# Unraveling the pathways influencing the berry color and firmness of grapevine cv. Flame Seedless treated with bioregulators using biochemical and RNA-Seq analysis under semi-arid subtropics

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## ABSTRACT

Plant bioregulators (PBRs) regulate developmental and physiological processes in plants. In this study, biochemical and transcriptomic analyses were conducted to evaluate the influence of PBRs [abscisic acid (ABA), benzothiadiazole (BTH), ethephon, and prohexadione-calcium (Pro-Ca)] on the grapevine cv. Flame Seedless under semi-arid subtropics. This study aims to see the effect of exogenous application of PBRs on overall berry quality, including uniformity of berry color. Uniform colored berries, the maximum total soluble solids (TSS) and total antioxidant activity (TAoA), and the highest total phenolics (TPC) and flavonoids (TFC) contents were obtained with the treatments, namely, 400 mg L$^{-1}$ ethephon and 400 mg L$^{-1}$ ABA. Further, RNA-Seq analysis has also identified some key DEGs like UFGT (*VIT_16s0039g02230*), GST (*VIT_04s0079g00690*), and chalcone synthase (CHS) (*VIT_05s0136g00260*) which were part of the anthocyanin biosynthesis pathway controlling grape berries color. Thus, ethephon (400 mg L$^{-1}$) and ABA (400 mg L$^{-1}$) were found promising for attaining greater uniformity in berry color development because of increased total anthocyanins content. In addition, they were also found associated with enhanced TAoA, TPC, and TFC. Hence, ethephon and ABA can be recommended for improving the berry quality.

## 1. Introduction

Grape (*Vitis vinifera* L.) is one of the popular fruits primarily used in the wine, fresh juice, and raisin industries (FAO, 2020). Grape berries are non-climacteric and highly perishable, which warrants careful postharvest handling. Moreover, several abiotic and biotic factors severely affect fruit set, berry development, and quality. Out of different factors, high temperature during vine growth affects both floral and berry development (Greer and Weston, 2010). The development of un-even berry color due to poor accumulation of anthocyanins is another severe concern of colored cultivars such as 'Flame Seedless', which is quite common, especially under semi-arid sub-tropical regions (Roberto et al., 2012). Poor color development affects consumer acceptability and

the overall market price of table grapes. High temperature during berry development and ripening is also detrimental to the berry's overall biochemical composition (Martinez de Toda et al., 2014). Therefore, improving berry quality, either through the development of high temperature-tolerant cultivars or the use of on-farm cultural practices, including plant bioregulators (PBRs), is of great importance.

The application of plant bioregulators is a common and effective solution for improving the quality of grapes. For instance, the exogenous application of abscisic acid (ABA) enhances anthocyanin content, resulting in proper berry color development in different colored grapes genotypes (Koyama et al., 2018). The application of ethephon on the developing bunches has shown promising effects on berry color development (Wang et al., 2022). The benzothiadiazole is involved in the

phenylpropanoid pathway, thereby improving the anthocyanins, proanthocyanidins, flavonol, and total phenolics in berries (Ruiz-Garcia et al., 2012). Pro-hexadione calcium is a new generation inhibitor of gibberellin biosynthesis, which blocks the dioxygenases activity resulting in reduced plant vigor (Rademacher, 2015) and improved berry quality.

The transcriptomics (or RNA-Seq) and other -omics approaches help find the genes and metabolic pathways involved in the plant–environment interaction (Guo et al., 2019; Ma and Yang, 2019). Systematic mapping of any plant genome to various phenotypes, growth and development stages, and the impact of the erratic weather, remains a significant challenge. A comprehensive understanding of gene expression and transcripts controlling the action of genes is fundamental to devising alternative strategies.

The expression of various genes and transcription factors involved in the anthocyanin biosynthesis of grape berries was upregulated by the external application of ethephon (Wang et al., 2022) and ABA (Roberto et al., 2012; Koyama et al., 2018). Further, a correlation between the increased anthocyanin accumulation and up-regulation of genes associated with abscisic acid has been observed (Koyama et al., 2010; Ma and Yang, 2019; Zhang et al., 2021).

Recently, the different effects of PBR treatments on grape berry quality have been reported. However, there is a shortage of information on their impact on berry quality under semi-arid subtropical conditions. Information about the effect of the exogenous application of ethylene and abscisic acid on the differential expression (DE) of specific anthocyanin biosynthesis pathway genes in grape berries, specifically under semi-arid subtropical regimes, is scarcely available. Poor berry quality and uneven color development are significant constraints in the semi-arid subtropics. Therefore, this research aimed to evaluate the effect of plant bioregulators such as prohexadione calcium (ProCa), benzothiadiazole (BTH), ABA, and ethylene on berry quality and biochemical characteristics content and correlate the effect with transcriptomic studies. A comprehensive study was conducted involving biochemical and transcriptomic approaches to unravel the DEGs and pathways associated with berry color development when exogenously treated with PBRs in grape cv. Flame Seedless under semi-arid sub-tropical conditions.

## 2. Materials and methods

### 2.1. Plant material and treatments

Five-year-old vines of 'Flame Seedless' planted at 3 m × 3 m and trained on the Bower system were used. The vineyard is located at an altitude of 228 m above mean sea level (AMSL) with a latitude of 28°38′51.8″N and longitudinal of 77°09′15.6″E. This region is characterized by a semi-arid, subtropical climate with hot summers and cool winters, and the soil is alluvial, having alkaline pH and clay loam texture. The average annual precipitation is around 650 mm, with most of it falling between July and September (Rudrappa et al. 2006; Feddema, 2005).

Plant bioregulators namely, abscisic acid (ABA) (Hi-Media, India), benzothiadiazole (BTH) (Sigma Aldrich, United States), ethephon (Hi-Media, India), and pro-hexadione calcium (Pro-Ca) (Sigma Aldrich, United States) were used for their evaluation at different concentrations along with the control (water spray). The nine treatments used in the study are as follows: (i) ABA 200 mg L$^{-1}$ and (ii) 400 mg L$^{-1}$, (iii) BTH 0.3 mM and (iv) 0.6 mM, (v) ethephon 200 mg L$^{-1}$ and (vi) 400 mg L$^{-1}$, (vii) Pro-Ca 200 mg L$^{-1}$ and (viii) 400 mg L$^{-1}$, and (ix) control (water spray). All these treatments were applied at the initiation of berry ripening (veraison stage) on whole vine canopies using a hand-held sprayer until runoff during the late evening.

### 2.2. Sampling and berry characteristics estimation

#### 2.2.1. Berry quality characteristics

Following harvest, three healthy bunches from each replication were collected to determine various berry quality characteristics. Berry firmness was estimated using a texture analyzer (Model: TA + Di, Stable Micro Systems Ltd., Surrey, UK). Further, the berry samples were compressed using pre-programmed settings using a cutting probe and a puncture probe (Yaman et al., 2002). The compression measurement force was determined at a pre-test speed of 5 mm/second, a test speed of 1 mm/second, and a 5 mm/second post-test speed. Berry hardness was defined as the maximum force (kg) during compression, as measured in Newtons (N).

After harvest, 100 berry samples were collected in three replications for each treatment and immediately stored at −20 °C until further biochemical analyses. The TSS (%) of juice was determined from these selected berries using a Fisher's hand-held refractometer (0–50) (Erma, Japan), adjusted at 20 °C. For titratable acidity (TA), the juice samples were titrated against 0.1 N NaOH (Zoecklein et al., 2000).

#### 2.2.2. Berry biochemical analysis

After harvest, 100 berry samples were collected at random in three replications for each treatment and immediately stored at −20 °C for biochemical analyses. The ascorbic acid content was determined using the method described in (Zoecklein et al. 2000). The pH-differential method (Wrolstad et al., 2005) was used to estimate the total monomeric anthocyanins in berries. Total flavonoid content (TFC) and total phenolic content (TPC) in grape berries were measured as described by Zhishen et al. (1999) and Singleton and Rossi (1965), respectively. The total antioxidant activity (TAoA) was calculated using the DPPH scavenging assay modified by Sanchez-Moreno et al. (1998). The spectrophotometric estimations were done using UV–VIS double-beam PC 8 scanning auto cell spectrophotometer (UVD-3200, Labomed, Inc., Culver City, USA).

### 2.3. RNA-seq analysis

#### 2.3.1. Transcriptome sequencing

For RNA-Seq analysis, along with control or water spray (hereafter, CTR), ethephon 400 mg L$^{-1}$ (hereafter, ETH), and ABA 400 mg L$^{-1}$ (henceforth, ABA) treatments were used. Following 6 d of exogenous application of plant bioregulators, 4–5 berries were collected in RNAlater (Qiagen, Germany) solution and stored at −80 °C for further analysis. Further, a separate collection of samples was snap-frozen in the liquid nitrogen and kept at −80 °C till further investigation. RNeasy Mini Kit (Qiagen) was used to extract total RNA from the samples using standard protocols. The RNA integrity was tested in agarose gel (1.0%), and the concentration and purity of RNA were estimated using a NanoDrop™ (Thermo Fisher Scientific Inc., USA). The integrity of total RNA was evaluated using a bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Total RNA (1.0 μg) having a RIN value >7.0 was used for the cDNA library preparation as per the manufacturer's instruction (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®), and quality was checked using Qubit 2.0. The RNA-seq was carried out by Nucleome Informatics Pvt. Ltd. (Hyderabad, India) using Illumina HiSeq 2500 for two treatments and control having three biological replicates. The clean reads were obtained by removing reads containing adapter or poly-N, and low-quality reads from raw reads using Fastp, an ultra-fast all-in-one FASTQ pre-processor (Chen et al., 2018).

#### 2.3.2. Quantification of gene expression level

The raw reads were deposited at the National Center for Biotechnology Information (NCBI), Short Read Archive (SRA) Sequence Database (PRJNA689573). HISAT2, a fast-spliced aligner, was used to compare clean reads with the reference genome (Kim et al., 2015). Subread aligner (v1.5.0), a read mapping tool, was used to quantify and

analyze the gene expression levels using the features count mode (Liao et al., 2013). Then, according to the gene length and the read counts mapped to the gene, the FPKM (fragments per kilobase of transcript per million fragments mapped) was estimated to show the gene expression level.

$$FPKM = \frac{cDNA\ fragments}{Mapped\ fragments(millions)\ \times\ Transcript\ length\ (kb)}$$

### 2.3.3. Differential gene expression analysis

The analysis of differentially expressed genes (DEGs) of all the samples was conducted using the edgeR, a Bioconductor package, and the genes were prioritized with $|\log2FC| > 1$, $p < 0.05$.

### 2.3.4. GO and KEGG pathway enrichment analysis of differentially expressed genes (DEGs)

The three major components of Gene Ontology (GO) classification are molecular function (MF), cellular component (CC), and biological process (BP). The GO enrichment analysis of DEGs was carried out using topGO, an R package version 2.28.0 (Alexa and Rahnenfuhrer, 2016). The WEGO 2.0, a web server tool, was used to summarize many GO terms by removing unnecessary terms and arranging related terms depending on semantic similarities. After that, the individual enriched annotation terms having an enrichment P-value threshold ($p < 0.05$) were identified.

KEGG (Kyoto Encyclopedia of Genes and Genomes), a database tool, was used to study the advanced levels of biological systems (such as cells, organisms, and ecosystems) from molecular-level knowledge (https://www.genome.jp/kegg/). ClusterProfiler, an R package, was used for comparing the biological themes among gene clusters for pathway enrichment using the information of the reference genome (p-value cut-off = 0.05).

### 2.3.5. Validation studies of DEGs using in-silico RT-PCR

The DEGs were first shortlisted from a list of about 20,000 genes individually depending upon the identified GO terms and were further narrowed down based on their FDR value ($p \leq 0.05$). The peptide sequences of shortlisted DEGs were extracted from the Uniprot database, BLASTP was run against *Arabidopsis thaliana*, and finally, Genevestigator® was run to find the expression details (Zimmermann et al., 2004).

### 2.4. Statistical data analysis

The study was conducted in a randomized block design, and univariate ANOVA (Analysis of variances) was used to analyze the data using SAS (Statistical Analysis System) (S.A.S., 1988). Three replications per treatment were used for berry quality and biochemical analysis. The least significant differences (LSD) values were used to compare the mean, and statistically significant differences were considered at $p \leq 0.05$.

## 3. Results

### 3.1. Berry quality and biochemical characteristics

TAC accumulation in berries got boosted most significantly when treated with the ethephon (400 mg L$^{-1}$) (553.65 mg kg$^{-1}$) and ABA (400 mg L$^{-1}$) (533.40 mg kg$^{-1}$) (Fig. 1). Ethephon and ABA treatments (200 and 400 mg L$^{-1}$, respectively) significantly raised ascorbic acid content compared to control. The highest TPC, TFC, and TAoA were recorded in the berries when treated with ethephon (400 mg L$^{-1}$) followed by ABA (400 mg L$^{-1}$) (Fig. 1).

When treated with ethephon (400 mg L$^{-1}$), the berries showed a significant reduction in firmness (2.64 N). The berry juice TSS improved with all of the treatments investigated, with ethephon (400 mg L$^{-1}$)

(20.77 %) and ABA (400 mg L$^{-1}$) showing considerable improvements (18.40 %). ABA (200 mg L$^{-1}$) and Pro-Ca (400 mg L$^{-1}$) treatments showed lower TA, while the rest also followed a similar trend. (Fig. 1).

### 3.2. RNA-seq

#### 3.2.1. Data overview of RNA-seq

The sequencing of three RNA samples (*viz.*, control, ETH, and ABA) have generated 150,798,082 raw reads, of which 149,744,180 (99.30%) were the clean reads (Table S2 and Fig. S1). The percentage of reads (>Q30) after filtering was >92% for each treatment (Table S2), while >80% of paired ends (PE) could be mapped uniquely for all the samples (Table S4). The overall alignment rate for ABA 400 mg L$^{-1}$ (ABA), ethephon 400 mg L$^{-1}$ (ETH), and control (CTR) were 92.7, 92.5, and 93.4%, respectively (Table S2; Fig. S2). The reads (%) assigned through feature count mode were 73.9, 72.7, and 81.6 % for ABA, ETH, and CTR, respectively (Table S5). The details of reading statistics are given in Table S2.

#### 3.2.2. DEGs analysis

The treatments were divided into three groups (ABA vs. CTR, ETH vs. CTR, and ETH vs. ABA) (Table S6), and Venn diagrams depicting the number of DEGs that were expressed both commonly and uniquely (Fig. 2). The edgeR software has identified 0 to 1604 DEGs ($p \leq 0.05$; fold change (FC) $\geq 1$) in the three libraries. A total of 532, 271, and 282 DEGs were found upregulated for ABA vs. CTR, ETH vs. CTR, and ETH vs. ABA, respectively (Fig. 2 a). Whereas 1105, 326, and 193 DEGs were found downregulated for ABA vs. CTR, ETH vs. CTR, and ETH vs. ABA, respectively (Fig. 2 b). Furthermore, the volcano plots (Fig. S3) also revealed a relationship between the three comparison groups and the numbers of up-and-down-regulated DEGs. The ABA vs. CTR and ETH vs. CTR treatment groups revealed the most widespread DEGs expression over ETH vs. ABA. Also, the sample distance heat map (Fig. 2 c) showed the distribution of DEGs across the treatment groups. The heat map representing the top 50 DEGs is presented in Fig. 2 (d). Few genes involved in the anthocyanin biosynthesis network, such as UFGT (*VIT_16s0039g02230*) and GST (*VIT_04s0079g00690*); belonging to the GST superfamily were also noted in this heat map indicating their significant upregulation after the treatments with plant bioregulators. UFGT (*VIT_16s0039g02230*) was highly upregulated for ETH vs CTR ($\log_2$FC = 10.24) and ABA vs CTR ($\log_2$FC = 11.17) while GST (*VIT_04s0079g00690*) was also significantly upregulated for ETH vs CTR ($\log_2$FC = 9.56) and ABA vs CTR ($\log_2$FC = 10.48).

#### 3.2.3. GO analysis of DEGs

GO analysis was performed to find DEGs' functions, and the most significant GO terms were selected and presented (Fig. 3 e-f). A maximum number of GO terms were recorded for MF, followed by BP and the least for the CC. The most significant GO terms were related to "response to abiotic stimulus" (GO: 0009628), "membrane protein complex" (GO: 0098796), "response to chemical" (GO: 0042221), "cellular component biogenesis" (GO: 0044085), "response to stimulus" (GO: 0050896), "response to stress" (GO: 0006950) and "hydrolase activity" (GO: 0016787) (Fig. S4). Amongst the upregulated genes, the maximum percentage of genes were associated with "metabolic process" followed by "cellular metabolic process," "response to stress," and "response to abiotic stimulus" for ABA vs. CTR and ETH vs. CTR. Furthermore, amongst the downregulated genes, the maximum percentage of genes belonged to the "cellular process" and "metabolic process" followed by "binding," "biological regulation," "cell," and "cell part" for both ABA vs. CTR and ETH vs. CTR.

#### 3.2.4. The KEGG pathway analysis of DEGs

The Kyoto Encyclopedia of Genes and Genomes (KEGG) provides an excellent integration of developmental pathway networks. To encompass the significant molecular, biological, and cellular processes
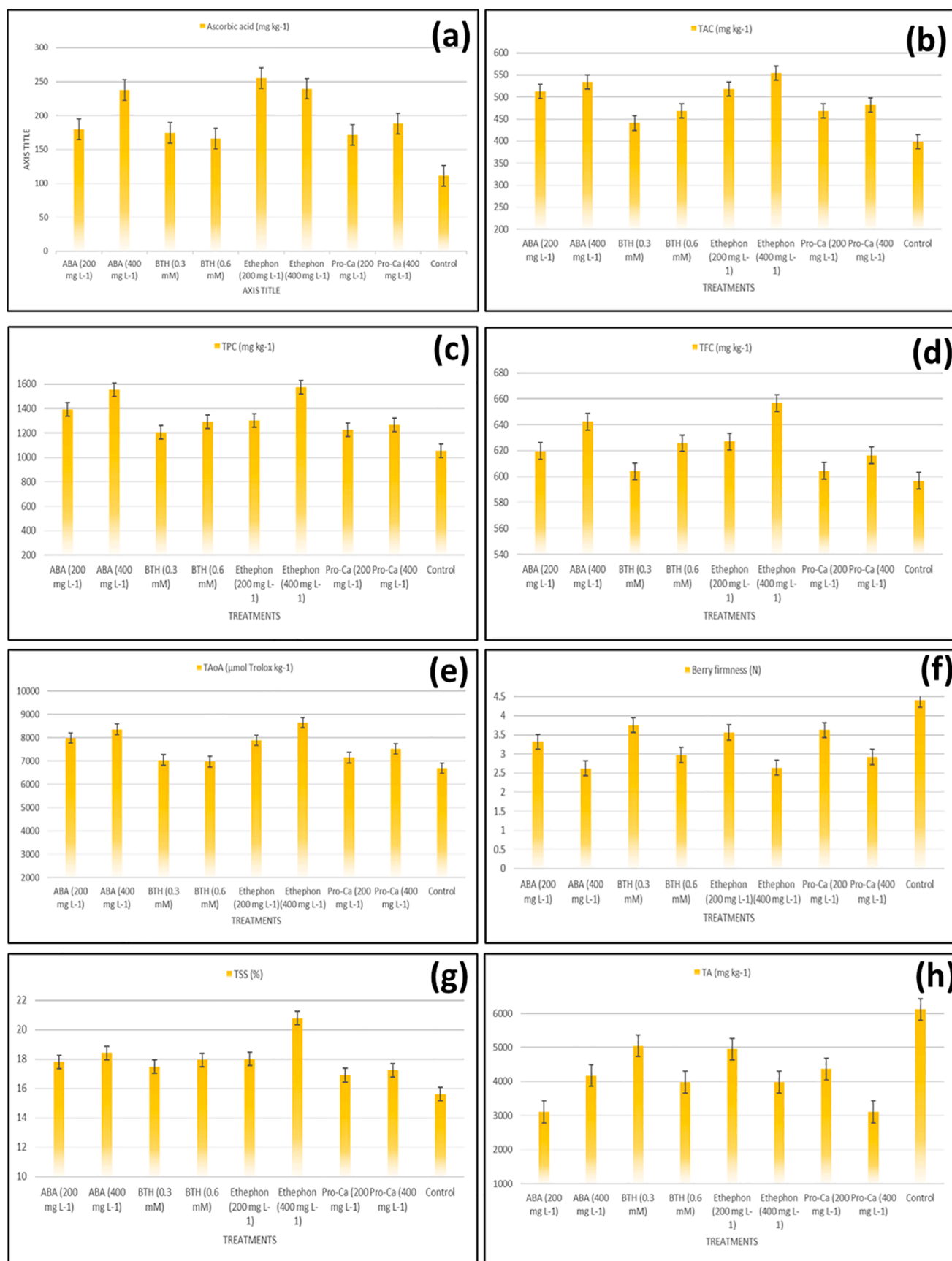
**Fig. 1.** Effect of plant bioregulators on berry metabolites and quality characteristics of cv. Flame Seedless (a) Ascorbic acid (b) Total anthocyanins content (TAC) (c) Total phenolics content (TPC) (d) Total flavonoids content (TFC) (e) Total antioxidant activity (TAoA) (f) Berry firmness (g) Total soluble solids (TSS) (h) Titratable acidity (TA).
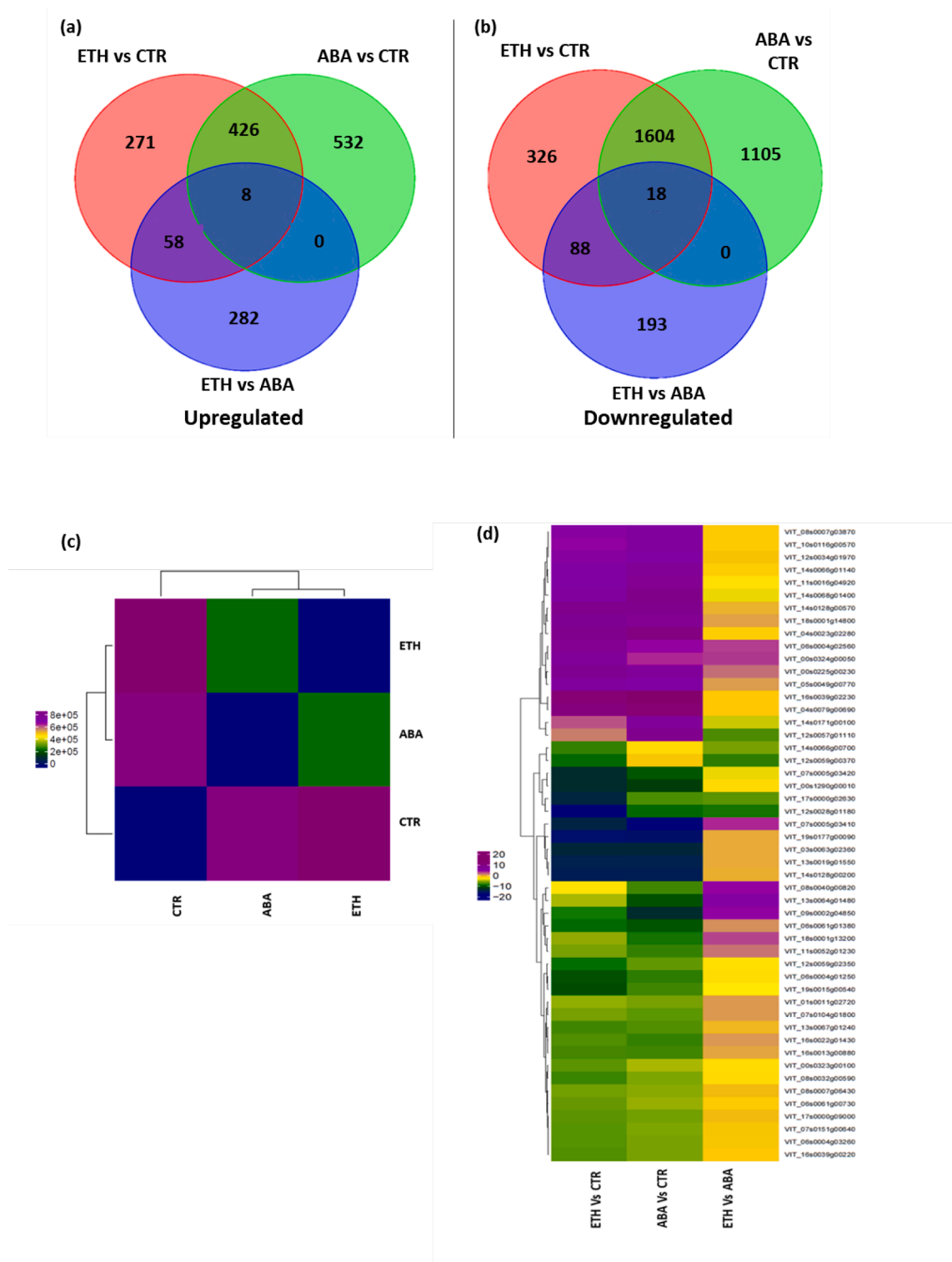
**Fig. 2.** Venn diagrams showing commonly and uniquely expressed DEGs under ABA vs CTR, ETH vs CTR, and ETH vs ABA combinations. (a) Distribution of upregulated DEGs, (b) Distribution of downregulated DEGs, (c) Sample distance heat map representing the overall expression of DEGs, (d) Heat map showing top 50 DEGs.

triggered after the treatment of berries with ABA and ethylene, DEGs were aligned to the KEGG database, and 19 most significantly enriched pathway entries were selected (Fig. 3 a-d). The most enriched pathways include "Flavonoid biosynthesis," "Circadian rhythm-plant," "Phenyl-alanine metabolism," "Carbon metabolism," "Protein processing in ER," "Peroxisome," "Glyoxylate and dicarboxylate metabolism," and "Carbon

fixation in photosynthetic organisms.".

*3.2.5. Validation studies using in-silico expression analysis*

*In-silico* validation analysis was performed using identified DEGs and *Arabidopsis thaliana* as the reference through Genevestigator® (Zimmermann et al., 2004). Initially, 31 genes could be identified, of which
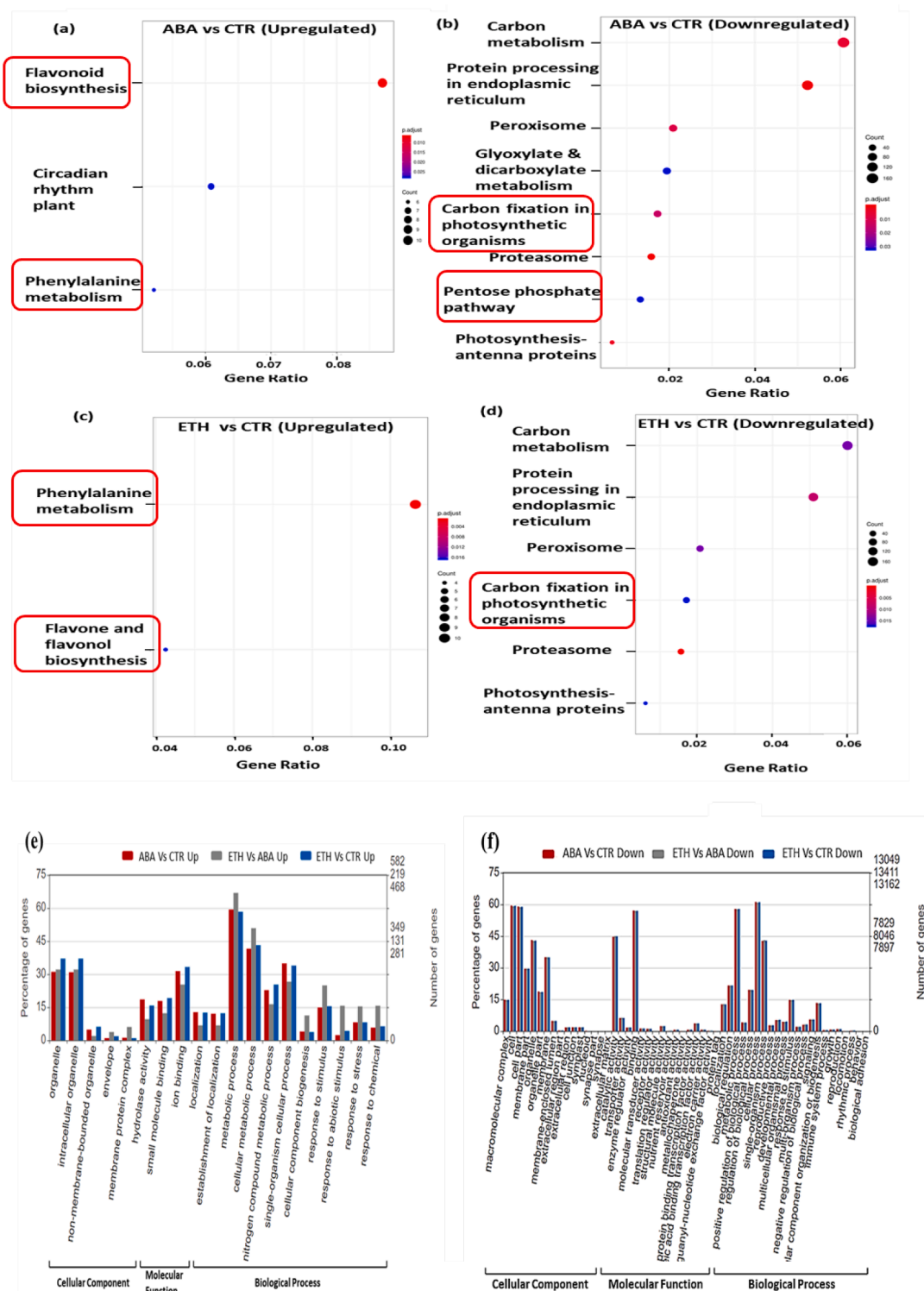
**Fig. 3.** Scattergrams (a-d) representing major KEGG pathways identified with significantly enriched DEGs (a). ABA vs CTR (Upregulated), (b). ABA vs CTR (Downregulated), (c). ETH vs CTR (Upregulated), (d) ETH vs CTR (Downregulated); (e) Highly upregulated GO terms due to plant bioregulators treatments; (f) Highly downregulated GO terms due to plant bioregulators treatments.

22 were unique, and 19 got validated against *Arabidopsis thaliana* (Fig. S5). The identified genes include *ABA2, ANN3, ANN5, CYS5, HSA32, HSFA6B, HSFC1, HSP17.4A, HSP17.6, HSP17.6C, HSP18.1, HSP26.5, HSP70-8, HSP81-1, MED37E, RBOHB, TDX, WRKY33*, and *ZEP*. Interestingly, *HSP81-1* and *WRKY33* genes showed high validation, especially at the flowering and fruiting stages (Fig. S5).

### 3.3. RNA Seq data and berry physical characteristics

The genes coding for pectin esterase (PE), polygalacturonase (PG), pectate lyase (PL) activity, polygalacturonate 4-alpha-galacturonosyl-transferase activity, xyloglucan endotransglucosylase/hydrolase (XTH) and several other genes involved in protein synthesis were found highly

expressed. In ABA vs CTR, a number of DEGs associated with PE (*VIT_05s0020g00420; −10.50 FC*), PL (*VIT_14s0219g00230; −5.25 FC*), pectin biosynthesis (*VIT_09s0002g02290; −1.03 FC*), β-galactosidase or β-GAL (*VIT_08s0007g04950l −9.74 FC*), and XTH (*VIT_11s0052g01230; −6.73 FC*) were found highly downregulated. Similarly, in ETH vs CTR, a number of DEGs could be identified for PE (*VIT_15s0048g00500, −5.49 FC; VIT_16s0022g00700, −5.09 FC*), PG (*VIT_15s0046g02000; −4.06 FC*), PL (*VIT_17s0000g09810, −2.98 FC*), pectin biosynthesis (*VIT_09s0002g02290; −1.10 FC*) and XTH (*VIT_11s0052g01230, −5.23 FC; VIT_11s0052g01200, −5.06 FC*) (Table S7; Fig. 4).

### 3.4. RNA Seq data and berry biochemical characteristics

The RNA-seq analysis identified several DEGs involved in berry phenolics, flavonoids, and anthocyanin biosynthesis pathways as highly upregulated (Table S1; Fig. 5). Similarly, the transcription factors (TFs) regulating the anthocyanin biosynthetic pathway in grapes, such as *VvMYBA1*, *VvMYBA2*, and *VvMYBA3*, were also reported to be upregulated (Table S1). Further, a relatively higher expression of DEGs in the stilbene biosynthesis pathway was observed. Interestingly, of the six genes coding stilbene synthase enzyme, three were upregulated while three were downregulated for ABA vs. CTR. However, for ETH vs. CTR,

all the six genes were downregulated (Table S1; Fig. 5).

### 4. Discussion

#### 4.1. Berry metabolites and quality characteristics

For different berry biochemical and quality attributes, the most significant findings were obtained by treating ethephon 400 ppm and ABA 400 ppm applied at the berry ripening (veraison) stage. In the present study, all the PBRs increased the berry TSS where ethephon 400 ppm and ABA 400 ppm produced the maximum TSS in berries. A few other studies have documented improvements in TSS due to using these PBRs, such as ethephon (Costa et al., 2016) and ABA (Balint and Reynolds, 2013). In the current investigation, all PBRs for TA produced varying outcomes. All treatments lowered the TA level of grape berries, but the ABA 200 ppm and Pro-Ca 400 ppm treatments had the lowest TA percentage. Recently, Gonzalez et al. (2018) studied the impact of ethephon on grape berry composition and observed that wines produced from treated vines had lower acid concentrations. PBRs are involved in improving the anthocyanin content in colored grape genotypes (Roberto et al., 2012). In the present study, the anthocyanins content in berries was most significantly improved by ethephon 400 ppm and ABA 400
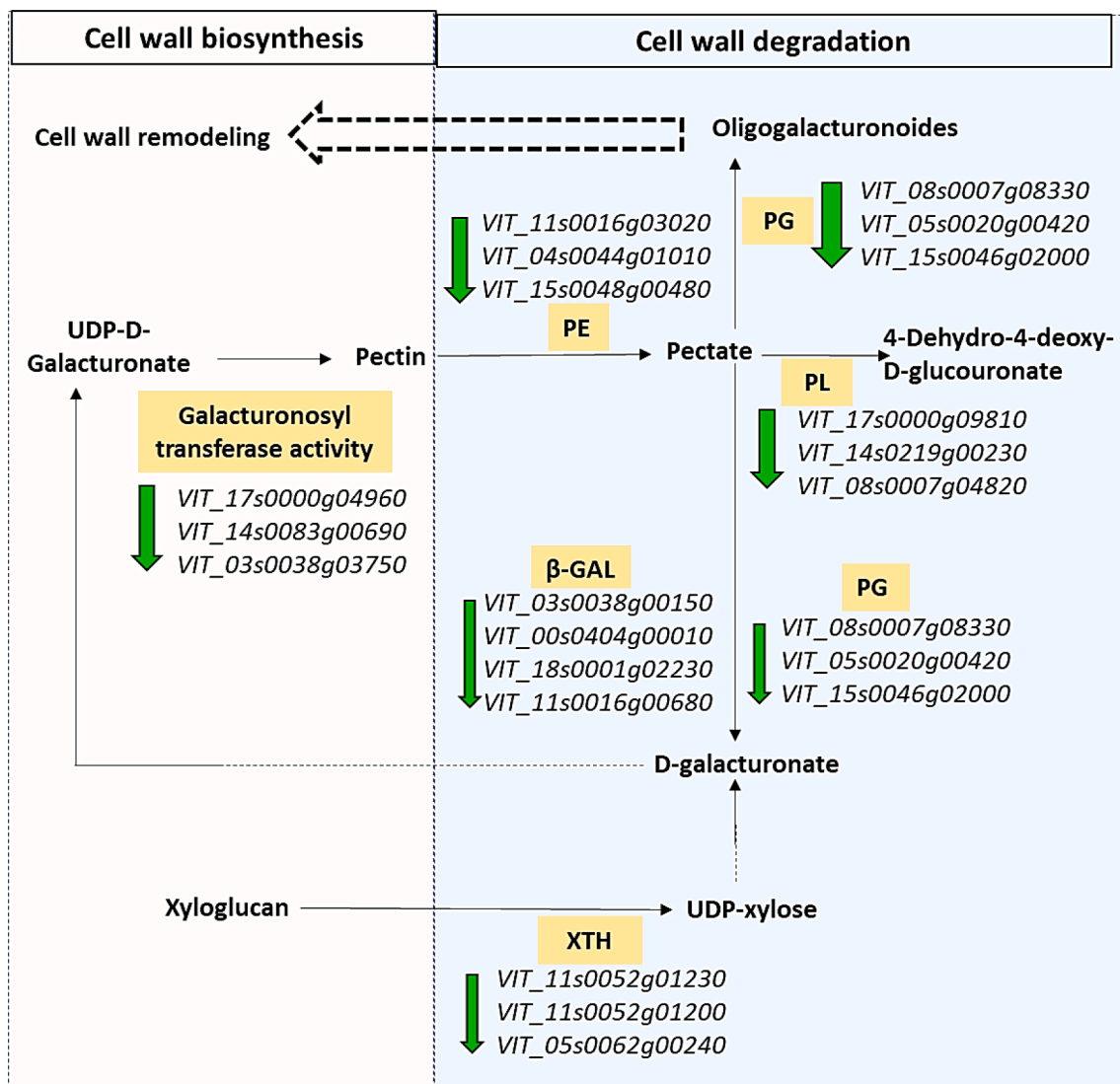


**Fig. 4. Berry firmness network as influenced by plant bioregulators treatments (ABA 400 mg L⁻¹ and ethephon 400 mg L⁻¹).** Where PE (pectinesterase), PG (polygalacturonase), PL (pectate lyase), β-GAL (β-galactosidase), XTH (xyloglucan endotransglucosylase/hydrolase).
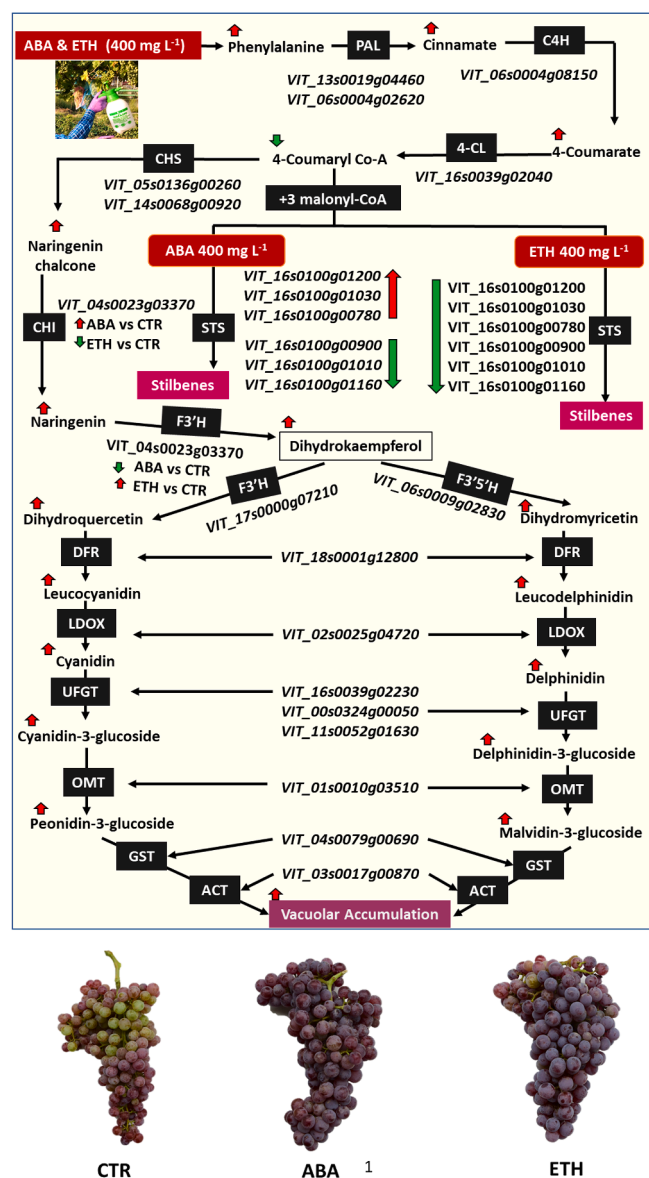
**Fig. 5.** Anthocyanins biosynthesis network as influenced by the application of plant bioregulators (ABA 400 mg L$^{-1}$ and ethephon 400 mg L$^{-1}$). Where PAL (phenylalanine ammonia-lyase), C4H (cinnamate-4-hydroxylase), 4-CL (4-coumarate: coenzyme A ligase), CHS (chalcone synthase), CHI (chalcone isomerase), F3′H (flavonoid-3′-hydroxylase), F3′5′H (flavonoid-3′-5′-hydroxylase), DFR (dihydroflavonol 4-reductases), LDOX (leucoanthocyanidin dioxygenases), UFGT (anthocyanidin 3-O-glucosyltransferase), OMT (O-methyltransferase), GST (glutathione S-transferase), ACT (anthocyanin acyltransferase); red and green arrows indicate upregulation and downregulation of genes, respectively.

ppm treatments compared to the control. Also, other PBRs treatments, BTH and Pro-Ca, enhanced the accumulation of anthocyanins significantly than the control. Earlier, the increase in anthocyanins content in grape berries by using the PBRs were reported, such as ethylene (Wang et al., 2022), ABA (Jia et al., 2018), Pro-Ca (Disegna et al., 2003), and BTH (Iriti et al., 2004). TPC increased significantly compared with control in all treatments, while application with ethephon and ABA led to the highest TPC in berries, followed by BTH and Pro-Ca treatments. The upregulation of genes coding for the action of certain enzymes involved in the biosynthesis mechanism of phenolics and flavonoids due to the treatment with ethephon and ABA to berries may be the possible reason for the increase in TPC in berries. Also, previously BTH has been

involved in improving berry phenolic content, as reported by Ruiz-Garcia et al. (2012). Exogenous application with ethephon 400 mg L$^{-1}$ and ABA 400 mg L$^{-1}$ resulted in the highest TFC content and TAoA in berries. Likewise, Xi et al. (2013) also noticed similar results. Both ethephon and ABA treatments showed reduced berry firmness, most prominently when treated with ethephon (400 mg L$^{-1}$). Similarly, ethephon treatment during berry ripening resulted in unappealing softening in the berries (Mhetre et al., 2021). The reduction in berry firmness is primarily due to the undesirable softening during berry development (Brummell and Harpster, 2001). It is caused by the action of specific cell wall degenerating enzymes like PG (Longhi et al., 2012).

### 4.2. RNA Seq analysis

This study could generate greater than 47 million raw reads with Q30 greater than 92% for each sample. Similarly, Ma and Yang (2019) reported greater than 42 million raw reads for each berry sample, while Guo et al. (2019) reported greater than 45.0 million clean reads with a Q30 value greater than 93.6% in grape samples. Thus, our reads were sufficient enough for the detailed transcriptomic analysis. Several DEGs were found upregulated as well as downregulated, and the highest was recorded for ABA vs. CTR (532 upregulated and 1105 downregulated), followed by ETH vs. CTR (271 upregulated and 326 downregulated) and the least for ETH vs. ABA (282 upregulated and 193 downregulated). The detailed analysis identified many genes involved in various pathways, including anthocyanins, phenolics, flavonoids, and berry firmness which got differentially regulated due to the application of different bioregulators. Similarly, transcriptomic analysis of CPPU treated grape berries Wang et al. (2017) found 149 –745 DEGs ($p \leq 0.05$; FC $\geq 2.0$) in the three different libraries (CPPU0, CPPU5, and CPPU10), and the highest numbers of DEGs (745 down and 492 upregulated) were found between CPPU0 and CPPU10 and total of 513 up- and 572 downregulated DEGs were identified between the CPPU5 and CPPU10 libraries.

The GO analysis revealed that amongst molecular function (MF), the genes associated with 'ion binding,' (more than 30% of genes), 'small molecule binding,' (more than 15% of genes) were significantly upregulated; while amongst biological process (BP), the genes related to 'metabolic process,' (nearly 60% of genes) and 'cellular process,' (nearly 45% of genes) were upregulated and under cellular component (CC), more than 30% of genes belonging to 'organelle,' and 'intracellular organelle' were found upregulated for both ABA vs. CTR and ETH vs. CTR combinations. Further, in the case of downregulated genes, GO analysis showed that amongst MF, the genes associated with 'binding,' (more than 50% genes), and 'catalytic activity' (nearly 45% genes) were significantly downregulated; while amongst BP, the genes related to 'cellular process,' (more than 60% of genes), 'metabolic process,' (more than 50% genes), 'single-organism process' (more than 40% genes) were downregulated and under CC, more than 60% of genes belonging to 'cell,' and 'cell part' were highly downregulated. Similarly, Han et al. (2021) in an RNA-Seq analysis of blueberry during veraison reported that amongst BP, more than 50% of DEGs were associated with 'metabolic process,' and 'biological process'; while amongst CC, nearly 10% of DEGs were related to 'protein complex'. Along the similar lines, Wei et al. (2015) during a comparative transcriptomic analysis showed that under BP category, the most common genes were associated with 'cellular process' (22.79% of genes), 'metabolic process' (21.34% of genes) and 'single-organism process' (11.08% of genes); whereas amongst CC, key genes were related to 'cell' (20.42% of genes), 'cell part' (20.38% of genes) and 'organelle' (14.32% of genes) and under MF group, genes were mainly classified into 'binding' (45.43% of genes), 'catalytic activity' (38.16% of genes) and 'macromolecular complex' (10.74% of genes). Moreover, a maximum number of GO terms in this study were associated with the 'response to abiotic stimulus', 'response to chemical', 'response to stimulus', and 'stress response'. This means plant bioregulators stimulate these processes, improving berry color,

phenolics, flavonoids, and antioxidant contents. Similar findings were also reported in a few other transcriptomics studies (Chang and Tong, 2020; Jiang et al., 2019).

The over-represented pathways identified in the KEGG analysis include 'Flavonoid biosynthesis,' 'Circadian rhythm-plant,' 'Phenylalanine metabolism,' and 'Flavone and flavonol biosynthesis'. Notably, most of these pathways are linked to the formation of phenolics, flavonoids, and anthocyanins, either directly or indirectly. Similarly, Guo et al. (2019) performed the transcriptome profiling of grapevine cv. 'Kyoho' at berry growth and developmental stages after the treatment with 5-azaC reported that the DEGs related to the processes such as 'photosynthesis,' 'photosynthesis antenna proteins,' 'protein processing in ER', and 'flavone and flavanol biosynthesis' got highly expressed. Also, the RNA-Seq analysis of grape berries, when treated with CPPU, showed some over-represented pathways like 'Biosynthesis of secondary metabolites', 'Phenylpropanoid biosynthesis', 'Metabolism pathway', 'Plant hormone signal transduction' and 'Flavonoid biosynthesis' (Wang et al., 2017).

This study could validate 19 genes using Genevestigator, and similar interpretations were also made by Mishra et al. (2021) while studying the gene co-expression analysis for disease gene prediction. Additionally, Guo et al. (2019) used qRT-PCR to confirm the accuracy and consistency of the transcriptome analysis results, finding that chosen genes had similar expression levels in treated and control grape berries. Furthermore, Wang et al. (2017) used qRT-PCR to check the correctness of the transcriptome data, finding that the qRT-PCR data closely matched the transcript levels calculated from the RNA-seq data, demonstrating that the RNA-seq data mirrored genuine expression patterns.

### 4.3. Correspondence between the RNA Seq and berry quality and metabolites studied

Interestingly, RNA Seq data has identified several DEGs encoding various cell wall degrading enzymes, viz., PE, PG, PL, β-GAL, hexosyltransferase, polygalacturonate 4-alpha-galacturonosyltransferase, and XTH indicating their role in regulating the berry firmness. Earlier, while attempting to reveal the genetic mechanism behind the changes in the berry firmness, the role of specific genes associated with cell wall degradation and multiple enzymatic pathways were proposed, which consist of XTH, β-GAL, and PG (Brummell and Harpster, 2001). Similarly, the genes coding for PL, xylosyltransferase activity, and calcium signaling were found to be involved in the regulation of berry firmness (Guo et al., 2019). The DE of these genes such as PE, PG, PL, β-GAL, hexosyltransferase, polygalacturonate 4-alpha-galacturonosyltransferase, and XTH might be the reason for the differences in the firmness level between ABA vs. CTR and ETH vs. CTR treatments as also proposed by Ma et al. (2020).

Three structural genes of the anthocyanin biosynthesis pathway include DFRs (dihydroflavonol 4-reductases), LDOXs (leucoanthocyanidin dioxygenases), and UFGT. The expression of UFGT mainly determines the color of berries. Various DEGs associated with these enzymes were highly upregulated, indicating that these got highly triggered when berries were treated with ABA and ethylene (both 400 mg L$^{-1}$). Similarly, multiple genes involved in the biosynthesis of anthocyanins, flavonols, and stilbenes were induced during berry ripening (Fortes et al., 2011). The relative expression of these genes was consistent with the rapid induction of anthocyanin concentration, indicating that the anthocyanin biosynthesis is regulated predominantly at the transcription level (Davies and Schwinn, 2003).

Earlier investigations provided evidence that a gene cluster on chromosome 2 is responsible for most of the berry color variation. The color's final expression is due to the combined additive effect of the *VvMybA* gene alleles (Fournier-Level et al., 2009). This locus constituted a cluster of three MYB-type TF genes, of which *VvMybA1* and *VvMybA2* are functionally involved in the berry pigmentation (Walker et al.,

2007). A third gene, *VvMybA3*, is also associated with berry color determination but is not yet functionally validated (Fournier-Level et al., 2009). Similarly, our RNA Seq data could also identify the transcripts related to the *VvMYB* TFs and were found overexpressed for ABA vs. CTR compared to ETH vs. CTR.

## 5. Conclusion

In the semi-arid subtropical area, uneven berry color development is critical in commercial viticulture, resulting in poor market prices. PBRs are beneficial in improving grape berry color uniformity. The current study suggests that ethylene and ABA improve berry color and overall quality. Our research will aid in the optimization of ABA and ethylene concentrations, particularly in semi-arid subtropics, by achieving a better compromise between berry color uniformity and firmness. Furthermore, RNA-seq investigations revealed candidate genes linked to berry color development. The knowledge gathered will undoubtedly aid in the future production of better and more evenly colored grapevine varieties.

## 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. **Acknowledgments**

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2022.100116.

## References

Alexa, A., and Rahnenfuhrer, J., 2016. topGO: Enrichment analysis for Gene Ontology. R package version 2.28. 0. *BioConductor.* https://www.bioconductor.org/packages/release/bioc/vignettes/topGO/inst/doc/topGO.pdf.

Balint, G., & Reynolds, A. G. (2013). Impact of exogenous abscisic acid on vine physiology and grape composition of Cabernet Sauvignon. *American Journal of Enology and Viticulture, 64,* 74–87. https://doi.org/10.5344/ajev.2012.12075

Brummell, D. A., & Harpster, M. H. (2001). Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Cell Walls, 47,* 311–340. https://doi.org/10.1023/A:1010656104304

Chang, H. Y., & Tong, C. (2020). Identification of candidate genes involved in fruit ripening and crispness retention through transcriptome analyses of a 'Honeycrisp' population. *Plants, 9*(10), 1335. https://doi.org/10.3390/plants9101335

Chen, S., Zhou, Y., Chen, Y., and Gu, J., 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics,* 34(17): i884-i890. doi:10.1093/bioinformatics/bty560.

Costa, T. V. D., Facanha, R. V., & Scarpare Filho, J. A. (2016). Ethephon sprays as a defoliant in 'niagara rosada' vine subjected to two crop productions per year. *Revista Brasileira de Fruticultura, 38,* e-297.

Davies, K. M., & Schwinn, K. E. (2003). Transcriptional regulation of secondary metabolism. *Functional Plant Biology, 30,* 913–925. https://doi.org/10.1071/FP03062

Disegna, E., Boido, E., Carrau, F., Farina L., Medina K., Mendez, M. and Dellacassa, E., 2003. Efectos de la aplicación del regulador de crecimiento 3, 5-Dioxo-4Propionil-ciclohexancarboxilato de calcio (BAS 125) en la producción de uvas, composición del vino y aroma del cv. Tannat (Primera comunicación). In *Jornadas GESCO, 13., 2003, Montevideo, Uruguay,* Universidad de la Republica; INIA.

FAO, 2020. https://www.fao.org/faostat/en/#data/QCL (accessed on May 19, 2022).

Feddema, J. J. (2005). A revised Thornthwaite-type global climate classification. *Physical Geography, 26*(6), 442–466. https://doi.org/10.2747/0272-3646.26.6.442

Fortes, A. M., Agudelo-Romero, P., Silva, M. S., Ali, K., Sousa, L., Maltese, F., & Pais, M. S. (2011). Transcript and metabolite analysis in Trincadeira cultivar reveals novel information regarding the dynamics of grape ripening. *BMC plant biology, 11,* 1–35. https://doi.org/10.1186/1471-2229-11-149

Fournier-Level, A., Le Cunff, L., Gomez, C., Doligez, A., Ageorges, A., Roux, C., & This, P. (2009). Quantitative genetic bases of anthocyanin variation in grape (*Vitis vinifera* L. ssp. sativa) berry: A quantitative trait locus to quantitative trait nucleotide integrated study. *Genetics, 183,* 1127–1139. https://doi.org/10.1534/genetics.109.103929

Gonzalez, R., Gonzalez, M. R., & Martin, P. (2018). Abscisic acid and ethephon treatments applied to 'Verdejo' white grapes affect the quality of wine in different ways. *Scientia Agricola, 75,* 381–386.

Greer, D. H., & Weston, C. (2010). Heat stress affects flowering, berry growth, sugar accumulation and photosynthesis of *Vitis vinifera* cv. Semillon grapevines grown in a controlled environment. *Functional Plant Biology, 37*, 206–214. https://doi.org/10.1071/FP09209

Guo, D. L., Li, Q., Ji, X. R., Wang, Z. G., & Yu, Y. H. (2019). Transcriptome profiling of 'Kyoho' grape at different stages of berry development following 5-azaC treatment. *BMC Genomics, 20*, 825. https://doi.org/10.1186/s12864-019-6204-1

Han, T., Wu, W., & Li, W. (2021). Transcriptome Analysis Revealed the Mechanism by Which Exogenous ABA Increases Anthocyanins in Blueberry Fruit During Veraison. *Frontiers in Plant Science, 12*, Article 758215. https://doi.org/10.3389/fpls.2021.758215

Iriti, M., Rossoni, M., Borgo, M., & Faoro, F. (2004). Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to Botrytis cinerea. *Journal of Agricultural and Food Chemistry, 52*(14), 4406–4413. https://doi.org/10.1021/jf049487b

Jia, H., Wang, S., Lin, H., Ampa, K., Todoroki, Y., & Kondo, S. (2018). Effects of abscisic acid agonist or antagonist applications on aroma volatiles and anthocyanin biosynthesis in grape berries. *The Journal of Horticultural Science and Biotechnology, 93*(4), 392–399. https://doi.org/10.1080/14620316.2017.1379364

Jiang, S. H., Sun, Q. G., Chen, M., Wang, N., Xu, H. F., Fang, H. C., & Chen, X. S. (2019). Methylome and transcriptome analyses of apple fruit somatic mutations reveal the difference of red phenotype. *BMC Genomics, 20*(1), 1–13. https://doi.org/10.1186/s12864-019-5499-2

Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: A fast-spliced aligner with low memory requirements. *Nature Methods, 12*, 357–360. https://doi.org/10.1038/nmeth.3317

Koyama, K., Sadamatsu, K., & Goto-Yamamoto, N. (2010). Abscisic acid stimulated ripening and gene expression in berry skins of the Cabernet Sauvignon grape. *Functional & integrative genomics, 10*(3), 367–381. https://doi.org/10.1007/s10142-009-0145-8

Koyama, R., Roberto, S. R., de Souza, R. T., Borges, W. F., Anderson, M., Waterhouse, A. L., & Blanco-Ulate, B. (2018). Exogenous abscisic acid promotes anthocyanin biosynthesis and increased expression of flavonoid synthesis genes in *Vitis vinifera × Vitis labrusca* table grapes in a subtropical region. *Frontiers in Plant Science, 9*, 323. https://doi.org/10.3389/fpls.2018.00323

Liao, Y., Smyth, G. K., & Shi, W. (2013). The Subread aligner: Fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Research, 41*, e108–e. https://doi.org/10.1093/nar/gkt214

Longhi, S., Moretto, M., Viola, R., Velasco, R., & Costa, F. (2012). Comprehensive QTL mapping survey dissects the complex fruit texture physiology in apple (*Malus* x *domestica* Borkh.). *Journal of Experimental Botany, 63*, 1107–1121. https://doi.org/10.1093/jxb/err326

Ma, L., Sun, L., Guo, Y., Lin, H., Liu, Z., Li, K., & Guo, X. (2020). Transcriptome analysis of table grapes (*Vitis vinifera* L.) identified a gene network module associated with berry firmness. *PloS One, 15*, e0237526.

Ma, Q., & Yang, J. (2019). Transcriptome profiling and identification of the functional genes involved in berry development and ripening in *Vitis vinifera*. *Gene, 680*, 84–96. https://doi.org/10.1016/j.gene.2018.09.033

Martinez de Toda, F., Sancha, J. C., Zheng, W., & Balda, P. (2014). Leaf area reduction by trimming, a growing technique to restore the anthocyanins: Sugars ratio decoupled by the warming climate. *Vitis, 53*, 189–192.

Mhetre, V. B., Patel, V. B., Singh, S. K., Verma, M. K., Mishra, G. P., Dahuja, A., & Kumar, C. (2021). Effect of new generation bio-regulators on anthocyanins and berry quality of grape cv. Beauty Seedless. *The Indian Journal of Agricultural Sciences, 91*, 116–119.

Mishra, G. P., Aski, M. S., Bosamia, T., Chaurasia, S., Mishra, D. C., Bhati, J., & Dikshit, H. K. (2021). Insights into the Host-Pathogen Interaction Pathways through RNA-Seq Analysis of Lens culinaris Medik. *Response to Rhizoctonia bataticola Infection. Genes, 13*(1), 90.

Rademacher, W. (2015). Plant growth regulators: Backgrounds and uses in plant production. *Journal of Plant Growth Regulation, 34*, 845–872. https://doi.org/10.1007/s00344-015- 9541-6

Roberto, S. R., de Assis, A. M., Yamamoto, L. Y., Miotto, L. C. V., Sato, A. J., Koyama, R., & Genta, W. (2012). Application timing and concentration of abscisic acid improve colour of 'Benitaka' table grape. *Scientia Horticulturae, 142*, 44–48. https://doi.org/10.1016/j.scienta.2012.04.028

Rudrappa, L., Purakayastha, T. J., Singh, D., & Bhadraray, S. (2006). Long-term manuring and fertilization effects on soil organic carbon pools in a Typic Haplustept of semi-arid sub-tropical India. *Soil and Tillage Research, 88*(1–2), 180–192. https://doi.org/10.1016/j.still.2005.05.008

Ruiz-Garcia, Y., Romero-Cascales, I., Gil-Munoz, R., Fernandez-Fernandez, J. I., Lopez-Roca, J. M., & Gomez-Plaza, E. (2012). Improving grape phenolic content and wine chromatic characteristics through the use of two different elicitors: Methyl jasmonate versus benzothiadiazole. *Journal of Agricultural and Food Chemistry, 60*, 1283–1290. https://doi.org/10.1021/jf204028d

Sanchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture, 76*, 270–276. https://doi.org/10.1002/(SICI)1097-0010(199802)76:2<270:AID-JSFA945>3.0.CO;2-9

S.A.S. Institute. (1988). *Users Guide: Statistic Version 6.03*. Cary, NC, USA: SAS Institute.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Walker, A. R., Lee, E., Bogs, J., McDavid, D. A., Thomas, M. R., & Robinson, S. P. (2007). White grapes arose through the mutation of two similar and adjacent regulatory genes. *The Plant Journal, 49*, 772–785. https://doi.org/10.1111/j.1365-313X.2006.02997.x

Wang, P., Yu, A., Ji, X., Mu, Q., Haider, M. S., Wei, R., & Fang, J. (2022). Transcriptome and metabolite integrated analysis reveals that exogenous ethylene controls berry ripening processes in grapevine. *Food Research International, 155*, Article 111084. https://doi.org/10.1016/j.foodres.2022.111084

Wang, W., Khalil-Ur-Rehman, M., Feng, J., & Tao, J. (2017). RNA-seq based transcriptomic analysis of CPPU treated grape berries and emission of volatile compounds. *Journal of Plant Physiology, 218*, 155–166. https://doi.org/10.1016/j.jplph.2017.08.004

Wei, H., Chen, X., Zong, X., Shu, H., Gao, D., & Liu, Q. (2015). Comparative transcriptome analysis of genes involved in anthocyanin biosynthesis in the red and yellow fruits of sweet cherry (*Prunus avium* L.). *PLoS One, 10*(3), e0121164.

Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., and Sporns, P., (Eds.) 2005. *Handbook of food analytical chemistry, volume 1: Water, proteins, enzymes, lipids, and carbohydrates*. John Wiley and Sons.

Xi, Z. M., Meng, J. F., Huo, S. S., Luan, L. Y., Ma, L. N., & Zhang, Z. W. (2013). Exogenously applied abscisic acid to Yan73 (*V. vinifera*) grapes enhances phenolic content and antioxidant capacity of its wine. *International Journal of Food Sciences and Nutrition, 64*(4), 444–451. https://doi.org/10.3109/09637486.2012.746291

Yaman, M., Atalay, V., Turker, M., Gerek, O., & Cetin, A. (2002). Texture segmentation by using adaptive polyphase subband decomposition. In I. Cicekli, N. Cicekli, & E. Gelenbe (Eds.), *Proceedings of the 17th international symposium on computer and information sciences*.

Zhang, R., Wang, Y., Li, S., Yang, L., & Liang, Z. (2021). ABA signaling pathway genes and function during abiotic stress and berry ripening in Vitis vinifera. *Gene, 769*, Article 145226. https://doi.org/10.1016/j.gene.2020.145226

Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., & Gruissem, W. (2004). GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiology, 136*, 2621–2632. https://doi.org/10.1104/pp.104.046367

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. S. (2000). *Wine Analysis and Production*. Maryland, Australia: Aspen Publishers.