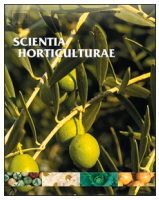
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Research Paper

A palindromic sequence on chromosome 4 of peach (*Prunus persica* L. *Batsch*) is flanking a large deletion related to a Slow Ripening Phenotype

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ABSTRACT

Maturity date (MD) represents one of the main goals of the peach breeding programs worldwide with the aim to extend the harvest calendar. A major QTL linked to the trait was previously identified in peach and other stone fruits on LG4, and NAC genes were identified as the best candidates for the MD control. Here, a bud mutation affecting MD was found in a tree of the nectarine 'Caldesi 2000'. The original variety (CW), its mutated sport (CM) and a segregating progeny obtained by selfing of CM was used to characterize the trait at phenotypic and molecular level. A panel of markers spanning the MD region was developed and a few recombinant plants were identified. A Non-ripening phenotype was observed in seedlings homozygous for one of the haplotypes, an effect probably caused by a large deletion of about 26 kb inactivating two transcription factors belonging to the NAC family (PpNAC072 and PpNAC1), similar, but independent, to a previously reported mutation associated to a slow ripening trait. The presence of palindromic sequences in the regions flanking the deletion may explain the occurrence of this phenomenon. The putative silencing of two members of the NAC family further supports the importance of these transcription factors in determining the MD phenotype. Further research is needed to elucidate functions and interactions between PpNAC072 and PpNAC1 in controlling the MD trait in peach and to determine the hierarchical expression of genes controlling fruit ripening.

1. Introduction

Peach (*Prunus persica* L.) is among the most produced temperate fruit crops in the world with a global production of 27.1 million tonnes (FAOSTAT, 2023). Peach fruits are climacteric, exhibiting a dramatic increase in respiration and an intense rise in ethylene production after harvest, which typically peaks within a few days, especially when fruits are collected for commercial purposes (Tonutti et al., 1991). Fruit maturation is a complex and highly coordinated set of processes that influence colour, firmness, aromas and flavour, where ethylene carries out a pivotal role (Barry and Giovannoni 2007; Trainotti et al. 2007; Osorio et al. 2011; Kumar et al. 2014) in all climacteric species, in an example of convergent evolution (Lü et al. 2018). The ethylene biosynthesis pathway is quite simple, as it consists of only 2 steps: first, the substrate S-adenosyl-l-methionine (SAM) is converted to 1-aminocyclopropane 1-Carboxylic Acid (ACC) by the enzyme ACC synthase (or *ACS*); ACC is further converted into ethylene by ACC oxidase (*ACO*), and both enzymes are encoded by multigene families (Barry and Giovannoni 2007). Though the ethylene biosynthesis pathway is straightforward, its regulation is far more complex and the interaction between this hormone and the ripening processes in whole fruit tissues is composite and involves many metabolic pathways (Quinet et al. 2019). In tomato, a range of TFs, like COLOURLESS NON-RIPENING (CNR), NON-RIPENING (NOR) and RIPENING INHIBITOR (RIN), act upstream of ethylene and are required for tomato ripening and for autocatalytic ethylene synthesis (Giovannoni, 2004; Vrebalov et al. 2002; Moore et al. 2002; Manning et al. 2006), although the role of RIN has been partially questioned (Ito et al. 2017). Epigenetics, and especially DNA

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methylation, also plays an important role and ripening is dynamically regulated by DNA methylation and demethylation (Liu et al. 2022). Although the model species for climacteric fruit is tomato, the mechanisms of fruit ripening have also been investigated in other species such as banana (Clendennen and May 1997) and peach (Dirlewanger et al. 1999). In this sense, a thorough analysis of 11 genomes and several transcriptome and methylation profiles showed that at least 3 different circuits controlling ethylene-based fruit ripening exist: a MADS-type circuit, identified in tomato, apple and pear, a NAC-type circuit, identified in peach, papaya and melon, and a dual-loop system in banana (Lü et al. 2018).

The pivotal role played by NAC Transcription Factors in peach fruit ripening is confirmed by a number of reports. The synergistic regulation of fruit quality and ripening by PpNAC1 and DNA demethylase PpDML1 was reported (Cao et al. 2023) as well as the interaction between PpNAC1 and PpNAC5 that could activate the transcription of the PpPGF to regulate peach softening during fruit ripening (Zhang et al. 2024). The NAC-like gene, NAP4, was also found to act with ethylene in a regulatory loop to modulate peach fruit ripening and softening (Dai et al., 2024). Moreover, a modification of fruit Maturity Date (MD) caused by modifications in the methylation status was identified in a bud sport mutation (Zhou et al. 2023).

In peach various natural mutations affecting commercially valuable traits have been deeply characterized to shed light on underlying genes, such as the flesh colour (white or yellow; Adami et al. 2013), fruit skin type (peach or nectarines; Vendramin et al. 2014), fruit flat shape (López-Girona et al. 2017) or fruit softening (Tatsuki et al. 2018). Interestingly, retrotransposons were often responsible for the emergence of such phenotypic characteristics; this phenomenon has been described in several species and is believed to be one of the drivers for phenotypic changes in plants (Han et al. 2017; Zhang et al. 2019; Alioto et al. 2020; Domínguez et al. 2020).

In peach, MD represents a valuable trait, given the limited shelf-life of the fruit. In this sense, the development of cultivars having different MD would be advantageous in that they could cover and extend the marketing season. For this reason, the trait was analysed in a wide panel of segregating progenies that were derived from parental lines such as 'Big Top', 'Nectalady', 'Ferjalou Jalousia', 'Fantasia', 'Bolero', 'Oro A', 'Contender', 'Ambra', 'NJ Weeping', 'Bounty', 'Belbinette', and 'Venus' to map the locus (Eduardo et al. 2011; Dirlewanger et al. 2012; Pirona et al. 2013; Eduardo et al. 2015; Meneses et al. 2016; Elsadr et al. 2019). These studies resulted in the identification of a cluster of QTLs on LG4 with several pleiotropic effects on other fruit traits (Eduardo et al. 2011; Pirona et al. 2013; Hernández Mora et al. 2017; Elsadr et al. 2019; Cao et al. 2023). In this region, a marker designed on a 9 bp deletion in the *PpNAC072* transcription factor was mapped indicating its role as candidate gene controlling the MD trait (Pirona et al. 2013). The discriminative power of this marker was tested on a panel of 140 peach accessions covering a wide range of MD. Despite the good correlation with MD the occurrence of some outliers clearly confirmed the complexity of this trait in peach (Pietrella et al. 2017).

In peach, a Slow Ripening phenotype (SR) has been described and associated with MD. Accessions carrying the SR trait show a block of the ripening process resulting in fruits that never ripe (Brecht et al. 1984; Brecht and Kader 1984). In a F2 population obtained from a self-pollination of 'Venus', the MD trait was located in LG4 and was correlated to a 26.6 kb deletion encompassing a NAC transcription factor locus (ANAC72), suggesting that the SR phenotype was a recessive effect of null alleles at the MD locus (Eduardo et al. 2015; Nuñez-Lillo et al. 2015; Meneses et al. 2016).

In this work we characterized at phenotypic and molecular level, a peach spontaneous mutation for MD that was identified in an orchard of the white-fleshed nectarine cv. 'Caldesi 2000′. On a tree with fruit ready for harvest (size, skin blush percentage and quality traits consistent with the characteristics of mature fruits of 'Caldesi 2000′; Conte et al. 1994), a single branch was bearing fruit small and green, clearly still immature. Vegetative buds from the mutated and the original (wild-type) branches of the tree carrying the mutation were grafted onto 'GF677' rootstock to propagate the mutation, to confirm its stability over time, and to investigate the differences from the original cultivar.

2. Materials and methods

2.1. Plant materials

Three trees of both cv. 'Caldesi 2000' (hereafter CW) and 'Caldesi 2000 mutant' (CM) were established at the experimental farm of CREA-Research Centre for Olive, Fruit and Citrus Crops, Forlì (44° N, 12°E, 32 m a.s.l.), Italy. Trees were spaced 5.0 m x 4.0 m respectively, between and along the row, and trained to a small vase system.

Two small populations from the selfing of CW (18 seedlings; CW-self) and CM (54 seedlings; CM-self) were planted on their own roots with a spacing of 1.2 m within and 4.5 m between the rows. All plant materials under investigation were subjected to local integrated pest management standardpractices.

2.2. Blooming and MD phenology, fruit development pattern and quality traits of CW vs CM

The dates of beginning, full and end of blooming (corresponding respectively to stages 61, 66 and 67 of the BBCH scale of Meier et al., 1994), MD, and the main fruit quality traits in both CW and CM plots were recorded for 5 consecutive seasons (2012–2016). In 2016, the development of the fruits on ten tagged 1-year old shoots on both CW and CM plots was regularly monitored, starting when the fruit size (average diameter at the fruit cheeks and suture) was $\geq$ 20 mm until MD. The MD (expressed in Julian days) was established when about 10 % of the fruit on the tree had reached the physiological stage of ready-to eat maturation (Valero et al., 2007) based on visual (ground colour change and blush percentage) and sensorial assessments, coupled with the measures of the I_{AD} (chlorophyll absorbance index, ranging 0.3–0.6), by using the portable DA-meter® device (Sintéleia, Bologna, Italy). A total of 10 fruits per genotype were sampled at the respective MD. The diameter, the weight, the I_{AD} index and the flesh firmness (FF), using an FTA penetrometer equipped with an 8 mm plunger (Güss instruments, Strand, South Africa) and the soluble solid content (SSC) of each fruit were measured. SSC was assessed by dispensing a drop of the entire fruit juice onto a digital Refractometer (Atago mod. DBX-55). Finally, the Titratable Acidity (TA) was measured on the juice pooled from groups of three fruits (3 replicates) by diluting 10 mL of juice with 25 mL of distilled water, titrating with NaOH 0.1 N to pH 8.4 and the results expressed as mEq l^{-1}.

2.3. MD, post-harvest ethylene emission and fruit quality evolution

The MD for each tree of the 3-years old 'CW-self' and 'CM-self' progenies was recorded during two productive seasons. Based on the observed ripening patterns of the 'CM-self' progeny, the ethylene emission and the fruit post-harvest quality was evaluated on five seedlings belonging to three MD classes: early (ER; < July 31), intermediate (IR; from August 1 to August 20), and very late (NR; still immature at the beginning of October). For this analysis, ten mature fruits were sampled based on both visual assessment and I_{AD} values in the range 0.3–0.6. For the seedlings of the very late MD class, the harvest date was arbitrarily set as October 5th. All the 10-fruit samples were taken to the laboratory, weighted, and divided into two groups of 5 fruits each (G1 and G2). On the same day of sampling (R0), G1 fruits were individually placed inside 1.7 L glass jars and hermetically sealed with Parafilm®, while G2 fruits were kept at room temperature (22 ± 1 °C) for four days before being sealed in the jars. After 24 h from sealing (both R0+1 and R0+4), the concentration of ethylene in the jars' headspace was measured by using the Ethylene Spy ES100 (Fruit Control Equipment Srl, Milano, Italy)

equipped with a suction needle. Ethylene concentration (ppm kg^{-1}) and flesh firmness (FF, kg) were measured in R0+1 (G1) and R0+4 (G2), whereas SSC (°Brix) and TA (mEq l^{-1}) were measured only in R0+1, since in previous years these parameters proved to be stable over the three-day interval (data not shown).

2.4. Fruit DNA isolation and whole genome sequencing

Fruit flesh samples from CW and CM were collected, sliced and immediately frozen in liquid nitrogen at the onset of S3 phase (second exponential fruit growth phase, Chalmers and van den Ende 1975). Samples were stored at −80 °C until high-quality genomic DNA extraction by a CTAB-based protocol developed by Zeng et al. (2002). Whole Genome Sequencing was performed by Genomix4Life (Salerno, Italy) using Illumina Nextera-Flex protocol and produced two paired-end reads of 150 bp. Reads were polished with bbduk, a software part of the BBTools suite (http://sourceforge.net/projects/bbmap/) to trim and remove low quality reads prior to mapping that was performed with bwa-mem v 0.7.17 (Li and Durbin 2009) on the Peach v2.0 genome release (Verde et al. 2017). The raw reads have been uploaded on the SRA database (BioProject PRJNA1087134). The alignment of CW and CM resequencing data to peach genome was used for markers development.

2.5. Leaf DNA isolation, genome scanning approach, marker development and analysis

Genomic DNA from CW, CM and each 'CMself' seedling was extracted from young leaves according to Mercado et al. (1999). A total of 63 genome-wide well-distributed SSR markers were tested on the CW genotype (Table S1). Heterozygous SSRs were genotyped on 15 plants belonging to the late harvesting phenotype (NR) by a "Genome Scanning Approach" (GSA) according to Patocchi et al. (2005) for identifying the genomic region in which the mutation was located.

Once the region putatively involved in NR trait was identified, candidate genes were searched on the Peach genome sequence v2.0 via the JBrowse tool available on Phytozome 13 website (https://phytozome-next.jgi.doe.gov/). For the identification of possible polymorphisms suitable for marker development, the software Tablet (Milne et al. 2013) was used on the aligned CW and CM sequences. Additional markers (Table S2) were designed within the genomic region involved in the NR trait and tested on each seedling. Primers have been designed by the Primer 3 software (https://primer3.ut.ee/).

SSR and SCAR PCR reactions were performed in a 10 µl volume containing 1 µl of peach genomic DNA (diluted to 50 ng/µl), 1.0 µl reaction buffer 10X, 0.6 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs (100 mM), 0.5 µl of each primer (10 µM) and 0.10 µl Taq polymerase (5 U/µl, Thermo Fisher Scientific, Waltham, MA, USA). The PCR conditions used were: 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 - 61 °C annealing temperature depending on primers (Table S2), and 1 min at 72 °C, with a 10 min final extension step at 72 °C. For Temperature-Switch PCR (TSP) markers, PCR reactions were performed with 1 µl of peach genomic DNA (diluted to 50 ng/µl), 1 µl reaction buffer 10X, 0.6 µl $MgCl_2$ (25 mM), 0.2 µl dNTPs (100 mM), 0.2 µl of each locus specific primer (10 µM), 0.5 µl for the allele-specific primer, 1 µl bovine serum albumin Fraction V (5 ng, Thermo Fisher Scientific) and 0.10 µl Taq polymerase (5 U/µl, Thermo Fisher Scientific, Waltham, MA, USA) in a total reaction volume of 10 µl. The PCR amplification protocol was carried out after Hayden et al. (2009).

SSRs were analysed in 5 % PAGE electrophoresis and silver stained (Benbouza et al. 2006). SCAR and TSP PCR products were separated in 1 % agarose gels in Sodium borate (SB) buffer using Gel Red for staining. PCR results were visualized through Kodak 440CF image analyser (Kodak, Rochester, NY, USA).

To validate PCR results, fragment sequencing has been carried out by the Mix2Seq Kit Eurofins service (Eurofins Genomics, Ebersberg, Germany) after ExoSAP-IT purification (Thermo Fisher Scientific, Waltham, MA, USA) to remove primers and dNTPs. Clustal Omega was used to align the sequences (https://www.ebi.ac.uk/Tools/msa/clustalo/). Analysis for the presence of palindromic sequences have been carried out with the palindrome tool available on EMBOSS explorer (https://www.bioinformatics.nl/cgi-bin/emboss/palindrome).

2.6. Synteny analyses

The CoGe platform for comparative genomics (Lyons et al. 2008) was used to detect orthologous genes between *P. persica* v2 and *S. lycopersicum* v2.40 genomes. To compute chains of syntenic genes found within complete genome sequences, the DAGchainer software with 'Relative gene order' option and maximum distance between two matches parameter set to 5 was used (Haas et al. 2004) together with Quota-Align algorithm with maximum distance between two blocks sets to 4 genes (Tang et al. 2011), both implemented to the SynMap function within CoGe. To reduce background noise due to successive duplication (s), Quota-Align ratios for coverage depth was set to 2:2.

3. Results and discussion

3.1. Phenotypic characterization of cv 'Caldesi 2000' and its bud sport mutation

The observations for five growing seasons (2012–2016) highlighted that CW and CM differed in the blooming and ripening phenology, as well as for major fruit quality traits (Table 1). CM blooming was on average delayed by 3 days (with a range of 2–5 days depending on the climatic trend of the year) as compared with CW. Moreover, CM fruits ripened with an average delay of 30 days compared to CW, with a range of 28 to 35 days, depending on the year course, confirming both the stability of the mutation originally identified in the 'Caldesi 2000' orchard, and the magnitude of its effect on MD. At a comparable physiological stage of maturity, as indicated by similar I_{AD} and flesh firmness values, CM fruits at harvest were larger, heavier, higher in SSC and in TA than CW ones. Additional differences observed on the mature fruits of CM vs. CW were a more extensive blush of the skin (85.5 % vs. 45.2 % in CW), a less elongated shape of the fruit, and a freestone (easy detachment of the flesh from the stone) phenotype, unlike the original genotype whose phenotype is clingstone. All these differences in fruit quality characteristics are presumably the result of the longer stay of the fruit on the tree.

Fruit size monitoring confirmed the typical double-sigmoidal peach fruit growth pattern (Chalmers and van den Ende 1975) in both genotypes. Differences in diameter were minimal in the S1 stage (intense cell division; Fig. 1 and Fig. 2a), becoming more and more pronounced from the end of the S2 (pit hardening) stage. In the sampling of June 1st, the endocarp lignification of CW fruits was well advanced, while in CM fruits the stone was still soft and easy to cut (Fig 2b). Differences in fruit

Table 1
Average blooming and maturity dates, and main fruit quality traits at harvest time of CM and CW (mean±standard deviation) in a 5-years period of records.

	CW	CM
Beginning of blooming (BBCH 61)	18-Mar	21-Mar
Full blooming (BBCH 66)	21-Mar	24-Mar
End of blooming (BBCH 67)	26-Mar	29-Mar
Maturity date (MD)	02-Jul	01-Aug
Fruit weight (g)	178.3 ± 18.1	208.9 ± 19.5
Fruit diameter (mm)	71.1 ± 3.4	75.6 ± 5.6
I_{AD} index	0.38 ± 0.1	0.42 ± 0.1
Fruit skin overcolour (%)	45.2 ± 9.1	85.5 ± 10.6
Flesh firmness (Kg/cm^2)	3.4 ± 0.5	4.0 ± 0.7
SSC (°Brix)	11.2 ± 1.5	13.9 ± 2.2
TA (mEq l^{-1})	116.5 ± 10.8	167.7 ± 19.3

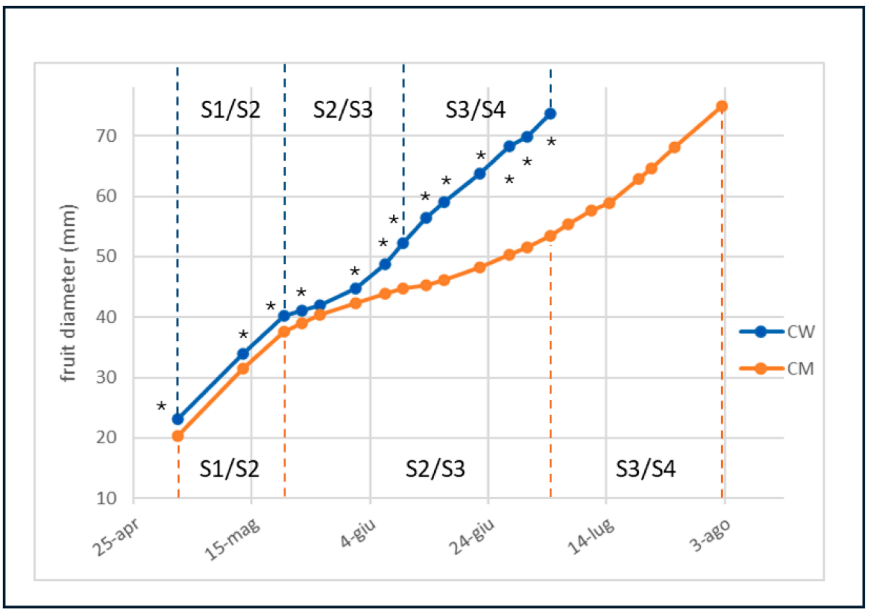


Fig. 1. Fruit size (average diameter at the cheek and suture, mm) development of CW and CM fruits from ≥ 20 mm until maturation (MD). Asterisk indicates that the difference is statistically significant (two-tailed *t*-test, p-value <0.05).

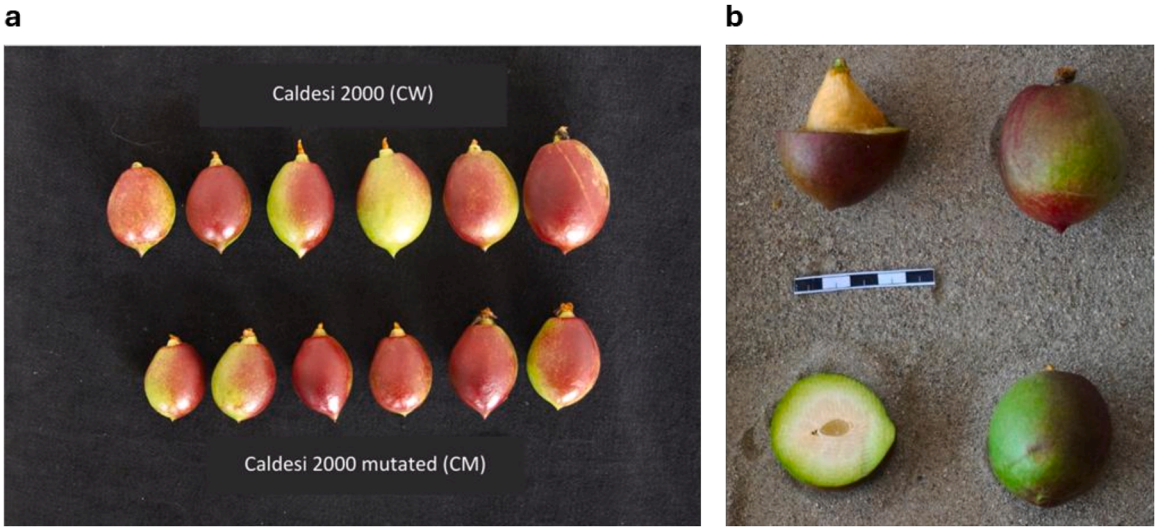


Fig. 2. Fruit phenotyping. a: Fruits of CW (above) and CM (below) sampled on May 2nd (S1 stage); b: CW and CM fruits sampled on June 1st, showing that in CW (above) the pit hardening phase (S2) was advanced, whereas in CM the stone was still soft (below).

size widened during the S3 (cell expansion) phase where fruit development in size progressed much faster in CW fruit, that ripened on July 4th (S4 stage), whereas CM was still in the S3 stage (Fig. 1). CM ripened 32 days after CW (on August 5). Interestingly, CM fruits at harvest showed an overripe flesh segment at the suture point, clearly visible even before MD (Fig 3). The CM suture over-ripening is clearly pointing out that this portion of the fruit ripened earlier than the rest of the fruit flesh. It is reported that fruit suture is originated by the L1 histogenetic layer that enters the fruit (Dermen and Steward 1973). All these results seem to indicate the chimeric nature of the observed 'Caldesi 2000' mutation. Analogous observations have been also reported for other traits, as the chimeric flesh colour mutations of 'White Redhaven' and 'Cristina' (Adami et al. 2013).

3.2. *Phenotypic characterization of cv CM-selfing population*

In the two-years of records, the fruits from the 'CW-self' segregating progeny ripened between July 1 and August 31 (data not shown). In the 'CM-self' population, 15 ER and 21 IR out of 54 seedlings (27.8 and 38.9 %, respectively) matured in the period July 2÷August 31, while 18 seedlings (33.3 %) never matured their fruits (Fig. 4). Indeed, at the beginning of October, the fruits of these seedlings had the high firmness and astringent taste typical of unripe fruits, though the I_{AD} value was within the 0.3–0.6 range established as one of the criteria for harvesting (Fig. S1). This segregation is consistent with a Mendelian segregation after selfing of a heterozygous genotype in which the NR represents the recessive phenotype. The chi-square test calculated on the three phenotypic classes (ER, IR and NR) supports this hypothesis (data not shown).

Fruit firmness clearly decreased after four days at RT (R0+4) in fruits



Fig. 3. 'Caldesi 2000' mutant (CM) fruits sampled 10 days before MD. The flesh at the suture point is in an advanced stage of melting, unlike the rest of the fruit which is still firm.

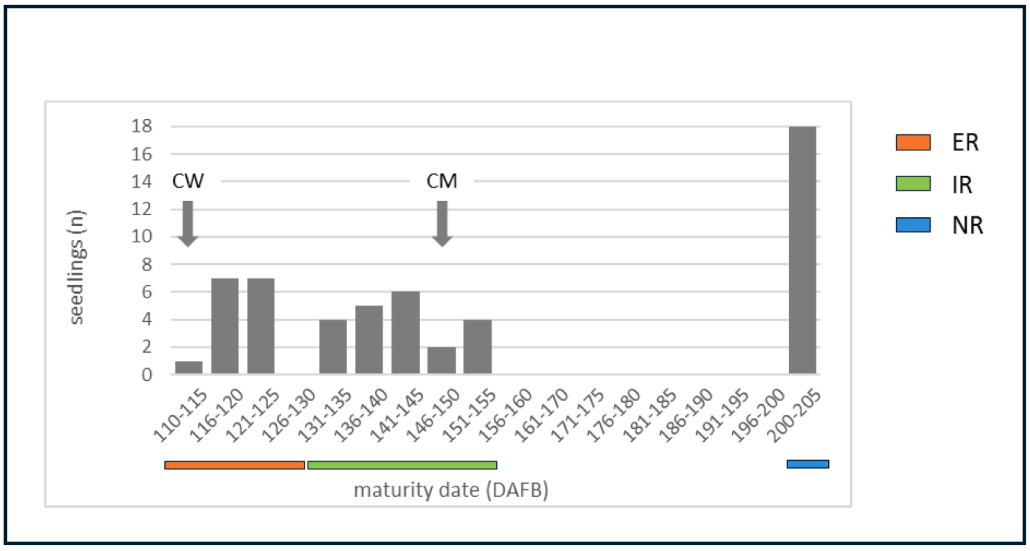


Fig. 4. Distribution of the ripening dates of the CM-selfing population, expressed in DAFB. ER = Early ripening; IR = Interm ediate ripening and NR = Non-ripening. The arrows identify the ripening date of CW and CM. Data were collected in 2019 and 2021 (I_{AD}= 0.3 - 0.6).

of the ER and IR seedlings while it remained unaffected in the NR ones. In addition, although the harvest time of each seedling was established based on the same I_{AD} range, flesh firmness both at R0+1 and R0+4 was consistently lower in ER than in the IR fruits, suggesting the presence of a possible additive effect at the MD locus (Fig. S2a). This observation is in agreement with data presented by Giné-Bordonaba et al. 2020, where allelic effects at the *Sr* locus were found to be involved in the fruit ripening physiology and in its postharvest behaviour.

Concerning the fruit ethylene emission, values increased significantly after 3 days of storage in the ER and IR seedlings while in the NR seedlings the ethylene emission remained close to zero (Fig. S2b). These analyses confirmed the findings of Tonutti et al. 1996, who evidenced that the higher the ethylene emission the lower is the observed firmness in fruits. Moreover, it has been observed that the presence of a dominant allelic variant at the *Sr* locus resulted in ripening fruits, while the recessive allele combination (*srsr*) produced little or no ethylene when harvested at $I_{AD} \geq 2$ (Giné-Bordonaba et al. 2020).

Finally, at R0+1, the average SSC and titratable acidity resulted lower in the NR fruits compared to that of the other seedling classes (Fig. S3).

3.3. Genetic characterization of the NR region

The Genome Scanning Approach (GSA) on the NR plants resulted in the identification of the SSR CPPCT003 as putatively linked to the NR region (data not shown). This SSR is located on chromosome 4 at 9.84 Mb of the Peach v2.0 genome assembly. Therefore, based on the maturity date literature and the annotated genes, a region between 9.0 and 12.0 Mb was identified for developing new markers. For marker design, the 'Caldesi 2000' (CW) and 'Caldesi mutant' (CM) Illumina sequences aligned to the peach reference genome (Verde et al. 2017) were used to develop new markers for fine mapping.

This region encompassed a total of 403 annotated genes of which 34 were putatively involved in fruit ripening (Table S3). Among the available polymorphisms, a total of 29 new markers (20 SSRs, 7 SCAR and 2 TSP) spanning the region of interest were designed and tested with fifteen of these markers designed on candidate gene sequences (Table S2). The marker analysis in the 'CM-self' progeny showed that most of the ER (13/15 plants) and IR (18/21) genotypes are either homozygous (aa) or heterozygous (ab) at all the loci while most of the NR plants (17/19) are homozygous for the alternative allele (bb). Then, a total of seven plants (2 ER, 3 IR and 2 NR) showed a recombination event inside the region of interest (Table 2). In the whole progeny, molecular results were in agreement with the known phenotypes of maturation and

Table 2
Markers' segregation for 'Caldesi 2000' (CW), 'Caldesi 2000 mutant' (CM) and seedlings (1–7) of the 'CM-selfing' population. Maturity date known for each seedling determines ripening class distribution. Orange: Early ripening (ER) segregations, blue: Intermediate ripening (IR) and green with Non-ripening (NR). CM ripening is reported as IR for the whole flesh except the fruit suture.

MARKER		CW	CM	SEEDLINGS						
position (Mbp)	name			1	2	3	4	5	6	7
9.22	MET_SSR	ab	ab	ab	ab	ab	ab	bb	aa	aa
9.7	UBI_SSR	ab	ab	bb	bb	ab	ab	bb	aa	aa
9.73	JAS_SSR	ab	ab	bb	bb	ab	ab	bb	aa	aa
9.84	CPPCT003B	ab	ab	bb	bb	ab	ab	bb	aa	aa
10.43	EREB_SSR	ab	ab	bb	bb	aa	ab	bb	aa	aa
10.62	EXO_SSR	ab	ab	bb	bb	aa	aa	bb	aa	aa
11.06	11.06_SSR	ab	ab	bb	bb	aa	aa	ab	ab	aa
	ripening	ER	IR	NR	NR	ER	ER	IR	IR	ER
11.118	Ppa008301_indel	a-	a-	--	--	a-	a-	a-	a-	a-
11.125	NAM2del_SCAR	a-	a-	--	--	a-	a-	a-	a-	a-
11.131	11.131_SSR	a-	a-	--	--	a-	a-	a-	a-	a-
11.135	4G.187000_SCAR	a-	a-	--	--	a-	a-	a-	a-	a-
11.137	NAM_TSP	a-	a-	--	--	a-	a-	a-	a-	a-
11.22	ULT_SSR	ab	ab	bb	bb	aa	aa	ab	ab	aa
11.62	11.62_SSR	ab	ab	bb	bb	aa	aa	ab	ab	ab

Genotype - Phenotype Inconsistencies (GPI; Patocchi et al. 2005) plants were not observed (Table 2).

The analyses on recombinant plants allowed to narrow the region of interest between position 11,060,000 and 11,220,000 (~160,000 bp; Fig. 5). Four markers (namely, NAM2_SCAR, 11,131_SSR, 4 G.187000SCAR; NAM TSP) were used to further genotype the NR region together with the Indel-specific marker (ppa008301_Indel), developed on the NAC gene sequence (ppa008301 or Prupe.4G186800 in the peach genome v.2.1) by Pirona et al. (2013).

The presence of null alleles for these five markers on NR plants suggested the existence of a deletion inside the region of interest, being the NR plants homozygous for this deletion; in this sense, the lack of one of the two copies of the gene would be most likely the reason for the one-month ripening delay. The three phenotypic classes, ER, IR, and NR, would thus be determined by three alleles configuration or haplotypes: two functioning DNA strands (ER), one functioning DNA strand and one with deletion (IR), and two DNA strands both with deletion (NR; Fig. S4).

3.4. Identification and characterization of the deletion in 'CM-selfing' related to the 'Non-ripening' trait

A visual control of the mapped reads on the CW and CM sequences by the software 'Tablet' evidenced an almost double amount of reads in CW

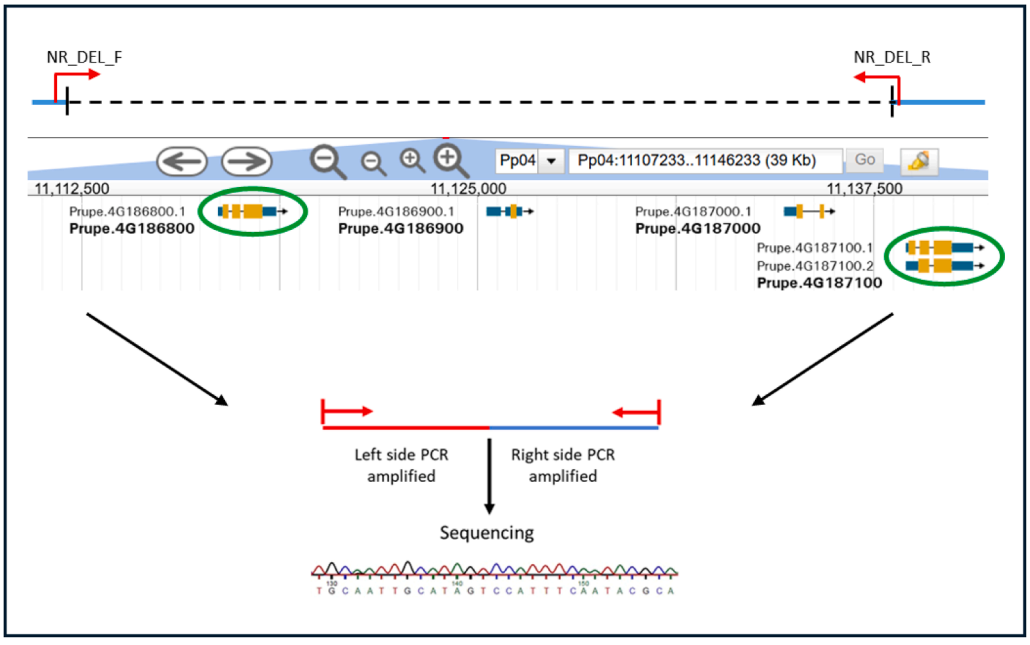


Fig. 5. Region of chromosome 4 encompassing the genomic deletion (evidenced as dotted line) involved in the 'non-ripening' phenotype. Candidate genes for NR are circled in green. Primers (NR_DEL_F and NR_DEL_R) were designed to individuate the breakpoint position and to identify seedlings carrying the deletion.

vs CM sequences in the deletion region. To determine the position and length of the deletion, and to discriminate between plants homozygous or heterozygous for the trait, three specific primers were used: a pair flanking the deletion (NR_DEL_F and NR_DEL_R) were used to identify the chromosome carrying the deletion, while an additional primer designed inside the deletion (WT_DEL_R) was used to identify the wild-type chromosome fragment (expected size of 351 bp); plants heterozygous for the deletion would amplify both fragments (Fig. S4). PCR analysis evidenced the presence of a product of 490 bp in the plants carrying the deletion, while the expected fragment of 351 bp was identified in ER and IR seedlings. Both fragments were amplified in heterozygous plants (Fig. S4).

Sequencing of the NR-specific amplification product and its alignment to the reference peach v. 2.1 genome allowed us to estimate the deletion's breakpoints, being between 11,111,954 - 11,138,018 bp resulting in a gap of 26,064 bp (Fig. 5).

Within the deletion region three predicted genes are present, Prupe.4G186800, Prupe.4G186900 and Prupe.4G187000. While Prupe.4G186900 and Prupe.4G187000 have no defined function, Prupe.4G186800 encodes a NAC Transcription Factor, PpNAC072 (PpNAC5 according to Zhang et al. 2024). Preliminary studies on this gene suggested a role in the regulation of maturity date (MD) (Pirona et al. 2013), and it was demonstrated to have pleiotropic effects on fruit enlargement and ripening, sugar accumulation and organic acid degradation (Zhang et al. 2024).

Interestingly, the deletion included part of the promoter of another NAC family Transcription Factor (PpNAC1, Prupe.4G187100) which is very close to the deletion breakpoint (645 bp upstream the start codon). This gene has been implicated in the early-ripening behaviour of a bud sport mutation of 'Zhong You 4' and of other early ripening cultivars (Zhou et al. 2023). This is somehow not unexpected, as these genes were identified as tandem duplicates, therefore they may have a common origin (data not shown). On the other hand, *PpNAC1* is syntenic (i.e. is the direct ortholog) with tomato NOR-like 1 gene (Solyc07g063420), whose silencing inhibited specific aspects of ripening in tomato, including ethylene biosynthesis, color change, and cell wall metabolism (Gao et al. 2018). Its role in fruit ripening was reported to be different with respect to NAC—NOR: while NOR-like 1 appears to be more important for fruit ripening initiation, the latter has a stronger influence on carotenoid accumulation (Gao et al. 2020).

A different role of these two genes in MD is possible since PpNAC072 and PpNAC1 show different expression specificities, with the latter being selectively expressed in fruit tissues, while the expression of the former was found to be more ubiquitous (Cao et al. 2023).

Due to its position, it is plausible that the deletion may have not only neglected PpNAC072 expression, but also hindered PpNAC1 promoter functionality, thereby influencing its activity.

26.6 kb deletion encompassing the NAC transcription factor locus (ANAC72) was also correlated to a Slow Ripening phenotype (Eduardo et al. 2015; Nuñez-Lillo et al. 2015; Meneses et al. 2016). The presence of two similar and independent deletions in the same genomic region in these two varieties and the occurrence of similar phenotypes raises the possibility of a "deletion hotspot" in this region.

Interestingly, performing a genomic scan of palindromic sequences using the EMBOSS software, two DNA palindromic sequences with a length of 54 base pairs (ATGCTAGGGAGACCAACTTTAGATACCAACTTGTGTACCAACTCTCTAATAGAG) were found both at the "start" and "end" of the deletion.

This palindromic sequence is highly repeated within the peach reference genome (cv Lovell, v2.0): at least 220 times, 100 % identity according to BlastN. A careful inspection within genomic reads of some peach varieties deposited in the SRA database in GenBank (https://www.ncbi.nlm.nih.gov/sra) confirmed the existence of such repeated sequences in different genomic backgrounds (data not shown). The presence of such palindromic sequences in unrelated varieties may explain the occurrence of independent deletions in the same genomic region. First reported in *Escherichia coli* (Dianov et al. 1991), the presence of long direct repeats can cause DNA deletions due to DNA misalignments or mispairings. This phenomenon has also been evidenced in the phases of strand annealing and replication in human and Rosaceae mitochondria DNA (Chen et al., 2011; Persson et al. 2019; Sun et al. 2022), *E. coli* (Bzymek and Lovett 2001), *Sclerotinia* (Xu et al. 2016). On the other hand, rearrangements, deletions and insertions are frequently found following DNA double-strand break repair and non-homologous end joining (Vu et al. 2017; Gorbunova et al. 1997; Schiml et al. 2016; Woodhouse et al. 2010; Puchta 2005) so that it can be speculated that the presence of the repeats identified in proximity of the deletion site may have fostered its occurrence.

4. Conclusions

This paper highlights, for the first time in peach, the presence and importance of palindromic sequences in producing the same deletion in independent mutation events. A Non-ripening phenotype has been obtained by the fixation in homozygosis of this deletion after selfing of the 'Caldesi 2000 mutated' genotype. The loss of this genomic region that results in the putative silencing of two members of the NAC family further supports the importance of these transcription factors in determining the MD phenotype. The mutation in the MD genomic region on chromosome 4 resulted to influence many other phenotypic traits and it is known that NACs have a variety of pleiotropic effects on many fruit quality traits. The co-localization of fruit quality QTLs in the MD region was widely reported in literature for several *Prunus* species. As consequence of this deletion the ethylene production in the NR genotypes was completely inhibited, affecting the whole ripening process. By conducting further research, we aim to differentiate the functions and interactions between *PpNAC072* and *PpNAC1* in our populations. This will help us determine the appropriate hierarchical order for the expression of ripening genes. Once validated, the markers developed on those genes will contribute to enhance MAS efficiency in the peach breeding programs aimed at extending the harvest calendar.

CRediT authorship contribution statement

Marco Pietrella: Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Leonardo Ferrari:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Luca Dondini:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Sara Alessandri:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Letizia Pondini:** Investigation, Formal analysis. **Francesco Tasini:** Investigation, Formal analysis. **Federica Brandi:** Investigation, Formal analysis. **Sandro Sirri:** Investigation, Formal analysis. **Giovanni Giuliano:** Investigation. **Iban Eduardo:** Writing – review & editing. **Stefano Tartarini:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Daniela Giovannini:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Reports a relationship with that includes:. Has patent pending to. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2025.114494.

Data availability

Sequencing data produced have been uploaded on the SRA database (BioProject PRJNA1087134).

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