



Identification and Expression Analysis of Fruit Weight Loci in Italian Plum (*Prunus domestica* L.) Varieties

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Abstract

Background Increasing global food demand is one of the greatest challenges of modern agricultural sciences. A crucial agronomic trait for plant breeding programs to increase yield production is fruit weight (FW). Although two major FW quantitative loci (FW2.2 and FW11.3) have been isolated and studied in several crop species, much still remains to be done for stone fruit trees, such as plum (*Prunus domestica* L.).

Results In this study, six Italian plum varieties were evaluated for fruit size-related traits and genotypic characteristics. Data brought out that Pappacona varieties were a good model for identifying molecular markers associated with fruit size. Using the recently released plum genome assembly, 73 cell number regulator (CNR) and 42 cell size regulator (CSR) genes were finely annotated. Two FW2.2 (PdCNR-1 and PdCNR-2) and one FW11.3 (PdCSR-1) homologs were identified. To provide a first transcriptional characterization, PdCNR-1, PdCNR-2, and PdCSR-1 were analyzed by real-time PCR. PdCNR-1 and PdCSR-1 expression patterns suggested a potential novel regulatory pathway in plum, which might contribute to variations in fruit size and weight.

Conclusions A better understanding of plum FW quantitative loci will promote progress in both basic research and practical breeding activities aiming to select large-fruited varieties.

Keywords Plum · Fruit size · CNR · CSR · Phylogenetic analysis · SNP

Background

Today, agriculture faces challenges with crucial implications for the global future. These include the need to increase crop yield production in response to the global demand for food.

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The size of edible organs, such as fleshy fruit, is an important agronomic trait and a target for plant breeding programs aimed at increasing food production for consumption worldwide (Butac 1937; Eremin et al. 2022). Final fruit morphology directly correlates to floral meristem development and organization processes (Kitagawa and Jackson 2019). Cell division rates and duration, cell shape and cell expansion are major processes that regulate fruit weight (FW). These cellular processes are typically defined to take place before or after anthesis (van der Knaap et al. 2014). Two fruit size quantitative trait loci, the Cell Number Regulator (CNR, also known as FW2.2 homolog) and the Cell Size Regulator (CSR, also known as FW11.3 homolog) genes were isolated and characterized in tomato (Frary et al. 2000; Mu et al. 2017). CNR changes cell layers in the ovary walls so that these organs are already larger at anthesis, while CSR increases fruit weight predominantly through enlargement of the pericarp areas (Su et al. 2021). Although FW2.2 and FW11.3 homologs have been studied in several crop plants (Mu et al. 2017; Su et al. 2021; Guo et al. 2010; De

Franceschi et al. 2013; Tian et al. 2016; Li and He 2015) their roles in determining fruit size in many plant species remain largely unexplored.

Among fruit trees, plum is one of the most important commodities consumed worldwide. There are 19–40 species, depending on taxonomists, that have originated in Europe, Asia and America. From this great diversity, only two species, the hexaploid European plum (*Prunus domestica*) and the diploid Japanese plum (*P. salicina* and hybrids), are of worldwide commercial significance. The European plums were cultivated in Roman times and stone remnants indicate human use 6,000 years ago. The rich diversity and history of European plums is reflected in the many pomological groups including Prunes, Gages, Mirabelles, Damsons, Bullaces and St. Juliens (Topp et al. 2012). European plum is a polymorphic allopolyploid (hexaploid) species ($2n = 6x = 48$) and derived through a natural crossing between *P. spinosa* L. (4x) and *P. cerasifera* Ehrh. (2x) (Mirheidari et al. 2020). *P. domestica* is a deciduous fruit tree bearing fruitstones, whose varieties show considerable variations in their fruit-related characteristics (Manco et al. 2019). Europe is the second world's largest producer of plums with 2199 million tons, which represents about 19% of world production (Butac 1937).

Modern plum varieties have a narrow genetic basis, whereas traditional varieties, landraces and related species represent a precious source of genes and allelic diversity for improving fruit quality, disease resistance, climatic adaptability, and rootstocks (Sottile et al. 2022). The growing interest in traditional varieties, progress in fruit tree genetics and genomics and the recent availability of plum genome sequence (Callahan et al. 2021) provide a real opportunity to identify CNR and CSR genes acting in determining fruit size. In this study, traditional Italian varieties were phenotypically evaluated for fruit size-related traits and genotypically characterized. We used the genome sequence of *P. domestica* to annotate the gene families of plum FW2.2 and FW11.3 homologs. Through phylogenetic analysis, we gained information concerning their genes, evolutionary history and function. Finally, to test the hypothesis that homologs of FW2.2 (CNR) and FW11.3 (CSR) may contribute to FW variation in traditional Italian plum varieties, we studied CNR and CSR expression patterns in three selected varieties using real-time qPCR.

Methods

Plant Materials

Six *Prunus domestica* varieties were used in this study: Calavrice, Marmulegna Nardo, Pappacona Fisciano (round fruit phenotype), Pappacona Gialla, Pappacona Verde and Sorriso

Primavera (elongated fruit phenotype) (Suppl. Figure 1). Plants were grown in Eboli (Salerno, Italy) at "Improsta" Regional Experimental farm (40°33'29''N; 14°58'28''E, at 15 m a.s.l.) using the standard agronomic practices for the area. A total of twenty fruits were collected for morphological analyses from three biological replicates per cultivar. Molecular analyses were carried out on two large-fruited varieties, 'Pappacona Fisciano' and 'Pappacona Verde', and a small-fruited variety, 'Pappacona Gialla' (Suppl. Figure 1). Young-leaf, flower and fruit samples of Pappacona Fisciano, Pappacona Verde and Pappacona Gialla were collected according to their developmental stages to identify the transcriptional changes of CNR and CSR genes during the early fruit development. In particular, flower and fruit samples were taken at four different time points (TP-1: inflorescence bud; TP-2: pre-anthesis flower; TP-3: flower at anthesis; TP-4: fruit at 14 days post-anthesis). The development stages were selected based on the literature (Mu et al. 2017; Su et al. 2021; Guo et al. 2010). Samples were immediately frozen in liquid nitrogen at the time of collection and then stored at -80°C .

Morphological Characterization

The phenotypic characterization was based on the measurement of 8 phenotypic traits on 30 fruits collected from 3 biological replicates (Suppl. Table 1). Specifically, the parameters considered were: the vertical fruit diameter, VFD (cm), the horizontal fruit diameter, HFD (cm), the fruit volume, FV (mm), the fruit weight, FW (g), the stone weight, SW (g), the vertical stone diameter, VSD (in centimeters), the horizontal stone diameter 1, HSD1 (cm) (flattened side) and the horizontal stone diameter 2, HSD2 (cm). Two R software packages of (v4.3) namely FactoExtra (Kassambara and Mundt 2020) and FactoMineR (Lê et al. 2008) were exploited to perform. Variable-plot and biplot R-functions were used for PCA graphical representation (Suppl. Table 2 and Suppl. Table 3).

Genotyping by ddRAD Sequencing and Maximum Likelihood Analysis

DNA was extracted from leaf tissue using the Qiagen DNeasy Plant Kit following the manufacturer's protocol. The quality and quantity of the extracted DNA were assessed using spectrophotometry and agarose gel electrophoresis. Double-digest restriction site-associated DNA sequencing (ddRADseq) libraries were prepared with respect to Peterson's protocol (Peterson et al. 2012). Libraries were sequenced with 150 cycles in paired-end mode on a NovaSeq 6000 instrument. Raw sequence reads were evaluated using FastQC to assess the data quality (Andrews 2020). Low-quality bases and adapter sequences were removed using

Trimmomatic (leading: 30 trailing: 30 minlen: 35 sliding-window: 10:30). Filtered reads were aligned to the plum reference genome by Minimap2 (Bolger et al. 2014; Li 2018), using default settings. The alignment files (BAM) were processed using Qualimap for quality assessment and Picard tools for removing duplicates and adding read group information (Ebbert et al. 2016; García-Alcalde et al. 2012). Single-nucleotide polymorphisms (SNP) were called using Freebayes (Garrison and Marth 2012), a haplotype-based variant detector with default cutoffs (Suppl. Table 4). The resulting VCF files were filtered and analyzed using VCFtools to retain high-confidence SNPs, using the following parameters: a minimum quality score (–minQ) of 10, a minimum depth (–minDP) of 6 reads per variant, a maximum missing genotype ratio (–max-missing) of 0.9 (at least 90% of genotypes must be present), and a minor allele frequency (–maf) of 0.05 (the alternative allele must be present in at least 5% of the samples) (Danecek et al. 2011). These high-confidence SNPs were subsequently analyzed to identify genomic variations and their potential impact on the relevant phenotypes.

To estimate the evolutionary divergence of our six varieties, we ran a phylogenetic analysis using nucleotide sequences generated from the concatenation of 57,815 SNPs. We used the Maximum Likelihood method and applied the General Time Reversible model. The alignments of these data sets were conducted using ClustalW 2.1 (Larkin et al. 2007), with the default setting. The model with the lowest BIC score (Bayesian Information Criterion) was considered to describe the substitution pattern as described by Andolfo et al. (Andolfo et al. 2020). The bootstrap consensus tree inferred from 100 replicates was taken to represent the evolutionary history of the sequences analyzed. The trees were drawn to scale, with branch lengths measured in the number of substitutions per site.

Taxa Data Set

To investigate the evolution of PLAC8 and FAF gene families into the plum genome, we used the *P. domestica* cultivar “HoneySweet” proteome (Callahan et al. 2021). The genomic data (assembly v1.0 and annotation v1.0.a1) were retrieved from Genome Database for Rosaceae (GDR: <https://www.rosaceae.org/>). For comparative purposes, we also added the members of *Zea mays* PLAC8 gene family (Guo et al. 2010) retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>) and 17 well-characterized reference CNR genes in other species. Moreover, the genomic data of *Solanum lycopersicum* and *Arabidopsis thaliana* (Araport11) were retrieved from Sol Genomics Networks (SGN: <https://solgenomics.net/>) and The Arabidopsis Information Resource (TAIR: <https://www.arabidopsis.org/>) databases, respectively (Suppl. Table 5). The tomato and Arabidopsis FAF families were identified

using the most recent gene annotations iTAG4.1 and Araport11, respectively. Finally, tomato CSR-like1 (Mu et al. 2017) was retrieved from iTAG3.0, as its protein sequence (Solyc06g073940.3.1) was absent in iTAG4.1.

Identification and Manual Curation of PLAC8 and FAF Gene Families

In order to identify predicted plum PLAC8 and FAF genes, we scanned the *P. domestica* cultivar “HoneySweet” proteome data set (Supplementary Table S1) with the hidden Markov model of the PLAC8 (Pfam: PF04749) and FAF (Pfam: PF11250) domains using HMMER (v3.0) software for sequence similarity searches with default parameters (Finn et al. 2011). In addition, a local BLASTp search (e-value < 1e-5) was performed by mapping the reference CNR (Su et al. 2021) and CSR (Mu et al. 2017) amino acid sequences as queries to *Prunus domestica* cultivar “HoneySweet” protein data set. The protein domain architecture of HMMER and BLAST outputs was annotated using InterProScan (Jones et al. 2014) and conserved domain search (CD-search) (Marchler-Bauer et al. 2017) with default parameters (Suppl. Table 6 and Suppl. Table 7). Similarly, the tomato and Arabidopsis FAF genes were annotated and classified using the above-mentioned hybrid strategy of gene annotation. In the first step, FAF-encoding genes were identified using HMMER (Finn et al. 2011). The resulting FAF proteins were used as queries against tomato and Arabidopsis proteomes to check for additional genes that had not been previously identified.

Multiple Sequence Alignments and Phylogenetic Analysis

Sequence similarities of PLAC8s and FAFs were determined performing two ClustalW (Larkin et al. 2007) multiple alignments with default settings using the conserved PLAC8 Pfam domain (PF04749) sequences predicted from CD-search (Marchler-Bauer et al. 2017) and the full-length FAF protein sequences as input data sets. Phylogenetic analyses were performed by using newly identified plum PLAC8 and FAF homologs. PLAC8 data set was completed with the *Z. mays* PLAC8s and reference CNRs, while FAF data set was enriched with tomato and Arabidopsis FAFs. Evolutionary relationships between PLAC8 and FAF proteins were inferred using the NJ method based on the Whelan and Goldman model (Whelan and Goldman 2001) using the MEGA6 software (<http://www.megasoftware.net>) (Koichiro et al. 2021). The model with the lowest BIC score was considered to describe the substitution pattern. The bootstrap consensus tree, obtained from 100 replicates, was taken to represent the phylogenetic histories of PLAC8 and FAF families (Felsenstein 1985).

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

Total RNA was isolated from 100 mg of ground plant tissues using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich) following the manufacturer's protocol with some modifications. Quantity and quality of the isolated RNA were measured using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). For cDNA synthesis, 100 ng of each RNA sample was reverse transcribed using the SuperScript® III cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Before use in Real-Time PCR experiments, cDNA reactions were diluted fivefold to 200 µL with UltraPure DNase/RNase-Free Distilled Water (Lonza Group, Basel, Switzerland). The expression study was conducted by real-time PCR analysis using an SYBR Green method on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each 15 µL PCR reaction contained 330 nM of each primer, 2 µL of diluted cDNA and 7.5 µL of Sybr Green Mix (Applied Biosystems). The $2^{-\Delta\Delta CT}$ method has been used as a relative quantification strategy for quantitative real-time polymerase chain reaction (qPCR) data analysis. The transcript abundances were calculated using the method of Livak and Schmittgen (Livak and Schmittgen 2001). The list of primers used for quantitative analysis is reported in Suppl. Table 9. To selectively amplify cDNA, primers were designed such that at least one primer from a pair spans an exon junction. Primers designed on the housekeeping gene actin (Act7) and a portion of Act7 mRNA sequence (Suppl. Table 9) were used as control reactions (Bastias et al. 2020).

Availability of Data and Materials

The datasets generated and analysed during the current study are available in the FIGSHARE repository [<https://figshare.com/s/347973bc7ed2594ac748>] and within the supplementary information files.

Results

Plum Varieties Exhibit Significant Morphological and Genotypic Diversity

Fruits displayed a wide range of diversity across the six varieties (Suppl. Table 1 and Suppl. Figure 1). A considerable variation in the proportion of stone weight (SW) to total fruit weight was found, ranging from 3% in Sorriso Primavera and Calavrice to as high as 9% in Pappacona Gialla. A strong positive correlation (Pearson's $r=0.87$) between

SW and stone horizontal diameter (HSD1) was found. Pulp weight also varied, with Calavrice fruits being the heaviest (on average about 74 g) and Pappacona Gialla the lightest (on average 12 g). PCA based on eight morphological parameters grouped the six varieties into three clusters (Fig. 1; Suppl. Table 2 and 3) and revealed that the Pappacona varieties exhibited the greatest morphological diversity for traits under evaluation.

Through SNP analysis, an average of 7.8 million (ranging from 6.6 to 10.8 millions) of high quality paired-end reads were mapped on *P. domestica* genome assembly. After filtering operations, a total of 671,707 SNPs were identified across all varieties relative to the reference genome, with 57,800 SNPs common to all varieties. Specifically, 254,966 SNPs were unique to Sorriso Primavera, 234,264 to Calavrice, 276,840 to Marmulegna Nardo, 238,088 to Pappacona Verde, 253,018 to Pappacona Gialla, and 251,233 to Pappacona Fisciano (Suppl. Table 4). A phylogenetic tree generated using common SNPs clearly separated round-shaped fruit varieties (Calavrice, Marmulegna Nardo and Sorriso Primavera) from elongated ones (Pappacona Fisciano, Pappacona Gialla and Pappacona Verde). A bootstrap index of 100 highlighted a strong clustering confidence (Fig. 2). The Pappacona varieties, while genetically similar, showed significant morphological differences, providing an ideal model for identifying molecular markers associated with fruit size.

Annotation of Cell Number and Size Regulator Gene Families in *P. domestica*

The observed diversity in the morphological characteristics of our plum varieties, along with the ML clustering that categorized them based on fruit size, prompted a detailed investigation on CNR and CSR. To this end, we first identified the genes encoding for both proteins in *P. domestica* through a protein domain search analysis into plum proteome. Drawing from existing literature (Wahl et al. 2010; Jimenez-Preitner et al. 2012), we know that CNR and CSR proteins are marked with the presence of a placenta-specific gene 8 (PLAC8) and a fantastic four (FAF) meristem regulator domains, respectively. In our study, we discovered 73 PLAC8 and 42 FAF genes, which we finely characterized (Table 1, Suppl. Table 6 and Suppl. Table 7). The average lengths of the PLAC8 and FAF loci we identified were 2.230 bp and 2.044 bp, respectively (Table 1, Suppl. Table 6 and Suppl. Table 7). The longest gene models within these families were Pd.00g1271850 for PLAC8 and Pd.00g1037160 for FAF, measuring 7.473 and 9.736 bp, respectively (Suppl. Table 6 and Suppl. Table 7). Despite this analysis, the partial assembly of the plum genome limited our ability to identify physical gene clusters (Callahan et al. 2021). However, we found seven tandem arrays and three triplets in the CNR family, along with three tandem

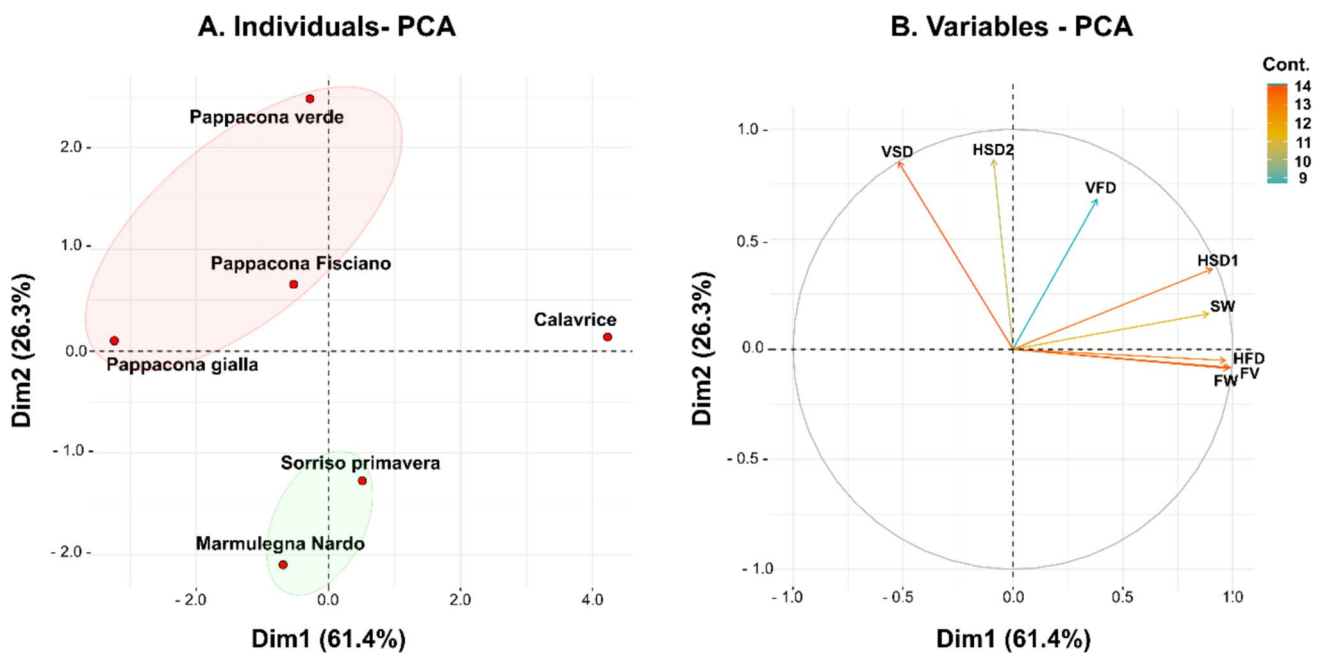


Fig. 1 Principal component analysis (PCA) of six *P. domestica* varieties on the base of eight fruit-related traits. **A)** PCA biplot graph representing *P. domestica* cultivars in two main principal components for eight fruit traits. These two components explained 61.4% (Dim1) and 26.3% (Dim2) of the variance, respectively. Genotypes were divided into three groups. **B)** Variable plot reporting the relationships

between all variables. The vectors show the contribution (direction and magnitude) of the eight fruit traits (FW, FV, VFD, HFD, SW, VSD, HSD1 and HSD2) to the first two components in the PCA. The color of the trait vectors represents the contribution to the variance in the dataset, ranging from 9% (light blue) to 14% (dark orange)

Fig. 2 Maximum-likelihood phylogenies based on SNP concatenated sequence alignment. Labels showing the bootstrap values (out of 100) are indicated above the branches. The tree is drawn to scale, with branch lengths proportional to the number of substitutions per site. For each cultivar is reported the fruit picture

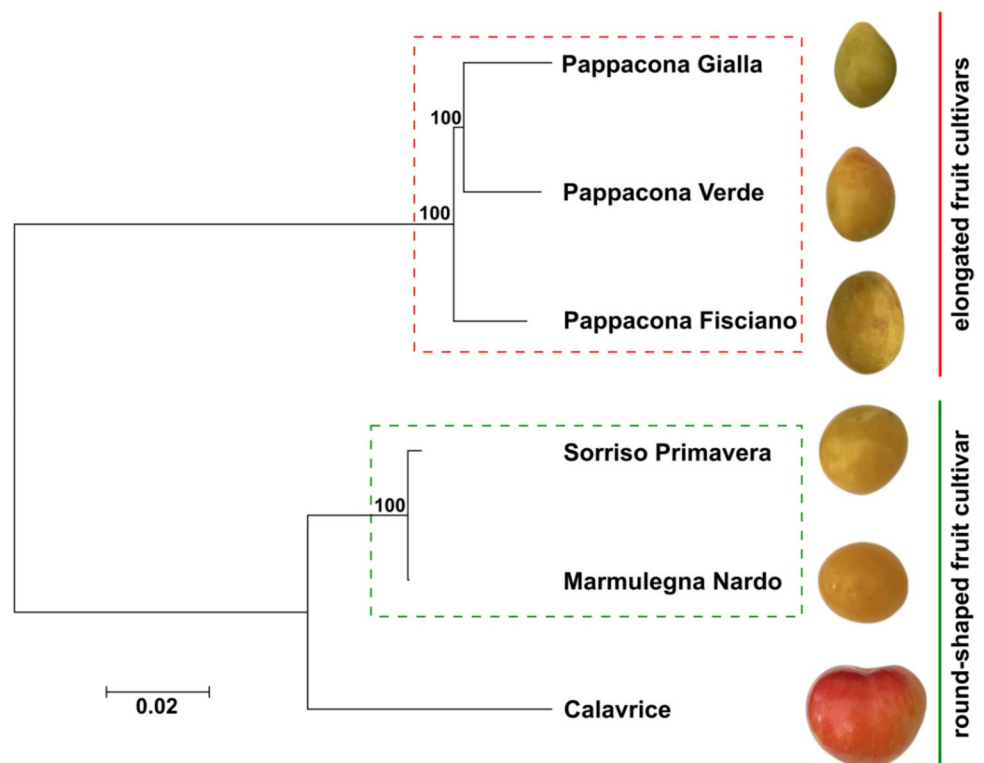


Table 1 Structural properties of PLAC8 and FAF gene models identified in *P. domestica* genome. Average gene, protein and domain lengths and number of exons are reported for each gene family. The average values refer to the arithmetic mean between the members of each gene family

Gene family	Number of loci	Gene length (bp)	Protein length (aa)	Domain length (aa)*	Number of exons for locus
PLAC8	73	2.230	117	100	5
FAF	42	2.044	337	52	3

*Length of PLAC8 (PF04749) and FAF (PF11250) Pfam domains predicted in this study by Interproscan

arrays and one triplet in the CSR family (Suppl. Table 8). On average, the number of amino acids in PLAC8 and FAF proteins were 117 and 337, while the number of exons was 5 and 3, respectively (Table 1). The average domain length was 100 amino acids for PLAC8 proteins and 52 for FAF ones (Table 1). Interestingly, 14 of the 73 PLAC8-like genes and 11 of the 42 FAF-like genes showed atypical protein domains (Suppl. Table 6 and Suppl. Table 7). In particular, about half of PLAC8 and FAF genes presented integrated domains encoding for the mixed lineage kinase domain-like (MLKL_NTD) and the vacuolar (H⁺)-ATPase (V-ATPases) domains, respectively (Suppl. Table 6 and Suppl. Table 7).

Identification of CNR and CSR Genes Associated with Fruit-Weight Traits

To retrace the evolutionary relationships of plum PLAC8s and FAFs and pinpoint the candidates involved in the fruit size formation, we inferred separate phylogenetic analyses for each gene family. Using the NJ (neighbor-joining) algorithm, we estimated the genetic distances between the 73 *P. domestica* PLAC8 proteins, and compared them with 17 functionally validated CNRs (also known as fruit weight regulators or FWs) from other plant species (Su et al. 2021) (Fig. 3a; Figure S1; Suppl. Table 5), including members of the *Zea mays* PLAC8 gene family (Guo et al. 2010). The ClustalW alignment of 93 PLAC8-Pfam domain (Pfam accession PF04749) sequences showed a global pairwise identity of ~39%. This analysis grouped the *P. domestica* proteins into nine phylogenetic clades (bootstrap index ≥ 50), of which six included a reference CNR gene (Suppl. Figure 2). Many evolutionary relationships remain uncertain, as the deeper phyletic divisions have weak statistical support (bootstrap index < 50). Thirteen plum proteins, along with the well-characterized CNRs (red triangle in Fig. 3; Suppl. Table 5) grouped into two phylogenetic clades (bootstrap index ≥ 64) (Fig. 3a). These clades were designated ZmCNR1-SIFW-like and PbFW-like, following the naming

convention of reference CNR homologs previously established (Su et al. 2021; De Franceschi et al. 2013). Sequences within these clades showed an average PLAC8 domain identity of 76.9% and 95.0%, respectively. Moreover, all members of these clades shared the conserved CXXXXXCPC motif (Su et al. 2021) with Rosaceae CNR-homologues, showing two additional conserved residues (CXITXXCPC) (Fig. 3b). Interestingly, a clade-specific pattern was enriched with two extra amino acids in ZmCNR1-SIFW-like and PbFW-like clades (Fig. 3b). We carefully investigated the phylogenetic relationships of *P. domestica* FAF proteins (Fig. 4). FAF proteins featured a very short conserved region (ranging from 32 to 60 residues) known as FAF domain (Pfam accession PF11250). Following the findings by Mu et al. (Mu et al. 2017), to distinguish the CSR candidates among the plum FAFs, we aligned the full-length sequences of 42 *P. domestica* FAF proteins with those of tomato and Arabidopsis FAFs, including their CSRs (Fig. 4). Five *P. domestica* proteins emerged as likely orthologs of the tomato and Arabidopsis CSRs (Fig. 4), forming a robust SICSR-like clade (bootstrap = 90). Five additional clades were pointed out within the plum CSR family, four of which included homologs from tomato and Arabidopsis (Suppl. Figure 3).

Overall, a total of 18 FW gene candidates were identified in our phylogenetic analyses (Table 2). These candidates collapsed into three distinct clades, likely representing alleles from three specific loci: PdCNR-1, PdCNR-2, and PdCSR-1 (Fig. 3 and Fig. 4). Given the hexaploid nature of the plum genome, the allelic variants within these clades showed a high degree of identity, ranging, on average, from 96.4% to 99.7%.

Transcriptional Characterization of CNR and CSR Genes

The three Pappacona varieties were chosen because they are genetically very similar but morphologically different in terms of fruit weight, providing an ideal model for investigating FW transcriptional regulation. To discover whether PdCNR-1, PdCNR-2 and PdCSR-1 could be putatively involved in the regulation of fruit size, we analyzed their expression levels at four checking time-points (TPs) in Pappacona Fisciano, Pappacona Verde and Pappacona Gialla (Fig. 5). PdCNR-1 was generally down-regulated in all plum varieties, except for an upregulation ($\text{Log}_2\text{FC} = 2.25$) in Pappacona Gialla during flower anthesis (Fig. 5B). Indeed, a very strong negative correlation (Pearson's $r^2 = -0.96$) was found between PdCNR-1 expression and FW of Pappacona cultivars at TP-3.

PdCNR-2 transcription patterns were up-regulated in Pappacona Verde and Pappacona Gialla, whereas it was generally down-regulated in Pappacona di Fisciano (Fig. 5C). PdCSR-1 was up-regulated from TP1 to TP4 in

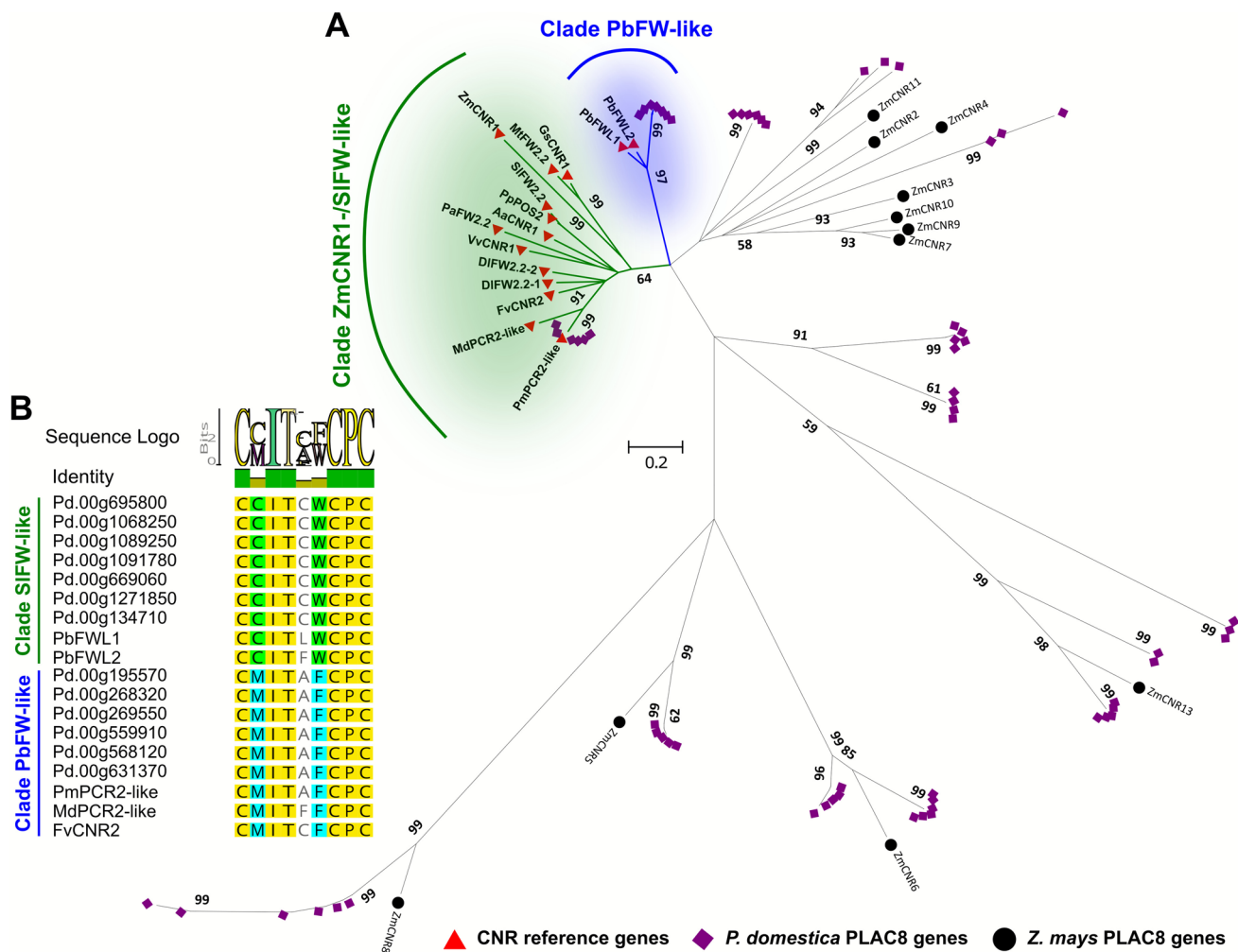


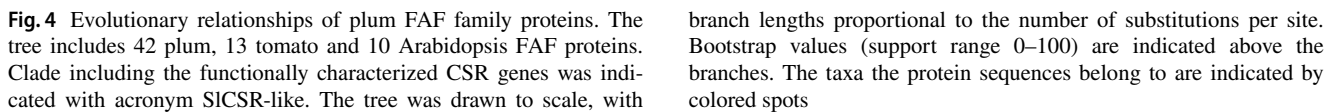
Fig. 3 Phylogenetic analysis of *P. domestica* PLAC8 family proteins and motif alignment of Rosaceae PLAC8 proteins. A) SIFW-like and PbFW-like clades are highlighted in green and blue respectively. The tree includes 73 plum CNRs and 20 reference CNR proteins already characterized in other species. Clades were indicated with acronyms (SIFW-like and PbFW-like) on the base of position reference CNRs. The tree was drawn to scale, with branch lengths proportional to the

number of substitutions per site. Bootstrap values (support range 0–100) are indicated above the branches. The taxa to which the protein sequences belong are indicated by colored spots. B) Motif (CXXXXXCPC) sequence alignment of proteins clustered in SIFW-like and PbFW-like clades belong to Rosaceae plant family. Identical amino acid residues are underlined in yellow and clade-specific residues in light green and cyan

the large-fruited varieties, while it was consistently down-regulated (Log_2FC ranging from -1.1 to -2.1) in Pappacona Gialla (Fig. 5D). In all time points, a strong positive correlation (Pearson's r^2 range $0.81 - 0.99$) between PdCSR-1 expression and Pappacona FWs was found. These patterns suggest that while large-fruited varieties generally exhibit lower PdCNR-1 and higher PdCSR-1 expression levels, particularly at TP3, PdCNR-2 exhibits a variable pattern, indicating a potentially complex role in fruit development (Fig. 5B and D).

Discussion

The phenotypic and genotypic evaluation of fruit tree germplasm could lead to the identification of genomic regions that influence traits of interest (Manco et al. 2019; Kellerhals et al. 2012). Here, we have characterized from a morphological and molecular perspective six *P. domestica* varieties with a focus on two fruit size quantitative trait loci, FW2.2 and FW11.3, which we identified for the



Locus Name	Phylogenetic clade	Putative alleles n	Gene identity (%)	Protein identity (%)	Coding DNA sequence identity (%)
PdCNR-1	ZmCNR1-/SIFW-like	6	97.9	99.2	99.4
PdCNR-2	PbFW-like*	7	96.4	90.2	88.8
PdCSR-1	SlCSR-like	5	99.7	90.6	97.2

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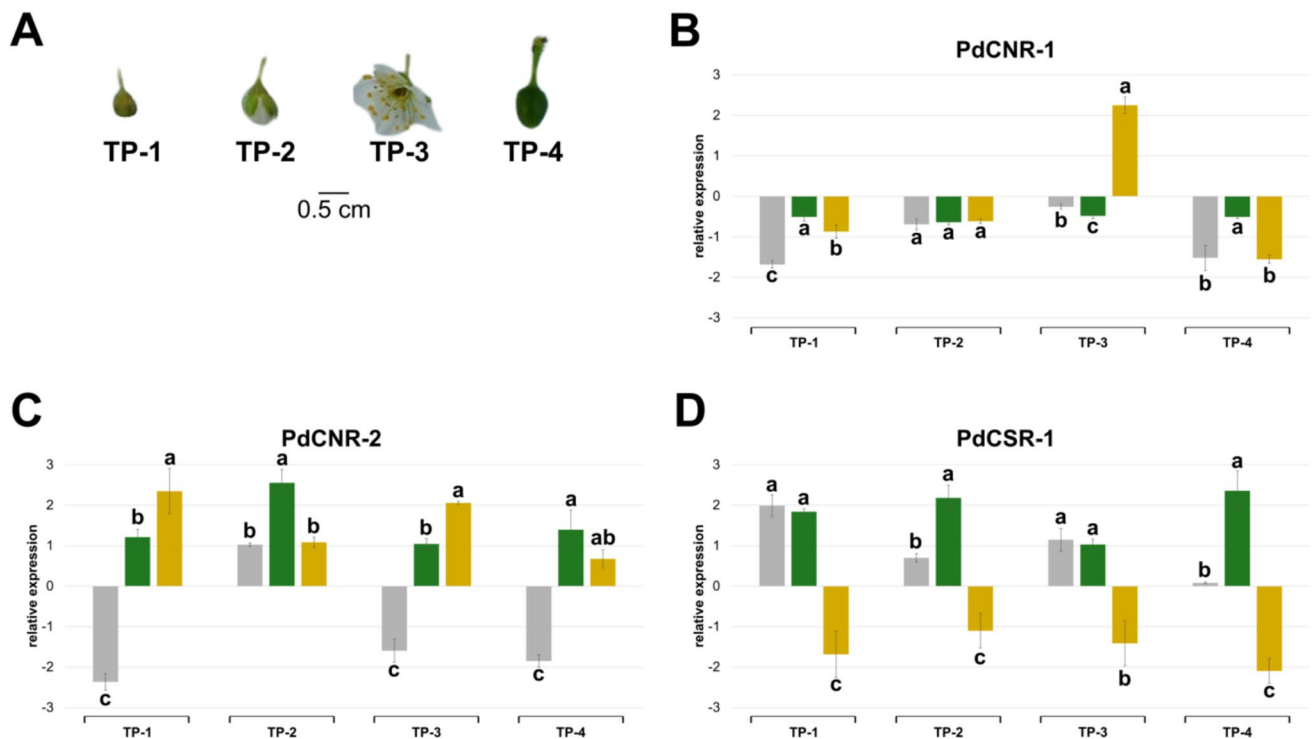


Fig. 5 Transcriptional changes of CNR and CSR genes in three plum cultivars. **(A)** Plant material was collected at four checking time-points (TPs) at different physiological ages during the early fruit development (TP-1: inflorescence bud; TP-2: pre-anthesis flower; TP-3: flower at anthesis; TP-4: fruit of 0.5 mm diameter). **(C-E)** Relative gene expression of PdCNR-1, PdCNR-2 and PdCSR-1 in Pappa-

cona Fisciano (gray), Pappacona Verde (green) and Pappacona Gialla (yellow). Data were analyzed by two-way analysis of variance following Duncan's test, and different letters represent a statistical difference ($p < 0.05$). Vertical bars represent the standard error of triplicate experiments

first time in plum and studied through a transcriptional characterization.

Our initial investigation on the morphological diversity revealed significant variations in fruit shape, size and stone weight. These traits are considered very important for conservation, management, and germplasm exploitation (Mirheidari et al. 2020). Phenotypic characterization of our varieties contributes to the exploration of Italian genetic resources. Morphological study is essential to evaluate prospective material to integrate valuable genotypes into breeding programs (Ternjak et al. 2023). At the European plum, the increasing fruit size is absolutely necessary, especially since most varieties with special tasting qualities have small fruits (Butac 1937; Eremin et al. 2022). Our plum varieties were clustered into two morphological groups (round and elongated) of fruit shape. Varieties It is interesting to note that three Pappacona varieties used here exhibited a marked pomological variability, which was 99% explained by fruit weight. Our fruit phenotypic characterization was associated to a whole-genome sequencing to investigate the genetic relationships among the six varieties, using a double-digest restriction site-associated DNA sequencing (ddRADseq) approach. Overall, we observed a relatively low

genetic diversity among varieties, all of which fell within the European pomological group previously proposed (Zhebentyayeva et al. 2019). SNP genotyping is a valuable tool for gene mapping, map-based cloning, and marker-assisted selection in crop breeding (Mammadov et al. 2012). The insights gained from SNPs will help us identify specific genetic variations that drive morphological diversity, thereby enhancing our ability to develop breeding programs for plum FW trait. Unlike morphological data, Pappacone varieties showed a strong clustering confidence supported by shared 98,386 SNPs. Most likely, they descend from a relatively-closed progenitor (Giovannini et al. 2011). By focusing on Pappacona varieties, we wanted to understand the underlying genetic factors contributing to their distinct fruit characteristics. Several genes (SUN, OVATE, CYP78A, TRM, FW2.2, FW11.3, YABBY, and WOX) have been identified as key determinant of fruit size, shape, and weight variation in Arabidopsis, tomato, and rice (Frery et al. 2000; Mu et al. 2017; Chakrabarti et al. 2013; Wu et al. 2011; Rodríguez et al. 2011; Muñoz et al. 2011; Lee et al. 2006; Liu et al. 2002; Bowman and Smyth 1999). FW2.2 and FW11.3 genes are characterized by the presence of PLAC8 and FAF protein domains, respectively (Mu et al. 2017; Su et al. 2021). The

recent publication of *P. domestica* genomic sequence (Callahan et al. 2021) has allowed us to identify 73 PLAC8 and 42 FAF domain-encoding loci. Our gene annotation greatly facilitates the studies of plant biology and functional genomics, as it provides a manually curated dataset. However, it should be pointed out that recent studies clarified that not all PLAC8 and FAF families members are involved in fruit weight regulation (Pan et al. 2020). For instance, some PLAC8 genes were reported to be responsive to environmental stresses (Li et al. 2019; Qiao et al. 2019a, 2019b). Similarly, tomato FAF genes were found expressed in different tissues compared to FW11.3, suggesting divergent roles beyond fruit weight regulation (Mu et al. 2017).

NJ clustering analyses of the PLAC8 and FAF gene families underlined distinct phylogenetic clades associated to fruit weight gene candidates: two clades (ZmCNR1-/SIFW-like and PbFW-like) within PLAC8 family and one (SICSR-like) within FAF family. Although *P. domestica* genome is hexaploid, only the PdCNR-1 locus has been linked to the six relative putative alleles, while seven and five putative alleles were identified for PdCNR-2 and PdCSR-1 loci, respectively. This is consistent with the BUSCO evaluation of the gene set predicted in the *P. domestica* genome assembly, where approximately only 2% of genes had six alleles (Callahan et al. 2021). It is interesting to note that, although PdCNRs collapsed in two phylogenetic clades, they are closely related to functionally characterized FW genes from other Rosaceae species, including PmPCR2-like from *P. mume*, MdPCR2-like from *Malus domestica* and PbFWs from *Pyrus betulifolia* (Su et al. 2021). This suggests a conserved role across species within the family. Moreover, two FW2.2 homologs were isolated and characterized in other species such as cherry, pear and loquat, underscoring the importance of the genetic component in fruit development across a range of fruit-bearing plants (Su et al. 2021; De Franceschi et al. 2013; Tian et al. 2016).

A first transcriptional validation was focused on the measurement of PdCNR-1, PdCNR-2 and PdCSR-1 expression levels in Pappacona Verde, Pappacona Gialla and Pappacona Fisciano. These varieties were selected based on their significant fruit weight differences (~40%) and the minimal genetic diversity (Fig. 1 and 2). Frary et al. (2000) previously reported that the FW2.2 accounts for approximately 30% of the variation in fruit size between cultivated tomato and its wild relatives. Meanwhile, the FW11.3 locus was identified as a minor quantitative trait locus, explaining only 8% of the phenotypic variance (van der Knaap and Tanksley 2003). We found an inverse relationship between the expression of PdCNR-1 and PdCSR-1, the latter being a positive cell cycle regulator of the cell cycle during G2 to M phase transition (Ito et al. 1998) in large- and small-fruited varieties. Specifically, during early developmental stages when the cortex greatly increases cell layers, the large-fruited

varieties Pappacona Fisciano and Pappacona Verde showed downregulation of PdCNR-1. On the contrary, these varieties displayed overexpression of PdCSR-1 at all time-points. The expression profiles of CdCNR-2 did not correlate with FW. Such results suggest that similar to the roles FW2.2 and FW11.3 in tomato, PdCNR-1 and PdCSR-1 are also involved in modulating fruit cell division in plum (Frary et al. 2000; Mu et al. 2017). The expression patterns of PdCNR-1 and PdCSR-1 suggest a potential novel regulatory pathway affecting cell division and enlargement (Mu et al. 2017; Su et al. 2021), and reveal how the heterochronic expression of plum FW-related genes in plum might contribute to variations in fruit size and weight across varieties. Understanding the genetic underpinnings of FW trait requires knowledge of the gene variants that contribute to phenotypic variability (Frary et al. 2000; Tian et al. 2016). As expected, the restricted number of analysed genotypes limits our ability to detect associations explaining a substantial proportion of the observed variance. Extending the analysis to a larger and more diverse number of plum varieties would increase the reliability of our findings. Moreover, although our transcriptional data support the involvement of PdCNR-1 and PdCSR-1, a functional validation of candidate genes for the establishment of biological plausibility will be essential to establish their biological roles in fruit development. In the near future researchers may consider these FW gene candidates to support marker-assisted breeding and potentially speed up the selection of new varieties for increasing plum production, although such applications will require further experimental confirmation.

Conclusions

In conclusion, the study undertaken here allowed us to explore the genetic bases of fruit size variation in *P. domestica* varieties. Traditional varieties represent a resource in breeding programs that can be used to recover diversity lost due to erosion and to increase genetic diversity. Maximum likelihood analyses of cell number and size regulator genes pinpointed three candidates (PdCNR-1, PdCNR-2 and PdCSR-1) involved in plum size formation. A strong correlation between PdCNR-1 and PdCSR-1 expression levels and Pappacona fruit weights was observed. Functional validation assays will be necessary to experimentally demonstrate their phenotypic effects.

Abbreviations *BIC*: Bayesian information criterion; *CD-search*: Conserved domain search; *CNR*: Cell number regulator; *CSR*: Cell size regulator; *ddRADseq*: Double-digest restriction site-associated DNA sequencing; *FV*: Fruit volume; *FW*: Fruit weight; *GDR*: Genome database for rosaceae; *HFD*: Horizontal fruit diameter; *HSD*: Horizontal stone diameter; *PCA*: Principal component analysis; *qPCR*: Quantitative polymerase chain reaction; *SGN*: Sol genomics networks;

SNP: Single-nucleotide polymorphism; **SW:** Stone weight; **TP:** Time point; **VFD:** Vertical fruit diameter; **VSD:** Vertical stone diameter

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Authors' Contributions R.G. and G.A. designed the study, collected the samples, and performed the data analyses. A.R.E. and R.S. executed the molecular analyses. R.G., R.A., D.C. and G.A. jointly interpreted data and made substantial contributions to conception and design of the manuscript. R.G. and G.A. wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Data Availability All data analysed and generated during this study are included in this published article and in supplementary information files.

Declarations

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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