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RESEARCH REPORT

Transcriptomic and metabolomic analysis of on-tree fruit maturation in the white, melting-flesh peach cultivar 'Lemonato'

Persefoni Maletsika[1] · Michail Michailidis[2] · Evagelia Mouchtaropoulou[3,4] · Vasiliki Liava[1] · Elpida Nasiopoulou[2] · Vaia Styliani Titeli[2] · Eirini Sarrou[5] · Stefan Martens[6] · Katerina Grigoriadou[5] · Anagnostis Argiriou[3,4] · Athanassios Molassiotis[2] · George D. Nanos[1]

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Abstract

The impact of different maturity levels of peaches on fruit quality, metabolic pattern and transcriptomic alternation was studied. To achieve this, 'Lemonato' peaches were sampled at commercial harvest time and then were separated based on their peel ground color into immature (light green exocarp), semi-mature (partly yellow exocarp) and mature (entirely yellow exocarp). Data indicated that several key maturity parameters, such as color index, fruit firmness, chlorophyll content, dry matter, total phenol content and antioxidant activity were notably induced by the maturity transition. Consistent with this observation, increased levels of phenolic compounds, including catechin, epicatechin, procyanidin B1/B2/B4 and chlorogenic acid, were identified at the last maturity stage. Mature fruit was also characterized by an elevated abundance of various primary metabolites (e.g. sorbitol, arabinose, xylose) and decreased levels of main organic acids and putrescine. Genes predominantly associated with flavonoid biosynthesis, calcium signaling, cell wall integrity, auxin metabolism, and aquaporins and chloroplast function were remarkably affected by maturity. Several genes were also identified (e.g. *EXORDIUM*, *PCC13-62* and *SDI1*), including transcription factors, such as *IBH1, homeobox ATHB-13* and *bHLH63*, undergoing major changes during fruit maturation. These results enhance understanding of on-tree metabolic and transcriptomic events that coordinate peach fruit maturation, helping to identify the optimal harvest time for best peach fruit quality.
Clinical trial number Not applicable.

Keywords Fruit quality · On-tree maturity · Peach · Polyphenols · Primary metabolites · Transcriptomics

1 Introduction

Peach fruit [*Prunus persica* (L.) Batsch] is an important and highly appreciated fruit by consumers due to its delicious flavor, attractive appearance and nutritional value (Minas et al. 2018). They are the world's third most economically important temperate tree fruit, after apples and pears, and more than 90% of their production is for the fresh market. Peaches are rich in a variety of vitamins and minerals, including carbohydrates, organic acids, pigments, phenolics, vitamins, volatiles, antioxidants, and small amounts of proteins and lipids (Dabbou et al. 2017). Facing overproduction of peaches and stable to decreasing consumption in world markets, fruit quality is becoming a main issue to enhance peach consumption and thus increase economic returns to growers (Crisosto and Crisosto 2005; Minas et al. 2021, 2018).

Peach fruit has a relatively large variation in the on-tree maturity; due to this variability, it is essential to apply successive harvests (Gonçalves et al. 2016). Maturity at commercial harvest is typically determined based on factors such as fruit size and diameter, background color, sugar-to-acidity ratio, flesh firmness, and non-destructive methods like DA-meter evaluation (Cascales et al. 2005; Crisosto and Costa 2008; Ziosi et al. 2008; Spadoni et al. 2016). Ideal harvest maturity has become a key factor in peach fruit production to better satisfy consumers in the marketplace as it determines fruit quality and postharvest performance.

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Indeed, peach fruit maturity has a great influence on flavor components, ripening potential, physiological deterioration issues, resistance to moisture loss, susceptibility to mechanical damage, and pathogen losses (Crisosto 1994). Overall, variable peach fruit on-tree maturity at harvest has practical and economic consequences for peach producers, marketers, retailers, and, ultimately, consumers (Crisosto and Costa 2008). Particularly, peach fruit position in the tree canopy can influence on-tree maturity with many fruit maturity-related metabolic shifts and consequently quality parameters (Minas et al. 2021; Lewallen and Marini 2003), because fruit is exposed to variable microclimatic conditions, such as light and temperature. This is an important feature because, when the fruits are harvested in one 'hand', then fruits of different maturity levels are simultaneously picked (Lurie et al. 2013). Therefore, the research on the mechanism of maturation during the last phase of on-tree development helps us better understand the changes in peach quality to finally promote peach industry development.

In recent years, high-throughput methods have been extensively applied to understand the peach fruit maturation dynamic. Integrated proteomic, metabolomic and transcriptomic analyses shed light on the relationship between the contents of various primary and secondary metabolites as well as the corresponding differentially accumulated proteins and expressed genes in peach fruit during development (Brummell et al. 2004; Karagiannis et al. 2016; Maletsika et al. 2023; Ramina et al. 2008; Tanou et al. 2017), but no published data exist concerning physico-chemical, transcriptomic and metabolomic changes in peach fruit that displayed distinct maturity status at harvest. In this study, we used the 'Lemonato' peach, a melting- and white-flesh Greek-originated cultivar that is susceptible to rapid postharvest softening and easy bruising leading to significant deterioration and losses (Maletsika et al. 2021). Thus, commercial harvest of relatively immature 'Lemonato' peach fruit is common practice to manage and extend its postharvest handling. This work aimed to evaluate the influence of the maturity stage at harvest [('immature', 'semi-mature' (commercial maturity stage), and 'mature'] on the fruit quality attributes, primary metabolites, and phenolic compounds along with transcription levels of 'Lemonato' peaches. Our study lays the groundwork for further elucidation of crucial players in peach maturation and offers a theoretical basis for providing a reference to produce high-quality fruit.

2 Materials and methods

2.1 Fruit material and sampling process

The experiment was conducted in a commercial orchard of 0.1 ha of peach 'Lemonato' (genotype 'Stamatis') at Niseli Imathias, Greece. The orchard consisted of five-year-old trees grafted onto GF677 rootstock, planted at 5 × 5 m, trained in an open vase system, and received standard pruning, fertilization, irrigation and plant protection practices. Twelve experimental trees ($n = 12$) representative of the orchard, were randomly selected for uniformity, avoiding border rows. From the 12 experimental trees, fruits were harvested (September 13, 2021) and mixed from at least partially lit positions within the tree canopy with comparable size (280 to 320 g). Fruit maturity was determined macroscopically by fruit skin color to obtain peaches with distinct phases of maturation, namely immature (light green exocarp, i.e. peel; RGB = 220, 240, 160), semi-mature (partly yellow exocarp; RGB = 220, 220, 120), and mature (entirely yellow exocarp; RGB = 230, 220, 110). Three 10-fruit replicates of exo- and meso-carp per maturity stage (immature, semi-mature and mature) were frozen in liquid nitrogen and stored at −80 °C for further analysis. Additionally, eight 10-fruit replicates per maturity stage were used for quality determination.

2.2 Peach quality determination

2.2.1 Quality attributes

Fruit quality measurements included exocarp color, dry matter, firmness, soluble solids concentration (SSC) and titratable acidity (TA). Color determination in the exocarp was performed using a Minolta colorimeter (Model CR-400, Minolta Ltd, Osaka, Japan) on the two opposite sides of the fruit. The dry matter analysis was performed on a 1 cm slice of exo-meso-carp tissue from each fruit. The fresh weight of the slice was measured immediately after slicing, and the dry weight was obtained after drying the slice in an oven at 65 °C for 72 hours. The dry matter content was then estimated by dividing the dry weight by the fresh weight and expressed in %. Firmness was also determined at two opposite sides of the fruit (after the exocarp was removed to a depth of 1 mm) using a digital penetrometer (model 53,205, Turoni Srl, Forli, Italy) equipped with an 8.9-mm plunger that was inserted into the mesocarp 1 cm deep, and the results were expressed in Newtons (N). The SSC and TA were estimated on the peach fruit juice by an Atago Refractometer (Model PAL-1, Atago, Tokyo, Japan) and after titration with 0.1 N NaOH until pH 8.2 (expressed in % malic acid), respectively, and the ratio SSC/TA was calculated.

The peach juice was extracted from one longitudinal slice of each fruit of the ten-fruit replication (Maletsika et al. 2023).

2.2.2 Antioxidant activity, total polyphenolic and chlorophyll contents

To measure the total antioxidant activity and total phenolic content of peach fruit (exo-meso-carp tissue) a sample of 5 g was homogenized and extracted with 25 mL methanol, and then centrifuged at 4000*g* for 10 min. The supernatant was used for the assay of total antioxidant activity and total phenolic content. The total antioxidant activity of the peach fruit (exo-meso-carp tissue) was assessed with the DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging activity) (Brand-Williams et al. 1995) and the FRAP (Ferric ion Reducing Antioxidant Power) (Benzie and Strain 1996) methods, and the results were expressed as mg of equivalent ascorbic acid per g fresh weight. The Folin–Ciocalteu colorimetric method was used to measure the total phenolic content of the peach fruit (exo-meso-carp tissue) with some modifications (Maletsika et al. 2023) and results were expressed as mg of equivalent gallic acid per g fresh weight. To determine the chlorophyll content of peach fruit (exo-meso-carp tissue), a sample of 2 g was homogenized and extracted with 20 mL ethanol (90%, v/v) in a flask for 24 h. The solution was measured at 665 nm and 649 nm using a UV-1700 PharmaSpec spectrophotometer (Shimadzu Corporation, Japan). Chlorophyll content was calculated by the equations: C_{a+b} (mg L^{-1}) = 6.1 OD_{665} +20.04 OD_{649} (1), and expressed in μg g^{-1} using the equation (C_{a+b} V)/m (2) with V the total volume and m is the sample weight (Sun et al. 2017).

2.3 Primary and secondary metabolites analysis

2.3.1 Primary polar metabolite analysis by gas chromatography–mass spectrometry

The primary metabolites of peach fruit (300 mg of ground frozen exo-meso-carp tissue) were extracted with methanol (1.4 mL) plus adonitol (0.1 mL, 0.2 mg mL^{-1}) as an internal standard at 70 °C for 10 min under constant agitation as previously described in detail (Polychroniadou et al. 2022). The metabolite analysis was implemented using a GC PerkinElmer Clarus® 590 equipped with MS Clarus® SQ 8 S (Perkin Elmer, NJ, USA) and a capillary type column (TR-5 MS) 30 m × 0.25 mm × 0.25 μm. Gas chromatography–mass spectrometry conditions and program, along with the identification and quantification of compounds, have been described in our previous study (Michailidis et al. 2024). The compounds were normalized based on the abundance of the internal standard of adonitol, and each compound was expressed as the relative abundance of adonitol (Table S1). Three biological replicates per maturity condition were used, while immature fruit served as control.

2.3.2 Individual polyphenolic compounds analysis by ultra-performance liquid chromatography-tandem mass spectrometer

For polyphenolic compound analysis, freeze-dried samples of exo-meso-carp tissue were used (Freeze-dryer Alpha 1–2 LD plus, Christ, Osterode, Germany; at − 24 °C). One hundred mg of sample were extracted with 4 mL methanol (80%). The solutions were sonicated for 20 min, shaken for 3 h at 20 °C, left at 4 °C overnight in the dark, filtered through 0.22 μm polytetrafluoroethylene membrane filters into glass vials, and injected directly for polyphenolic analysis. Polyphenolic compound determination was performed by an ultra-performance liquid chromatography-tandem mass spectrometer (UPLC—MS/MS) on a Waters Acquity system (Milford, MA, USA) using a Waters Acquity HSS T3 column (1.8 μm, 100 × 2.1 mm^2, set at 40 °C) and separation conditions as previously described (Vrhovsek et al. 2012). Data processing was performed using Mass Lynx Target Lynx Application Manager (Waters). The experiment was performed using three biological replicates per maturity stage, while immature fruit served as control. The metabolites were quantified based on reference standards and expressed as mg/100 g.

2.4 Analysis and validation of RNA-seq data

2.4.1 RNA extraction, library construction and analysis

Total RNA was extracted from exo-meso-carp tissue at different peach maturity stages in three biological replicates using the Spectrum™ Plant Total RNA Kit (St. Louis, Missouri, United States) (Michailidis et al. 2020). Additionally, BioSpec Zirconia/Silica beads (0.1 mm diameter, 11079101Z) were used, and the samples were homogenized in a Retsch MM 300 TissueLyser Mixer Mill (Qiagen) at maximum speed (30 Hz) to ensure complete homogenization. The isolation of mRNA was performed using the NEBNext® Poly(A) mRNA Magnetic Isolation Module from New England Biolabs Inc., (Ipswich, MA, USA). Libraries were generated using the NEBNext® Ultra™ II RNA Library Prep Kit for Illumina® (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's instructions. The libraries were quantified and qualified using the Agilent 5300 Fragment Analyzer and QIAGEN Rotor-Gene Q Real-Time PCR System. Ultimately, each library was sequenced using an Illumina® NextSeq 500 platform 2x150. Sequences underwent trimming to remove adaptors

and were filtered to exclude low-quality reads (Q > 28) and unknown sequences (N) using Trim Galore. The readings were filtered and aligned to the *Prunus persica* reference genome (GCF_000346465.2 Prunus_persica_NCBIv2) using Hisat2. Differential gene expression analysis (DGE) was statistically evaluated using the Ballgown package in the R programming language. Functional annotation analysis of transcriptome data was conducted using the package 'biomaRt' (Durinck et al. 2005, 2009) and the ShinyGO.080 (Ge et al. 2020), a web-graphical gene-set enrichment tool for animals and plants. Raw sequencing data are available on GenBank under project acc. number PRJNA1114023. The differentially expressed genes (DEGs) are presented in Table S2.

2.4.2 Real-time qPCR analysis

Peach exo-meso-carp RNA extraction at three maturity levels was conducted in three biological replicates using the RNeasy® Plus Mini Kit from Qiagen (Valencia, CA, USA). The process of constructing cDNA involved the reverse transcription of 10 ng of RNA using the LunaScript® RT SuperMix Kit provided by New England Biolabs Inc. The real-time PCR was conducted using 2 µL of cDNA, 0.5 µL each of forward and reverse primers, and following the protocol provided by Luna® Universal qPCR Master Mix (New England Biolabs Inc.) in a QuantStudio® 5 Real-Time PCR System (96-well, Thermo Fisher Scientific). The primers were generated using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and are listed in the Table S3. The qPCR program was carried out according to Michailidis et al. (2023). The Ct value was determined at 0.2 ΔRn, and a melt curve was generated to confirm the PCR products. The data were analyzed using the ΔΔCt approach, as previously described (Livak and Schmittgen 2001).

2.5 Statistical analysis

One-way analysis of variance (ANOVA) was conducted using SPSS v29.0. Mean values of peach quality traits, metabolites, and gene expressions (from RT-qPCR) were compared by Tukey's multiple range test ($p \leq 0.05$). The association between RNA-seq data with qRT-PCR Ct values and quality traits with metabolites was assessed by the Pearson correlation coefficient (SPSS v29.0). Principal coordinates analysis, GO and KEGG enrichment analysis was employed using R software and principal component analysis (PCA) was employed using ClustVis software 2.0 (Metsalu and Vilo 2015).

3 Results

3.1 Quality traits of peach fruit exhibiting three distinct maturity levels at harvest

Peach maturity level at harvest had a significant effect on fruit quality parameters. Although fruit firmness was similar among immature and semi-mature peaches (31.4 N and 34.5 N, respectively), the mature fruit displayed a significantly lower firmness value reaching 14.3 N (Fig. 1A). In addition, the color index a^* of the fruit exocarp increased gradually as macroscopic color became less green (Fig. 1B). Another parameter related to fruit quality is dry matter content, which was found to increase from the immature to semi-mature stage without any further increase at the mature stage (Fig. 1C). Titratable acidity decreased, while SSC and the ratio SSC/TA increased gradually from immature to the mature stage (Fig. 1D–F). Meanwhile, the total antioxidant activity and the total phenolic content were influenced by the peach fruit maturity stage (Fig. 1G–I). The lowest antioxidant activity in both DPPH and FRAP assays as well as the lowest total phenolic content were recorded in immature fruit and the highest in mature fruit. On the contrary, the highest chlorophyll content was detected in immature fruit and the lowest in mature fruit (Fig. 1J).

3.2 Changes in primary metabolites and polyphenolic compounds in peach fruit exhibiting different on-tree maturity levels at harvest

We examined the patterns of primary and secondary metabolite changes among evaluated peach phenotypes to understand how the maturity levels could lead to different metabolic responses. Although physiological analysis reveals several quality changes in peach fruit with different maturity levels (Fig. 1), relatively low differences were found among the different maturity conditions regarding the primary metabolites (Fig. 2). The most abundant sugars present in 'Lemonato' peach fruit were sucrose, fructose, glucose, and the sugar alcohol sorbitol (Table S1). The levels of glucose, fructose and sucrose contents slightly increased with maturity. Regarding the rest of the sugars, mature fruit had lower concentrations of xylose compared to both semi-mature and immature fruit as well as arabinose compared to semi-mature peaches (Fig. 2). Furthermore, cellobiose increased, while trehalose decreased in the semi-mature stage (Fig. 2). The levels of alcohols were generally suppressed by maturity since sorbitol decreased in mature compared to both semi-mature and immature phases, while myoinositol decreased in both semi-mature and mature fruit (Fig. 2).

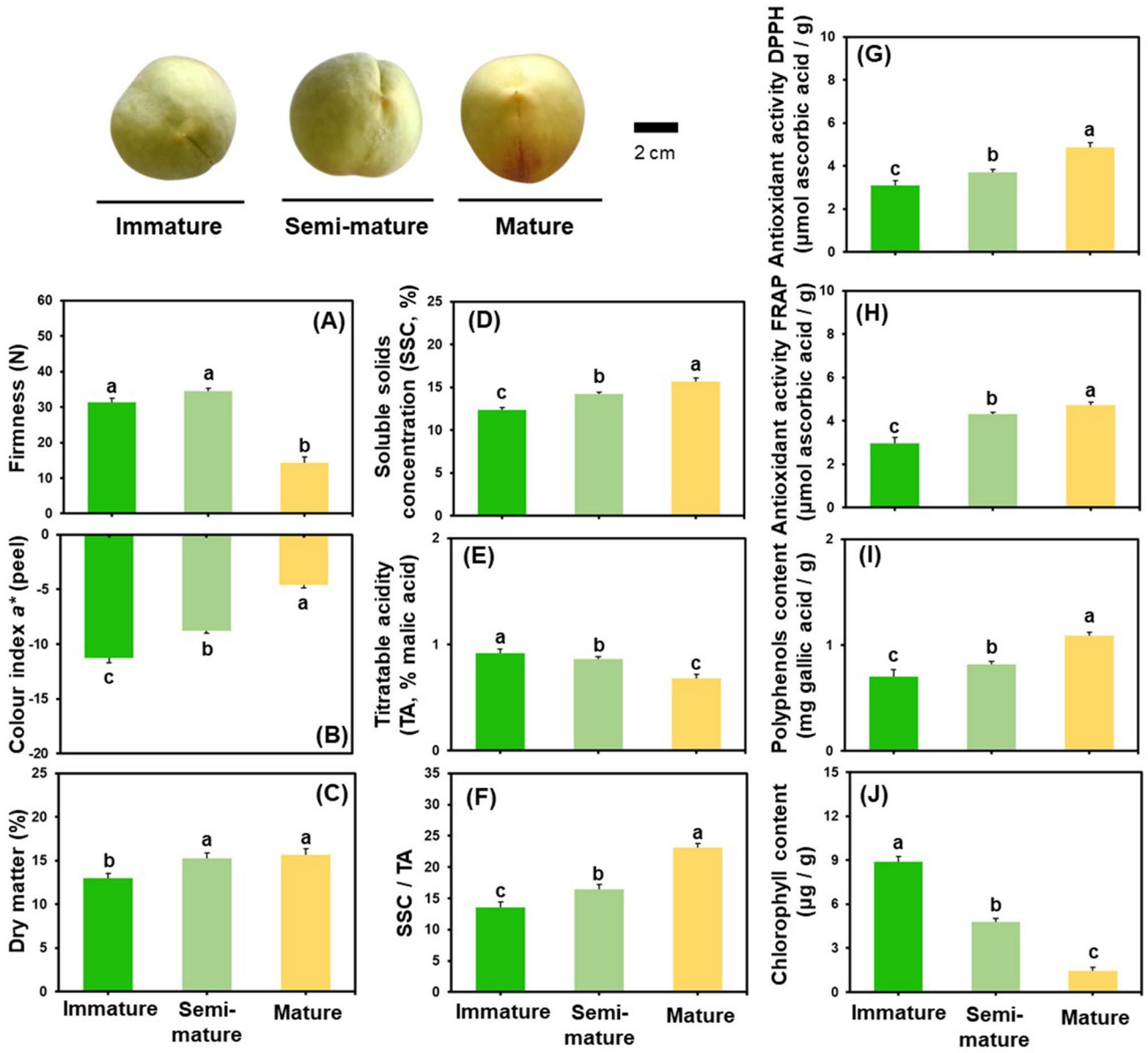

Fig. 1 The influence of maturity levels (immature, semi-mature, and mature) on fruit quality traits of 'lemonato' peach. Determination of firmness (**A**), color index a* (**B**), % dry matter (**C**), soluble solids concentration (**D**), titratable acidity (**E**), SSC/TA (**F**), antioxidant activity based on DPPH (**G**) and FRAP (**H**) assays, total polyphenolic content (**I**), and chlorophyll content (**J**) at commercial harvest stage. Different letters at each maturity phase indicate significant differences at $p \leq 0.05$ according to Tukey's multiple range test ($p \leq 0.05$). Error bars indicate the SEM of 30 fruit (**A**-**F**) or 3 replicates of 10 fruit (**G**-**I**) per maturity level

The analysis of organic acid profile of the peach fruit showed that malic acid was the predominant organic acid followed by quinic acid and then citric acid (Table S1). Malic acid and citric acid were decreased in the mature fruit (Fig. 2). The quinic acid was not influenced by fruit maturity (Fig. 2) while other organic acids detected in low amounts such as threonic, fumaric, pyruvic, oxalic and succinic, showed no changes among the maturity stages (Fig. 2). The free amino acids found in peach fruit were valine, threonine, glycine, beta-alanine, pyroglutamic acid, glutamic acid, and proline, while no changes were detected among the fruit maturity conditions (Fig. 2). Moreover, the polyamine putrescine decreased in the mature stage, whereas the level of phosphoric acid was depressed in the semi-mature fruit (Fig. 2).

The composition of polyphenolic compounds was affected by the on-tree maturity phases, especially in the final stage of maturity (Fig. 2, Table S1). Particularly, the concentration of catechin, epicatechin, procyanidin B1, B2, and B4 as well as neochlorogenic acid, chlorogenic acid

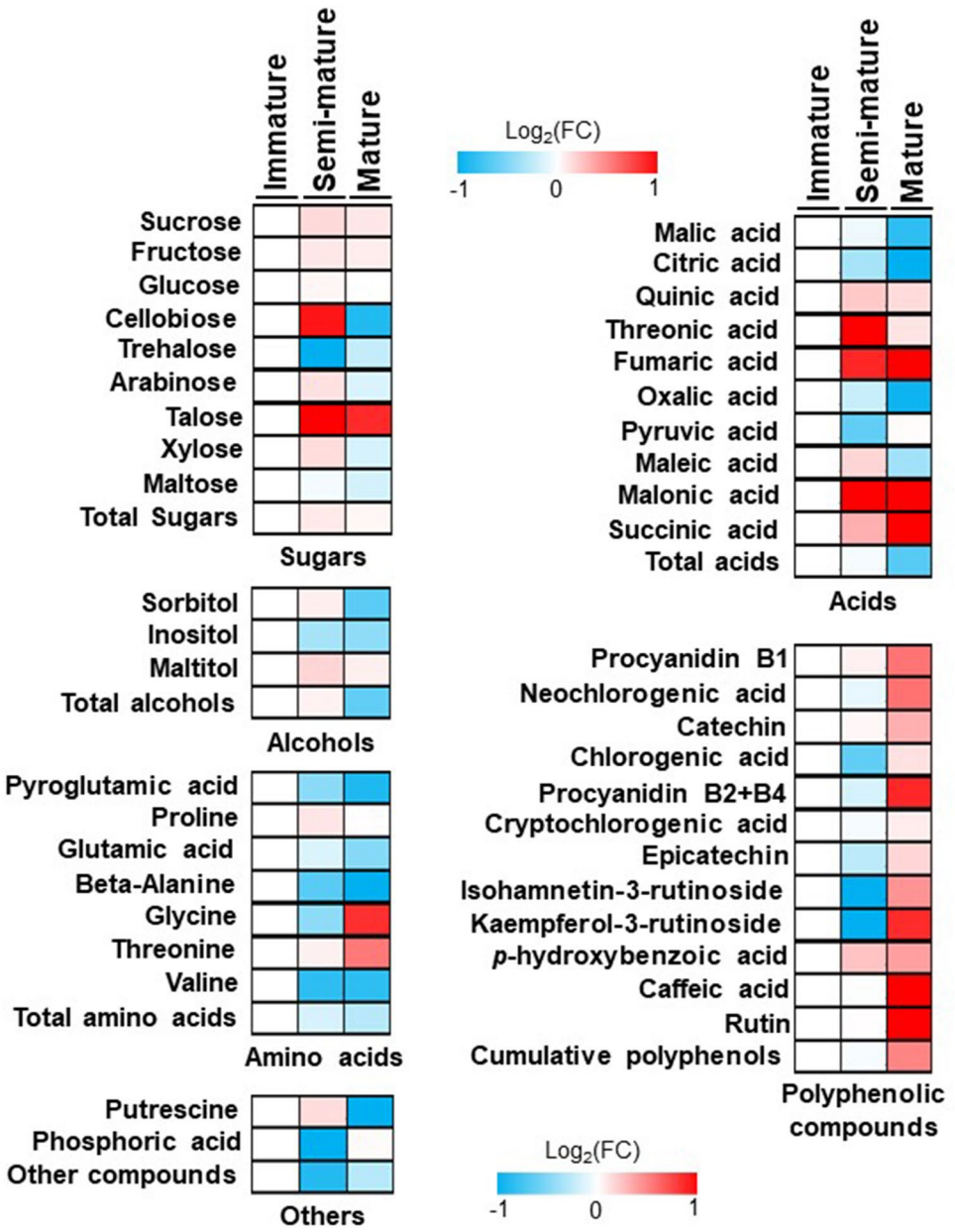

Fig. 2 Heatmap diagram of the primary polar metabolites and polyphenolic compounds in peach fruit at different maturity levels (immature, semi-mature, and mature). The blue color indicates a decrease, and the red color indicates an increase of metabolites. Data and statistical analysis are provided in Supplementary Table S1

and cryptochlorogenic acid increased in mature peaches compared to the rest stages (immature or semi-mature fruit) (Fig. 2, Table S1). The current analysis also showed that procyanidin B1 was the main polyphenol detected in 'Lemonato' peach fruit, followed by neochlorogenic acid and catechin, while rutin, isorhamnetin-3-rutinoside, kaempferol–3–rutinoside, p–hydroxybenzoic acid and caffeic acid were also detected in low concentrations (< 1 mg per 100 g dry weight) and increased in the mature fruit (Fig. 2, Table S1). Furthermore, in semi-mature fruits, several polyphenolic compounds, including chlorogenic acid, procyanidin B2 + B4, epicatechin, isorhamnetin-3-rutinoside, and kaempferol-3-rutinoside, were found in lower abundance compared to both other maturity levels (Fig. 2, Table S1).

Principal component analysis (PCA) was performed to verify the distribution of primary metabolites among the peach fruit's different maturity levels (Fig. 3A). According to the PCA, the primary metabolites of the three maturity stages (immature, semi-mature, mature) were separated into three distinct groups; based on PC1, mature fruit were separated from both immature and semi-mature peaches, which was explained by 29% of metabolic variance, and based on PC2, a discrimination of immature with semi-mature fruit was observed, which was explained by 18% of metabolic variance (Fig. 3A).

To associate fruit quality traits with identified metabolites at the three different fruit maturity levels, a Pearson correlation analysis was employed ($p \leq 0.05$). As depicted

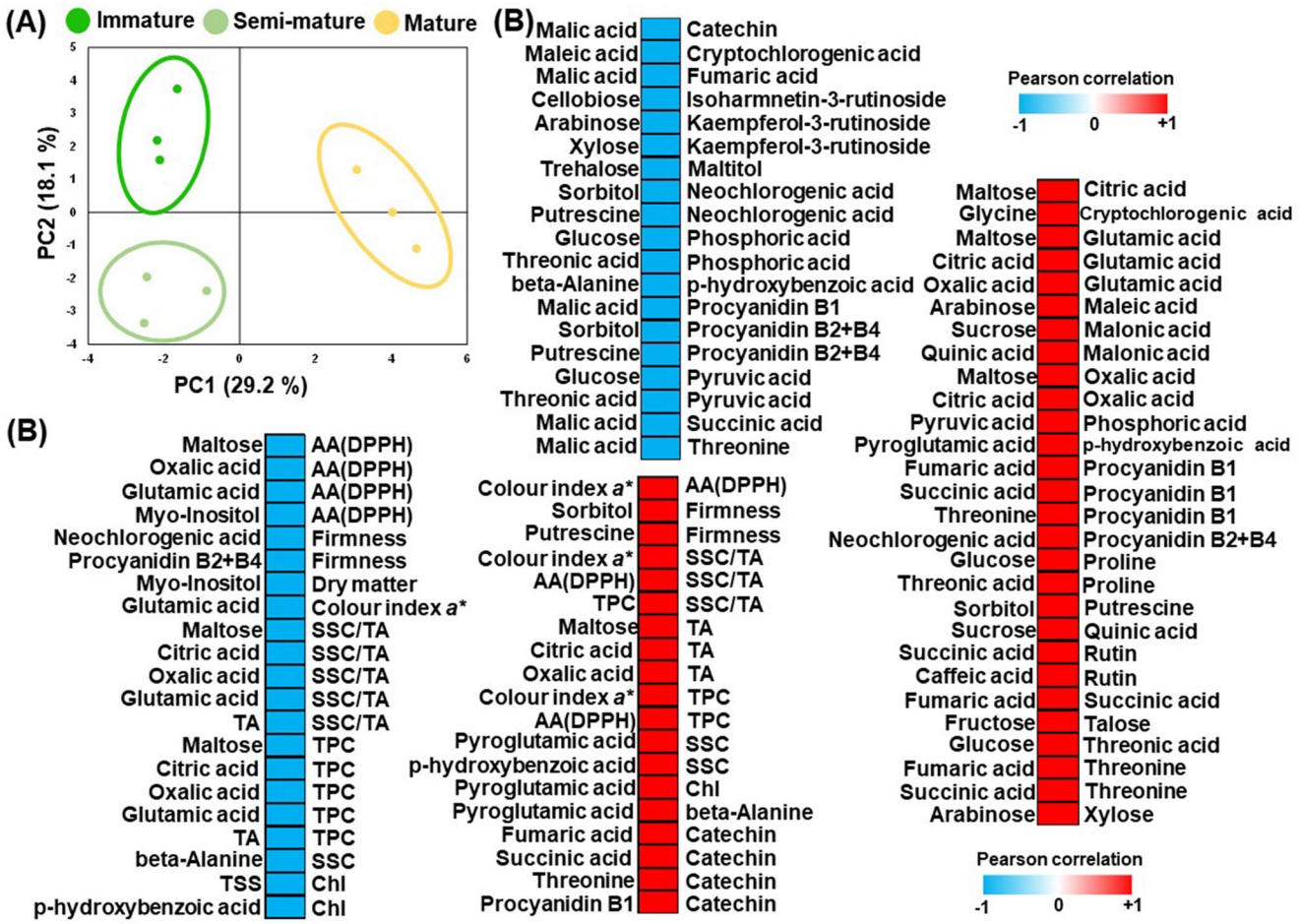

Fig. 3 (**A**) Principal component analysis (PCA) of the primary polar metabolites in immature, semi-mature and mature fruit of 'lemonato' peach. (**B**) Pearson correlation analysis between quality attributes and metabolite pattern in peach fruit across different fruit maturity levels at the commercial harvest stage. Only statistically significant correlations are displayed with $R^2 \geq |0.98|$. The blue color indicates negative correlation, while the red color indicates positive correlation ($p \leq 0.05$). Abbreviation list: AA, antioxidant activity; TPC, total polyphenols content; SSC, soluble solids concentration; TA, titratable acidity; chl, chlorophyll content

in Fig. 3B, 40 negative and 47 positive correlations were identified among the variables. Focusing on the association between quality traits and metabolites, 18 negative correlations were observed, such as antioxidant activity (DPPH) with maltose, oxalic acid, and glutamic acid; antioxidant activity (FRAP) with myo-inositol; firmness with neochlorogenic acid and procyanidin B2 + B4; dry matter with myo-inositol; color index *a** with glutamic acid; SSC/TA and polyphenolic content with maltose, citric acid, oxalic acid and glutamic acid; SSC with beta-alanine; chlorophyll content with *p*-hydroxybenzoic acid (Fig. 3B). In contrast, 8 positive correlations were detected between quality traits and metabolites, namely firmness with sorbitol and putrescine; titratable acidity with maltose, citric acid, and oxalic acid; SSC with pyroglutamic acid and *p*-hydroxybenzoic acid; chlorophyll content with pyroglutamic acid (Fig. 3B). Similarly, the association between polyphenols and primary metabolites at the three different fruit maturity levels based on Pearson correlation analysis revealed 11 negative and 9 positive correlations (Fig. 3B). These negative correlations were catechin and procyanidin B1 with malic acid; cryptochlorogenic acid with maleic acid; isorhamnetin-3-*O*-rutinoside with cellobiose; kaempferol-3-*O*-rutinoside with arabinose and xylose; neochlorogenic acid and procyanidin B2 + B4 with sorbitol and putrescine; p-hydroxybenzoic acid with beta-alanine. On the other hand, the positive correlations were catechin and procyanidin B1 with fumaric acid, succinic acid and threonine; cryptochlorogenic acid with glycine; p-hydroxybenzoic acid with pyroglutamic acid; and rutin with succinic acid (Fig. 3B).

3.3 Identification of the transcriptional changes in response to diverse on-tree maturity levels in peach fruit

A next-generation RNA-sequencing (RNASeq) analysis was conducted to examine the gene expression patterns. The objective of this analysis was to gain insights into how on-tree fruit maturity might influence the expression of peach genes at harvest. To achieve this, mRNA-enriched analysis was performed on exo-meso-carp tissues from the three fruit groups (immature, semi-mature, mature) collected at harvest. Using the principal coordinates analysis, the global gene expression data were separated into three groups according to the fruit maturity status (Fig. 4A). The three maturity levels were distinguished based on PCo1, with positive values corresponding to immature peaches, negative values to mature peaches and values near zero to semi-mature peaches (Fig. 4A). To further explore the Differentially Expressed Genes (DEGs) in the three distinct peach fruit maturity phases at harvest, the 10 highest and 10 lowest DEGs (in the corresponding comparisons mature vs immature, mature vs semi-mature and semi-mature vs immature) were depicted in Fig. 4B (based on Table S2, there were differences in some of DEGs below). It is noteworthy that in the three comparisons (mature vs semi-mature, mature vs immature, and semi-mature vs immature) among 10 highly expressed DEGs, an increase was found in the expression of *EXORDIUM* [3.3-, 3.4-, and 1.5-fold change (FC), respectively]. It is also interesting to note that in the mature fruit, compared to immature and semi-mature, 5 DEGs were strongly elevated. These genes include *BSP1* (4.4 and 3.9 FC), *SULFUR DEFICIENCY-INDUCED 1* (4.3 and 4.1 FC), *PCC13-62* (4 and 3.8 FC), *UN(LOC18780569)* (3.7 and 3.6 FC) and *Purple acid phosphatase 22* (3.2 and 2.8 FC). On the contrary, 6 DEGs in the mature fruit were strongly downregulated in comparison to immature and semi-mature fruit, which were included in the top 10 downstream genes based on mature fruit. The above group of the six genes include the *Linoleate 13S-lipoxygenase 2–1* (−4.1 and −3.5 FC), *Auxin-responsive protein IAA27* (−3.1 and −2.8 FC), *Oxygen-evolving enhancer protein 1* (−3.1 and −2.8 FC), *Histidine 1* (−3.1 and −3 FC), *Cyclic nucleotide 1* (−3.5 and −2.9 FC), and *Cyclic nucleotide 4* (−3.6 and −2.7 FC) (Fig. 4B). Validation of RNA-seq data was carried out in genes of interest, such as *EXORDIUM*, *SULFUR DEFICIENCY-INDUCED 1*, *Linoleate 13S-lipoxygenase 2–1*, and *Auxin-responsive protein IAA27* (Table S3) using qRT-PCR, and the results are illustrated in Fig. S1.

In total, RNA-seq data analysis revealed 531 DEGs in mature vs immature, 513 DEGs in mature vs semi-mature, and 57 DEGs in semi-mature vs immature as presented in the Table S2. In order to group and to further analyze DEGs of each comparison, a KEGG enrichment analysis was performed (Fig. 5A). When comparing DEGs among the samples with diverse maturity levels, several apparent differences were found. For example, in both comparisons of mature peaches, the lysosomal cystine transporter was strongly enriched whereas RNA-dependent RNA polymerase was highly induced with the transition from the immature to semi-mature stage. Other enriched KEGG pathways were the proteins EXORDIUM and BYPASS in mature vs immature fruit, chromatin silencing by small RNA and auxin-mediated signaling pathway in semi-mature vs immature fruit, and sulfur/L-cystine transmembrane transporters in mature vs semi-mature fruit (Fig. 5A). To further cluster and study DEGs regarding gene ontology and specific biological processes (BP) that genes were involved, a GO enrichment analysis BP-based of DEGs in each comparison was employed (Fig. 5B). Among the different maturity peach phases, DNA transcription regulation

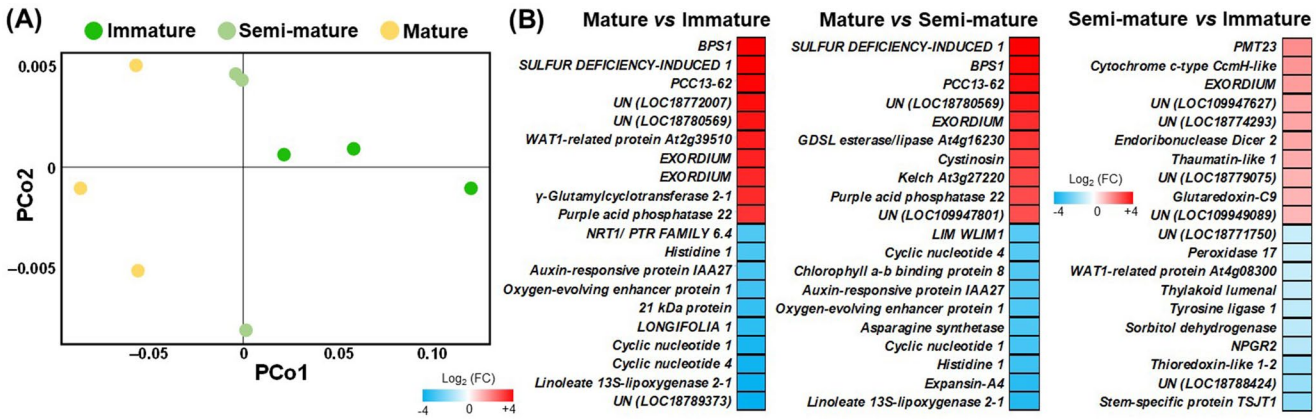

Fig. 4 (**A**) Principal coordinates analysis (PCoA) of RNA-seq data in immature, semi-mature and mature fruit of 'lemonato' peach. (**B**) Heatmap of the differential expression gene (DEG) analysis of 10 high and 10 low DEGs among the different maturity levels. The blue color indicates a decrease, while the red color indicates an increase ($p \leq 0.05$), based on the comparison mature vs both semi-mature and immature and/or semi-mature vs immature

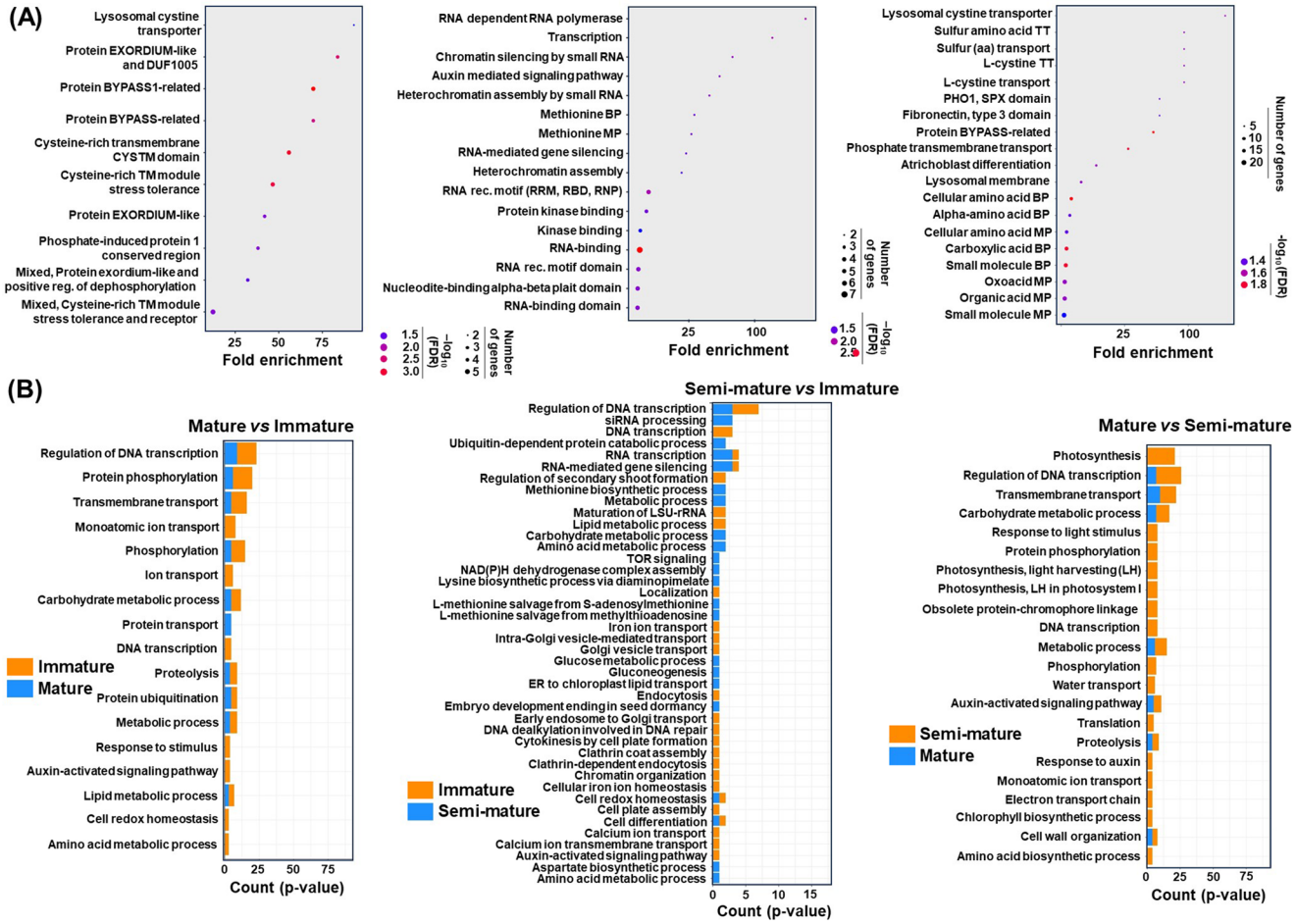

Fig. 5 KEGG pathway enrichment analysis of DEGs (**A**) and GO enrichment analysis of the biological process of the identified DEGs (**B**) in the comparisons of mature vs immature, mature vs semi-mature and semi-mature vs immature peaches

was significantly enriched. In mature vs immature fruit, protein phosphorylation and transmembrane transport categories were strongly enriched, while the top categories in mature compared to semi-mature peaches were photosynthesis, transmembrane transport, and carbohydrate metabolic process. Despite the high number of biological process categories (42) in the comparison between semi-mature and immature fruit, the enrichment score was relatively low; however, they were distinguished categories such as siRNA processing, ubiquitin-dependent protein catabolic process, and RNA transcription (Fig. 5B). Moreover, GO enrichment analysis regarding the cellular component (CC) and molecular function (MF) of DEGs was carried out (Fig. S2). This analysis revealed that the membrane and nucleus were enhanced from CC, whereas protein binding was enriched from MF among all comparisons evaluated.

Having analyzed the transcriptomic data, we focused on 7 categories of maturity-related genes, which were cell wall, aquaporins, calcium, flavonoid biosynthesis, auxin, chloroplast, and transcription factors (Fig. 6). In particular, cell wall-related genes had a controversial response to mature fruit as some of them increased such as *polygalacturonase* (4 out of 6 isoforms), *xyloglucan endotransglucosylase*, *pectate lyase 4* and *beta-galactosidase 16*, whereas other genes like *expansins* and *galactosidases* (*alpha- 1* and *beta-5*) decreased. Upregulation of genes related to calcium signaling and flavonoid biosynthesis was also observed (Fig. 6). By focusing on calcium signaling, we found that *calcium-binding proteins* such as *CML27*, *CML45*, *CML31*, and *At1g02270*, *calcium kinase 24*, *calmodulins* (*like 3*, *binding 60 E*, and *kinase 2*) displayed higher expression in mature fruit, and *CML23* in semi-mature fruit, whereas *calcium kinase SK5* exhibited lower expression in the comparison of mature vs immature phases (Fig. 6). Regarding DEGs involved in flavonoid biosynthesis, we detected a rise in the expression of *flavonol synthase*, *isoflavone reductase*, and *cinnamoyl-CoA-reductase-like SNL6*, while a decline of *anthocyanin-3-O-glucosyltransferase 5* expression was evidenced in the mature fruit. An obvious down-regulation of aquaporins-related genes, such as *PIP2-5*,

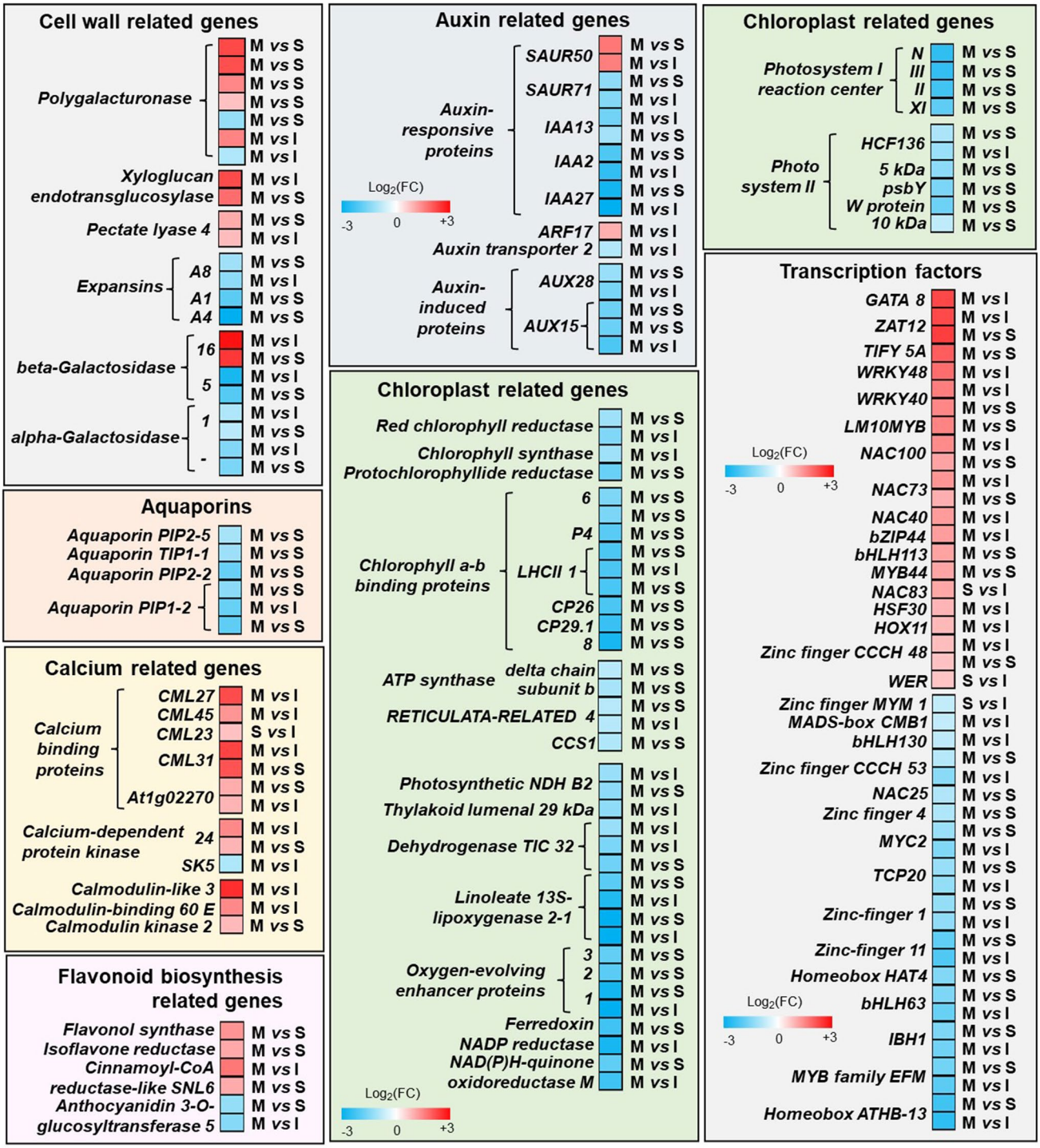

Fig. 6 Heatmap of the differential expression gene (DEG) analysis, which corresponds to the main seven gene-related classes, namely cell wall, aquaporins, calcium, flavonoid biosynthesis, auxin, chloroplast, and transcription factor among the different maturity levels (immature, semi-mature, and mature). The blue color indicates a decrease, while the red color indicates an increase ($p \leq 0.05$) based on the comparison mature vs immature (M vs I), mature vs semi-mature (M vs S) and semi-mature vs immature (S vs I)

TIP1-1, *PIP2-2* and *PIP1-2* was observed in mature fruit (Fig. 6). Furthermore, auxin-related and chloroplast-related genes have also been downregulated in mature fruit compared to immature and semi-mature. Interestingly, from the 46 chloroplast-related DEGs reduced in mature fruit, most of them (32 DEGs) were identified in the comparison of mature vs semi-mature and only 14 DEGs were downregulated in mature vs immature fruit (Fig. 6). Regarding

14 chloroplast-related DEGs, 8 of them were shared DEGs during the transition to mature fruit indicating a potential role of these DEGs as peach maturity biomarkers. We also noticed a lower expression pattern in genes related to auxin-responsive proteins (e.g., *SAUR71*, *IAA13*, *IAA2* and *IAA27*), induced-proteins (e.g., *AUX28* and *AUX15*) and transporter 2 in mature fruit, while *SAUR50* and *ARF17* displayed higher expression in mature peach fruit (Fig. 6).

Information regarding the impact of different on-tree peach fruit maturity levels on the regulation of transcription factors (TFs) is provided in Fig. 6. Data indicated that 32 TFs were differentially expressed, among them 15 upregulated and 17 downregulated in the comparison between mature and semi-mature fruit (Fig. 6). In the mature fruit, the 5 TFs that upregulated compared to semi-mature and immature fruit were *ZAT12*, *WRKY40, NAC100*, *NAC73* and *Zinc finger CCCH 48*. Also 6 TFs, including *WRKY48*, *NAC40*, *GATA 8*, *bZIP44*, *HOX11*, and *HSF30* were upregulated in mature compared to immature fruit. In addition to this, *TIFY 5A*, *LM10MYB*, *bHLH113* and *MYB44* were increased in mature compared to semi-mature fruit, whereas *NAC83* and *WER* were upregulated in semi-mature vs immature samples (Fig. 6). In contrast, we noticed that 9 TFs, including *homeobox ATHB-13*, *MYB family EFM, IBH1*, *bHLH63*, *Zinc-finger 11*, *Zinc-finger 1*, *TCP20*, *MYC2*, and *Zinc finger CCCH 53* were downregulated in the mature fruit compared to both semi-mature and immature peaches. Additionally, *bHLH130* and *MADS-box CMB1* were downregulated in the mature compared to immature fruit. Finally, *homeobox HAT4, NAC25* and *Zinc-finger 4* were found in lower expression in mature fruit compared to semi-mature ones, whereas *Zinc-finger MYM 1* was downregulated in semi-mature vs immature fruit (Fig. 6).

To accomplish an integration analysis between highly expressed transcripts in the mature phase of peaches with both metabolites and physiological/biochemical traits of fruit, a Pearson correlation analysis was employed (Fig. 7). The analysis indicated that 10 transcripts [*protein EXORDIUM* (*LOC18789063*), *purple acid phosphatase 22* (*LOC18769867*), *LOC18772007*, *WAT1-related protein At2g39510* (*LOC18784854*), *probable peroxygenase 4* (*LOC18789647*), *universal stress protein in QAH/OAS sulfhydrylase 3'region* (*LOC18769705*), *calcium-binding protein CML31* (*LOC18777511*), *LOC18787151*, *LOC109949847*, *protein CDI* (*LOC18769371*)] were positively correlated to 4 polyphenols (procyanidin B1, catechin, caffeic acid, rutin), 2 acids (fumaric and succinic acids) and an amino acid (threonine), whereas they were negatively associated with malic acid and total acids (Fig. 7). Furthermore, 3 transcripts [*mannan endo-1,4-beta-mannosidase 7* (*LOC18771386*), *tryptophan aminotransferase-related protein 4* (*LOC18766943*), *BURP domain-containing protein 3* (*LOC18779587*)] were positively correlated to several metabolites, including oxalic acid and glutamic acid, whereas they were negatively associated with physiological/biochemical traits, such as skin a*, antioxidant activity (DPPH), total polyphenolic compounds and SSC/TA (Fig. 7).

4 Discussion

Harvesting peach fruit at once, instead of two or three harvest rounds, undoubtedly leads to harvesting fruit with different maturity levels, which may lead to considerable variation in their external and internal maturity status (Minas et al. 2021). Despite our knowledge of the biochemical and molecular changes during peach fruit development, maturity and ripening (Brummell et al. 2004; Gonçalves et al. 2016; Karagiannis et al. 2016; Maletsika et al. 2023; Ramina et al. 2008; Tanou et al. 2017), the novelty of the present study is that it deals with the physiological and molecular alternations of peaches among different maturity stages at the same harvest date. In the current study, using the traditional Greek-origin 'Lemonato' peach fruit as a model, we conducted a comprehensive physico-chemical, metabolomic and transcriptomic analysis of maturity-related responses in peaches experiencing different levels of on-tree maturity to gain a real-time picture of maturity transition.

During peach fruit development and maturity, fruit firmness undergoes significant changes (Karagiannis et al. 2016). The observed firmness loss in the fully mature peaches at harvest (Fig. 1) would be associated with the activation of cell wall-related genes, particularly *polygalacturonase*, *pectate lyase 4* and *beta-galactosidase 16* (Fig. 4). Previous studies demonstrated that in melting-flesh peaches such as 'Lemonato', a major loss of firmness during maturity is primarily due to a change in cell wall carbohydrate metabolism, resulting in a reduction in certain structural components, and the increased activity of the cell wall enzyme endo-polygalacturonase, which is coincident with the melting phase (Brummell et al. 2004).

In several peach cultivars, an increase in SSC and a decrease in acids were found during on-tree fruit maturity (Gonçalves et al. 2016; Maletsika et al. 2023), and a similar tendency was also observed among the three distinct fruit maturity phases in our study Fig. (1, ED). In line with the observed reduction of TA (Fig. 1E), malic and citric acids were also found in lower concentrations in the mature fruit (Fig. 2). The observed increase in SSC was accompanied by a significant rise in free sugars, including sucrose, fructose, and glucose, during the transition from immature to mature fruit (Fig. 2). Notably, the typical threshold levels for SSC (10%) and TA (0.7%) were already met even in less mature

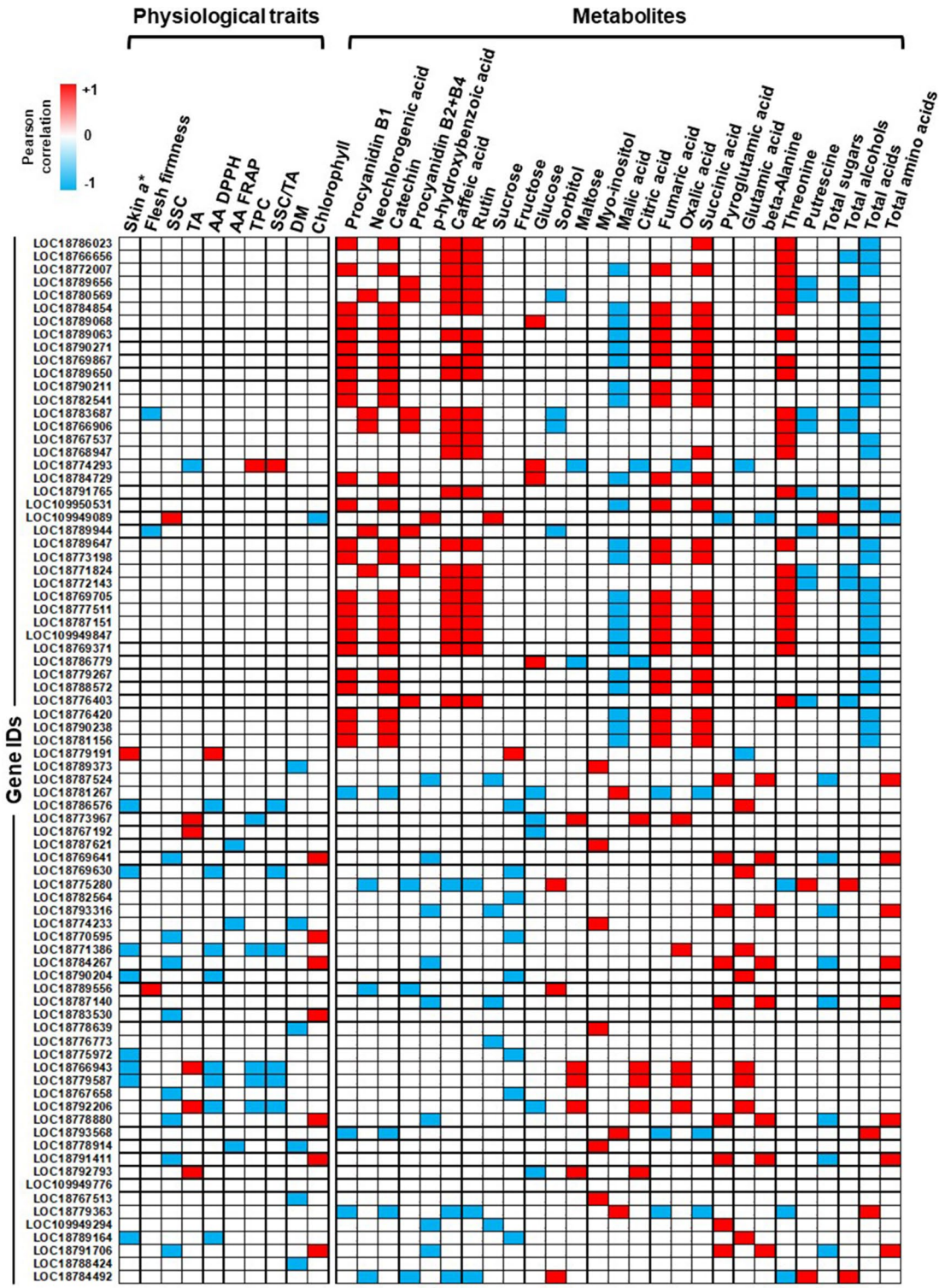

Fig. 7 Heatmap of Pearson correlation between highly expressed DEGs and physiological traits as well as metabolites among the different maturity levels at harvest (immature, semi-mature, and mature). Only statistically significant correlations are displayed with $R^2 \geq |0.98|$). The blue color indicates a negative correlation whereas the red color indicates positive correlation ($p \leq 0.05$)

fruits (Fig. 1, ED), satisfying the acceptable consumer standards for melting-flesh peaches (Crisosto and Crisosto 2005). As peaches contain no starch, it seems that at the mature stage the peach fruit does not accumulate any more sugars, but, due to acid breakdown, SSC/TA ratio increases and the human sense of sweetness increases.

Oxidative stress is known to markedly increase during the last stages of normal fruit development (Karagiannis et al. 2020). It has been proposed that maturity process facilitates metabolic changes and may alter defense metabolism enabling peach fruit to withstand maturity-induced oxidative stress conditions (Camejo et al. 2010; Dabbou et al. 2017; Hodges 2003). During fruit maturity, chloroplasts develop into chromoplasts accumulating high amounts of antioxidant compounds, which presumably will protect the chromoplast and fruit cells (Sadali et al. 2019). In this regard, we noticed an increase in total antioxidant activity, total polyphenolic content and individual polyphenolic compounds through the passing of immature to semi-mature and then to mature fruit stage (Fig. 1G–I and Fig. 2). This accumulation of polyphenolic compounds during the peach fruit maturity transition was accompanied with a strong increase in the expression of several genes related to flavonoid biosynthesis, including *flavonol synthase, isoflavone reductase, and cinnamoyl-CoA reductase-like SNL6* (Fig. 6), that may be associated with maturity-inducible fruit defense responses at the late stage of peach development (Crisosto et al. 1999; Karagiannis et al. 2016). Also, it has been shown that the decrease in chlorophyll content in the mesocarp tissue is tightly linked to maturity in peaches, and therefore, tools to determine it non-destructively were developed (Gonçalves et al. 2016). This was also confirmed in our study (Fig. 1J), as peach chlorophyll was found to be negatively associated with SSC (Fig. 3B), and more than 40 chloroplasts-related DEGs were downregulated in mature fruit (Fig. 6).

Primary metabolites analysis revealed that a few shifts occur in their abundance at harvest under three different peach fruit maturities (Fig. 2). For instance, sorbitol and myo-inositol were reduced in mature fruit, especially as sorbitol, after phloem unloading in the fruit, is rapidly metabolized to glucose and fructose by sorbitol oxidase and sorbitol dehydrogenase, respectively (Ramina et al. 2008). Interestingly, the observed reduction of cellobiose in the mature fruit (Fig. 2) is probably associated with fruit softening (Fig. 1A). Peach softening is generally attributed to the disassembly of the cellulose and hemicellulose network through depolymerization of pectin and hemicellulose involving the action of hydrolytic cellulases that break down the cellulose found in plant cell walls into simple sugars (Brummell et al. 2004). In this regard, further research is needed to investigate the observed strong changes in the abundance of several primary metabolites, such as cellobiose and trehalose, as well as of several polyphenols, in semi-mature fruit compared to the other two maturity levels (Fig. 2, Table S1). Our analysis further showed that putrescine levels were remarkably reduced in mature fruit (Fig. 2), suggesting that this component could be classified as having maturity-specific expression patterns. Previous studies have already pointed out that higher concentrations of putrescine have been detected at the early stages of peach fruit development, followed by a gradual decrease as fruit matures through increased putrescine oxidation, while putrescine oxidation, in turn, affected the expression of numerous fruit ripening–related genes (Wang et al. 2021). A detailed genetic approach needs to be employed to investigate the specific role of putrescine in peach fruit maturation and ripening syndromes.

As this was the first study to comprehensively characterize the maturity-associated transcriptome in peach fruit harvested at the same time, current data provide new insights into gene expression in the context of maturity transition in developmental fruit biology. Predominantly, this study revealed DEGs that were highly up- or down-regulated in response to the maturity process (Fig. 4). Of particular interest is the strong upregulation of *EXORDIUM* across all peach fruit maturity transition stages examined (Fig. 4). EXORDIUM is a regulator of brassinosteroid-responsive gene that is involved in the mediation of brassinosteroid-promoted growth in plant tissues (Sun et al. 2010). Intriguingly, two EXORDIUM and EXORDIUM-like proteins were found to be significantly up-regulated when banana fruit underwent the transition from the mature green stage to the maturity stage (Xiao et al. 2019). Several proteomics approaches also identified EXO, EXL1, and other members of the EXORDIUM protein family as part of the cell wall proteome changes in fruit undergo during maturity (Jamet et al. 2006). Peach fruit maturity may, therefore, be associated with brassinosteroid signaling mediated by EXORDIUM, whereas a strong positive correlation with polyphenolic compounds has been found in the current study (Fig. 7). Previous findings also showed that; abscisic acid (ABA) plays a relevant role in the control of peach fruit maturity since it can modulate its biosynthesis as well as the biosynthesis and signaling of ethylene and auxin, by strongly affecting related gene expression (Soto et al. 2013). In this study, the *PCC 13–62*, an ABA-responsive gene, was intensely upregulated in mature fruit (Fig. 4), which indicates its role related to ABA response in peach fruit maturity passage, as suggested in mangoes (Chin et al. 2019). In addition, a high increase of *SULFUR DEFICIENCY-INDUCED 1* gene (*SDI1*) in the mature phase at harvest (Fig. 4) is possibly associated with the interplay between ethylene and sulfur metabolisms, notably through the sulfur-containing metabolite methionine (Wawrzynska et al. 2015). This link between peach fruit maturity transition and hormonal metabolism is

further supported by the fact that several genes related to auxin-responsive and induced proteins (AUX/IAA) were sharply downregulated in mature fruit (Fig. 6). Auxin signaling is activated when auxin content in cells is high, which causes degradation of AUX/IAA by TIR1/AFB and releases auxin response factors (ARFs) that bind to DNA (Cancé et al. 2022). Hence, the observed downregulation of *IAA2* and *IAA27* genes may be linked with the upregulation of *ARF17* (Fig. 6), even though both genes (*IAA2* and *IAA27*) have been demonstrated to be involved in fruit size (Bassa et al. 2012).

Membrane lipid peroxidation has been suggested as a characteristic of a senescing system, which is initiated by either enzymatic activities or reactive oxygen species (ROS) (Brummell et al. 2004). In our results, *linoleate 13S-lipoxygenase 2–1* which belongs to LOXs family and catalyzes the oxidation of linoleate into 13-HPODE, was greatly downregulated from immature and semi-mature to mature phase (Fig. 4) consistently with the result in sweet cherry and peach research, in which it was found to be more abundant in the immature fruits and decreased in mature fruits (Prinsi et al. 2016). This profile of linoleate *13S-lipoxygenase 2–1* may reflect either the influences of light exposure and the lessening of chloroplastic functionality during maturity or the depression of degradation of membrane lipids at the final step of the on-tree maturity (Prinsi et al. 2016). Current data further uncovered that the *cyclic nucleotide-gated ion channel 1* (*CNGC1*) and *4* (*CNGC4)* genes, which are classified as hyperpolarization, voltage, and ligand-gated cation channels (Saddhe et al. 2021), were promptly downregulated in mature fruit (Fig. 4). This is in line with the results of a recent study, where external calcium application stimulated *CNGC4* activation in sweet cherry fruit (Michailidis et al. 2022). In addition to this, other genes related to Ca^{2+}-binding proteins, calcium kinases, and calmodulins were upregulated in the mature phase and associated with polyphenols accumulation (Figs. 6, 7), indicating that Ca^{2+} signaling plays a significant role in peach fruit maturity, as was recently documented in 'Lemonato' peach fruit (Maletsika et al. 2023). Another interesting outcome of this study was the observation that various aquaporin (AQ) genes, such as aquaporins PIP2-5, TIP1-1, PIP2-2 and PIP1-2 displayed a downstream tendency during the transition from immature to mature phase (Fig. 6). As peach fruit growth may continue after maturity has been initiated (Perkins-Veazie, et al. 1995), it is possible that this growth needs fruit turgor, which may be provided by an enhanced water accumulation. Once maturity has started from the immature to mature stage, the fruit continues accumulating water and solutes, contributing by this way to the maintenance of fruit turgor. This increment in AQs expression in the maturity initiation, therefore, could lead to fast water accumulation to support growth, while its suppression at the mature stage may be crucial for the developmental strategies to support maturity orchestration, as proposed by Mut et al. (2008).

Transcription factors (TFs) play critical roles in the regulation of fruit growth and development by modulating target gene transcription that is involved in specific fruit life periods. Here, it has been demonstrated that maturity outcome stress elicits substantial TFs changes in 'Lemonato' peach fruit. Among them, *ZAT12*, *NAC100*, and *WRKY40* exhibited higher expression levels in mature fruit at harvest time (Fig. 6). It has been established that *ZAT12*, a member of the Zinc-finger (C2H2-type) family, exhibited a strong activation potential on defense responses via flavonoid/polyphenols biosynthesis. It has also been found to be co-expressed with *WRKY40* TF (Davletova et al. 2005), leading us to the hypothesis that *ZAT12* may play a role in maturity responses through *WRKY40*-dependent pathway. Considering the vital role of ethylene in peach fruit maturity initiation (Brummell et al. 2004) together with the fact that *WRKY40* is associated with the activation of ethylene biosynthesis in kiwifruit through binding to *SAM* and *ACS* gene promoters (Gan et al. 2021), while *NAC100* acts downstream to ethylene signaling, it is likely that these TFs might be a regulatory module involved in ethylene production during on-tree peach fruit maturity. An important result revealed by our study was that the mature fruit had substantially lower expression levels of four TFs, namely *IBH1*, *MYB EFM*, *Homeobox ATHB-13* and *bHLH63* (Fig. 6). *IBH1* acts as transcriptional repressor that negatively regulates cell and organ elongation in response to gibberellin (GA) and brassinosteroid signaling, whereas *AtHB13* was shown to be a regulator of plant tissue development in response to carbon availability in the late developmental stages (Hanson et al. 2002) pointing their active participation in the growth depression of mature fruit. It is also notable that; *bHLH63* is ethylene-sensitive since external ethylene treatment inhibits the transcriptional activity of *bHLH63* in cut chrysanthemum varieties, while 1-methylcyclopropene (1-MCP, an inhibitor of ethylene action) induces its expression (Liu et al. 2022), thus proposing a possible role of *bHLH63* in climacteric peach fruit maturity. Meanwhile, there is convincing evidence that the MYB transcription factor, EARLY FLOWERING MYB PROTEIN (MYB EFM), mediates *Arabidopsis* responses to temperature and light to determine the timing of reproduction in response to changing environments (Yan et al. 2014); however, the role of *MYB EFM* in fruit maturity is totally unknown. Thus, the present results provide a foundation for exploring the characterization of the *MYB EFM* genes in peach fruit biology and offer insights for additional maturity studies in the frame of climatic changes. Interestingly, most of the observed maturity-affected genes (Figs. 4 and 6; Table S2) are either uncharacterized or unappreciated

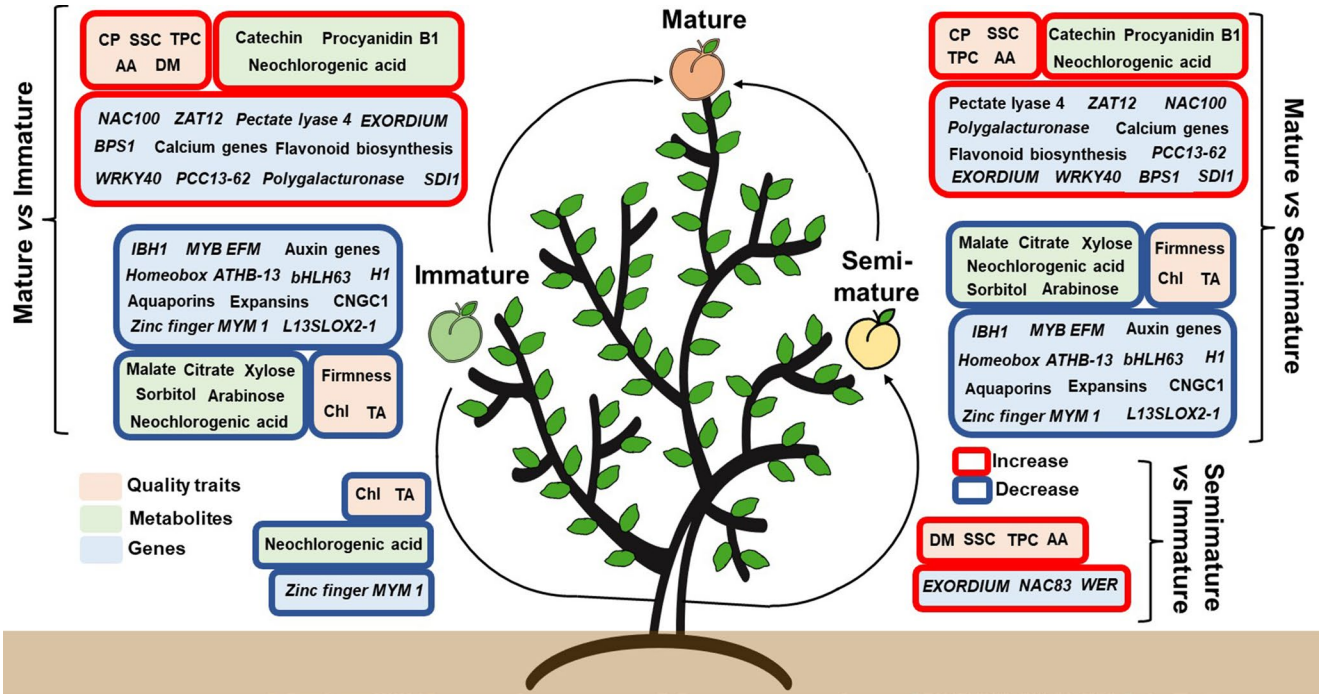

Fig. 8 Schematic illustration of key changes in maturity traits, metabolite levels and gene expression dynamic among the distinct on-tree maturity phases (immature, semi-mature, and mature) of 'Lemonato' peach fruit at the commercial harvest stage. Abbreviation list: CP, cumulative polyphenols; TPC, total polyphenols content; SSC, soluble solids concentration; AA, antioxidant activity; DM, dry matter; TA, titratable acidity; Chl, chlorophyll content; *SDI1, SULFUR DEFICIENCY-INDUCED 1; CNGC1*, cyclic *nucleotide-gated ion channel 1; L13SLOX2-1, linoleate 13S-lipoxygenase 2-1*

for their role in on-tree peach fruit maturity physiology, thus supporting the novelty of the present work. To summarize our findings regarding the peach on-tree maturity transition, a schematic figure was constructed (Fig. 8) to point out the crucial alternations of quality traits, metabolites and genes among the different maturation stages.

5 Conclusion

This study provides the first information concerning the peach fruit quality parameters along with metabolomic and transcriptomic datasets that were evaluated on the same trees and were related to the different fruit maturation levels, from the immature and semi-mature to the mature level. Maturity variability is characterized by differences in quality traits, including organoleptic properties, antioxidant activity, dry matter, chlorophyll content and fruit softening that become more pronounced as fruit matured. Accordingly, several primary metabolites, such as sorbitol, arabinose, xylose, malate and citrate along with polyphenolic compounds, like catechin, epicatechin, procyanidin B1/B2/B4 and chlorogenic acid were altered by the distinct maturity stage. The maturation transition includes also diverse transcriptomic changes associated with flavonoid biosynthesis, calcium signaling, cell wall integrity and auxin metabolism. We also identified several peach genes, including *EXORDIUM*, *PCC13-62*, *SDI1 and bHLH63* undergoing major changes at the evaluated stages that might be used as biomarkers for delineating the on-tree maturation process. These findings provide the basis to understand the regulation of on-tree fruit maturation, highlighting the link between key metabolic and transcriptomic changes and fruit quality traits. This knowledge can help optimize the harvest period and offers the potential to control peach fruit maturity transition to achieve high quality and reduce post-harvest losses.

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Data availability All data are included in the tables and the Supplementary Materials.

Declarations

Ethical approval Not applicable.

Conflicts of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Authors and Affiliations

Persefoni Maletsika[1] · Michail Michailidis[2] · Evagelia Mouchtaropoulou[3,4] · Vasiliki Liava[1] · Elpida Nasiopoulou[2] · Vaia Styliani Titeli[2] · Eirini Sarrou[5] · Stefan Martens[6] · Katerina Grigoriadou[5] · Anagnostis Argiriou[3,4] · Athanassios Molassiotis[2] · George D. Nanos[1]

✉ Persefoni Maletsika
pmalets@uth.gr

Michail Michailidis
msmichai@agro.auth.gr

Evagelia Mouchtaropoulou
eva.mouchtaropoulou@certh.gr

Vasiliki Liava
vasiliki.liava@gmail.com

Elpida Nasiopoulou
elpinasi@agro.auth.gr

Vaia Styliani Titeli
vgtiteli@gmail.com

Eirini Sarrou
esarrou@elgo.gr

Stefan Martens
stefan.martens@fmach.it

Katerina Grigoriadou
grigokat@outlook.com

Anagnostis Argiriou
argiriou@certh.gr

Athanassios Molassiotis
amolasio@agro.auth.gr

George D. Nanos
gnanos@uth.gr

[1] Laboratory of Pomology, Department of Agriculture Crop Production and Rural Environment, University of Thessaly, Fitoko Str., Nea Ionia, Magnisia 38446, Greece

[2] Laboratory of Pomology, Department of Horticulture, Aristotle University of Thessaloniki, Thessaloniki-Thermi 57001, Greece

[3] Department of Food Science and Nutrition, School of Environment, University of the Aegean, Myrina, Greece

[4] Institute of Applied Biosciences, Centre for Research and Technology Hellas, Thessaloniki, Greece

[5] Institute of Plant Breeding and Genetic Resources, Hellenic Agricultural Organization-DIMITRA, Thessaloniki-Thermi 57001, Greece

[6] Fondazione Edmund Mach, Centro Ricerca e Innovazione, Department of Food Quality and Nutrition, Via E. Mach, 1, 38010 San Michele all' Adige, TN, Italy