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ORIGINAL ARTICLE

Identification of candidate genes associated with mealiness and maturity date in peach [*Prunus persica* (L.) Batsch] using QTL analysis and deep sequencing

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Abstract Peach and nectarine quality traits such as flavor, texture, and juiciness are important for consumer acceptance. Maturity date (MD) also plays a role in the fruit-ripening process and is an important factor for marketing fresh fruit. On the other hand, cold storage produces a physiological disorder known as chilling injury where the most important symptom is a lack of juice in the flesh or mealiness (M). In this study, we analyzed an F2 population obtained from a self-pollination of "Venus" nectarine that segregates for MD and M. We built a linkage map with 1,830 SNPs, 7 SSRs and two slow-ripening (SR) morphological markers, spanning 389.2 cM distributed over eight linkage groups (LGs). The SR trait was mapped to LG4 and we compared the whole genome sequences of a SR individual and "Venus" and identified a deletion of 26.6 kb containing ppa008301m (ANAC072) co-localized with the SR trait. Three Quantitative Trait *Loci* (QTL) for MD were detected; they all co-localize on LG4 between 31.0 and 42.0 cM. Four co-localizing QTLs on LG4 between 33.3 and 40.3 cM were detected for M, explaining 34 % of the phenotypic variation. We identified five and nine candidate genes (CGs) for MD and M from the QTL regions, respectively. Our results suggest that the transcription factors (TFs) ANAC072 and ppa010982m (ERF4) are CGs for both traits. LG4 contains a cluster for genetic factors that possibly regulate M and MD, but functional validation is necessary to unravel the complexity of genetic control responsible for fruit traits.

Keywords Genetic linkage map · Slow ripening · ANAC072 · ERF4

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Introduction

Peach [*Prunus persica* (L.) Batsch] is the third most economically important temperate fruit tree species that has been used as a model for genetic and genomic studies within its genus (Abbott et al. 2002; Shulaev et al. 2008; Arús et al. 2012). Peach is a self-compatible fruit tree with a relatively short juvenile period (2–3 years) and a small genome (220 Mb; Verde et al. 2013). Peach has been sequenced, and the data have been available since 2010 (Verde et al. 2013). Several breeding programs are currently focused on the development of new varieties where fruit quality and post-harvest performance appear to be targeted traits (Infante et al. 2006). The conventional fruit-breeding process is time-consuming and costly, so the development of genomic tools to enable marker-assisted selection is a key step to improving the efficiency of fruit-breeding programs.

Maturity date (MD) plays an important role for marketing fresh fruit because cultivar selection with differing MD would be useful to span and extend the marketing season particularly in fruits with a short shelf life period such as peaches and nectarines (Pirona et al. 2013). Besides, maturity process involves many metabolic pathways that regulate traits such as the softening rate, covering color, and sugar/acid balance (Dirlewanger et al. 2012). Another important fruit quality trait is susceptibility to mealiness (M), which is a fruit texture disorder characterized by a lack of juice in the flesh due to cold storage at 0–5 °C for at least 2 weeks (Lurie 1992; Lurie and Crisosto 2005). M has been associated with an imbalance in the activities of cell wall degrading enzymes due to an accumulation of de-methyl esterified pectins that are not depolymerized (Lauxmann et al. 2012; Obenland et al. 2003). Pectins form a gel structure that captures free water from the flesh, resulting in a mealy phenotype of the fruit (Zhou et al. 2000). Pectin methylesterase, polygalacturonase (PG), endo-l,4-glucanase, and expansine are genes associated with cell wall metabolism. These genes have been reported to be related to M, but the exact mechanism of their functioning is still not clear (Lurie and Crisosto 2005). On the other hand, there is a relationship between MD and M susceptibility. In general, early-season cultivars are not susceptible to M; late-season cultivars are very susceptible to M (Mitchell and Kader 1989; Crisosto and Valero 2008).

The construction of genetic maps and Quantitative Trait *Loci* (QTL) analysis are good strategies for identifying candidate genes (CGs) associated with quality traits. In the past 20 years, numerous genetic maps of economically important plant species have been developed (Collard et al. 2005), including peach (Chaparro et al. 1994; Sosinski et al. 1998; Yamamoto et al. 2005; Dirlewanger et al. 2006; Eduardo et al. 2011) and sweet cherry (Stockinger et al. 1996; Olmstead et al. 2008; Klagges et al. 2013). QTLs identified in peach related to soluble sugars have been mapped to linkage groups (LGs) 2, 3, 4, and 5 (Abbott et al. 1998; Etienne et al. 2002; Quilot et al. 2004; Cantín et al. 2010) and QTLs related to organic acids have been mapped to LGs 1, 4, 5, and 6 (Dirlewanger et al. 1999; Etienne et al. 2002; Cantín et al. 2010). QTLs associated with chilling injury (M, browning, and bleeding) and MD were detected on several LGs with different levels of reliability (Peace et al. 2005; Ogundiwin et al. 2009; Cantín et al. 2010). Before next-generation sequencing (NGS) technology became available, genetic maps had a low density and were constructed using only a few markers and small populations due to the high costs of genotyping. This approach resulted in the detection of large QTLs containing a high number of putative CGs, making their identification and functional validation difficult.

Thanks to technological advances in the field of the DNA sequencing (e.g., NGS), it is now possible to obtain thousands of molecular markers at low costs (Ganal et al. 2009). Genotyping costs can be reduced due to the availability of high-throughput platforms for SNPs genotyping. For peach, a high-density SNP array, the Illumina 9 k array v1 (Verde et al. 2012; Illumina Inc., San Diego, CA, USA), was produced by the International Peach SNP Consortium using a discovery panel of 56 peach accessions. Using this array, several saturated genetic maps have been constructed for peach (Eduardo et al. 2013; Yang et al. 2013; Romeu et al. 2014).

Our goal was to identify genes involved in the expression of traits associated with M and MD in peach. We analyzed the correlation between phenotype and genotype in a population derived from self-pollination of "Venus" nectarine. QTL analysis using a dense genetic map was carried out during three seasons. Additionally, we sequenced the entire genomes of a parent "Venus" and a sibling of the "Venus" × "Venus" population showing a fruit typology known as slow ripening (SR). The genome data were used to identify structural variants (SVs) in CGs and SVs associated with the SR phenotype.

Results

Fruit quality trait phenotyping

The 151 siblings of an F2 population obtained from a self-cross of the nectarine "Venus" were phenotyped in 2012, 2013, and 2014 for different physiological parameters (firmness, soluble solids contents, titratable acidity, weight, and absorbance of chlorophyll values (I_{AD})) at harvest stage (Supplementary material; Table S1). The harvesting index (I_{AD}) ranged between 0.8 and 1.5. The average values for firmness were between 46.7 and 56.9 N. Soluble solids content (SSC) and titratable acidity (TA) did not show significant differences among seasons. On the other hand, the average fruit weight increased during the 2014 season ($p<0.01$). This increase may be explained by abnormal fruit abortion due to

an uncommon frost in the spring; the frost affected the fruit setting, resulting in fewer larger fruits per tree.

The MD trait is expressed as the number of days from September 1st until the harvest date. The average MD values for seasons 2012, 2013, and 2014 were 137, 133, and 130 days, respectively (Table 1). The minimum and maximum values obtained in all seasons were between 112 and 145 days. The Pearson correlation between seasons for this trait was very high (r=0.96 between seasons 2012 and 2013; Table 1). A bimodal distribution was observed for MD for the three evaluation seasons (Fig. 1).

The average juiciness (expressed as the percentage of juice in the flesh) over the three evaluated seasons was 32.6 % with observed; minimum and maximum values of 15 and 51 %, respectively (Table 1). An unimodal distribution was recorded for all seasons (Fig. 1). Pearson correlations for fruit juiciness between seasons ranged from 0.66 (2012 vs. 2013) to 0.75 (2012 vs. 2014) with 0.68 between seasons 2013 and 2014.

Slow ripening (SR)

Approximately 25 % of the siblings of the mapping population (33 individuals) produced fruits with a typology slow ripening (SR; Brecht et al. 1982). These trees produce fruits that remain firm on the tree until the winter. Furthermore, these fruits did not produce ethylene and their respiration rate was very low compared to that of their siblings from the same population with melting or normal fruits. The frequency of SR individuals in the population suggests that this phenotype is controlled by one gene (75 % melting fruit and 25 % SR; 3:1 Mendelian segregation).

Table 1 Statistics of maturity date (MD) and mealiness (M) on the progeny of the F2 population for three seasons

Traits	Maturity date[+]			Mealiness[++]		
Seasons	2012	2013	2014	2012	2013	2014
Mean	131.7	133.3	130.2	29.9	36.8	31.3
Median	137.0	137.5	133.0	30.0	36.0	31.0
Maximum	145.0	144.0	143.0	49.0	51.0	50.0
Minimum	112.0	116.0	114.0	15.0	20.0	21.0
SD	10.3	9.1	9.8	6.2	5.7	5.7
Seasons	2012	2013	2014	2012	2013	2014
2012	–	*0.0**	*0.0*	–	*$1.0e^{-12}$*	*0.0*
2013	0.96**	–	*0.0*	0.66	–	*0.0*
2014	0.97	0.97	–	0.75	0.68	–

[+] Days after September 1

[++] Juiciness in the flesh fruit (%)

*Italic numbers are correlation p value

**Normal letters are coefficient of Pearson correlation

Genetic linkage map construction

The F2 population was genotyped using SSRs and SNPs. Of the 25 SSR markers tested in the parents, 7 were polymorphic and 18 were monomorphic in "Venus." The seven polymorphic SSRs were used to genotype the entire population. Ten off-types siblings were identified and were not considered as part of the population. One hundred and forty-one siblings of the population plus "Stark Red Gold," "Flamekist," and "Venus" were genotyped using the peach Infinium array (Verde et al. 2012). Of the 8,144 SNPs of the Infinium array, 1,984 (24.4 %) were identified as heterozygous in "Venus." Of the remaining SNPs, 4,939 (60.6 %) were monomorphic and 1,221 (15.0 %) did not pass the score quality filters (GenTrain and GenCall).

The 1,984 SNP markers plus 7 microsatellites and 2 SR morphological markers (dominant and codominant) were used to construct a genetic linkage map (Fig. 2). The genetic map spans 389.2 cM in 332 genetic clusters (co-localized SNPs) with an average interval of 1.15 cM/cluster and 0.21 cM/marker pair. The map includes 1,839 markers of which 1,830 SNPs, 7 SSRs, and 2 morphological markers. Of the total SNPs used to build the map, 154 were unlinked. The number of markers mapped ranged from four (LG5) to 482 (LG4), with an average of 230 markers per LG. Eight LGs were detected corresponding to the T × E LGs (Dirlewanger et al. 2004) and to the number of chromosomes in peach (Verde et al. 2013). LG5 spans 4.8 cM and includes only four markers (two are co-localized), which are distributed throughout the LG. The SR phenotype was recorded as a dominant and co-dominant morphological marker that co-localized at 36.5 cM on LG4 (Fig. 2).

QTL for mealiness and maturity date

Seven QTLs for M were identified using phenotypic data from three seasons (Table 2; Fig. 3). Four co-localized QTLs that explain approximately 30 % of the phenotypic variance were identified on LG4 from 33.3 to 40.3 cM (Fig. 3). This region includes a total of 560 annotated genes. Out of them, nine genes with at least one structural variant (two alleles) in "Venus" and related to cell wall synthesis, ethylene signaling, or cold stress were selected as putative CGs (Table 3). Two QTLs on LG2 and one on LG7 were detected using phenotypic data from the last season (2014) with a Logarithm of Odds (LOD) score equal to 2.4, 2.6, and 3.0, respectively (Table 2). These QTLs were not identified using data from other seasons (2012 and 2014), and consequently, were not considered in the selection of CGs associated with the trait.

Three QTLs for MD were detected on LG4 using phenotypic data from three evaluation seasons (Fig. 3), which co-localized between 31 and 42 cM and explained between 75 and 80 % of the phenotypic variability (Table 2). In this

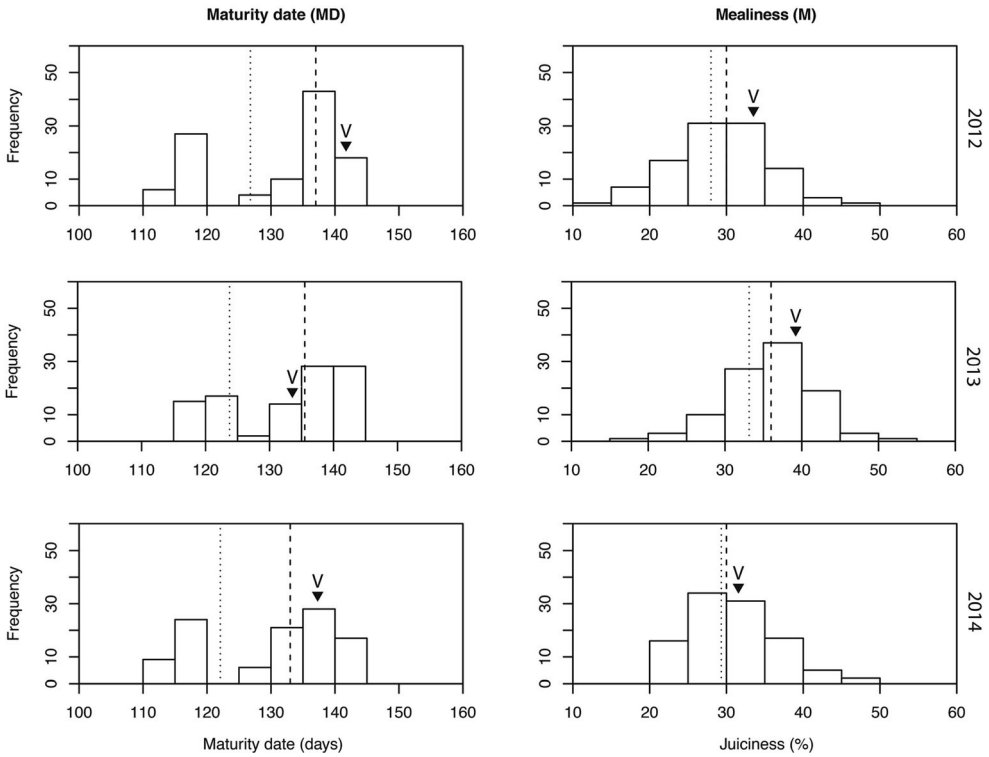

Fig. 1 Histograms representing the measured fruit quality traits: maturity date (MD, *left*) and mealiness (M, *right*) in the population during three seasons (2012, 2013, and 2014). The letter "V" indicates the values measured for the parental line "Venus." The *dotted lines* are the average and the *dashed lines* are the median

region, 346 annotated genes were identified and five genes related to cell wall synthesis, ethylene signaling, or cold stress were selected (Table 3).

Whole genome sequencing

The whole genome sequencing of "Venus" produced over 144 million reads for a total of 36.8 Gbp (Supplementary material; Table S2). About 74 % of the reads were mapped on the peach reference genome (Verde et al. 2013) obtaining a coverage of 93X. The whole genome sequencing of the F2 population sibling (p152-sr) produced over 39 million reads for a total of 9.0 Gbp (Supplementary material; Table S2). About 90.14 % were mapped against the reference genome, and the coverage of 28X was obtained.

We compared the genome sequences of p152-sr with "Venus" and identified a deletion of 26.6 kb in the position scaffold_4:11,101,110–11,127,721 (Fig. 4), which co-localized with the SR markers (dominant and co-dominant morphological markers). Thus, within this region we identified two genes: ppa008301m (ANAC072) and ppa021959m (putative transposase).

Using the "Venus" sequence, we designed primers CBV_ANAC072F and CBV_ANAC072R for the gene ANAC072 to genotype the entire population. Polymerase chain reaction (PCR) conditions were optimized with an annealing temperature of 65 °C. Amplification failure indicates the absence of the gene ANAC072, while the presence of a band of 892 bp in the gel shows the presence of the gene. We observed that all melting fruit siblings exhibited the band; none of the SR siblings exhibited the band of ANAC072 (Supplementary material; Fig. S1).

We identified a deletion of 16 bp (heterozygous on "Venus" sequence) in the promoter region of the candidate gene ERF4 in the scaffold_4:10,424,016–10,424,031. This structural variant was genotyped in the entire population using the primers CBV_ERF4F and CBV_ERF4R flanking the deletion, which were designed from the "Venus" sequences. PCR conditions were optimized with an annealing temperature of 58 °C. The deletion in homozygosity (genotype B) was associated with early-season siblings, the deletion in heterozygosity (genotype H) was associated with mid-season individuals, and the homozygous for the no-deletion genotype (genotype A) was associated with SR siblings (Supplementary material; Fig. S2). The genotyping results showed 25.8 % of genotype A, 50.0 % of genotype H, and 24.2 % of genotype B.

Discussion

Phenotyping

Phenotyping data from three seasons (2012, 2013, and 2014) were recorded for an F2 population derived from self-pollination of "Venus." We observed a unimodal distribution for M in the progeny of the population, which is a characteristic for polygenic inheritance and consistent with previously

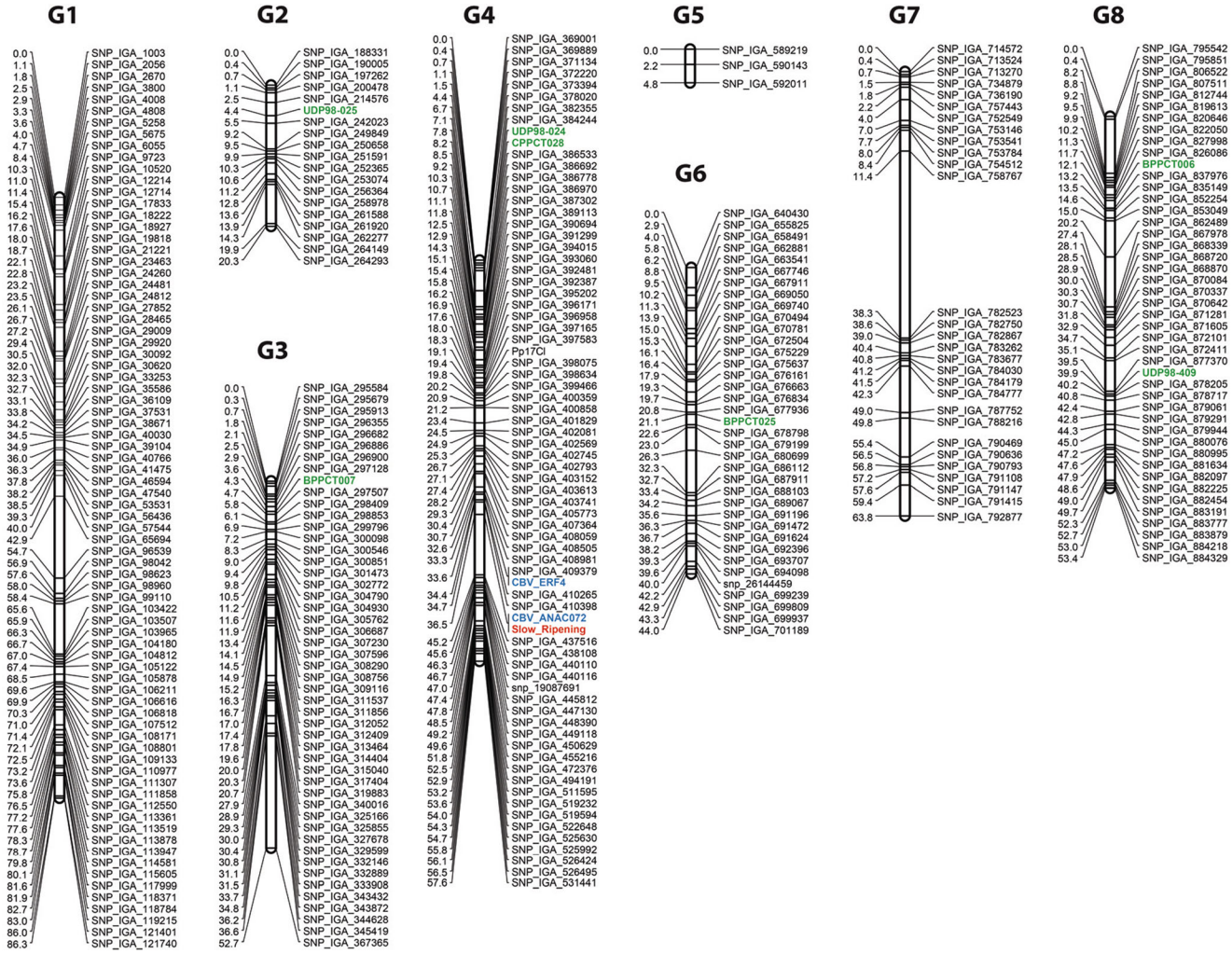

Fig. 2 Genetic linkage map. Linkage groups (LGs) are represented by *vertical bars* and are referred to as *G1*, *G2*, *G3*, *G4*, *G5*, *G6*, *G7*, and *G8*. Genetic marker names are listed to the *right* of each LG. The SR morphological marker is shown in *red*, SSR markers in *green*, and SNP markers in *black*. The PCR markers CBV_ANAC072 and CBV_ERF4 are shown *blue*. The numbers on the *left side* refer to the distance in centimorgans (cM) from the top of each LG

published works (Campos-Vargas et al. 2006; Cantín et al. 2010; Martínez-García et al. 2013). The juice content in fruits of the siblings varied between 5 and 65 %, which is similar to the values observed in other melting flesh cultivars such as

Table 2 QTL for maturity date (MD) and mealiness (M)

Traits	QTL name	Linkage group	Max. LOD peak (cM)	Nearest marker	Max. LOD value	% Variation explained
Mealiness	QTL_{M2012}	4	27.5	SNP_IGA_403613	6.6	32.5
	$QTL_{M2013LG2a}$	2	2.5	SNP_IGA_214576	2.4	12.0
	$QTL_{M2013LG2b}$	2	14.3	SNP_IGA_262277	2.6	13.0
	$QTL_{M2013LG4a}$	4	25.5	SNP_IGA_402745	3.0	14.5
	$QTL_{M2013LG4b}$	4	52.4	SNP_IGA_472376	2.3	11.3
	$QTL_{M2013LG7}$	7	51.0	SNP_IGA_455216	3.0	14.0
	QTL_{M2014}	4	35.0	SNP_IGA_410398	16.0	55.0
Maturity date	QTL_{MD2012}	4	36.5	CBV_ANAC072	30.0	80.0
	QTL_{MD2013}	4	37.5	CBV_ANAC072	30.0	75.0
	QTL_{MD2013}	4	37.5	CBV_ANAC072	30.0	75.0

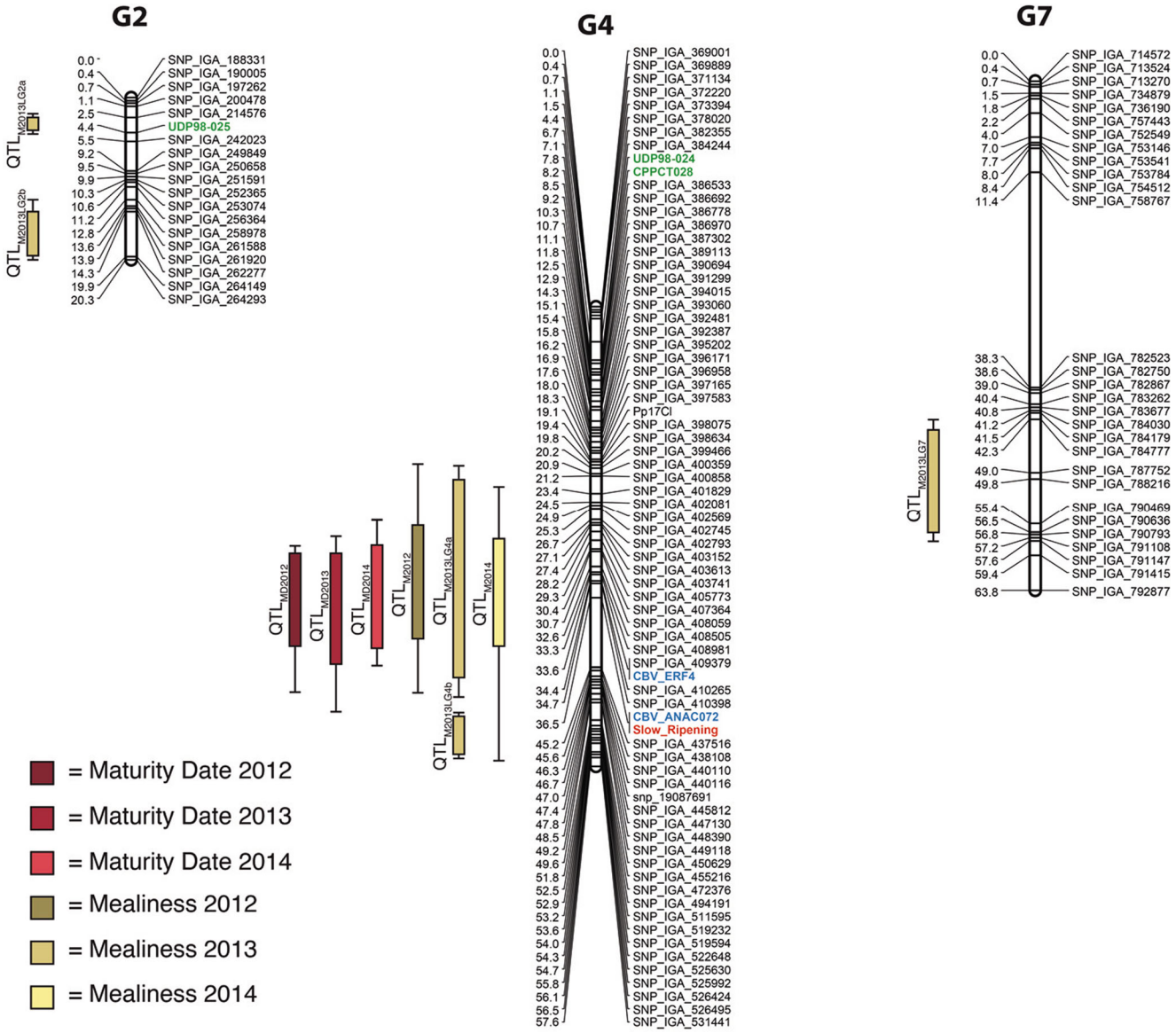

Fig. 3 QTLs in the genetic linkage map detected during three phenotype seasons. QTLs are shown with *bars* on the right of the linkage groups (LG). Maturity date (MD) QTLs (QTL_{MD2012}, QTL_{MD2013}, and QTL_{MD2014}) are represented as *red bars* and mealiness (M) QTLs (QTL_{M2012}, QTL_{M2013}, and QTL_{M2014}) are represented as *yellow bars*

"O'Henry" and "Elegant Lady" evaluated using the same method (Infante et al. 2009). The environmental effect on the mealy phenotype has been described (Campos-Vargas et al. 2006), and the contribution to phenotypic variance has been estimated to be between 36.5 and 57.5 % in peach bi-parental populations (Peace et al. 2005; Cantín et al. 2010). These results are consistent with the correlation values obtained between phenotyping seasons for M in this work (approximately 0.7 between seasons).

MD showed a bimodal distribution (two peaks) in the population, with peaks corresponding to early-season and mid-season siblings. In the F2 peach population obtained from the cross "Contender" × "Ambra," a trimodal distribution (early, mid, and late season) was observed by Eduardo et al. (2011). In both populations, the F1 individuals were mid-season individuals, but the segregation in the siblings differed (bimodal vs. trimodal). Perhaps the third missing peak in the population presented here corresponded to the siblings whose fruits did not ripen (SR). This phenotype is similar to that of a mutant of *P. persica* described before (Brecht et al. 1984), which was identified in two nectarine populations, obtained from the selfing of "Fantasia" and "Flamekist" (Ramming 1991). The idea that the third missing peak corresponds to SR siblings is supported by the haplotype of the markers between 32.5 and 36.5 cM on LG4, the region that includes the SR markers (dominant and co-dominant markers). In this region, a different genotype for each phenotypic class was found (Supplementary material; Fig. S3).

Table 3 Candidate genes (CGs) for maturity date (MD) and mealiness (M)

Trait	Peach model	Pfam hit in *Prunus persica*	Blastp hit in *Arabidopsis*	e-value	Structural variant[+]	Position (bp)
Mealiness	ppa010982m	AP2 domain	ATERF-4	2×10^{-26}	Indel	Scaffold_4:10,424,016–10,424,031
	ppa022385m	bZIP transcription factor	ATBZIP61	8×10^{-99}	Indel	Scaffold_4:10,177,257–10,177,260
	ppa019380m	Myb-like DNA-binding domain	ATT2	2×10^{-52}	SNP	Scaffold_4:12,746,223
	ppa020620m	No apical meristem (NAM) protein	ANAC032	6×10^{-61}	SNP	Scaffold_4:19,292,603
	ppa025660m	Glycosyl hydrolase family 1	BGLU47	3×10^{-172}	Indel	Scaffold_4:11,079,934–11,079,937
	ppa022465m	Myb-like DNA-binding domain	ATMYB101	5×10^{-69}	SNP	Scaffold_4:13,456,323
	ppa025596m	No apical meristem (NAM) protein	ANAC101	6×10^{-18}	Indel	Scaffold_4:14,794,895–14,794,920
	ppa008301m	No apical meristem (NAM) protein	ANAC072	2×10^{-128}	Indel	Scaffold_4:11,107,278–11,107,279
	ppa003830m	Protein kinase domain; EF hand	CPK9	0.0	Indel	Scaffold_4:13,345,475–13,345,495
Maturity date	ppa010982m	AP2 domain	ATERF-4	2×10^{-26}	Indel	Scaffold_4:10,424,016–10,424,031
	ppa022385m	bZIP transcription factor	ATBZIP61	8×10^{-99}	Indel	Scaffold_4:10,177,257–10,177,260
	ppa019380m	Myb-like DNA-binding domain	ATT2	2×10^{-52}	SNP	Scaffold_4:12,746,223
	ppa022465m	Myb-like DNA-binding domain	ATMYB101	5×10^{-69}	SNP	Scaffold_4:13,456,323
	ppa008301m	No apical meristem (NAM) protein	ANAC072	2×10^{-128}	Indel	Scaffold_4:11,107,278–11,107,279

"Venus" has both alleles for the structural variant (heterozygous genotype).

[+] Structural variant detected on "Venus" sequence

Genetic linkage map

We constructed the genetic map with eight LGs, including 1,993 *loci* spanning 389.2 cM using the 9 k SNP array for peach. Recently, other genetic linkage maps have been constructed using the same genotyping array and the number of polymorphic SNPs was similar to other works that used the same genotyping tool (Yang et al. 2013). The map published by Martínez-García et al. (2013) spans 454.6 cM in 546 clusters with 0.81 cM/cluster. Eduardo et al. (2013) and Yang et al. (2013) published peach genetic maps with a density of 1.62 and 1.64 cM/cluster, respectively. The average marker density between previously mentioned genetic maps was similar. In our genetic map, three gaps of 16.1, 8.7, and 16.9 cM on LG3, LG4, and LG7, respectively, were observed. The lengths of the gaps are similar to those found in other published maps based on the same genotyping strategy, but their genetic positions are different. Despite the large number of mapped markers, the presence of gaps is related to the high level of homozygosity in some regions in the genome of peach

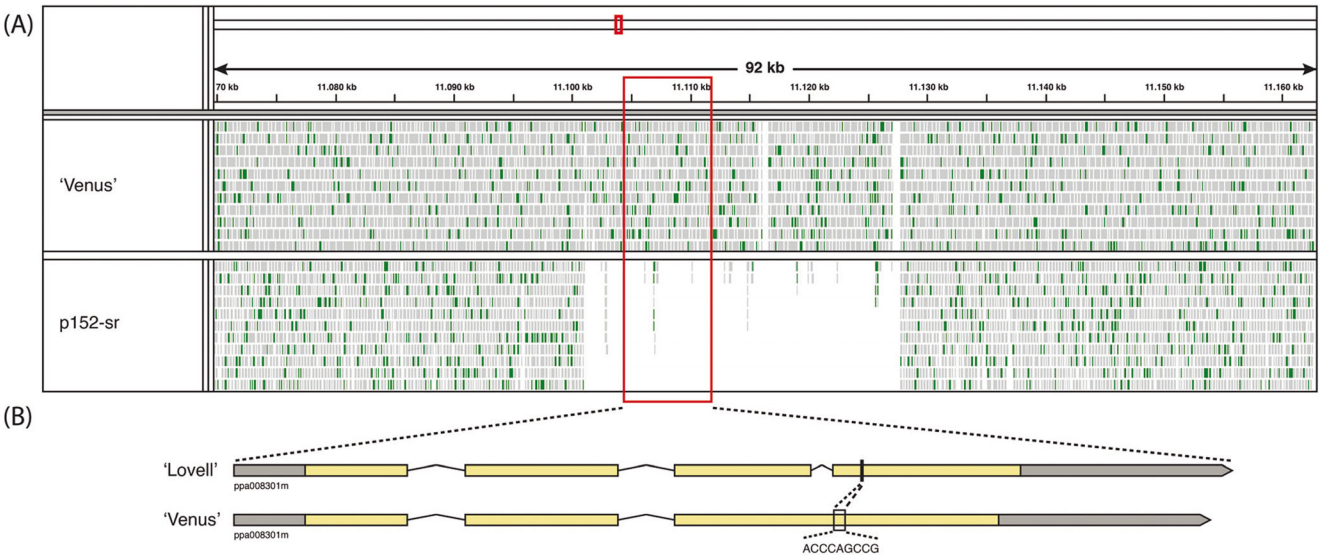

Fig. 4 Structural genomic organization of ppa008301 transcription factor. **a** Comparison of the DNA sequence of the LG4 region between "Venus" and the SR individual p152-sr. A deletion of 26.6 kb in the position scaffold 4:11,101,110–11,127,721 bp was detected. **b** Structural variation of the ppa008301 genomic sequence between reference genome for peach and "Venus"

(Aranzana et al. 2012; Verde et al. 2013). Moreover, the population size is not large enough considering the low recombination rate observed in peach (Collard et al. 2005; Arús et al. 2012). In relation to LG5, a low coverage of markers in the entire LG has been described before for the LG8 of the genetic map of a peach "Ferjalou Jalousia®" × "Fantasia" F2 population, which was genotyped by SSR (Dirlewanger et al. 2006). Although unlikely, the lack of coverage could be due to the use of low power genotyping technology. On the other hand, Yang et al. (2013) also obtained a low coverage in the LG5 and LG6 using a 9 k Illumina Array. These homozygous regions were likely fixed due to successive natural events of selfing or by the use of the same recurrent parents in peach breeding programs. The extensive genome homozygosity (Verde et al. 2013) can be explained in the same way.

QTL cluster on LG4

Using phenotyping from the three seasons and the dense genetic linkage map, we detected seven QTLs for M and three QTLs for MD during all seasons. Interestingly, seven QTLs (four for M and three for MD) were located on LG4 for the 3 years of our evaluation (Fig. 4). In the case of MD, the physical position of the SNP closest to the LOD peak for the co-localized QTL was SNP_IGA_410398, which is close to the SNP_IGA_411147 that was associated with the LOD peak of QTL identified by Romeu et al. (2014) for the same trait. On the other hand, QTL for M co-localized with QTL detected by Cantín et al. (2010) on LG4 in an unrelated peach progeny population. In addition, Dhanapal and Crisosto (2013) identified SNPs significantly associated with M by association mapping using 51 individuals on LG4. Different approaches clearly yield a significant genetic factor associated with this trait on LG4.

Previously, other QTL analyses for fruit quality traits were carried out on peach (Etienne et al. 2002; Quilot et al. 2004; Dirlewanger et al. 2006). QTLs for MD in most cases were co-localized with QTLs for SSC, TA, firmness and flesh weight on LG4 (Dirlewanger et al. 1999; Eduardo et al. 2011). Furthermore, Kenis et al. (2008) identified QTLs associated with MD in apple, which co-localized with QTLs for the same fruit quality traits (CSS, TA, weight) on chromosome 10. As reported by Illa et al. (2011), chromosome 10 in apple and chromosome 4 in peach share homologous segments with a high level of conservation. This cluster of QTLs for fruit quality traits on LG4 can be explained either by a strong link between QTLs or the existence of a single QTL with pleiotropic effects (Dirlewanger et al. 1999; Kenis et al. 2008). In fact, some CGs identified for MD and M by QTL analysis could participate in the regulation of both traits (ERF4 and ANAC072).

MD could have a pleiotropic effect on fruit quality characters because (1) the fruits with a longer ripening process have more time to grow in different environmental conditions than the fruits with shorter ripening process (indirect effect) or (2) fruit quality genetic factors are being directly controlled by a master gene that regulates ripening through the MD (direct effect). This pleiotropic effect and the low population size could eventually hinder the identification of QTLs in other regions of the genome that control characteristics associated with fruit quality (Collard et al. 2005).

As previously reported, there is a correlation between susceptibility to M and MD (Mitchell and Kader 1989), with early-season cultivars being tolerant to M after cold storage at 0–5 °C for at least 2 weeks followed by shelf storage at room temperature, and mid-season and late-season cultivars being susceptible to M. We found a co-localization between QTLs for MD and M; they both co-localized with the SR marker. In fact, the CGs ERF4 and ANAC072 were selected as the best candidates for both traits considering putative functioning and structural variants associated with the phenotypic classes.

Candidate genes for M, MD, and SR

We identified nine CGs for M and five CGs for MD in the QTL region for each trait. The CGs were selected considering the position (genes were within the QTL region), putative function *in silico*, and the presence of polymorphism in the gene sequence (at least one structural variant in the parent genome of the population as identified). Because of the co-localization of the QTLs between traits, the CGs for MD were a subset of the CGs for M.

The identified CGs are involved in several processes associated with MD, ripening, ROS detoxification, proanthocyanidin accumulation, ethylene signaling, and secondary cell wall biosynthesis. The gene ppa010982m is an ethylene responsive TF 4 (ERF4) related to ripening in apple, tomato, and kiwifruit (Wang et al. 2007; Chen et al. 2008; Yin et al. 2010) and is a transcriptional repressor modulating ABA and ethylene responses in *A. thaliana* (Yang et al. 2005), which is upregulated during fruit ripening (Wang et al. 2007; Manning et al. 2006). Besides, ERF4 has been proposed as a CG for MD in peach, apricots, and sweet cherry (Dirlewanger et al. 2012). The marker closest to the LOD score peak of QTL_{M2012} is SNP_IGA_410398, which is just 0.2 Mbp away from this gene. The gene ppa022385m is a bZIP tanscription factor (bZIP61), and its orthologs in tobacco and rice encode for phloem-specific TFs (Yin et al. 1997; Jakoby et al. 2002). The gene ppa019380m encodes for the MYB TF TRANSPARENT TESTA2 (TT2). In *A. thaliana*, TT2 regulates proanthocyanidin synthesis in the seed coat (Bogs et al. 2007). Pons et al. (2014) proposed that another TF (TT19) is related to the accumulation of anthocyanin and proanthocyanin, and is involved in cell wall composition and sensitivity to chilling. The genes ppa020620m, ppa025596m,

and ppa008301m encoding for NAC TFs are specific to plants and are involved in MD (Pirona et al. 2013; Romeu et al. 2014), ripening (Giovannoni et al. 1995; Thompson et al. 1999; Giovannoni 2007; Shan et al. 2012; Zhu et al. 2014), biotic and abiotic stress (Fujita et al. 2004; He et al. 2005; Nakashima et al. 2007; Hu et al. 2008; Nakashima et al. 2012; Jin et al. 2013). Furthermore, these TFs are involved in the ABA-dependent response to dehydration, reactive oxygen species detoxification and defense (Fujita et al. 2004). The ppa003830m encodes for calmodulin-domain protein kinase 9 (CPK9). This family of enzymes in plants is characterized by a C-terminal calmodulin (CaM)-like domain (Suresh-Kumar and Jayabaskaran 2004) and is involved in the phosphorylation of serine residues in different types of ACS in tomatoes (Tatsuki and Mori 2001; Sebastià et al. 2004). Zhou et al. (2000) proposed that the M in nectarines is related to an impaired ability to produce ethylene. The CGs (NAC, ERF4, and CPK9) are related to ethylene pathways and structural variants of their body regions or their promoter regions could modify their expression level, and thus, susceptibility to M.

In relation to SR phenotype, we detected a deletion of 26.6 kb in the sequence of a slow ripening individual compared against the "Venus" sequence genome. This deletion co-localized with QTLs for MD and the SR morphological markers. We have genotyped the deletion by PCR using custom primers in the entire F2 progeny and all slow ripening individuals showed the absence of the region of 26.6 kb. These results strongly suggest that the deletion of 26.6 kb, and specifically at least one or both genes spanned in this region, may be responsible for the SR phenotype. However, since SR behaves as a Mendelian trait controlled by one gene (*Sr*/*sr*), ANAC072 is a more likely candidate to be responsible for the SR phenotype than ppa021959m, considering the putative gene function. The gene ppa021959m may be involved in deletion events since it encodes a putative transposase. On the other hand, SR individuals in the F1 population of the cross between "Belbinette"and "Nectalady" (Bb × Nl) were identified by Eduardo et al. (2015). The Sr gene was located in LG4 on the Bb × Nl genetic map in the same region of the gene ANAC072 and co-located with the CPP15636 and PSR2 markers. These authors observed a null allele in the parents and a Bb × Nl population using PSR2, which in homozygosis was associated with the SR phenotype. This result is consistent with the relationship between the deletion of 26.6 kb and the SR character detected in the same region in our work.

Conclusions

We have identified genetic factors associated with MD and M in peach. We have identified consistent QTLs on LG4 using phenotypic data based on 3 years of phenotyping for both traits. In addition, the SR Mendelian trait segregates in the population and was mapped to LG4 of the genetic map as a dominant morphological marker. Nine and four genes from QTL regions were selected as CGs for M and MD, respectively. Regarding M, the TFs of the NAC family, ERF4 and CPK9, seem to be the best candidates. Screening of the structural variants and expression profiles in the entire population and commercial cultivars are important to validate the relationship between phenotypic classes and structural variants.

Regarding MD, the best candidates are ANAC072 and ERF4, which were previously described to be associated with the same trait. We also have observed the co-localization between QTLs for the MD and SR markers. The comparison between the whole genome sequence of "Venus" and the SR sibling of the "Venus" × "Venus" progeny showed a deletion of 26.6 kb in the QTL region for MD. This deletion includes two genes one of which (ANAC072) was genotyped in the population and found to be associated with the SR trait. These results strongly suggest that the absence of ANAC072 is responsible for the SR phenotype.

The most significant CGs found in this work are TFs involved in the regulation of M and MD which target genes should be identified to help unravel the genetic mechanisms that define these traits. RNA-seq and eQTL could be suitable techniques for such investigations. The pleiotropic effect observed in this work on LG4 is very promising and requires further research, including physiological assays and functional validation of the CGs to observe the effect on the different traits and to dissect the role of the genetic factors.

Experimental procedures

Mapping population

An F2 population with 151 individuals was used in this study. This population was obtained from the self-pollination of a nectarine cultivar [*P. persica* (L.) Batsch cv. "Venus"]. "Venus" was obtained from the intra-specific cross between "Stark Red Gold" and "Flamekist." Both parents produce melting yellow-fleshed nectarine fruit and "Venus" produces freestone melting yellow-fleshed nectarines. The mapping population ("Venus"×"Venus") consists of 6-year-old trees grown on G × N rootstock in an experimental orchard located at 34°24′S latitude and 70°50′W longitude (INIA-Rayentué, VI Region, Chile). This population segregates for peach fruit quality traits including M, SSC, TA, and MD.

Phenotyping of the F2 population

One hundred fruits with a green-yellow background color (commercial maturity determined using a color table) for each F2 individual were harvested and sorted in the lab considering

the index of absorbance difference (I_{AD}; Ziosi et al. 2008). Fruits with an I_{AD} between 0.8 and 1.5 were selected (Lurie et al. 2013). We measured physiological parameters and calculated their averages for nine fruits at the harvest stage for weight, flesh firmness, SSC, and TA. MD was phenotyped in the November–March (southern hemisphere) 2012, 2013, and 2014 seasons. The MD was determined as a number of days starting from September 1st until the harvest date.

I_{AD} was measured using a portable Vis/NIR DA-Meter (Sinteleia, Bologna, Italy) considering the average between two measurements per fruit on the cheeks (one per each cheek). Flesh firmness was measured with a penetrometer Fruit Pressure Tester (Effigi, Alfonsine, Italy) on both cheeks of the fruit. TA was determined by titration of 5 mL fruit juice with 0.1 N NaOH until a pH close to 8.2 was achieved; the acidity was expressed as a percentage of malic acid. The SSC content was assessed using a temperature-compensated refractometer (Atago, Tokyo, Japan).

M was determined on fruits stored after harvest for 21 days at 4 °C and then 3 days at 20 °C following Infante et al. (2009) protocol, which is based on the absorption of juice on a paper towel after the sample fresh fruit is squeezed by two metal cylinders. Nine fruits were measured for each sibling and averaged.

Slow ripening phenotyping

An SR phenotype, identified in the F2 progeny of the selfed "Venus" population, was phenotyped by visual inspection. SR was recorded as a dominant morphological marker with two phenotypic classes and was coded in the mapped matrix as "D" (melting or normal fruit) or "B" (SR fruit). Furthermore, a co-dominant morphological marker with three phenotypic classes was used in the map and was coded as "A" (early-season siblings), "H" (mid-season siblings) or "B" (SR siblings).

DNA isolation and quantification

Genomic DNA of 151 F2 individuals from "Venus" × "Venus" progeny, "Stark Red Gold," "Flamekist," and "Venus" was isolated from 50–100 mg of young leaves. DNA extraction was performed using a DNeasy 96 Plant Mini Kit (QUIAGEN, Düsseldorf, Germany) according to the manufacturer's protocol. The integrity of the DNA was determined by electrophoresis on 1 % agarose gels. DNA quantification was done using a Qubit® 2.0 Fluorometer (Invitrogen™, Eugene, Oregon, USA) and a Qubit® dsDNA BR Assay Kit (Invitrogen™).

SSR genotyping

Twenty-five SSRs distributed over the eight chromosomes of peach (Supplementary material; Table S3) were used to genotype "Stark Red Gold," "Flamekist," and "Venus" plus six individuals of the mapping population. Polymorphic SSRs were selected and the entire population was genotyped for these SSRs. PCRs were performed using PCR GoTaq® Master Mix (Promega, Madison, Wisconsin, USA) in a final volume of 10 uL containing 1X reaction buffer, 1.5 mM $MgCl_2$, 0.25 uM forward primer, 0.25 uM reverse primer, and 40 ng of DNA. Temperature profile was the following: 94 °C for 4 min, then 35 cycles of (94 °C for 40 s, Tm for 30 s and 72 °C for 30 s), and finally, 72 °C for 5 min in a thermocycler PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The PCR products were detected by electrophoresis in 1.5 % agarose gels and stained with GelRed (Biotium, Hayward, CA, USA) to confirm the SSR amplification. For the analysis using the Fragment Analyzer™ Automated CE System (Analytical Advanced Technologies, Ames, IA, USA), the PCR products were separated using a dsDNA Reagent Kit 35–500 bp (Analytical Advanced Technologies), following the manufacturer's recommendations. We used ProSize 2.0 software (Analytical Advanced Technologies) to determine the number and size of the alleles in the electropherograms.

SNP genotyping

The "Venus" × "Venus" progeny, "Stark Red Gold," "Flamekist," and "Venus" were genotyped using the peach Illumina 9 k SNP array v1 following the standard Illumina protocol (Verde et al. 2012). The beadchips were scanned on the lllumina HiScan (Illumina Inc., San Diego, CA, USA) at the Fondazione Edmund Mach (San Michele all'Adige, Trento, Italy) following Illumina-published standard operating procedures (www.illumina.com). The SNP data were analyzed using GenomeStudio Data Analysis software (Illumina Inc.) with a GenCall threshold of 0.15. SNPs with GenTrain score <0.6 and those showing severe segregation distortion (χ^2 test, $p<10^{-6}$) and more than 1 % of missing data were excluded from further analyses.

Map construction and QTL analysis

The genetic linkage map was build using the software TMAP (Cartwright et al. 2007). Grouping was carried out using a LOD score between 5 and 8. QTL analysis was performed with MapQTL (Van Ooijen 2009) using the Interval Mapping (IM) test (normal distribution trait). QTLs were detected considering a LOD score greater than 2 for the IM test.

Whole genome sequence of "Venus" and a slow ripening individual

Genomic DNA of "Venus" and one SR sibling (p152-sr) were sonicated by Covaris (model M220) and fragments of 400–

500 bp were then repaired, adenilated, ligated with adaptors, and amplified using TruSeq DNA Sample Prep kit reagents (Illumina Inc.), following the protocol of the Illumina's kit. The final size of the library was determined by a Fragment Analyzer and quantified using a Kapa Library Quantification kit (Kapa Biosystems, Boston, MA, USA). The library was sequenced in one run of MiSeq platform (Illumina Inc.) and 2×300 bp pair-end reads were obtained. The raw data were trimmed using Flexbar (QC>20) and adapters removed. Filtered reads were mapped against the peach reference genome v1.0 (Verde et al. 2013) using Bowtie2 (Langmead and Salzberg 2012) allowing one mismatch. Mapped reads were visualized on the Integrative Genomic Viewer (Robinson et al. 2011).

Structural variant genotyping

The indel of 16 bp (heterozygous in "Venus" sequence) on the promoter region of ERF4 was genotyped in entire F2 progeny. We designed primers CBV_ERF4F (5′ GCCACATTAGGCTAACATTGTGC3′) and CBV_ERF4R (5′TTTGGAGTTTGGGCTCGGGATT3′) using Primer3 (Rozen and Skaletsky 2000). PCRs were performed using PCR GoTaq® Master Mix (Promega, Madison, Wisconsin, USA) in a final volume of 10 uL using the same conditions described above for SSR genotyping with an annealing temperature of 58 °C. The PCR products were separated using the Fragment Analyzer™ Automated CE System and the data were analyzed by ProSize 2.0 software.

For genotyping of the presence of the 26.6 kb deletion in entire F2 progeny, primers CBV_ANAC072F (5′-ATGGGTGTGCCAGAAACCGACCCA-3′) and CBV_ANAC072R (5′-CCGAGCTTGCTGTCCTCCTGCT-3′) were designed using Primer 3. PCRs were performed using PCR GoTaq® Master Mix (Promega, Madison, Wisconsin, USA) in a final volume of 10 uL using the same conditions as described above for SSR genotyping with an annealing temperature of 65 °C. The PCR products were separated by electrophoresis in 2 % agarose gels and stained with GelRed™ (Biotium, Hayward, CA, USA). The absence of a band of 892 bp in the gel is associated with the presence of the 26.6 kb deletion.

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Authors' contributions GNL carried out the experimental design, DNA extraction of the siblings of the F2 population, phenotypic data analysis, SSR genotyping, genetic map construction, QTL analysis, mapping of reads against the reference genome and SNP detection using the "Venus" sequence. ACE carried out the sibling phenotyping of the F2 population, phenotypic data analysis and structural variants genotyped. MT carried out the SNP genotyping with the Infinium bead array for peach, the SNP data analysis, drafted the manuscript and made substantive intellectual contributions to the manuscript. DM carried out the SNP genotyping with the Infinium bead array for peach, the SNP data analysis and drafted the manuscript. RI supported the phenotypic evaluation of fruit quality traits and made substantive intellectual contributions to the manuscript. RCV carried out the experimental design, the phenotypic data analysis and made substantive intellectual contributions to the manuscript. AO carried out the experimental design and made substantive intellectual contributions to the manuscript. FBH carried out the experimental design, CG selection, drafted the manuscript and made substantive intellectual contributions to the manuscript. CM carried out the experimental design, the data analysis, drafted the manuscript and made substantive intellectual contributions to the manuscript. All authors read, revised the manuscript critically and approved the final version of the manuscript.

Data archiving statement The data reported here are archived and publicly available at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) and Genome Database for *Rosaceae* (GDR; http://www.rosaceae.org/). Sequence raw data of "Venus" in fastaq format (accession numbers SRR1867740, SRR1867794 and SRR1867795) and sequence raw data of slow ripening sibling p152-sr in fastaq format (accession number SRR1867806) are available at the NCBI. The mapped markers were submitted to GDR (tfGDR1015).

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