

Differentially methylated DNA regions in apricot (*Prunus armeniaca* L.) and Japanese plum (*Prunus salicina* L.) during fruit ripening after ethylene-related treatments

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ABSTRACT

Apricot (*Prunus armeniaca* L.) and Japanese plum (*Prunus salicina* L.) are two of the most important stone fruit species, and their climacteric nature is a major determinant in terms of fruit shelf-life period. The role of the main genes involved in the ripening process of these species has been extensively studied, however there is a need to expand our molecular knowledge at epigenetic level. To achieve this, postharvest monitoring of 'Goldrich' apricots and 'Santa Rosa' Japanese plums after 1-MCP (ethylene inhibitor) and Ethrel (ethylene precursor) treatments was carried out to delve deep into the epigenetic level using Whole Genome Bisulfite Sequencing (WGBS). Results showed over 12,000 and 9,000 genes with differentially methylated regions (DMRs) in 'Goldrich' and 'Santa Rosa' cultivars respectively. 1-MCP-treated apricot 'Goldrich' exhibited more hypomethylated regions, suggesting lower methylation levels compared to Ethrel treatment. While starch and sucrose metabolism exhibited the greatest number of partially methylated genes, other pathways showcased more significant differences. Thus, 4-coumarate-CoA ligase in phenylpropanoid biosynthesis, DELLA protein in hormone signal transduction, GDP-L-fucose synthase in amino sugar metabolism, D-lactate dehydratase in pyruvate metabolism, and hydroxymethylglutaryl-CoA reductase in 'Goldrich', as well as homocysteine S-methyltransferase in the cysteine and methionine metabolism, 1-phosphatidylinositol-4-phosphate 5-kinase in the inositol phosphate metabolism, pectinesterase in the pentose and glucuronate interconversions, malate dehydrogenase in the carbon metabolism, photosystem I subunit VI in photosynthesis, and aquaporin-4 as a membrane transporter in Japanese plum showed inverse gene expression-methylation patterns. These findings reveal variations in epigenetic regulation during fruit ripening in apricot and plum, influenced by ethylene modulating treatments, suggesting significant role of DNA methylation in this process.

1. Introduction

Prunus includes a wide range of stone fruit species many of them extensively cultivated for their attractive and delicious fruits and their beneficial and healthy properties. Apricot (*P. armeniaca* L.) and Japanese plum (*P. salicina* L.) represent an annual world production of 16 Mt about 40 % of the total stone fruit production. However, the perishable nature of these species due in part to their climacteric stage restricts their commercialization. Thus, in the current context of international trade, it is interesting to improve not only fruit quality traits but also the perishable of the fruit, which would allow us to address more distant markets (Salazar et al., 2022a).

The study of biological processes affecting various phenological

traits in *Prunus*, including fruit development, growth, and physiological ripening (Galimba et al., 2020; García-Gómez et al., 2020; Xu et al., 2021), as well as fruit postharvest behavior (Salazar et al., 2021; Sanhueza et al., 2015; Ying et al., 2019) have been systematically studied at the molecular level through the study of the gene expression linked to the main metabolic pathways (Gismondi et al., 2020; Salazar et al., 2021). To date, many efforts have been focused on the study of fruit development and ripening at transcriptomic, proteomic, or metabolomic levels in order to unravel the regulatory mechanisms involved in these processes (García-Gómez et al., 2021).

At the transcriptome level, a recent study in apricot using RNA-seq confirmed that fruit softening during ripening is strongly correlated with the upregulation of polygalacturonase, pectin methylesterase, and

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pectin lyase activities, as well as the gradual increase in the expression of ACC, ACS, and ACO enzymes (Xu et al., 2021). These enzymes are closely involved in ethylene biosynthesis and, consequently, the fruit ripening process. Furthermore, the study identified novel key genes associated with ethylene signal transduction, which provided insights into the molecular mechanism of ethylene metabolism.

Proteins from peach fruits after harvest and treated by heat combined with 1-MCP and stored at room temperature, were found to be associated with pathways linked to defense and response, energy and metabolism, or ripening and senescence (Ma et al., 2014). Most recently, different analyses between mature and ripe peach fruit showed that at least 15 % of proteins identified were involved by the ripening process, including cell wall modification in ripe fruit (Nilo-Poyanco et al., 2021). Furthermore, this study identifies potential biomarkers for the ripening process, including pectin methylesterase inhibitor, gibberellin 2-beta-dioxygenase, omega-6 fatty acid desaturase, homeobox-leucine zipper protein, ACC oxidase, as well as NAC and Myb transcription factors. Additionally, other studies have used a combination of transcriptomic and metabolomic analyses to elucidate molecular processes such as fruit flesh coloration in *Prunus mira*. In this study, the authors constructed a regulatory network that illustrates the interactions between anthocyanins and transcripts involved in flesh coloration (Ying et al., 2019). Moreover, recent investigations in sweet cherry utilizing an integrative analysis of the metabolome and transcriptome have revealed novel key genes implicated in sugar metabolism, organic acid metabolism, and flavonoid metabolism (Yang et al., 2021).

In addition, the effect of ethylene-related treatments such as 1-MCP ethylene inhibitor has been extensively studied in *Prunus* species including apricot (Cao et al., 2009; Fan et al., 2018) and Japanese plum (Khan and Singh, 2008; Velardo-Micharet et al., 2017), as well as the ethylene precursor effect of ethephon, is well known in these species (Ximena Rozo-Romero et al., 2015). Thus, the use of these ethylene regulators together with the lack of knowledge about epigenetics mechanisms involved in the ripening process is an interesting approach in order to study the role of DNA methylation regions on fruit ripening processes in *Prunus* species such as apricot and plum.

However, the implications of epigenetic control at DNA methylation level on the expression of these genes and its potential consequences in fruit development and ripening remain poorly understood. Epigenetic regulation encompasses various mechanisms, including DNA methylation, where methyl groups are added to the C5 position of DNA through the action of DNA methyltransferase (DNMT) enzymes (Goldberg et al., 2007). As an essential type of epigenetic modification, DNA methylation is widely studied. It collaborates with histone modifications to significantly regulate gene expression, chromatin conformation, and other essential processes. DNA methylation predominantly occurs at CpG dinucleotides (cytosine phosphate-guanine sites), where a cytosine is followed by a guanine in the DNA sequence (Goldberg et al., 2007). However, there is a large proportion of non-CG methylation (CHH, CHG, H represents A, C, T) in plants (Jackson et al., 2002). In general, high DNA methylation could repress gene expression, and demethylation would recover gene expression. In animals, DNA methylation participates in many cell activities, including cell differentiation, tissue-specific expression, genomic imprinting, X-chromosome inactivation, and genomic stability maintenance, as well as environmental adaptation (Bird, 2002; Jones and Takai, 2001; Reik and Dean, 2005).

In horticultural crops, most of the knowledge regarding epigenetic control of fruit growth and ripening has been primarily elucidated in tomato (Farinati et al., 2017). However, there are limited studies on DNA methylation in fruit trees, particularly in *Prunus* species. Notable examples in this field included the methylation of *Sf* locus in almond (Fernández i Martí et al., 2014), the methylation changes as a strategy for the study of Noninfectious Bud Failure (Fresnedo-Ramírez et al., 2017), or the DNA methylation analysis in almond flower buds using epi-genotyping sequencing (Prudencio et al., 2018), where they identified methylation markers associated with early and late flowering in

specific coding regions. More recently, a study has investigated the relationship between transcriptome profiles, DNA methylation, and peach mealiness. This research demonstrates the existence of epigenetic regulation of gene expression that leads to the development of flesh mealiness during later stages of ripening (Rothkegel et al., 2021).

The main objective of this study is to evaluate DNA methylation patterns in two climacteric species, such as apricot and Japanese plum, during their postharvest behavior and after the application of treatments related to ethylene regulators, by using Whole Genome Bisulfite Sequencing (WGBS).

2. Material and methods

2.1. Plant material, experimental design and treatments

Samples of apricot and Japanese plum fruits were collected for a postharvest monitoring assay at the CEBAS-CSIC experimental orchard in Cieza-Calasparra, Murcia, Spain, when they reached a firmness exceeding 40N and full fruit coloration (commercial maturity). The apricot cultivars 'Moniquí,' 'Goldrich,' and 'Dorada,' as well as the Japanese plum cultivars 'Golden Japan,' 'Santa Rosa,' 'Black Splendor,' and 'Angeleno,' were selected based on their significant variability in fruit quality and postharvest behavior. These sample fruits were treated with different ethylene-related treatments and divided into three fruit sets (control, 1-MCP-treated, and Ethrel-treated) (Fig. S1).

All treatments including the control were stored at 20 °C and 80 % of relative humidity, applying 1-MCP and Ethrel in these conditions. The 1-MCP treatment (T1) involved the gasification of SmartFresh™ product at a concentration of 0.14 % (625 ppb) in a watertight chamber for 20 h, while ethephon (T2) (Acid-2-chloroethyl-phosphonic) was applied by immersing the fruits in buckets of distilled water using Ethrel® 48 SL product (Ethrel) at 300 ppm for apricots and 600 ppm for Japanese plum cultivars, by immersion lasting 15 min and 20 min, respectively. In this study, the control (T0: stored at 20 °C with 80 % of RH) and 1-MCP treatment (T1: stored at 20 °C with 80 % of RH + 1-MCP) were not immersed in distilled water. We made this decision considering that, on one hand, the optimal control condition (T0) aimed to simulate the conditions of a commercial fruit without submerging it in water. On the other hand, the 1-MCP gasification (T1) was implemented following the recommendation of the SmartFresh TM (Crouch, 2003). In contrast, the Ethrel treatment group (T2: stored at 20 °C with 80 % RH + ethephon) involved the application by immersion, mixed with water, in accordance with the product recommendation, mirroring a common commercial practice of immersion or spraying (Ethrel® 48 SL). The primary objective of these treatments was to create substantial differences between T1 and T2, while including a T0 group untreated with any product or water.

Therefore, the control group (T0), 1-MCP treatment (T1), and Ethrel treatment (T2) were stored under the same conditions of temperature and relative humidity (20 °C and 80 %) to monitor fruit quality and postharvest characteristics. Six fruit quality traits were assessed, including fruit weight (Blausscal digital balance AH-600), skin and flesh color (CR-300; Minolta, Ramsey, NJ, USA), soluble solids content (ATAGO®, Tokyo, Japan), and acidity (Acid-base titration Metrohm® system) at harvest, mid-period, and final stage, based on fruit maturity.

On the other side, these fruits were monitored in their post-harvest period considering fruit firmness in N (texturometer: TA.XT plus), CO₂ in ml kg⁻¹ h⁻¹ (CO₂ analyzer: Thermox® CG 1000), and ethylene emission in µl kg⁻¹ h⁻¹ (gas chromatography: ethylene chromatograph Konik®). Finally, considering firmness and ethylene emission as the most contrasting criteria, 'Goldrich' and 'Santa Rosa' emerged as the most promising candidates for gaining a deeper understanding of the molecular-level mechanisms involved in the fruit ripening process (Figs. S2 and S3). Details of the fruit monitoring assay are described in a previous work carried out in both species (Salazar et al., 2022b).

2.2. Library construction

DNA for methylation analysis was extracted from the same fruits previously evaluated for RNAseq and stored at -80 °C, focusing on the most contrasting day of the assay in terms of firmness and ethylene emission (day 4 in both species). Three biological replicates were considered for the 'Goldrich' apricot and 'Santa Rosa' Japanese plum varieties, with each replicate composed of a pool of three fruit halves. Total DNA was extracted using a modified CTAB method (Doyle, 1990), with agarose gel electrophoresis used to assess DNA degradation and potential RNA contamination. Subsequently, the DNA was quantified using the Qubit 2.0 fluorometer. Library preparation and Whole Genome Bisulfite Sequencing (WGBS) were performed by NOVogene in Cambridge, United Kingdom. Following DNA testing, 5.2 micrograms of genomic DNA, spiked with 26 ng of lambda DNA, were sonicated to 200-400 bp using the Covaris S220 focused-ultrasonicator (Woburn, Massachusetts, USA). DNA underwent repair, adenylation, and ligation of methylated barcodes. After bisulfite treatment and PCR amplification, library concentration was quantified with Qubit® 2.0 and quantitative PCR. Insert size was verified using the Agilent 2100 system.

2.3. Clustering and whole genome Bisulfite sequencing

The index-coded samples were clustered using a cBot Cluster Generation System with PE Cluster Kit cBot-HS (Illumina) following the manufacturer's instructions. After cluster generation, library preparations were sequenced on an Illumina HiSeq/NovaSeq platform, producing paired-end reads. Image analysis and base calling were done using the standard Illumina pipeline, yielding paired-end reads with three technical replicates per sample. To obtain qualified clean data, raw reads (FASTQ) underwent trimming with the Trimmomatic multi-threaded command line tool to remove low-quality reads.

2.4. Alignment to reference genome

In this study, bisulfite-treated reads underwent alignment to the *P. armeniaca* v1.0 genome (Jiang et al., 2019) using Bismark software v0.12.5 with default parameters (Krueger and Andrews, 2011). The reference genome was converted to a bisulfite-converted version (C-to-T and G-to-A) and indexed using bowtie2 (Langmead and Salzberg, 2012). Sequence reads were similarly transformed into fully bisulfite-converted versions before directional alignment. Unique best alignments from the two processes were compared to the normal genomic sequence, and the methylation state of all cytosine positions in the read was deduced. Duplicated reads aligning to the same genomic regions were considered duplicates. Deduplicated reads were used to summarize sequencing depth and coverage. Methylation extractor results were converted to bigWig format for visualization in the IGV browser. Finally, the sodium bisulfite non-conversion rate was calculated as the percentage of sequenced cytosines at cytosine reference positions in the lambda genome.

2.5. Methylation level and differentially methylated region analysis

The methylated and unmethylated counts at each site were tested by a binomial distribution. Assuming the number of methylated cytosines is x in a certain site, where the read coverage is n and BS un-conversion rate is p , the reliability of x methylated cytosine needs to be tested in the above conditions. In order to find accurate methylated sites, a set of thresholds were set in the analysis process (Gifford et al., 2013; Habibi et al., 2013): (1) the sequencing depth ≥ 5 ; (2) q -value ≤ 0.05 . We employed a sliding-window approach, which is conceptually similar to approaches that have been used for bulk BS-Seq. With window size $w = 3,000$ bp and step size 600 bp (Smallwood et al., 2014), the sum of methylated and unmethylated read counts in each window was calculated. The methylation level (ML) for each C site showed the fraction of

methylated Cs, which is defined as $ML = mC/(mC+umC)$ where ML represents the methylation level, and mC and umC represent the number of methylated and unmethylated cytosines, respectively. Dispersion Shrinkage for Sequencing data (DSS) was designed for differential methylation analyses for bisulfite sequencing (BS-seq) data. The core of DSS is a procedure based on a Bayesian hierarchical model to estimate and shrink CpG site-specific dispersions, then conduct Wald tests for detecting differential methylation. Regions satisfying the following criteria were reported as DMRs by using DSS Bioconductor package: (1) Called DMRs must exceed a minimum length (50bp by default) and cover more than a minimum number of CpG sites (three by default); (2) The percentage of CpG sites in the DMR with threshold P -values $\leq 1e-5$ must exceed a user-specified value (50 % by default).

2.6. Enrichment analysis of DMR-related genes

Gene Ontology (GO) enrichment analysis of genes related to DMRs was implemented by the clusterProfiler R package. Most importantly, clusterProfiler applies biological term classification and enrichment analyses to gene cluster comparison, helping to better understand higher-order functions of biological systems. The genes with their promoter and gene body region overlapped with DMR were selected to perform GO enrichment analysis Gene Ontology (GO, <http://www.geneontology.org/>). GO enrichment analysis of DMR-related genes contributes to dig out the biological processes involved in fruit ripening. GO enrichment analysis is used by Goseq (Young et al., 2010), which is based on Wallenius non-central hyper-geometric distribution. Thus, this method could estimate GO Term enrichment probabilities of DMR-related genes. GO terms with corrected P -values ≤ 0.05 were considered significantly enriched by DMR-related genes.

On the other hand, KEGG (Kyoto Encyclopedia of Genes and Genomes) is the main public database of the related pathways (Kanehisa et al., 2008). Pathway enrichment analysis significantly identifies enriched metabolic pathways using super geometry inspection compared with the whole genome background. Thus, KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism, and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high throughput experimental technologies (<http://www.genome.jp/kegg/>). In the current work, we used KOBAS software to test the statistical enrichment of DMR-related genes in KEGG pathways.

2.7. RT-qPCR validation

RNA extractions were conducted from a pooled fruit sample of each treatment and species, following the protocol established by Le Provost et al.'s (2007). Subsequently, the RNA samples were purified using the UltraClean Plant RNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. The quantity and quality of RNA were assessed using a spectrophotometer and agarose gel. RT-qPCR validation was performed using three biological and two technical replicates of cDNA. Ripening-related gene linked to carbon metabolism, 3-hydroxyisobutyryl-CoA hydrolase (HIBCH), and associated with the cysteine and methionine pathway, 1-aminocyclopropane-1-carboxylate synthase (ACS), were analyzed to compare gene expression and methylation patterns in 'Goldrich' apricot and 'Santa Rosa' Japanese plum, respectively (Fig. 5). RNA Polymerase II (RPII) and Ubiquitin 10 (UBQ10) housekeeping reference genes were selected because they have demonstrated constant expression in tested *Prunus*-related species (Tong et al., 2009). The cDNA was synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), and RT-qPCR experiments were conducted in the One Step Plus real-time PCR system (Applied Biosystems). Prior to initiating the gene expression quantification of the target genes, efficiency curves were calculated for each gene, including housekeeping genes. This involved generating a standard curve with

concentrations of 1.25, 2.5, 5, and 10 ng/μl from reverse-transcribed cDNA. For all genes, the primer efficiency obtained ranged between 1.9 and 2.1. For all RT-qPCR reactions, a 10 μL mix was prepared, including 5 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems), 0.5 μL of each primer (5 μM), and 2 μL of cDNA (2.5 ng/μl). Amplification conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, and for the melting curve, 15 s at 95 °C, 1 min at 60 °C, with an increase of 0.3 °C until 0.15 sec at 95 °C.

Normalized Relative Quantification (NRQ) for gene expression levels was performed using the modified Pfaffl method (Pfaffl, 2001), which relies on the expression levels of the target gene in comparison to a housekeeping gene. In this case, the geometric average of housekeeping genes was utilized to normalize the gene expression of target genes. Additionally, the relative error of the biological replicates' average for each treatment was calculated. The Tukey test with a significance level of $P \leq 0.05$ was applied to discern significant differences between treatments.

3. Results

3.1. Fruit quality and postharvest monitoring

This study is based on previous research involving the monitoring of fruit quality and postharvest traits in apricot ('Moniquí,' 'Goldrich,' and 'Dorada') and Japanese plum cultivars ('Golden Japan,' 'Santa Rosa,' 'Black Splendor,' and 'Angeleno') after treatments with 1-MCP and Ethrel (Salazar et al., 2022b). The principal component analysis reveals substantial phenotypic variability among the evaluated apricot and Japanese plum cultivars (Fig. S2). In terms of fruit quality traits, 'Goldrich' exhibited the highest fruit weight, an orange skin colour and the lowest SSC/Malic ratio attributable to its elevated acidity. On the other side, 'Santa Rosa,' of medium size, presented significant variations between treatments and days in terms of skin and flesh colour. Although other cultivars exhibited differences among treatments, we ultimately decided to choose apricot 'Goldrich' and Japanese plum 'Santa Rosa', based primarily on firmness and ethylene differences among treatments for further analysis. On day 4, 'Goldrich' showed high differences, especially in the comparison of the control and Ethrel application with 1-MCP treatment, coinciding with an increase of ethylene in the control and Ethrel treatment, and no emission for the 1-MCP treatment (Fig. S3). Similarly, on the same day 4, 'Santa Rosa' exhibited a climacteric peak for the Ethrel treatment, the onset of ethylene emission for the control, and no emission for the 1-MCP treatment (Fig. S3). Consequently, 'Goldrich' and 'Santa Rosa' cultivars were specifically chosen on day 4 to discern changes attributed to ethylene-related treatments for methylation analysis.

3.2. Sequencing quality control, coverage, and mapping

DNA methylation analysis was conducted through whole-genome bisulfite sequencing to elucidate the methylome of three sample fruits: control, 1-MCP, and Ethrel-treated, from both the 'Goldrich' apricot and 'Santa Rosa' plum. Approximately 85 to 100 million clean reads were generated for each sample group, resulting in a total of 280 million raw reads for 'Goldrich' and 275 million raw reads for 'Santa Rosa', including three biological replicates. The quality control (QC20) yielded a high rate of around 97-98 % with a conversion rate of 99 % for both species (Table 1). In terms of coverage, the 'Goldrich' DNA samples exhibited an average coverage of 39.08, while 'Santa Rosa' showed a slightly lower average coverage of 17.20 (Table 2). Notably, the coverage for 'Santa Rosa' was comparatively lower than that for 'Goldrich' (Table 2). The apricot genome (Jiang et al., 2019) was used as common reference for both species for mapping. Regarding the reads uniquely mapped to the reference genome, 'Goldrich' achieved 45-50 % matches, whereas 'Santa Rosa' only exhibited 15-20 %. Both species demonstrated a similar duplication rate of approximately 15 % (Table 3).

3.3. Methylation level and distribution of DMR

Genomic regions with different DNA methylation patterns are termed "differentially methylated regions" (DMRs). DMRs identified under distinct biological conditions can offer valuable insights into various crucial biological processes. DSS (Dispersion Shrinkage for Sequencing data) was specifically developed for analyzing differential methylation in bisulfite sequencing (BS-seq) data (Wu et al., 2015).

In this study, we identified over 12,000 and 9,000 genes with differentially methylated regions (DMRs) in the 'Goldrich' and 'Santa Rosa' cultivars, respectively, between various treatment comparisons (Table S1). Analysis of DMR distribution in the 'Goldrich' apricot cultivar indicated a higher number of hypomethylated regions in response to 1-MCP treatment compared to Ethrel treatment (Fig. 1A). Similarly, comparison between control samples and 1-MCP treatment revealed hypermethylation in control samples compared to 1-MCP treatment. In particular, no significant differences in the number of hypo- and hypermethylated regions were observed when comparing control and Ethrel treatment.

These findings suggest that fruits in a more advanced ripening state, induced by Ethrel application, tend to exhibit higher levels of methylation, while 1-MCP treatment promotes a less advanced maturation state associated with lower levels of methylation. It is essential to highlight that these observations were primarily observed in the 'Goldrich' apricot cultivar, as the Japanese plum cultivar 'Santa Rosa' showed minimal methylation level variations between treatments. Therefore, we

Table 1

Summary of sequencing quality control for 'Goldrich' apricot (GD) and 'Santa Rosa' Japanese plum (SNR) under each treatment (T0, control; T1, 1-MCP; and T2, Ethrel).

Samples	Raw reads ¹	Raw base(G) ²	Raw Q20 (%) ³	Raw Q30 (%) ⁴	Raw GC (%) ⁵	Total Raw data	Effective rate (%)	Clean reads	Clean base(G)	Clean Q20 (%)	Clean Q30 (%)	Clean GC (%)	Conversion rate (%) ⁶
GD_T0	93,354,825	280,064	97.11	91.89	22.72	751,760	90.53	92,233,825	253,406	97.82	92.71	21.52	99.48
GD_T1	90,572,335	271,716	97.20	92.10	22.69	637,934	90.68	89,551,349	246,176	97.90	92.89	21.48	99.49
GD_T2	100,183,187	300,549	97.05	91.75	22.87	601,098	90.40	98,805,442	271,288	97.80	92.61	21.64	99.47
SNR_T0	91,959,915	275,878	96.90	91.59	24.19	827,643	89.20	89,576,447	245,811	97.76	92.57	22.27	99.48
SNR_T1	96,338,679	289,016	97.23	92.16	23.13	867,048	90.70	95,327,114	262,226	97.91	92.93	21.96	99.67
SNR_T2	86,879,829	260,639	97.41	92.64	23.94	709,782	90.60	85,780,005	236,315	98.07	93.38	22.61	99.25

¹ The original sequencing read counts (sum of three replicates).

² Raw read counts multiplied by read length, saved in G unit (sum of three replicates).

³ The proportion of raw bases with quality score more than 20 (average of three replicates).

⁴ The proportion of raw bases with quality score more than 30 (average of three replicates).

⁵ The proportion of G and C in all raw bases (average of three replicates).

⁶ The bisulfite conversion rate for C converting to T.

Table 2
Summary of sequencing coverage of apricot ‘Goldrich’ (GD) and Japanese plum ‘Santa rosa’ (SNR) under each treatment (T0, control; T1, 1-MCP; and T2, Ethrel) and biological replicate.

Samples	sites_num ¹	CovgMean ²	numCovg1(%) ³	numCovg5(%) ⁴	numCovg10(%) ⁵
GD_T0_1	220,557,992	37.03	99.39	97.11	90.39
GD_T0_2	220,581,748	37.79	99.41	97.15	91.22
GD_T0_3	220,572,267	39.54	99.40	97.55	92.10
GD_T1_1	220,501,529	38.22	99.37	97.48	91.87
GD_T1_2	220,690,076	37.56	99.45	97.03	91.09
GD_T1_3	220,508,172	35.39	99.37	97.09	89.82
GD_T2_1	220,932,604	54.83	99.56	98.47	95.54
GD_T2_2	220,428,736	33.26	99.34	96.54	89.55
GD_T2_3	220,532,759	38.11	99.38	97.47	92.08
SNR_T0_1	213,487,107	18.69	96.21	71.51	50.16
SNR_T0_2	212,264,290	15.34	95.66	67.41	42.42
SNR_T0_3	211,436,692	15.61	95.28	68.27	46.14
SNR_T1_1	212,970,003	16.84	95.97	69.97	47.43
SNR_T1_2	213,460,393	17.72	96.20	71.17	47.68
SNR_T1_3	214,133,640	20.15	96.50	73.96	52.67
SNR_T2_1	210,121,294	14.23	94.69	64.85	40.50
SNR_T2_2	213,192,914	18.36	96.08	71.85	50.36
SNR_T2_3	213,075,200	17.94	96.02	72.19	49.84

¹ The total detected genome base amount.
² The average base coverage of the genome.
³ The percentage of bases with a minimum 1x coverage of the genome.
⁴ The percentage of bases with a minimum 5x coverage of the genome.
⁵ The percentage of bases with a minimum 10x coverage of the genome.

Table 3
Summary of mapping of apricot ‘Goldrich’ (GD) and Japanese plum ‘Santa rosa’ (SNR) under each treatment (T0, control; T1, 1-MCP; and T2, Ethrel) and biological replicate.

Samples	Total reads ¹	Mapped reads ²	Mapping rate (%) ³	Duplication rate (%) ⁴
GD_T0_1	29,063,081	13,511,395	46.49	15.12
GD_T0_2	30,218,439	14,310,021	47.36	15.35
GD_T0_3	32,952,305	15,271,400	46.34	17.76
GD_T1_1	31,227,852	14,481,094	46.37	14.81
GD_T1_2	30,094,935	13,856,748	46.04	16.33
GD_T1_3	28,228,562	12,813,011	45.39	16.18
GD_T2_1	41,952,496	19,151,534	45.65	18.09
GD_T2_2	26,491,720	12,148,455	45.86	14.24
GD_T2_3	30,361,226	14,055,165	46.29	16.33
SNR_T0_1	30,680,491	5,293,506	17.25	15.24
SNR_T0_2	30,289,482	3,888,588	12.84	15.42
SNR_T0_3	28,606,474	4,907,096	17.15	19.44
SNR_T1_1	31,912,220	4,660,889	14.61	16.69
SNR_T1_2	32,732,003	4,581,590	14.00	16.87
SNR_T1_3	30,682,891	5,885,967	19.18	16.98
SNR_T2_1	23,826,290	3,484,695	14.63	14.36
SNR_T2_2	32,060,000	5,796,867	18.08	20.66
SNR_T2_3	29,893,715	4,658,983	15.59	14.69

¹ The filtered clean reads used for mapping.
² The uniquely mapped reads.
³ The percentage of uniquely mapped reads in the filtered clean reads used for mapping.
⁴ The percentage of duplication reads.

could suggest that the ethylene-related treatments have dual effects on apricot maturation state. On one hand, they may slow down the gene methylation level (1-MCP, associated with less advanced maturation), and on the other hand, they may increase the genes methylation level (Ethrel, associated with more advanced maturation).
Methylation distribution analysis revealed a larger fraction of total mC for a methylation level of 100 %, reaching 0.15 and 0.10 in ‘Goldrich’ and ‘Santa Rosa’, respectively (Fig. 1B). The CG context was predominant, and no differences were observed between treatment comparisons. Furthermore, the methylation level was assessed for each gene region, indicating a higher methylation ratio in the CG context,

particularly in the exonic and intronic regions, ranging between 0.3 and 0.5 in ‘Santa Rosa’ and ‘Goldrich,’ respectively (Fig. 2). Despite the lower methylation levels observed in the CHG and CHH contexts, the promoter and intronic regions were identified as the most significant for both species.

3.4. GO and KEGG enrichment analysis of DMRs related genes

By conducting enrichment analysis on the DMR-related genes, we can uncover the biological functions or pathways significantly associated with differential DMR-related genes. Incorporating biological ontologies is a common strategy for identifying shared functions among genes. Gene Ontology (GO) annotates genes to biological processes, molecular functions, and cellular components within a directed acyclic graph structure, while the Kyoto Encyclopaedia of Genes and Genomes (KEGG) annotates genes at the pathway level. Our GO enrichment analysis revealed that the CHH context exhibited the highest number of genes with DMR between treatments in both species (Fig. S4 and S5).
Focusing on the most contrasting comparison of ethylene-related genes (1-MCP treatment vs. Ethrel treatment) in the ‘Goldrich’ apricot cultivar, we identified helicase activity ($n = 24$) in the CG context and carbohydrate metabolic process ($n = 186$) in the CHH context as the most significant. In the CHH context, ion binding ($n = 836$) and cellular nitrogen compound metabolic process ($n = 779$) were particularly abundant. Similarly, in Japanese plum ‘Santa Rosa,’ kinase activity in the CH context ($n = 66$) and DNA metabolic process in the CHH context ($n = 244$) were prominent. Ion binding ($n = 805$) and cellular nitrogen compound metabolic process ($n = 720$) in the CHH context were also significant in both apricot and Japanese plum. Additionally, signal transduction ($n = 221$ and $n = 239$) was abundant and significant in both species, as were transferase or methyltransferase activities in the CHH context.
Our pathway enrichment analysis by KEEG revealed several significant metabolic pathways for each treatment comparison in both species (Fig. 3). In ‘Goldrich’ apricot, significant pathways included sesquiterpenoid and triterpenoid biosynthesis, plant-pathogen interaction, glycan degradation, and ABC transporters, while in Japanese plum ‘Santa Rosa,’ pathways such as glucosinolate biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, cyanamino acid metabolism, and ribosome biogenesis in eukaryotes were identified. Notably, the

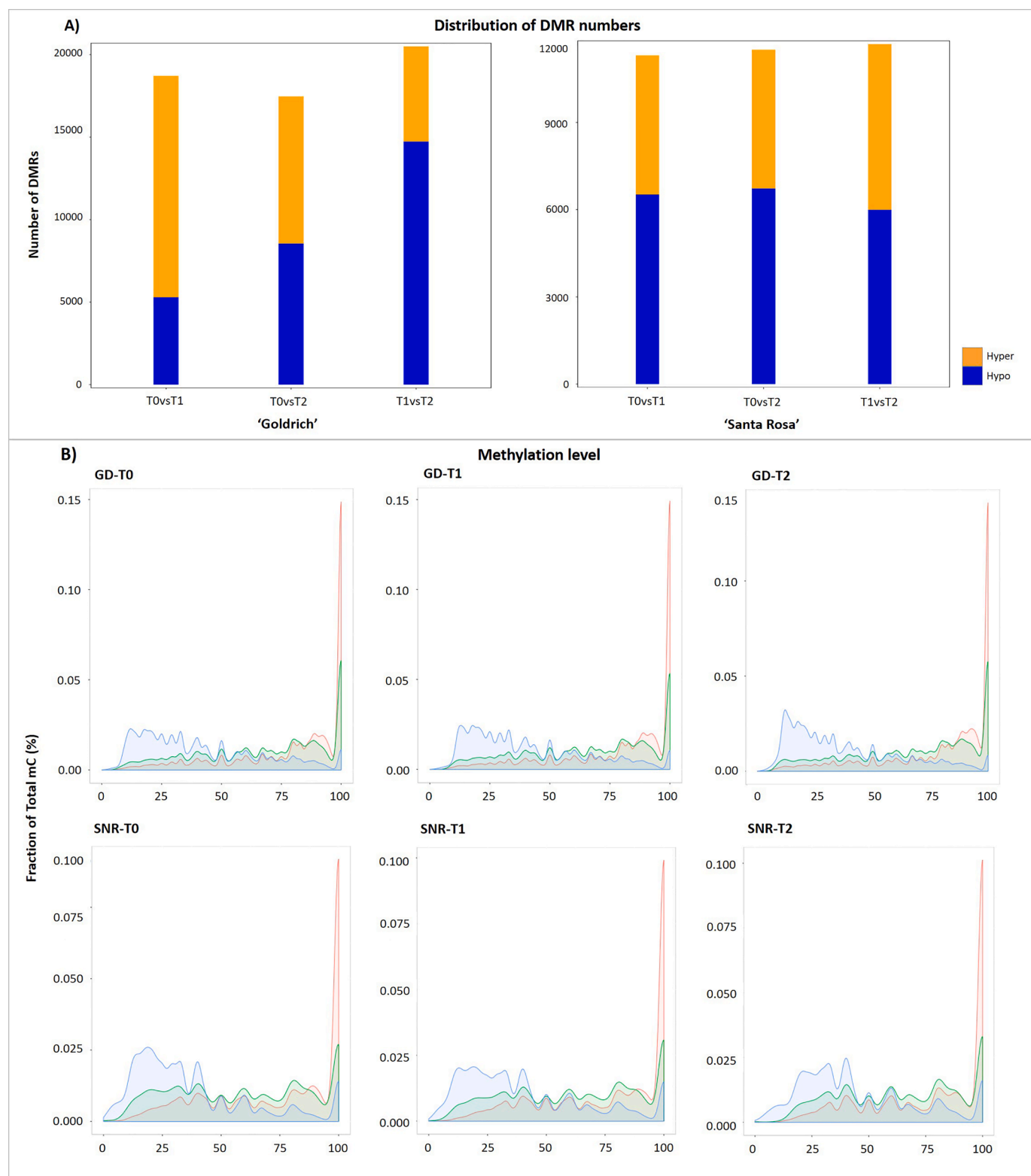


Fig. 1. A) Number of differentially methylated regions (DMRs) between treatments (control, T0; 1-MCP, T1 and Ethrel, T2) in 'Goldrich' (GD, left) and 'Santa Rosa' (SNR, right). B) Level of methylation for each C context (CG in red, CHG in green, and CHH in blue) for 'Goldrich' (GD) and 'Santa Rosa' (SNR) after treatments (T0, control; T1, 1-MCP; T2, Ethrel).

starch and sucrose metabolism pathway emerged as the most significant across both species and all treatment comparisons (control vs. 1-MCP treatment, control vs. Ethrel treatment, and 1-MCP treatment vs. Ethrel treatment), particularly in the CHG context. This fact highlights the involvement of ethylene-related treatments in fruit ripening through

methylation-mediated epigenetic events, potentially influencing the expression of key genes associated with the critical starch and sucrose metabolism pathway during fruit ripening.

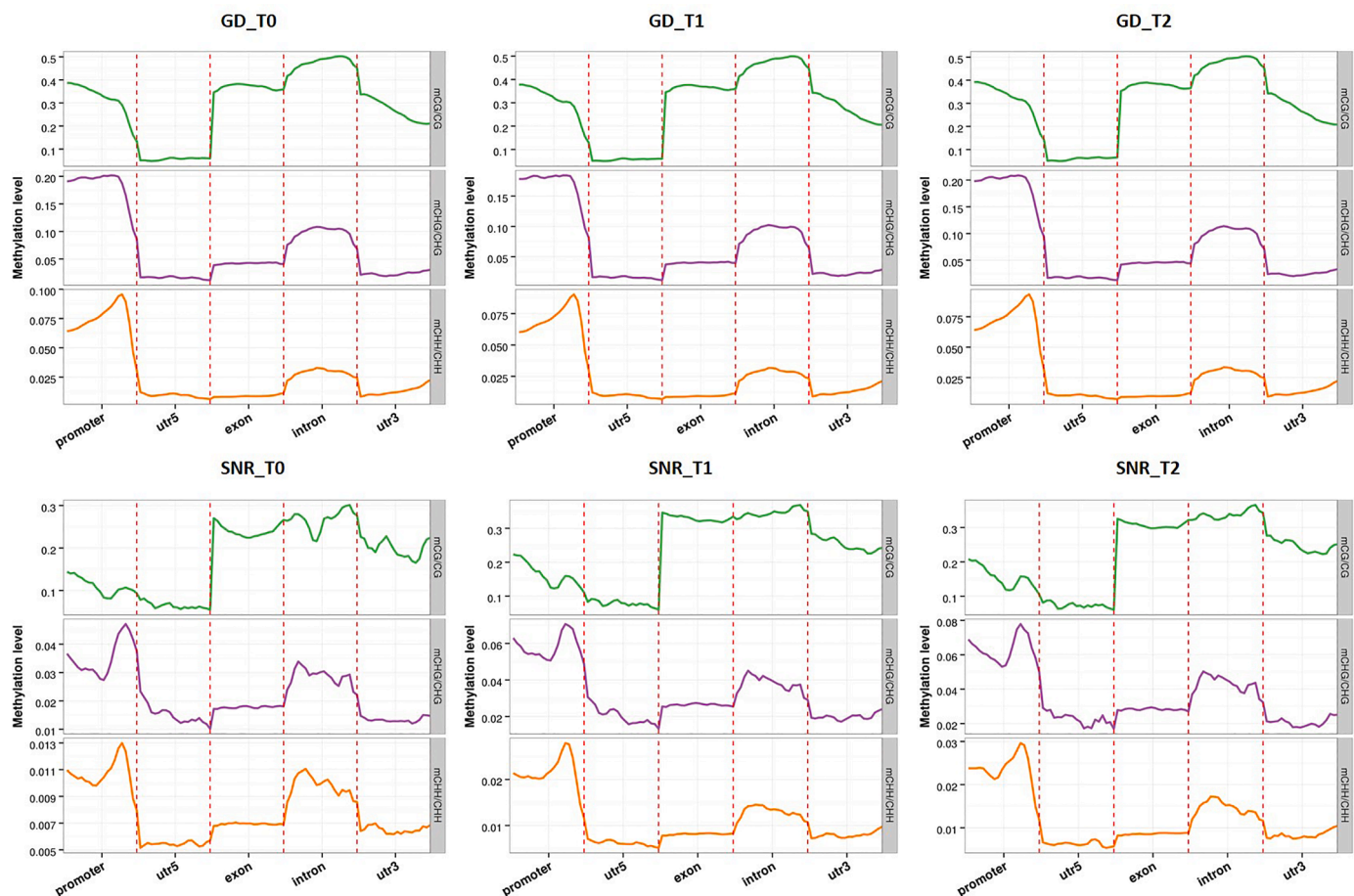


Fig. 2. Methylation levels in apricot 'Goldrich' (GD) and Japanese plum 'Santa Rosa' (SNR) in response to each treatment (T0, control; T1, 1-MCP; T2, Ethrel) across different gene regions.

3.5. Methylation level vs. differentially gene expression

Epigenetic mechanisms, particularly DNA methylation, plays an important role in gene regulation and silencing within specific metabolic pathways. In this context, we conducted a cross-analysis of data from a prior RNAseq study (Salazar et al., 2022b) using the same samples, comparing expressed genes (cDNA regions) to genes exhibiting partial methylation (DNA regions). This analysis identified 2,372 common genes in both species. Initially employing Pearson coefficients to discern the biological weight of the DMRs over the DEGs, we found correlations barely reaching -0.1 (data not shown). Consequently, we filtered the most significant genes, considering only DEGs with LogFC exceeding 1 or below -1. This selection process yielded 411 differentially expressed and methylated genes in both species, with 332 mismatched genes in 'Goldrich' and 405 mismatched genes in 'Santa Rosa' (Fig. 4).

In this study, focusing on the most contrasting comparison (1-MCP treatment vs. Ethrel treatment), along with the identification of genes with inverse methylation-expression patterns and functional annotations, we identified 10 genes for 'Goldrich' apricot and 17 genes for 'Santa Rosa' Japanese plum, based on their inverse methylation-expression patterns and functional annotations (Fig. 5 and Table S2). Notably, in 'Goldrich' apricot, several significant genes exhibited an inverse correlation between gene expression and methylation level in the comparison of differentially expressed genes (DEGs) and differentially methylated regions (DMRs).

For instance, 4-coumarate-CoA ligase [EC:6.2.1.12] involved in phenylpropanoid biosynthesis, DELLA protein in plant hormone signal transduction, GDP-L-fucose synthase [EC:1.1.1.271] in amino sugar and nucleotide sugar metabolism, and D-lactate dehydratase [EC:4.2.1.130]

in pyruvate metabolism showed inverse correlation in terms of gene expression and methylation level between the contrasting treatments (1-MCP treatment vs. Ethrel treatment). These genes exhibited over-expression in response to the ethylene suppressor treatment (1-MCP), indicating a lower methylation level compared to the ethylene precursor treatment (Ethrel) (Fig. 5 and Table S2). Furthermore, GDP-L-galactose phosphorylase, VTC2.5, associated with amino sugar and nucleotide sugar metabolism, also showed gene overexpression upon 1-MCP treatment, as confirmed through RT-qPCR validation (Fig. 6). On the contrary, hydroxymethylglutaryl-CoA reductase (NADPH) [EC:1.1.1.34] exhibited higher gene expression in the Ethrel treatment, coinciding with a lower methylation level (Fig. 5 and Table S2). A similar CoA-related gene 3-hydroxyisobutyryl-CoA hydrolase, (HIBCH) linked to valine, leucine, and isoleucine degradation, showed a similar trend during RT-qPCR validation (Fig. 6).

If we focus on the gene expression and methylation levels of the control treatment in the mentioned genes, 4-coumarate-CoA ligase, GDP-L-fucose synthase, and D-lactate dehydratase exhibited similar behavior to Ethrel treatment. In contrast, DELLA protein control exhibited a response similar to 1-MCP treatment, while hydroxymethylglutaryl-CoA control showed intermediate gene expression and methylation levels (Fig. 5).

As for 'Santa Rosa' Japanese plum, genes encoding homocysteine S-methyltransferase [EC:2.1.1.10] in cysteine and methionine metabolism, 1-phosphatidylinositol-4-phosphate 5-kinase [EC:2.7.1.68] in inositol phosphate metabolism, and pectinesterase [EC:3.1.1.11] in pentose and glucuronate interconversions were identified as overexpressed in the Ethrel treatment, with lower methylation levels compared to the 1-MCP treatment (Fig. 5 and Table S2). In this instance, 1-

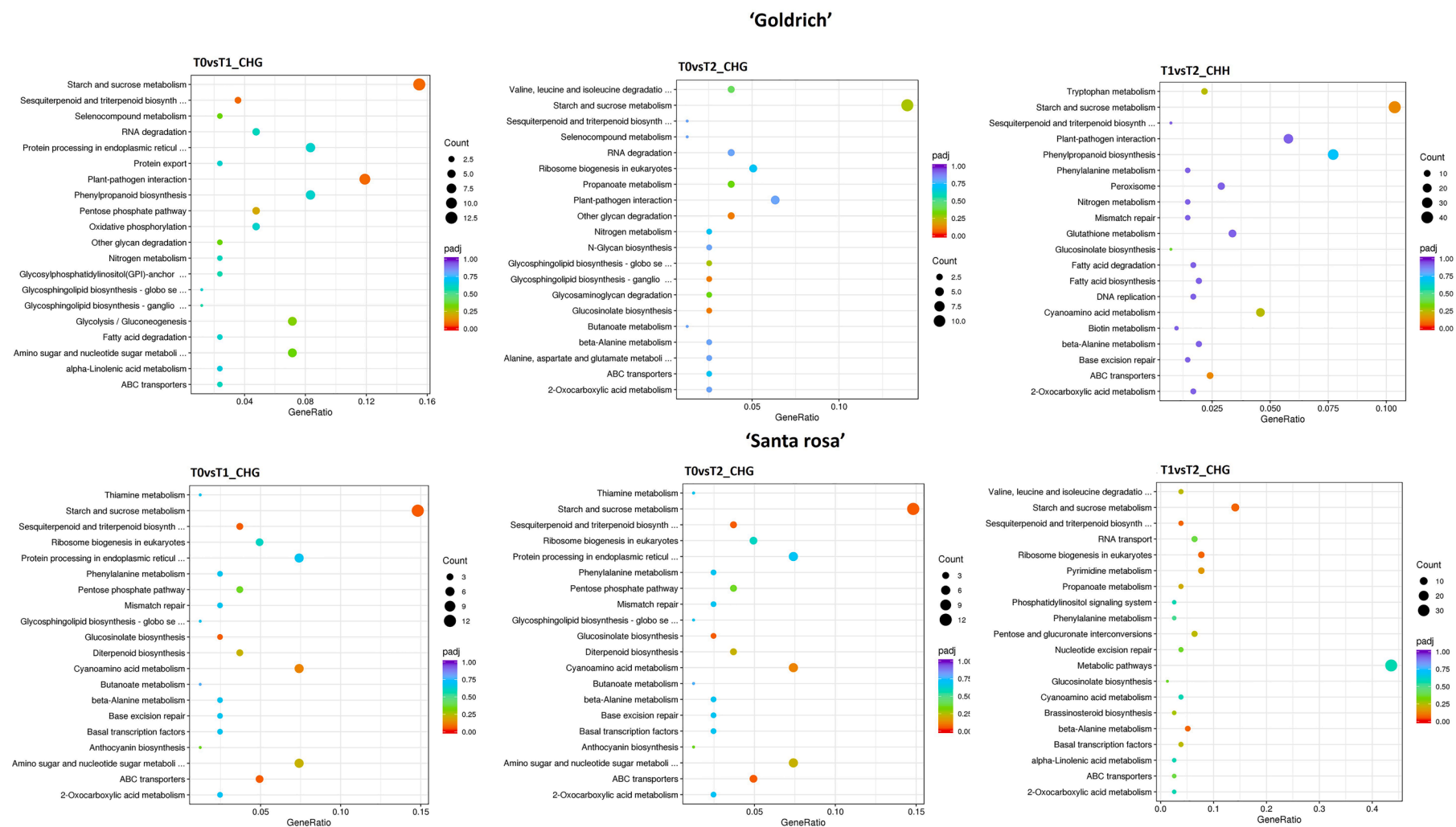


Fig. 3. Metabolic pathway analysis of differentially methylated regions (DMRs) in both species, comparing the effects of each treatment. The advanced bubble chart visualizes the enrichment of DMRs in specific signaling pathways. The y-axis represents the pathways, while the x-axis represents the gene ratio. The size of each bubble corresponds to the number of DMRs enriched in each pathway (counts), and the color range indicates the significance of enrichment (adjusted p-value).

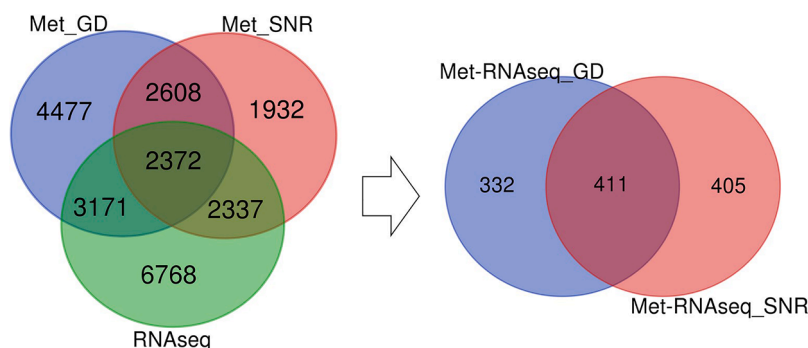


Fig. 4. Venn diagram illustrating the overlap between previous RNA-seq analysis and the current DNA methylation analysis, focusing on the most significant genes. The selected genes are differentially expressed genes (DEGs) with $\log_2$ fold change ($-1 < \log_2FC < 1$) and a P-value ≤ 0.005 in both species.

aminocyclopropane-1-carboxylate synthase (ACS), closely associated with cysteine and methionine metabolism, along with homocysteine S-methyltransferase and the pectinesterase gene in pentose and glucuronate interconversions, showed a similar pattern of overexpression upon Ethrel treatment, as demonstrated by RT-qPCR validation (Fig. 6 and Table S2).

Genes encoding malate dehydrogenase (oxaloacetate-decarboxylating) (NADP+) [EC:1.1.1.40] in carbon metabolism, aquaporin-4 as a membrane transporter, and photosystem I subunit VI in photosynthesis exhibited, however, an overexpression in response to 1-MCP treatment and were found to be hypomethylated compared to the Ethrel treatment (Fig. 5 and Table S2). In this case, for the mentioned genes, the control exhibited behavior similar to the Ethrel treatment for homocysteine S-methyltransferase, 1-phosphatidylinositol-4-phosphate 5-kinase, pectinesterase, and photosystem I subunit VI. The control emulated a more similar response to 1-MCP for malate dehydrogenase and aquaporin-4.

4. Discussion

4.1. Quality control, coverage and mapping

The Whole-Genome Bisulfite Sequencing (WGBS) technique, developed by Illumina® in 2014, has emerged as an advanced tool for DNA methylation analysis. While initially applied in animal and plant models such as *Arabidopsis*, its usage has expanded to include various plant species, including almond (D'Amico-Willman et al., 2021; Fresnedo-Ramírez et al., 2017; Prudencio et al., 2018), strawberry (Cheng et al., 2018), sweet cherry (Rothkegel et al., 2017), and peach (Rothkegel et al., 2021). However, its application in apricot and Japanese plum remains unexplored. In this study, we employed WGBS to investigate the methylation patterns between contrasting ethylene-related treatments (1-MCP and Ethrel) in 'Goldrich' apricot and 'Santa Rosa' Japanese plum.

As a result of this WGBS analysis close to 100 million raw reads were sequenced to each sample group for both species, similar results to Cheng et al. (2018) in strawberry. The mean coverage obtained in our study was over 30 for apricot and around 15 for Japanese plum. A previous study conducted in peach (Rothkegel et al., 2021) reported lower coverage, as they focused on uniquely mapped reads ranging from 15 to 25 million. In the present study, the mapped reads reached similar values in apricot 'Goldrich' (around 15 million), but lower in 'Santa Rosa' Japanese plum (approximately 5 million), resulting in a lower coverage. Despite the differences in coverage, we were able to identify a substantial number of partially methylated high-quality reads associated with genes and relevant pathways, even with the lower coverage in 'Santa Rosa' Japanese plum. The use of the same *P. armeniaca* genome as a reference and the lower coverage of Japanese plum 'Santa Rosa' contributed to the disparity in the percentage of mapped reads, which

ranged from 40 to 50 % in apricot and 15 to 20 % in Japanese plum.

4.2. Methylation level and distribution of DMR

In light of the aforementioned results, more than 10,000 differentially methylated genes have been identified in both species, using a procedure based on the Bayesian hierarchical model and then performing Wald tests to detect differential methylation (Feng et al., 2014). In detail, this method involves the development of a hierarchical Bayesian model that takes into account both technical and biological variations between and within replicates. It employs a beta-binomial model, a shrinkage procedure to enhance the estimation of dispersion parameters, and a Wald test procedure that considers depth of coverage and within-group variation. These findings indicated that methylation-mediated epigenetic mechanisms in the context of GC are predominantly observed in introns and exons. On the other hand, for the CHG and CHH contexts, the promoter regions and introns emerge as the predominant contexts. Similar results were obtained in red and white petals of Japanese apricot (*Prunus mume*) (Jiang et al., 2020). Furthermore, it is noteworthy that although the CHH context exhibited the lowest overall methylation level, it showed the highest degree of methylation variation. This observation aligns with previous studies on contrasting varieties of Japanese plum, such as 'Royal Dawn' and 'Kordia', which investigated differential methylation and chilling requirement (Rothkegel et al., 2020).

On the other hand, 'Goldrich' showed predominantly hypomethylated regions for 1-MCP as opposed to Ethrel, as well as the control was hypermethylated as opposed to 1-MCP, therefore 1-MCP caused a lower methylation level contrary to Ethrel. This behavior could be associated with less advanced fruit ripening time for 1-MCP-treated fruits (lower methylation level), and contrary with a more advanced ripening for Ethrel-treated fruits (higher methylation level). In the case of Japanese plum these differences were not observed. This difference between species could be due to the differences in the shelf-life period between species (Japanese plum longer than apricot) since the samples were collected in both cases after four days of storage because was the most contrasting day in terms of firmness and ethylene differences. Therefore, we could presume more hypermethylated regions of control and Ethrel in contrast to 1-MCP in Japanese plum if we sample some days after day 4.

In other species such as tomato, it is important to note that the developmental process of fruit ripening is partially regulated by epigenetic processes (Gallusci et al., 2016) so it would be expected that this model would be fulfilled in other fruit species such as apricot and Japanese plum. Thus, Hadfield et al. (1993) had already reported a decrease of DNA methylation linked to genes highly expressed in tomato which would explain in the present assay a lower methylation level in the 1-MCP treatment (less advanced fruit ripening state) conversely with the Ethrel treatment (more advanced fruit ripening state). However, it is not

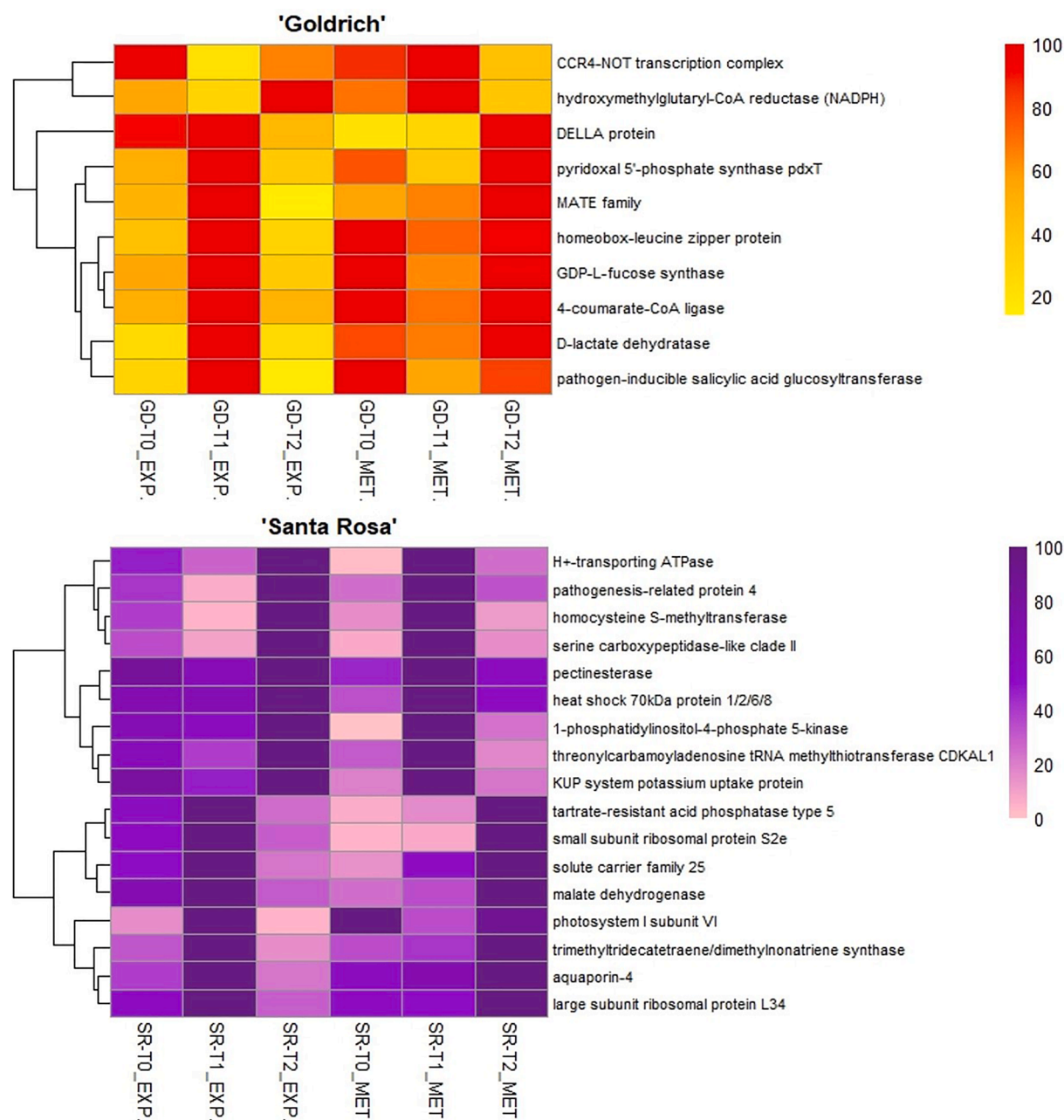


Fig. 5. Heatmap of genes from apricot 'Goldrich' (GD) and Japanese plum 'Santa Rosa' (SR) under each treatment (T0, control; T1, 1-MCP; T2, Ethrel), depicting their distinct gene expression (EXP.) and methylation level (MET.) patterns. The graduation bars show expression and methylation values relative to each gen as a percentage.

until 2006 that Manning et al. (2006) definitively achieves the identification of DNA methylation marks in the gene colorless non-ripening in tomato. Recent studies integrating transcriptomic and epigenetic methodologies have revealed the downregulation and hypermethylation of specific genes during the ripening process in peach fruits, leading to the development of a mealy texture (Rothkegel et al., 2021).

4.3. GO and KEGG enrichment analysis of DMRs related genes

GO and KEGG analysis aimed to reveal whether biological functions or pathways are highly associated with DMR-related genes. Helicase activity in the CG context and carbohydrate metabolic process in apricot 'Goldrich' were the most significant in the 1-MCP vs. Ethrel comparison. It is important to note that despite DNA helicase activity not having been thoroughly studied in eukaryotic systems such as plants, we can

speculate that these helicases may be involved in many biological processes, including DNA recombination, DNA replication, or even the regulation of genomic methylation patterns (Tuteja, 2003). Therefore, epigenetic modifications, such as DNA methylation, have the potential to influence the helicase activity, which plays a crucial role in gene expression and genome maintenance (Kumar and Mohapatra, 2021). Regarding the carbohydrate metabolic process, previous studies, provided evidences that epigenetic mechanisms, including histone acetylation, methylation, and DNA methylation, play a significant role in plant sugar signal transduction (Chen et al. (2022)). This process encompasses the regulation of carbohydrates such as sucrose and glucose.

On the other hand, the kinase activity was also significant for Japanese plum 'Santa Rosa' in the CH context. In previous studies such as Shen et al. (2012) in tomato, the kinase SnRK1 is known as a regulator of many physiological processes interacting with phosphorylate β C1,

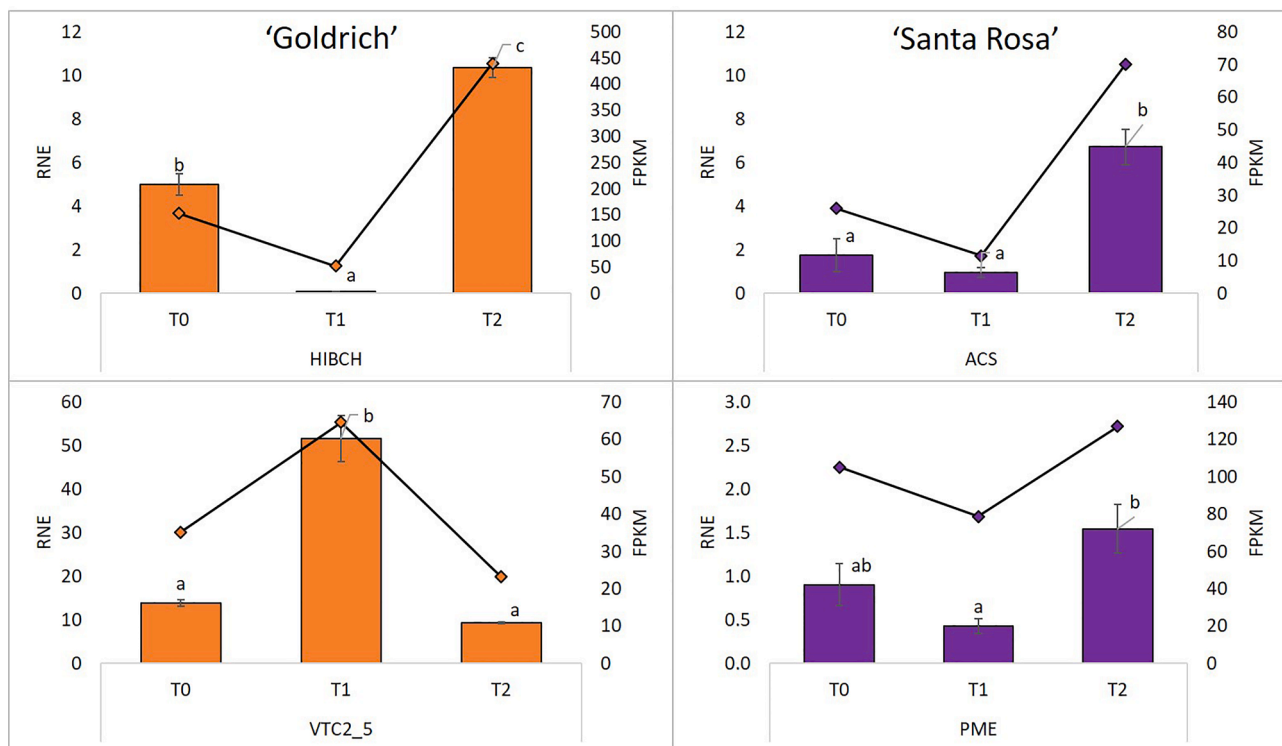


Fig. 6. RNAseq validation using Relative Normalized Expression (RNE, lines) from RT-qPCR analysis and Fragment per Kilobase Million (FPKM, bars) from RNAseq of 4 genes using three biological and two technical replicates. The orange color shows 3-hydroxyisobutyryl-coa hydrolase (HIBCH) and GDP-L-galactose phosphorylase (VTC2_5) validated in apricot 'Goldrich', and purple color 1-aminocyclopropane-1-carboxylate synthase (ACS) and pectinesterase (PME) in Japanese plum 'Santa Rosa'.

which is capable of reducing plant infection of geminivirus by decreasing the viral DNA accumulation. Moreover, β C1 protein could inhibit gene silencing due to the methylation mechanism. Thus, the interaction between SnRK1 and β C1 would be partly regulated by epigenetic mechanisms. Additionally, the cellular nitrogen compound metabolic process was also significant in the CHH context of both species. Currently, the epigenetic regulation of important nutrients such as nitrogen and phosphorus has been discussed mainly in model plants such as *Arabidopsis*, maize, or rice (Li et al., 2021). However, in stone fruits, there is a lack of studies evidencing this regulation.

According to KEGG analysis, starch, and sucrose metabolism was the most contrasting pathway in any treatment comparison for both species, which would partly explain the involvement of DNA methylation in the carbohydrate metabolic process. Moreover, in the *Arabidopsis* plant model, it has been observed that methylation can influence certain sugar signaling sensors, such as hexokinase and fructose (Sakr et al., 2018). These findings suggest that methylation plays a role in modulating the response of these sensors to sugar signaling in plants. In other studies, lower DNA methylation leads to a disruption of sugar and ROS, triggering microspore sterility in *Arabidopsis* (Ma et al., 2018).

Finally, it is worth noting that to date, there is limited research focusing specifically on the epigenetic regulation of starch and sucrose metabolism. However, the present study provides valuable evidence suggesting that DNA methylation is one of the epigenetic mechanisms involved in the regulation of this important metabolic pathway during fruit ripening.

4.4. Methylation level vs. differentially gene expression

It is important to note that epigenetics mechanisms, are involved in gene silencing. However, a very low correlation between DEGs and DMRs was observed in this study. This could be attributed to the complexity of the genome, as there are other mechanisms regulating

gene expression, such as post-translational histone modification, gene silencing mediated by non-coding RNA, ATP-dependent chromatin remodeling, and Polycomb and Trithorax group proteins. Similar low correlations were also reported by other researchers, such as Jiang et al. (2020) in *Prunus mume*.

Indeed, the intricate nature of DNA methylation patterns in genes from different genomic regions adds complexity to the analysis of correlations between DNA methylation and gene expression. Variations in DNA methylation levels among different regions of the same gene, including introns, exons, promoters, 3' untranslated regions (UTR), and 5' UTR, can complicate the identification of direct correlations between DNA methylation and gene expression based solely on DNA methylation levels.

It is worth mentioning that previously, starch and sucrose metabolism has been identified as the most important pathway, showing a generally higher proportion of methylated genes and significant differences appreciable between treatments. However, if we strictly consider those methylated genes that correlate inversely with their methylation status, other metabolic pathways related to fruit ripening stand out. The fact that many methylated genes related to starch and sucrose metabolism do not appear to be the most significant in terms of differences in gene expression between treatments is in part due to the low correlation between gene expression and methylation. As mentioned, methylation levels change even for the same gene, creating a web of complexity that is difficult to decipher.

Therefore, due to the low correlations observed between DNA methylation and gene expression in the present study, the approach was adjusted to focus on the most contrasting genes in terms of differentially expressed genes (DEGs) between the most contrasting treatment comparisons contrasting (1-MCP vs Ethrel). This filtering process resulted in a narrowed-down gene list consisting of 10 genes for 'Goldrich' and 17 genes for 'Santa Rosa'. By emphasizing on the most contrasting genes, the analysis aimed to capture genes that show significant differences in

gene expression between treatments, while also considering the potential impact of DNA methylation. This approach enables a more focused exploration of specific genes that may be particularly relevant to the observed differences between treatments.

In the case of apricot, in the current assay, a gene encoding coumarate CoA ligase [EC:6.2.1.12] was found to be differentially methylated among treatments, with lower methylation level and higher expression observed for the 1-MCP treatment. This enzyme has been associated with fruit quality in other species, such as raspberry (*Rubus idaeus*), with its coding genes involved in various metabolic pathways that produce phenylpropanoid-derived metabolites responsible for the flavor and color of these fruits (Kumar and Ellis, 2003). A current study of monitoring apricot ripening (García-Gómez et al., 2020) shows this enzyme linked to anthocyanins biosynthesis, being 4CL one of the most significant genes in the statistical model. On the other side, a DELLA protein was also overexpressed by 1-MCP treatment and hypermethylated by Ethrel in the intronic region. These proteins are mediators of Gibberellic acid (GA) which promotes plant growth and development. In other species such as tomato, fruit development is regulated by DELLA proteins, interacting with some auxin-signaling components in order to regulate fruit initiation (Xue et al., 2022).

DELLA proteins appear to be involved in fruit ripening, exhibiting overexpression and hypomethylation in response to 1-MCP treatment. Similarly, the same pattern is observed in the case of GDP-L-fucose synthase [EC:1.1.1.271] in the promoter region and D-lactate dehydratase [EC:4.2.1.130] in the intronic region, both associated with amino sugar and nucleotide sugar metabolism as well as pyruvate metabolism, respectively.

On the one hand, GDP-L-fucose corresponds to the active form L-fucose, which is incorporated into the cell wall in the form of xyloglucans, rhamnogalacturonan II, and arabinogalactans, being deeply involved in protein glycosylation. Another study in *Arabidopsis* shows that this protein is linked to plant development concretely in the leaf shape development (Gonçalves et al., 2017). On the other hand, D-lactate dehydratase is also known as Glyoxalase 3 and catalyzes the reaction where D-lactate produces methylglyoxal (2-oxopropanal) and a molecule of water (Pohanka, 2020). The role of this enzyme in fruit development or fruit ripening is still unknown, but in our assay the gene encoding this enzyme was overexpressed and hypomethylated by 1-MCP. On the contrary to previous enzymes hydroxymethylglutaryl-CoA reductase (NADPH) [EC:1.1.1.34] was downregulated and hypermethylated by 1-MCP. This enzyme has been exclusively reported as a key in the early fruit development of tomato but not for fruit ripening (Narita and Grisse, 1989). However, in the same study, although hydroxymethylglutaryl-CoA reductase (NADPH) activity is higher in the fruit development early stages in tomatoes, some enzyme activity was also observed during fruit ripening. In spite of the fact that the role of this enzyme in ripening time has not been extensively studied in *Prunus* species, it is well known that HMGR catalyzes the reductive deacylation of HMG-CoA to mevalonic acid in the mevalonate pathway for isoprenoid biosynthesis (Campos et al., 2014). This HMG-CoA reductase is regulated by a wide range of external and endogenous stimuli being detected in every plant tissue in agreement with other studies such as (Salazar et al., 2021) in kiwifruit.

According to the results obtained in the 'Santa Rosa' Japanese plum, malate dehydrogenase (NADP+) [EC:1.1.1.40] was up-regulated and hypomethylated by 1-MCP in the promoter region. This enzyme is involved in malate metabolism also playing a committed role during plant growth depending on light conditions and darkness. This fact acts as an evolutionary "redox switch" regulating enzymes related to photosynthesis (Yokochi et al., 2021). The same trend for photosystem I subunit VI and aquaporin-4 in its promoter regions was shown. Photosystem I (PSI) is involved in light energy conversion, mediating in the light-driven electron transfer from plastocyanin to ferredoxin (Amann et al., 2004). However, the role of genes encoding photosynthetic proteins should be scarce and more considering the low levels of expression

shown in fruits in spite of the high logFC between 1-MCP and Ethrel treatments. On the other hand, aquaporins are membrane channels playing an important role in cell water control. Current studies on sweet cherry show an overall expression higher in the flesh than in the skin and showing a downward trend toward ripening (Chen et al., 2019). In our assay, in spite of the gene expression was very low, 1-MCP and Ethrel fostered a high logFC between treatment comparison being Ethrel treatment hypermethylated.

In contrast, 1-phosphatidylinositol-4-phosphate 5-kinase [EC:2.7.1.68] in the inositol phosphate metabolism was downregulated and hypermethylated by 1-MCP in the promoter and intronic regions. This enzyme plays an important role in signal transduction being widely identified in the whole genome in several plants and participating in plant development and growth, as well as in biotic and abiotic stresses (Munir et al., 2020). However, the level of knowledge about its involvement in processes related to fruit ripening is still scarce, although it has been shown that some related enzymes such as myo-inositol are positively regulated by 1-MCP in *P. salicina*, which favors a stage of early ripening and extends the shelf-life period (Farcuh et al., 2018). Pectinesterase [EC:3.1.1.11] in the pentose and glucuronate interconversions has been widely reported in climacteric species including *Prunus* as a key enzyme involved in the cell wall degradation being downregulated (Salazar et al., 2022b) and hypermethylated (current study) by 1-MCP. Recent studies in the peach species (Zheng et al., 2023), similar to Salazar et al. (2022b), demonstrate that treatments with 1-MCP reduce the activity of polygalacturonase (PG) and pectin methylesterase (PME), promoting a lesser degradation of the cell wall and therefore extending the fruit's shelf life period.

On the other hand, another study has analyzed the de-methylation during the storage of peach finding little changes in methylation level in the pectins of the cell wall (Lurie et al., 2003). In the current assay, pectinesterase was down-regulated and hypermethylated by 1-MCP in the promoter region slowing down the fruit senescence and suggesting that the gene encoding this enzyme is partially methylated which could be repressing its gene expression. The same effect was shown by homocysteine S-methyltransferase [EC:2.1.1.10]. This enzyme plays an important role in providing methionine for plant growth development converting homocysteine to methionine using S-methylmethionine (SMM) or S-adenosylmethionine (SAM) as methyl donors (Zhao et al., 2018). At the same time, some of the methyl donor enzymes such as SAM are deeply involved in the synthesis of ACC, which is converted in turn to ethylene, CO₂, and cyanide, by the enzyme ACC oxidase. The significance of this enzyme has been widely reported in climacteric species such as *Prunus* including *Prunus mume* (Mita et al., 1999), *Prunus persica* (Ziosi et al., 2003), *P. salicina* (Sadka et al., 2019) or apricot (García-Gómez et al., 2022; Muñoz-Robredo et al., 2012), playing an important role in order to induce the fruit ripening process.

In light of the results obtained, it is important to note that the relationship between DNA methylation and gene expression is complex and multifaceted. Although methylation is generally often associated with gene expression repression, there are many other elements and factors influencing this process. The presence of other epigenetic modifications, interactions with proteins, and the three-dimensional structure of the genome itself can modulate the relationship between methylation and gene expression. The specific location of methylation in the DNA sequence is also crucial (promoter, intronic, and exonic regions). While methylation in promoters is often associated with gene repression, methylation in other regions, such as gene bodies, can have different effects, even activating transcription in some cases (Lucibelli et al., 2022). However, we can affirm that treatment with 1-MCP slows down fruit ripening, while treatment with Ethrel accelerates this process, as evidenced in the same trial through the previously published de novo transcriptome (Salazar et al., 2022b). Furthermore, this assay demonstrates that the least advanced ripening state (1-MCP) and the most advanced ripening state (Ethrel) correlate with lower and higher methylation levels, respectively, at least in the case of apricots,

specifically the 'Goldrich' variety. The lowest methylation level (1-MCP) is associated with higher gene expression levels, while conversely, the highest methylation level (Ethrel) is associated with lower gene expression levels. Furthermore, identifying the specifically methylated part of the gene most related to its expression, as well as other mechanisms involved, would require more specific studies. Therefore, further discussion is needed to explore the role of partially methylated regions in gene expression specifically within *Prunus* species.

5. Conclusions

This study compared DNA methylation patterns in post-harvest apricot ('Goldrich') and Japanese plum ('Santa Rosa') after ethylene-related treatments. The results reveal different methylation patterns: CG context is highly methylated in exonic and intronic regions, while CHG and CHH contexts show heightened methylation in the promoter region. Following methylation and gene expression comparison in apricot 'Goldrich', significant genes were found to be hypomethylated and overexpressed by 1-MCP. On the other hand, the starch and sucrose pathway showed the highest number of partially methylated genes. However, we highlight genes involved in other routes with inverse expression and methylation patterns. Notably, in 'Goldrich' apricot, CoA ligase in phenylpropanoid biosynthesis, DELLA protein in hormone signal transduction, GDP-L-fucose synthase in amino sugar metabolism, and D-lactate dehydratase in pyruvate metabolism were identified. Meanwhile, hydroxymethylglutaryl-CoA reductase (NADPH) was hypomethylated and upregulated by Ethrel. In Japanese plum, genes encoding homocysteine S-methyltransferase in the cysteine and methionine metabolism, 1-phosphatidylinositol-4-phosphate 5-kinase in the inositol phosphate metabolism, and pectinesterase in the pentose and glucuronate interconversions, were hypomethylated and upregulated by Ethrel. On the contrary, malate dehydrogenase in the carbon metabolism, aquaporin-4 as membrane transporter, or photosystem I subunit VI in the photosynthesis were hypomethylated and overexpressed by 1-MCP treatment. These results reveal that ethylene-related treatments induced different methylation patterns, providing evidence that epigenetic mechanisms are undoubtedly involved in the fruit ripening process in *Prunus* species.

Data availability statement

The raw reads from the Illumina NovaSeq 6000 platform were deposited into NCBI in the sequence reads archive (SRA) under the BioProject accession number PRJNA953594.

CRedit authorship contribution statement

Germán Ortuño-Hernández: Writing – review & editing, Visualization, Methodology, Formal analysis. **David Ruiz:** Validation, Supervision, Resources, Funding acquisition. **Pedro Martínez-Gómez:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization. **Juan Alfonso Salazar:** Writing – review & editing, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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