM and HH should be NMF. Conversely, supposing H2 (M^2) is a dominant haplotype that determines MF texture, as assumed by Gu et al. (2016), the H2H2 genotype shown in DD, “OroA” (OA), and MZ cannot explain their NMF phenotypes (Peace et al., 2005; Morgutti et al., 2006, 2017; Yoshioka et al., 2011).

Re-evaluation of M locus in association with MF/NMF phenotypes in this study revealed that M^0 and M^1 were likely to function dominantly over M^2 and M^3. To our knowledge, M^0M^3 was linked to MF, as shown in TH, SM, and BT. LL (M^1M^3) was also reported to exhibit MF phenotype (Fonti and Forcada et al., 2013), whereas NJF16 (M^2M^3) and MZ (M^2bM^2) had NMF phenotype (Clark and Finn, 2010; Yoshioka et al., 2011; Table 1). Although PGM-M^1 was present in M^2, its expression level seemed to be suppressed as reported in OA, whose genotype was determined as M^2M^2 in this study (Morgutti et al., 2006). Thus, the low expression of PGM-M^1 was consistent with the feature of M^2, namely, recessive against M^1 and M^0, and comparable to M^3. The hypothesis cannot be excluded that M^2 haplotypes in NMF accessions are specific haplotypes possessing additional mutation(s) that result in the disruption of M^2 function. The sequences of PGM-M^1 and its surrounding region on M^2 and M^3 haplotypes were identical with each other. As it was predicted that M^2 was generated from M^1 by deletion of the region including PGF, it seemed reasonable to consider that PGM-M^1 had lost its function before the emergence of M^2 haplotype and, thus M^2 haplotype in general was not functional. This prediction was supported by Morgutti et al. (2017), who reported that all accessions harboring flf (M^2M^2) or flfnull (M^2M^2) exhibited NMF phenotype, as well as previous reports showing the existence of accessions exhibiting NMF phenotype but not completely lacking endoPG genes (Lester et al., 1994, 1996; Callahan et al., 2004; Peace et al., 2005; Morgutti et al., 2006).

It was hypothesized that two tandem endoPG genes at M locus, PGM, and PGF, were responsible for peach flesh texture regulation (Gu et al., 2016). In this study, we suggested that PGM-M^0 and PGF in particular would affect flesh textural quality whereas PGM-M^1 would have no effect. Considering the lack of sequence diversification and the relatively recent divergence between M^1 and M^2, it would not be possible that PGM-M^1 on M^1 retains its function. Although 11 haplotypes were characterized in this study, it might not be necessary to identify correctly all the haplotypes in order to estimate flesh textural quality in a breeding program. Only an analysis to confirm the presence of PGM-M^0 and PGF should be sufficient. This means we only need to test whether the PGM/F primer set (Figure 8 and Supplementary Figures S9, S10B) amplifies PGM-M^0 or PGF fragments to estimate MF/NMF phenotypes in individual accessions and progenies.

M locus is strongly linked to freestone/clingstone (F) locus. It seems that PGM-M^0 is not responsible for the determination of F trait because most M^0M^0 and M^0M^3 accessions have clingstones. Gu et al. (2016) hypothesized that not PGM but only PGF on H1 (M^1) haplotype is associated with the freestone phenotype. We re-evaluated the relationship between M genotypes and reported freestone/clingstone phenotypes. We found some accessions harboring M haplotype but being reported to have not freestone but clingstone phenotype (data not shown). Further studies are required to unravel the role of PGF in the regulation of stone adhesion.

The classification of M haplotypes based on genomic structure and the re-evaluation of M locus in association with flesh melting traits in this study are expected to provide valuable information for studies on controlling fruit softening and textural quality. For example, BT is a well-known cultivar having slow softening behavior, but reaches MF texture at full maturity (Bassi and Monet, 2008; Ghiani et al., 2011). The M genotype of BT was found to be M^0M^3 in this study, which was the same as that of SM, a famous Japanese MF cultivar that softens rapidly (Yamamoto et al., 2003b). Therefore, we suggest that the slow softening behavior of BT is controlled by loci other than M locus. Even with the re-evaluated genotypes in this study, there are few incongruities between M genotypes and MF/NMF phenotypes.
revealed four main haplotypes: M1, M2, and M3. Further classification of M1 haplotype designated as M3 and lacked two tandem endoPG genes, PGM and PGF, at M locus. On the other hand, TH was a heterozygote of M0 and lacked a structurally unidentified haplotype designated as M0 that consisted of only PGM-M0 and was responsible for determining MF texture. Further classification of M haplotypes in 412 peach accessions revealed four main haplotypes: M0; M1 consisting of PGM-M1 and PGF; M2 consisting of PGM-M2 and M1; and M3 was widely spread among MF accessions. We proposed the scenario that combinations of M0 to M3 determined flesh texture, and M0 and M1 dominantly controlled MF texture over M2 and M3. These suggested the possibility that PGM-M0 and PGF could confer MF phenotype and PGM-M1 of M1 and M2 haplotypes may have lost its function. This scenario was supported by the evolution history of each M haplotype assumed from the structural features of M locus in Prunus species, in which the ancestral haplotypes of M0 and M1 diverged before subgenus divergence and evolved independently of each other, whereas M2 and M3 were assumed to be derived from M3 in recent age by deletion mutations.

CONCLUSION

We found that two ultra-late maturing cultivars, DJ and TH, showed different postharvest properties. DJ did not soften at all during ripening in spite of significant ethylene production, whereas TH showed rapid fruit softening leading to MF texture. Resequencing analyses of DJ and TH demonstrated that DJ was a homozygote of M haplotype designated as M3 and lacked two tandem endoPG genes, PGM and PGF, at M locus. On the other hand, TH was a heterozygote of M3 and a structurally unidentified haplotype designated as M0 that consisted of only PGM-M0 and was responsible for determining MF texture. Further classification of M haplotypes in 412 peach accessions revealed four main haplotypes: M0; M1 consisting of PGM-M1 and PGF; M2 consisting of PGM-M2 and M1; and M3 was widely spread among MF accessions. We proposed the scenario that combinations of M0 to M3 determined flesh texture, and M0 and M1 dominantly controlled MF texture over M2 and M3. These suggested the possibility that PGM-M0 and PGF could confer MF phenotype and PGM-M1 of M1 and M2 haplotypes may have lost its function. This scenario was supported by the evolution history of each M haplotype assumed from the structural features of M locus in Prunus species, in which the ancestral haplotypes of M0 and M1 diverged before subgenus divergence and evolved independently of each other, whereas M2 and M3 were assumed to be derived from M3 in recent age by deletion mutations.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found in the DDBJ Sequenced Read Archive database (https://www.ddbj.nig.ac.jp/dra/index-e.html), accession numbers DRR248809-DRR248811 and DRR249197-DRR249201. The contigs A and B of M0 haplotype will appear in the DDBJ/EMBL/GenBank databases under the accession numbers LC592228 and LC592229, respectively.

AUTHOR CONTRIBUTIONS

RN, TK, KU, and FF designed the study and drafted the manuscript. YF, DT, and MS performed sampling and phenotyping. RN, TK, YF, KA, and SW investigated postharvest ethylene production and flesh firmness. KU, RN, TK, and TA analyzed the genomic sequences of peach accessions and determined the genotypes of M locus in various peach accessions. All authors have contributed to manuscript revision and have read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.554158/full#supplementary-material
Arabidopsis NADH indicated that the original NADH could be composed of five exons as shown in NADH3/1 of M0b and NADH of Japanese apricot (Supplementary Figures S5, S6).

Supplementary Figure 5 | Amino acid sequence comparison of NADH. Putative amino acid sequences of peach NADHs and Arabidopsis NADH (AtNADH; AT3G03080) were aligned by CLC Genomics Workbench.

Supplementary Figure 6 | Comparison of NADH gene structures among Prunus species. Gene structures of 11 NADHs from eight Prunus species were compared. Considering exon composition, NADH3 and NADH2 of peach were regarded as one gene (NADH3/2), although they were annotated as different genes in reference genome. Pkn NADH gene was divided into different contigs whose linkages were unknown. Disrupted structures were found in five NADHs: PknNADH, NADH3/2, PmNADH, PdLN1, NADH3/2, and PdTX-NADH. The other links were intact structures. The M1 insertion was found at third intron of NADH3/2 and PmNADH.

Supplementary Figure 7 | Nucleotide sequence comparison of PGM-M1 and -M0b. Sequence comparison showed six mutations in PGM of TH (designated as PGM-M0b in this figure). Furthermore, a large insertion was predicted at the upstream region of PGM-M0b. Of the six mutations, only one was located in CDS region and it was a synonymous substitution. AAC and AAT at 346–348 encode Asn. Therefore, we did not discriminate PGM-M1 and PGM-M0b in this study. In the study of Morgutti et al. (2017), PGM-M0b was detected by CAPS analysis using BstXII. This restriction enzyme site was caused by the nucleotide substitution at 348 bp of M0b haplotype and the fragment from PGM-M2 was expected to be insensitive to BstXII. PGM-M0b could confer the MF phenotype because flesh texture was melting in both TH (M0b-M0b) and BT (M0b-M0b, Morgutti et al. (2017)) proposed four alleles, PG-M, PGb, PGb1, and PG2, at M locus from OA (M0b-M0b), Bolero (M0b-M0b), Yumyeong (M0b-M0b, “Ghiaccio” (M0b-M0b), and BT (M0b-M0b). Based on Figure 7, our classification suggested that PG-M and PGb were derived from M0b or M1 haplotype and these were designated as PGM-M1 and PGM-Y, respectively. These might be strictly different alleles because of the nucleotide substitution, but flesh texture was melting in both SM (M0b-M0b) and TH (M0b-M0b), suggesting that their effects on flesh texture were the same and they were not different functionally.

Supplementary Figure 8 | Primers for PCR genotyping. Three primer sets were designed for the discrimination of four main haplotypes, M0b, M1, M2, and M3. (A) Primer position at M1 haplotype. (B) Position of PGM-F primer set.

Supplementary Figure 9 | M2 and O3 possessed M2b haplotype. M2 and O3 possessed PGM-M1 and no PGF. This pattern indicated the M1 haplotype, but no amplification to detect the M2 deletion was observed by PCR (Figure 8). In addition to M2b haplotype, we found the same combination in M0b and M2b haplotypes, which were rare haplotypes compared with M0b. To determine the genotypes of M2 and O3, we designed three primer sets to discriminate M0b, M2b, and M2 haplotypes on the basis of the sequences of NADH genes (Supplementary Figure S4). M2b amplified one fragment from M0b, M2b or M2b1 haplotype. NDP1 was expected to amplify two fragments: upper for M2b, and lower for M0b, M2b, and M2b1. NDP2 was also expected to amplify two fragments: upper for M2b or M2b1, and lower for M0b or M2b1. The amplification of M2b2 fragment indicated M2b had M2b, M2b or M2b1, but not M0b and M2. The lower NDP1 fragment in M2b excluded the possibility of M2b. Furthermore, the upper NDP2 fragment was amplified in PCR, indicating that M2b possessed M2b haplotype. O3 also had M2b, but two fragments were amplified in NDP1 and NDP2 because O3 had M2b haplotype (Figure 8).

Supplementary Figure 10 | Peach cultivars bred in Japan shared M0b haplotype. HT, Hakuto; HH, Hakuko; AK, Akatsuki; BH, Benihakuto; YZ, Yuzora; TS, Tosui; KN, Kawanakajima-Hakuto; OB, Okubo; SM, Shinizu-Hakuto; O3, Okayama-5; HK, Hakawa-Hakuto; LL, Lovelli; CC, Chinese cling (Shanhai Suimitsudo); TW, Tachibananawase; UNK, unknown cultivar. (A) Genealogy of peach cultivars in Japan. Green cultivars were the five leading cultivars in Japan in 2016 (e-stat Japan, https://www.e-stat.go.jp/). Their cultivation areas accounted for more than 60% of the total peach cultivation area in Japan. Haplotypes in parentheses were presumed from haplotypes of offspring and another parent. (B) PCR haplotyping. CC was imported from China to Japan in the late nineteenth century. HT was reported to be found and selected as a chance seedling of CC in 1899 (Yamamoto et al., 2003a). HT was frequently used as seed parent in breeding programs and many Japanese peach cultivars were the progeny of HT, as described in Supplementary Figure S10A. We selected 11 Japanese MF cultivars and carried out PCR genotyping to determine the genotypes of their M loci. All cultivars shared M0b haplotype and all cultivars except OB and SM were M2b homozygous. OB was M0bM0b and SM was M2bM2b. SM was found as a chance seedling at a mixed orchard of HT and O3. SSR analysis supported the hypothesis that SM was a progeny of HT (Yamamoto et al., 2003a). Because of male sterility of HT, O3 had been regarded as the pollen parent of SM. M0b haplotype of SM was inherited from HT and seed parent should possess M2b haplotype, because HT was M2b homozygous. These indicated that O3 was not the parent of SM.

Supplementary Figure 11 | Structural comparison of M loci among Prunus species. Circos plots show sequence similarities among M0b, M1, and M2 haplotypes of other Prunus species. Ribbons link homologous regions between haplotypes. "Indicates genes that did not have intact CDS sequence. PG1 of P. mume was translocated to the > 2 Mbp downstream region. To determine the M locus region, reference genomes of almond (“Laurrene” and “Texas”), P. kansuensis, P. mira, Japanese apricot, apricot, sweet cherry, and P. x yedoensis were searched by Blastn analysis using PG1, PG2, PG2M, NADH, and F-box genes as query. M locus was found at the right arm of chromosome 4 or LG3 in all Prunus species except P. kansuensis, in which no pseudomolecule was released. Nucleotide sequences of M locus were compared by nuclemer and the relationships were drawn by Circos.

Supplementary Table 1 | Climate conditions in peach production areas in Okayama and Fukushima Prefectures, Japan in 2018. 

Supplementary Table 2 | Relationships between flesh penetration force measured by the system used in this study and other fruit maturity indexes.

Supplementary Table 3 | Primers used in this study.

Supplementary Table 4 | Comparison of SNP number in regions from PG1 to PG2. Intergenic region 1 was from end of PG1 to 19,026,186 bp (outside M3 deletion region). Intergenic region 2 was from 19,026,186 bp (inside M3 deletion region) to start of PG2. Values in parentheses are those of heterozygous SNPs.

Supplementary Table 5 | M genotypes of 412 peach accessions.

Supplementary Table 6 | Differences in flesh texture predicted by genotype and reported phenotype. *The reported phenotype matched the predicted one in this study. The 11 accessions showed differences between predicted and reported phenotypes. All except “Early Gold” (EG) were reported by Yoon et al. (2006) and Caò et al. (2016). EG was a canning peach and its parent was “Nehishi” (NK). EG should possess at least one M0b haplotype because NK was an M0b homozygote, but resequencing analysis showed that EG was M2bM2b.

Supplementary Table 7 | Correlation of PGM/MF genes in this study with those in previous reports. PG2 in Morgutti et al. (2017) was BstXI-sensitive (Supplementary Figure S6).

Supplementary Table 8 | Correlation of haplotypes in this study with those in previous reports. *M0b was misidentified as H2 haplotype in Gu et al. (2016), f haplotype structure in Morgutti et al. (2017) was postulated from M1 haplotype. PG gene composition was correct but the genome structure was not the same as M0b haplotype in this study. PGM-M2b and PGM-M0b were allelic.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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