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Effect of brassinosteroids on grape berry ripening by coordinating transcriptomic and metabolic analysis

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Abstract

Brassinosteroids (BRs) are essential for the regulation of plant growth, development, and stress responses. However, a comprehensive understanding of the transcriptional regulatory network governed by BRs that orchestrates grape berry ripening remains limited. Herein, the application of exogenous epibrassinolide (EBR) increased endogenous BRs content while down-regulating the expression of BRs biosynthetic genes, indicating feedback transcriptional regulation of BRs biosynthesis. EBR treatment accelerated coloring and enhanced anthocyanin accumulation, which was closely associated with the up-regulation of anthocyanin biosynthetic genes. In addition, EBR significantly increased water-soluble pectin (WSP) content and concurrently reduced protopectin and cellulose levels, resulting in berry softening. The study also delved into the intricate cross-talks between BRs and other plant growth regulators, for instance, ethylene, abscisic acid (ABA), auxin, and cytokinin. Moreover, transient overexpression of *VvDWF4* (CYP90B1 steroid 22-alpha-hydroxylase) in strawberry increased BRs content, leading to anthocyanin accumulation and fruit softening. These findings provide compelling evidence that the pre-véraison application of BRs provide an effective strategy for enhancing grape berry quality.

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Introduction

Grape (*Vitis*. spp) is one of the most appreciated and extensively planted fruit crops, boosting economic significance and nutritional benefits. The growth, development, and ripening process of grape berry involve a complex interplay of biochemical and physiological events, following a typical biphasic sigmoidal profile that encompasses three different phases[1]. Phytohormones intricately regulate grape berry development and ripening. Notably, abscisic acid (ABA), ethylene, and BRs emerge as pivotal promoters in the ripening process, while auxin, cytokinin, and gibberellin are proposed as ripening inhibitors[1,2]. In grape, the level of endogenous BRs increased markedly at the initiation stage of berry ripening. Conversely, treatments with brassinazole (Brz), a BR synthesis inhibitor, significantly delay berry véraison and ripening process[3], suggesting a crucial role of BRs in grape berry ripening process.

BRs represent natural steroid-like hormones in the plant kingdom that play fundamental roles in various processes of plant growth and development, such as cell division and elongation, plant architecture, vascular differentiation, photomorphogenesis, flowering, senescence, tolerance to biotic and abiotic stresses[4–6]. More than seventy different BR compounds and their derivatives have been verified from the plantae, of which brassinolide (BL) and castasterone (CS) are emerging as the most actively and prevalently class of BR compounds[6,7]. BL and CS arise from the conversion of campesterol (CR), a prominent plant sterol, through two parallel routes known as C-6 oxidation pathways at the early and late phases, respectively[6,8]. A cytochrome P450 monooxygenase, denoted as DWF4, plays a pivotal role in this biosynthetic cascade by catalyzing multiple C-22 hydroxylation steps. This enzyme, recognized as a rate-limiting factor, significantly influences the BR biosynthesis route[9]. In Arabidopsis, the *dwf4* mutant exhibits a dwarf phenotype, attributed to impaired BRs biosynthesis, but hydroxylated BRs can restore the defective phenotypes from the *dwf4* mutant of Arabidopsis[9,10]. In autotetraploid apple, the reduction in BR levels and the dwarf phenotype are speculated to result from the significant down-regulation of *MdDWF4* expression[11].

The signaling pathways of BRs are initiated through a binding of BRs with a leucine-rich repeat (LRR) receptor-like kinase known as *BRASSINOSTEROID-INSENSITIVE 1* (*BRI1*), which operates in conjunction with its co-receptor *BRI1-associated receptor kinase* (*BAK1*)[12]. For example, the Arabidopsis *bri1* mutant exhibits elevated endogenous BR levels[13] and manifests multiple developmental deficiencies, including a profound reduction in height, notably dark green, thick leaves, male sterility, and diminished apical dominance[14]. Subsequently, this signaling cascade culminates in the activation of a group of transcription factors, including *BRASSINAZOLE RESISTANT 1* (*BZR1*)[15] and *BRI1-EMSSUPPRESSOR 1* (*BES1*)[16]. *BZR1* and *BES1* not

only directly regulate the transcription of numerous BR-responsive genes governing various plant growth and development processes but also contribute to feedback inhibition by binding directly to the promoter regions of BR biosynthetic-related genes, thereby inhibiting their transcription levels[17]. This regulation occurs through the modulation of light reactions, cell-wall-related metabolism, and plant hormone pathways[18].

In grapes, several previous investigations have confirmed the positive impact of BRs on the berry ripening process. The exogenous spray of epibrassinolide (EBR) not only extensively induced the accumulation of soluble sugars, anthocyanins, proanthocyanidins, and other phenolics but also promoted the enzyme activities and gene expression associated with anthocyanin and sugar biosynthetic pathways[19,20]. However, there are limited reports regarding the interrelationship between BRs-induced ripening and gene regulatory networks in grapes. Herein, this study has further confirmed the functions of BRs in promoting berry ripening and accelerating secondary metabolite accumulation. Transcriptomic analysis unveiled the regulatory networks governing berry ripening organs and the cross-talk among hormones in response to EBR treatment. The present results not only enhance understanding related to the roles of BRs during the regulation network of the ripening process in berry but also provide an excellent tool for improving the berry quality in commercial vineyards.

Materials and methods

Plant material and epibrassinolide (EBR) treatment

Six-year-old Muscat Hamburg grape (*Vitis vinifera* L.) was considered as the experimental material, which was grown on a grape farm at Qingdao Agricultural University, Qingdao, China. Berries were soaked with deionized water (control) containing a trace amount of ethanol (< 0.1%) and 0.5 mg·L^{-1} EBR (Sigma catalog no. E1641)[21] with 0.05% Tween 20 at one week before véraison (eight weeks after full bloom), respectively. The stock solution of EBR was prepared by dissolving its compound in 1 mL ethanol (98%), and the control stock solution only contained 1 mL ethanol (98%) but without EBR. One hundred berries were randomly harvested at 0, 5, 10, and 15 d after EBR application from three biological replicates. Thirty berries at each sampling date were selected for the physiology and biochemical indices as per the previous method[22], including longitudinal diameter (mm), transverse diameter (mm), fresh berry weight (g), content of total soluble solids (Brix°), content of titratable acidity (%). These left berries were frozen in liquid nitrogen and kept at −80 °C for the next analysis.

Determination of endogenous BRs level

The levels of endogenous BRs were quantified using the method described by Xu et al.[23]. One gram of each of the berry skin samples was ground and homogenized in PBS extraction solution. The extracts were then centrifuged at 12,000 rpm for 30 min, and the supernatant subsequently was collected and stored at 4 °C until enzyme-linked immunosorbent assays (ELISAs), which are manufactured by Shanghai Yanqi Biotechnology Co., Ltd (Shanghai, China).

Quantification analysis of soluble sugars and organic acids

The concentrations of soluble sugar (fructose and glucose) and organic acid (malic acid, tartaric acid, and citric acid) were determined by high-performance liquid chromatography (HPLC) as a previous method with some modifications[24]. Six hundred mg of berry samples were ground and extracted with 1.5 mL of 80% ethanol. These samples were vortexed and centrifuged at 12,000 rpm for 30 min, and then the extract was filtered through a 0.22 μm water filter for injection. The next analysis was manipulated by the liquid chromatography-mass spectrometry (LC-MS) system, which is a combination of Waters Acquity I-Class PLUS ultra-high performance liquid tandem Waters (Shanghai, China) and an AB Sciex Qtrap 6500+ high-sensitivity mass spectrometer. The chromatographic conditions of soluble sugars were as follows: Prevail Carbohydrate ES 5μ column (4.6 mm × 100 mm, 5 μm); mobile phase: acetonitrile/water, 80/20 (v/v); flow rate: 1.0 mL/min; column temperature: 50 °C; injection volume: 20 μL. Furthermore, the chromatographic conditions of organic acids were as follows: Discovery C18 column (4.6 mm × 250 mm, 5 μm); mobile phase: 50 mM K_2HPO_4 solution (pH adjusted to 2.4 with phosphoric acid); flow rate: 0.5 mL/min; column temperature: 30 °C; injection volume: 20 μL.

Quantification analysis of anthocyanin

Grape anthocyanins were extracted using the methanol containing 0.1% HCl (v/v). The components and contents of anthocyanin were determined by LC-MS according to the previous method[25], and the LC-MS system used has been mentioned above. The chromatographic conditions were as follows: aqueous mobile phase: 0.1% formic acid in water; organic mobile phase: 0.1% formic acid in acetonitrile; flow rate: 0.4 mL/min; injection volume: 2 μL. All mass spectrometry (MS) were equipped with turbo ion spray sources in both positive and negative ion modes, with a selected mass range of 50−1,200 m/z. The ionization parameters were set as the previous method[22]. Malvidin-3-O-glucoside (Extrasynthese, Genay, France) was used as the external standard for the quantitation of anthocyanins. Furthermore, the total anthocyanin contents of strawberry fruits were measured according to the pH differential method.

Measurement of fruit firmness and cell wall components

Firmness was measured by using a Texture Analyzer (TA. XT, Stable Micro Systems, Surrey, UK) with a 2 mm diameter penetration probe (needle P/2). The penetration test of the whole grape berry (with skin) was performed at 1 mm/s for 6 mm after contacting the surface of the flesh, and the results were expressed in N. Protopectin, water-soluble pectin (WSP) and cellulose contents were measured using the test kits (Suzhou Comin Biotechnology Co., Ltd. Suzhou, China) according to the manufacturer's instructions.

RNA extraction, cDNA library construction and sequencing

The CTAB method was used to total RNA isolation from berry skins. RNA integrity and concentration were measured by using a Bioanalyzer 2100 system (Agilent Technologies, CA, USA) and a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, CA, USA), respectively. Then, 3 μg total RNA was used to prepare a cDNA library using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, MA, USA). All the library preparations were sequenced by an Illumina Hiseq platform (Hiseq 4000, 150PE) at Novogene Bioinformatics Technology Co. Ltd (Beijing, China).

Transcriptome analysis

Clean reads were aligned to the grape reference genome (www.grapegenomics.com/pages/PN40024) by HISAT 2.0. The gene expression level was presented by using the fragments per kilobase of exon model per million mapped fragments (FPKM) values. Differentially expressed genes (DEGs) between BR-treated and control samples (two biological replicates per sample) were identified by the DESeq 1.8.3 package. The resulting *p* values were adjusted using the Benjamini-Hochberg approach for controlling the false discovery rate (FDR). The DEGs were collected by the |log2(fold change)| ≥ 1 and FDR < 0.05 as select standard. Furthermore, GO enrichment analysis of DEGs was conducted using the GOseq *R* package, and

KEGG pathway analysis was implemented using KOBAS software. The function and biological pathway with a *q*-value ≤ 0.05 were considered significantly enriched.

Quantitative RT-PCR validation

Twelve DEGs were chosen to validate the results of transcriptome sequencing by using qRT-PCR. Total RNA was extracted by the CTAB method from every single sample, and 1 μg of total RNA was used for cDNA synthesis by using PrimeScript™ RTase (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instruction. qRT-PCR was implemented by using SYBR®Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China) with the Bio-Rad iQ5 thermocycler (Bio-Rad, CA, USA). The grape *Actin* gene (AY680701) was used as the reference gene for normalization, and all primers pairs were shown in Supplementary Table S1. All experiments were implemented with three independent biological replicates for every sample. The relative expression values were calculated by the $2^{-\Delta\Delta C_T}$ method.

Transient overexpression of *VvDWF4* using the strawberry fruit

The full-length coding sequence (CDS) encoding *VvDWF4* (VIT_04s0023g01630) was cloned into a pRI101 binary vector to generate the plant expression constructs. The construct was introduced into the *Agrobacterium tumefaciens* strain GV3101 and then transformed into the fruits (as previously described[26]) of strawberry (*Fragaria* × *ananassa* Duch. L. cv. Albion) plants which were grown under the greenhouse at 25 °C, approximately 70% relatively humidity. Strawberry fruit infiltrated with an empty PRI101 vector was used as the control. The primers for construction were listed in Supplementary Table S1.

Statistical analysis

Statistical analysis was carried out with SPSS software ver. 22.0 (IBM, NY, USA). Data were expressed as means ± standard deviation (SD). Comparisons between groups were carried out by one-way ANOVA corrected with Tukey's multiple comparison test at a significance level of $p < 0.05$.

Results

BRs modulate the physiochemical parameters during berry ripening in grape

The exogenous BRs treatment remarkably facilitated ripening process of the berries, encompassing berry coloration, sugar accumulation, and organic acid degradation (Fig. 1). Within 15 d of BRs treatment, the berry clusters exhibited full coloration, whereas the control groups showed significantly poor color development compared to the BRs-treated berries (Fig. 1a). To further validate the correlation between endogenous BRs content and berry ripening, the BRs concentrations were detected. The results displayed that the content in BRs was obviously increased at 5 and 10 d after treatment. However, there is no significant difference found at 15 d between the control and treated fruit, suggesting BRs might be a potent inducer and started the cascade of events leading to ripening (Fig. 1b).

Furthermore, BRs application significantly accelerated the accumulation of soluble solids, glucose and fructose (Fig. 1c–e), indicating the participation of BRs onto sugar accumulation. During the berry ripening process, the content of titratable acids in BRs-treated berries, including tartaric, malic, and citric acid, exhibited a significant reduction in comparison to the control group (Fig. 1f–i). All these results suggested that the appropriate level of epibrassinolide (EBR) treatment is an effective strategy for promoting the grape berry ripening process.

BRs application promotes anthocyanin accumulation in grape

Anthocyanin composition and content were measured through the LC-MS system, leading to the identification and quantification of seven distinct anthocyanin compounds[27], including peonidin-3-O-glucoside (Pn-3-G), malvidin-3-O-glucoside (M-3-G), cyanidin-3-O-glucoside (C-3-g), petunidin-3-O-glucoside (Pt-3-G) and delphinidin-3-O-glucoside (D-3-G), peonidin-3-O-(6-p-coumaroyl)-glucoside (Pn-3-coum-G) and malvidin-3-O-(6-p-coumaroyl)-glucoside (M-3-coum-G). (Fig. 2). Application of exogenous BR resulted in a significant increase in total anthocyanins contents, spanning from 85.47 to 179.43 mg/kg and 274.91 to 385.44 mg/kg at 10 and 15 d post-BR treatment, respectively (Fig. 2h). Furthermore, the concentration of individual anthocyanins was also significantly enhanced due to BRs treatment. Among these, peonidin-3-O-glucoside (Pn-3-G), a 3'-substituted anthocyanin, stood as the highly abundant compound within the individual anthocyanin constituents of Muscat Hamburg, accounting for around 50.0% of the total anthocyanins level (Fig. 2a). Malvidin-3-O-glucoside (M-3-G), the key 3',5'-substituted anthocyanins, exhibited concentrations ranging from 19.61 to 31.83 mg/kg and 52.63 to 77.55 mg/kg at 10 and 15 d post-BR application, respectively (Fig. 2c). In addition, the contents of two p-coumaroyl anthocyanins, namely Pn-3-coum-G and M-3-coum-G, was notably lower than monoglucosidic anthocyanins. These findings robustly underscore the capacity of exogenous EBR application to trigger anthocyanin accumulation and promote berry coloration in grape.

Identification of DEGs between BRs-treated and non-treated groups during berry ripening

To identify DEGs in response to BRs, seven samples of BRs-treated and non-treated groups across four stages (0, 5, 10, and 15 d) were subjected to RNA sequencing, with each sample having two biological replicates. A total of 100.01 Gb of clean reads were acquired, with more than 90% of clean reads for every library successfully mapped to the grape reference genome (Supplementary Table S2), suggesting that sequencing data can accurately represent the differences among these samples in the transcription levels. Furthermore, the principal component analysis (PCA) demonstrated the combined effect of developmental stage and EBR treatment on the transcriptome differences among the samples (Supplementary Fig. S1). In total, 4,578, 3,397 genes and 3,569 DEGs were verified post-5, 10, and 15 d of EBR application, respectively (Supplementary Fig. S2). The KEGG analyses of the DEGs displayed that pathways related to the plant hormone signal transduction and phenylpropanoid biosynthesis were significantly enriched (*q*-value ≤ 0.001) at the véraison stage (10 d after EBR treatment) (Supplementary Fig. S3) and these pathways were essential for the anthocyanin synthesis and berry ripening. Subsequently, there were five main pathways in the berries at the ripening stage (15 d after EBR treatment), including carbon metabolism, biosynthesis of amino acids, phenylpropanoid biosynthesis, plant-pathogen interaction, and flavonoid biosynthesis (Supplementary Fig. S3). Taken together, these findings underscore that BRs treatment regulated a series of secondary metabolic pathways that underpin berry ripening at the transcription level.

BRs application influences the gene expression related to soluble sugar and organic acid metabolism

Herein, this study also found the differential expression of several genes related to the soluble sugar and organic acid metabolism

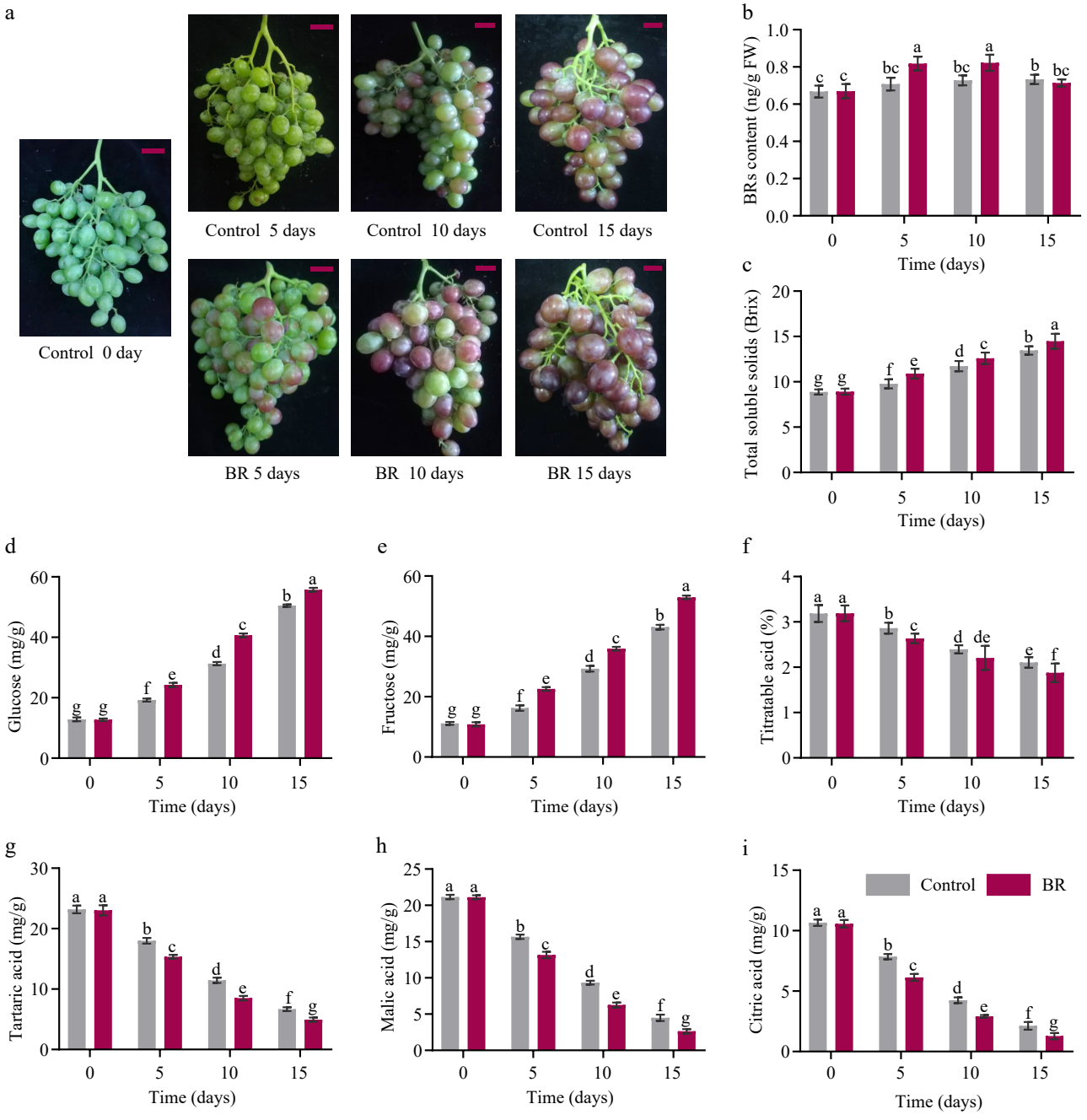

Fig. 1 The effect of exogenous 2,4-epibrassinolide (EBR) treatment on the berry ripening indexes of grape 'Muscat Hamburg'. (a) Color development and anthocyanin accumulation phenotypes under EBR treatment; The red bar indicates 5 cm, (b) BRs content, (c) total soluble solids, (d) glucose, (e) fructose, (f) titratable acid, (g) tartaric acid, (h) malic acid, and (i) citric acid were qualified post-EBR application. Data are expressed as the means ± standard deviation. Different letters indicate significant differences at $p < 0.05$ according to one-way ANOVA and Tukey's test.

under BR treatment. The expression of one sucrose synthase gene (*VIT_11s0016g00470*) which catalyzes the reversible reaction between sucrose and glucose, displayed nearly 10-fold lower at 15 d after BR application. Otherwise, one sucrose phosphate synthase (*VIT_18s0089g00410)* gene expressed five times higher than control fruits, and the other one sucrose phosphate synthase gene also showed nearly two times higher than control. In addition, BR application also significantly increased the expression of one starch synthase (*VIT_16s0098g01780*) (Supplementary Table S8).

Regarding to the organic acid metabolism-related genes, the expression levels of several genes like one NAD-dependent isocitrate dehydrogenase gene (NAD-ICDH: *VIT_03s0038g03120*), one fumarase gene (FUM: *VIT_07s0005g00880*), two succinate thiokinase gene (SAT: *VIT_07s0005g03790* and *VIT_17s0053g00120*), one GDP-l-galactose pyrophosphatase gene (GalPP: *VIT_10s0405g00030*), one phosphoenolpyruvate carboxylase gene (PEPC: *VIT_12s0028g02180*), one UDP-glucose-dehydrogenase gene (UGDH: *VIT_17s0000g06960*), were found to be significantly inhibited at 15 d after BR application (Supplementary Table S8). In general, the transcription profiles of sugar and organic acid metabolism genes were also significantly altered by the BR treatment.

Exogenous BRs treatment strongly stimulates anthocyanin biosynthetic-related genes in grape

To further evaluate the BRs-mediated anthocyanin accumulation, we scrutinized the transcription profiles of anthocyanin biosynthetic

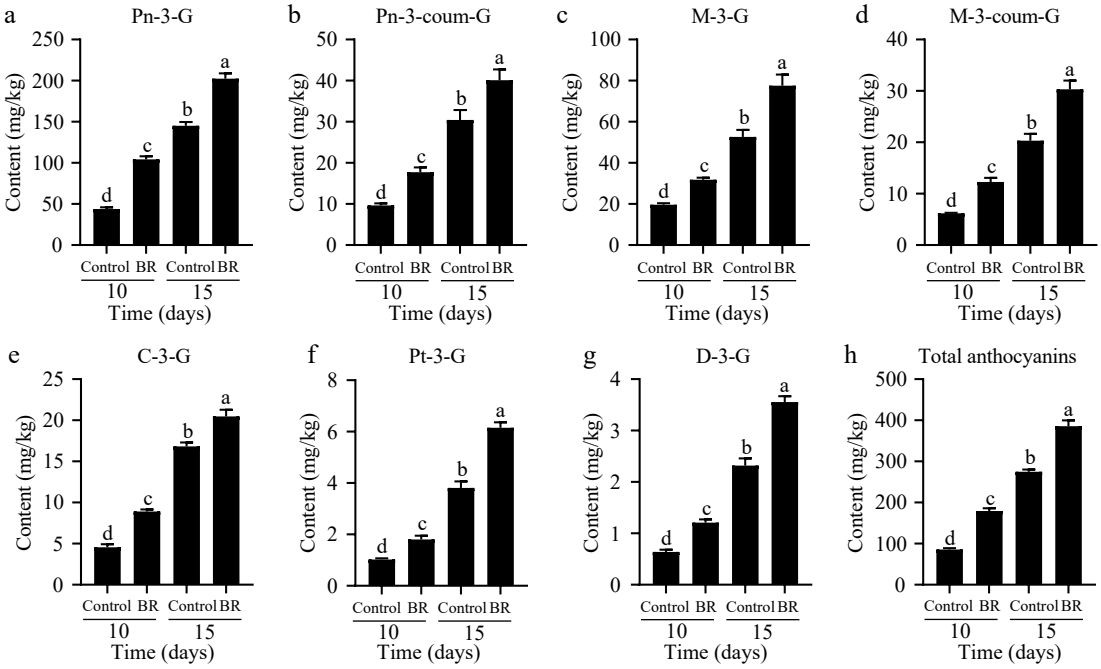

Fig. 2 The effect of exogenous 2,4-epibrassinolide (EBR) treatment on the content and constitute of anthocyanin during berry ripening process. (a) Pn-3-G, peonidin-3-O-glucoside. (b) Pn-3-coum-G, peonidin-3-O-(6-p-coumaroyl)-glucoside. (c) M-3-G, malvidin-3-O-glucoside. (d) M-3-coum-G, malvidin-3-O-(6-p-coumaroyl)-glucoside. (e) C-3-G, cyanidin-3-O-glucoside. (f) Pt-3-G, petunidin-3-O-glucoside. (g) D-3-G, delphinidin-3-O-glucoside. Data are shown as the means ± standard deviation of three biological replicate assays. Different letters indicate significant differences at $p < 0.05$ according to one-way ANOVA and Tukey's test.

genes. A group of 12 phenylpropanoid pathway genes, including nine phenylalanine ammonia-lyase *(VvPAL)*, one cinnamate 4-hydroxylase (*Vv4CH*), and two 4-coumarate-CoA ligases *(Vv4CL)* showed increased expression at 10 d, followed by a decline in expression in 15 d post-EBR application (Fig. 3, Supplementary Table S3). BRs treatment notably enhanced the expression levels in a lot of flavonoid biosynthesis genes. For example, two chalcone synthases (*VvCHS*: *VIT_05s0136g00260* and *VIT_14s0068g00920*), two flavanone 3-hydroxylase (*VvF3H: VIT_04s0023g03370* and *VIT_16s0098g00860*), one flavonoid 3'-hydroxylase (*VvF3'H: VIT_17s0000g07210*), one flavonoid 3',5'-hydroxylase (*VvF3'5'H*: *VIT_06s0009g03010*), one flavonol synthase (*VvFLS*: *VIT_18s0001g03430*) and one leucoanthocyanidin dioxygenase (*VvLDOX*: *VIT_02s0025g04720*) exhibited increased expression in 5 d post-EBR application (Fig. 3, Supplementary Table S3). However, dihydroflavonol 4-reductase *(VvDFR*: *VIT_18s0001g12800*) shows no discernible response for the exogenous EBR treatment (Fig. 3, Supplementary Table S3).

Furthermore, the transcription of UDP-glucose: flavonoid-3-O-glucosyltransferase (*VvUFGT: VIT_16s0039g02230*), which was involved in the final process of anthocyanin biosynthetic pathway, was robustly activated at 5 d after BRs treatment. In contrast, other *VvUFGT* (*VIT_12s0034g00130*) showed down-regulated expression at 5 and 15 d after treatment. An anthocyanin O-methyltransferase (*VvAOMT*: *VIT_01s0010g03510, VIT_07s0031g00350,* and *VIT_03s0063g00140*) involved in anthocyanin methylation and two glutathione-S-transferase (*VvGST*: *VIT_04s0079g00690* and *VIT_01s0026g01340*) involved in anthocyanins transport, exhibited induced transcript level in response to BRs application compared to control berries (Fig. 3, Supplementary Table S3). These results suggested that BRs promoted skin coloring and anthocyanin accumulation in berry by inducing the transcript level of anthocyanin biosynthetic-related genes.

BRs application affects the gene expression of plant hormone biosynthesis and signaling

Plant hormones have crucial effects on berry development and ripening. At the transcriptomic analysis, a multitude of genes related to biosynthesis and signaling of different plant hormones, such as BRs, ethylene, abscisic acid (ABA), auxin (IAA), and cytokinin (CTK), were detected responding to EBR application (Fig. 4, Supplementary Table S4). For example, one steroid 22-alpha-hydroxylase (*VvDWF4: VIT_04s0023g01630*), one 3-epi-6-deoxocathasterone 23-monooxygenase (*VvROT3*: *VIT_04s0023g02650*), and one brassinosteroid-6-oxidase (*VvBR6OX1: VIT_14s0083g01110*) involved in BR biosynthesis were down-regulated under EBR treatment. In BR signaling pathway, three brassinosteroids insensitive 1-associated receptor kinase 1 (*VvBAK1: VIT_12s0055g01160, VIT_12s0055g01280, VIT_12s0121g00300*), one BES1/BZR1 homolog protein (*VvBES1*: *VIT_10s0003g01790*), and one BES1-interacting Myc-like protein 1 (*VvBIM1*: *VIT_17s0000g04790*) all demonstrated down-regulated expression patterns upon BR application (Fig. 4a, Supplementary Table S4), suggesting a loop regulation network for regulation in BR biosynthesis pathway.

EBR application evoked pronounced effects on the genes associated with the ABA biosynthetic and signaling pathways. EBR treatment highly induced the transcript level of ABA biosynthesis-related gene (*VvNCED: VIT_10s0003g03750*) while concurrently leading to a decrease in the transcript level of ABA metabolism-related gene (*VvCYP707A1: VIT_02s0087g00710*) (Fig. 4c, Supplementary Table S4). In ABA signaling pathway, a notable reduction was observed in 9 *VvPP2Cs*, known as negative regulators of ABA. Among them, *VvPP2C15* (*VIT_08s0007g00310*) and *VvPP2C55* (*VIT_06s0004g06840*) consistently exhibited down-regulation at all time points. Another three ABA signaling genes, including two *VvPYR/PYL* (*VIT_02s0012g01270* and *VIT_15s0046g01050*) and one *VvABF1* (*VIT_12s0055g00420*), manifested increased expression following

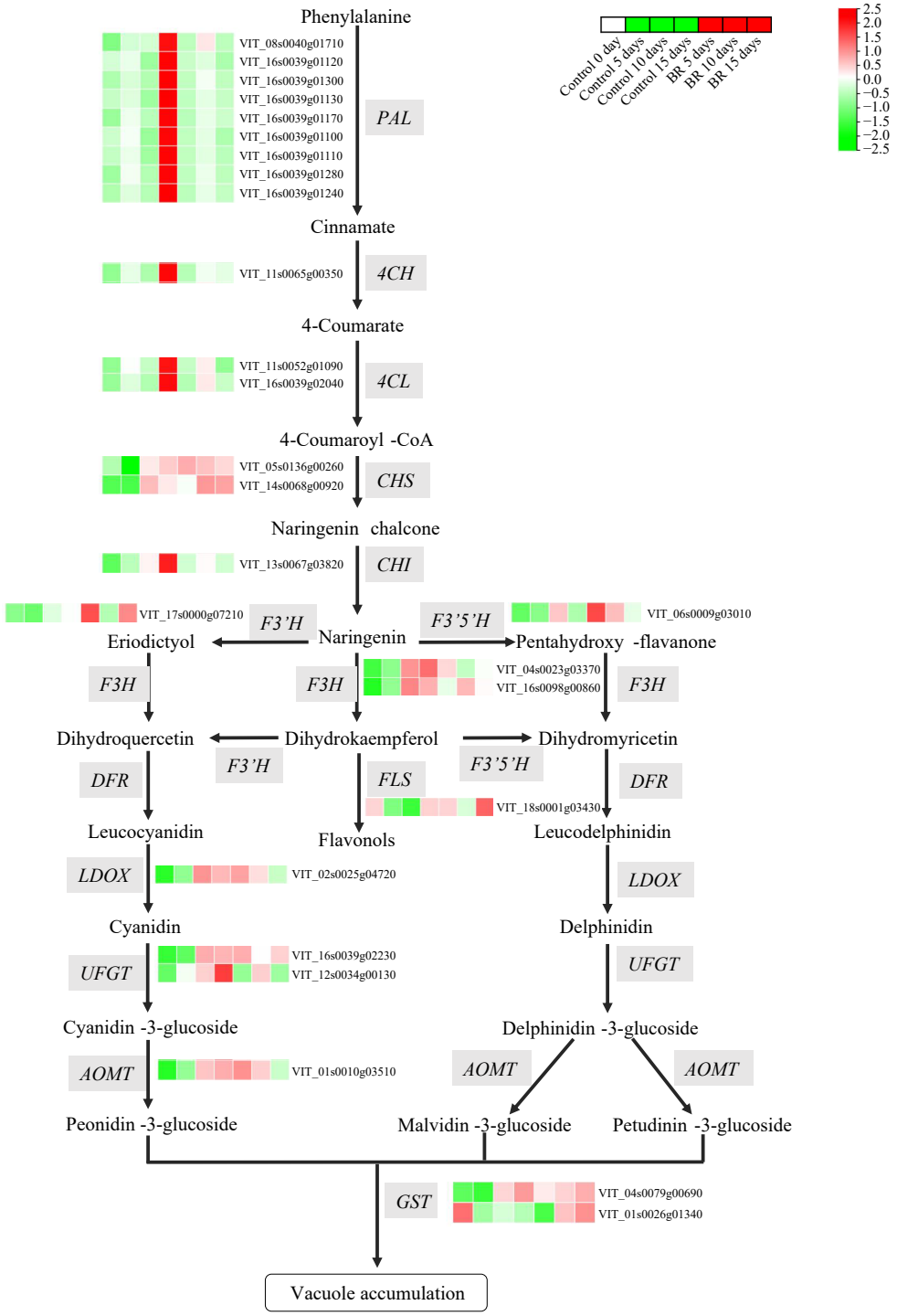

Fig. 3 The effect of exogenous 2,4-epibrassinolide (EBR) treatment on the anthocyanin biosynthetic pathway in grapevine berries. The heatmap was constructed based on relative log2 (FPKM) values obtained by RNA-Seq data during berry development. Green to red color indicates a gradual increase in gene expression level.

exogenous BRs treatment (Fig. 4c, Supplementary Table S4). Furthermore, two ethylene receptors, ethylene response sensor 1 (*VvERS1*: *VIT_07s0005g00850*) and ethylene response 2 (*VvETR2*: *VIT_05s0049g00090*), recognized as negative regulators of ethylene responses, exhibited reduced expression under BRs treatment when compared with control berries. However, ethylene response factor 1 (*VvERF1: VIT_05s0049g00510*) was up-regulated in expression (Fig. 4b, Supplementary Table S4).

Four auxin synthesis genes, including one tryptophan aminotransferase related *(VvTAR2*: *VIT_17s0000g08990*), two *VvYUCs* (*VIT_04s0023g01480* and *VIT_07s0104g01250*), and one IAA-amido synthetases (*VvGH3: VIT_03s0091g00310*) showed significant down-regulation in response to BRs treatment. Only one *VvTAR1* (*VIT_00s0225g00230*) was obviously up-regulated in all samples. Furthermore, the transcription levels of 13 genes related to auxin, including three Auxin/Indole-3-acetic acid-inducible proteins (*VvIAAs*), seven auxin response factors (*VvARFs*), and three auxin-responsive SAUR signaling, underwent significant attenuation following BRs treatment (Fig. 4d, Supplementary Table S4). In the CTK biosynthesis and metabolism pathway, two isopentenyltrans-

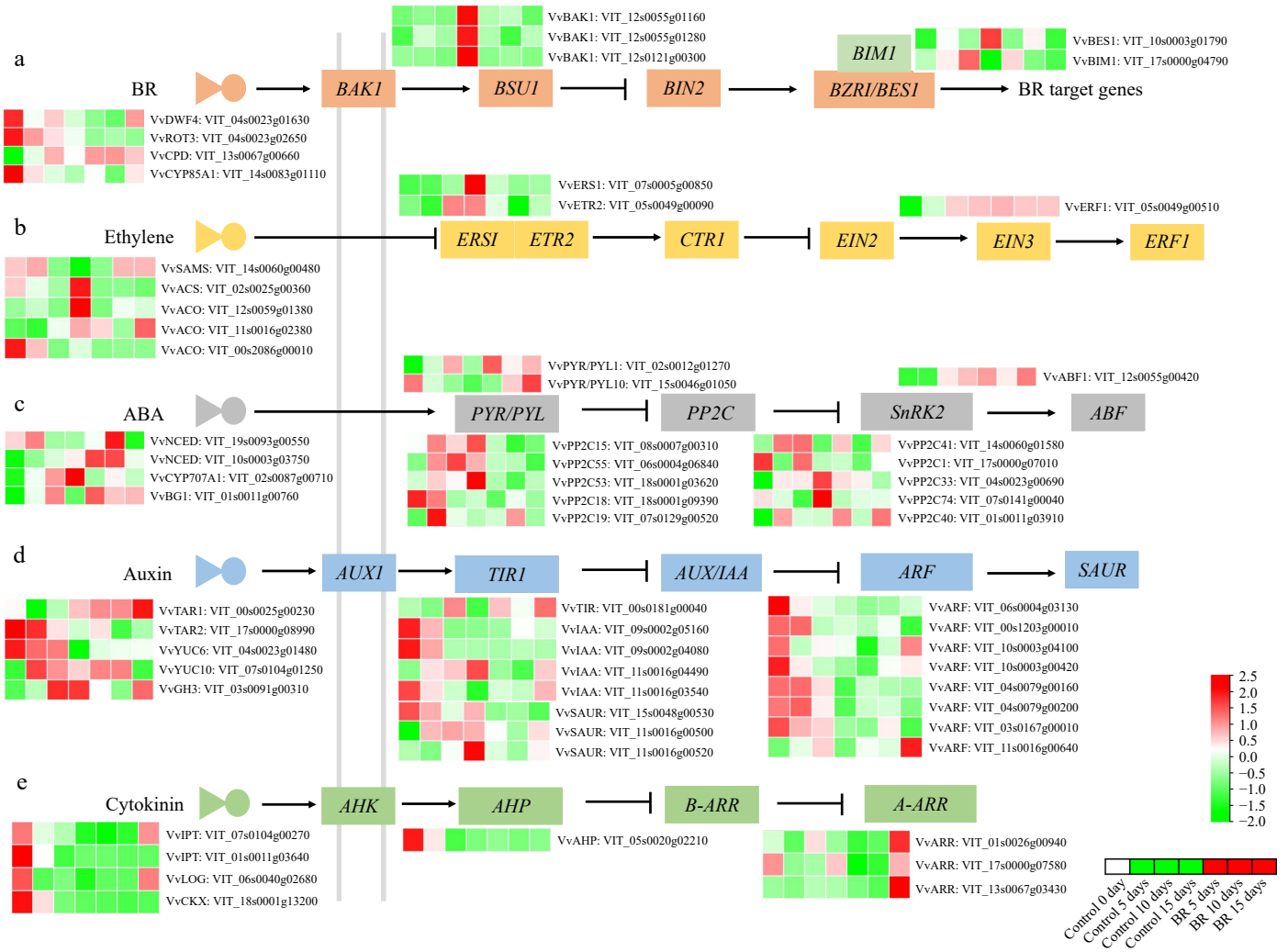

Fig. 4 The effect of exogenous 2,4-epibrassinolide (EBR) treatment on the phytohormones biosynthesis and signaling pathways in grapevine berries. (a) BRs, (b) ethylene, (c) abscisic acid (ABA), (d) auxin, and (e) cytokinin biosynthesis and signaling-related genes responded to EBR application, respectively. The heatmap was constructed based on relative log2 (FPKM) values obtained by RNA-Seq data during berry development. Green to red color indicates a gradual increase in gene expression level.

ferases (*VvIPTs*: *VIT_07s0104g00270* and *VIT_01s0011g03640*) and one cytokinin oxidase gene (*VvCKX*: *VIT_18s0001g13200*) showed decreased expression in 5 d post-BRs application (Fig. 4e, Supplementary Table S4). Additionally, one histidine-containing phosphotransfer protein (*VvAHP*: *VIT_05s0020g02210*), the positive regulator of CTK signaling, expression of auxin and CTK biosynthetic and signaling pathway genes.

BRs application enhances berry softening through regulating the transcription of cell wall-related genes

The effect of BRs in grape berry softening was thoroughly investigated, including the evaluation of berry firmness, protopectin content, soluble pectin content, and cellulose content. BRs application significantly accelerated the decline in berry firmness and promoted berry softness (Fig. 5a). The cellulose and protopectin contents were lower in BRs-treated samples compared with the control group (Fig. 5b & c), while the water-soluble pectin (WSP) content was higher at all time points (Fig. 5d). These results showed that BRs treatment accelerated berry softening and cell wall degradation by decreasing the cellulose and protopectin content, and increasing the WSP content.

Transcripts related to cell-wall degradation during ripening were found in response to EBR application (Supplementary Table S5). At first, the transcription level of two pectin methylesterases (*VvPMEs: VIT_15s0048g00500* and *VIT_12s0035g01900*) was robustly induced by EBR treatment at two stages (Fig. 5e). The expression levels of *VvXET* (*VIT_06s0061g00550*), a prominent gene of xyloglucan endotransglucosylase family, exhibited significant induction (Fig. 5f). However, seven *VvXETs* (*VIT_11s0052g01300, VIT_11s0052g01260, VIT_11s0052g01180, VIT_11s0052g01190, VIT_11s0052g01280, VIT_11s0052g01200 and VIT_11s0052g01270*) demonstrated inhibited expression due to BRs application at all three stages (Fig. 5f), indicating *VvXETs* might have opposite role in BRs-mediated berry softening. Furthermore, 10 cellulose synthases (*VvCES*) (Fig. 5i) and three cellulases (*VvCEL*) (Fig. 5h), respectively, displayed down- and up-regulated expression responding to BRs application, which was consistent with the reduction in cellulose content (Fig. 5b). Additionally, six expansin (*VvEXP*) genes, which are responsible for the extension of cell walls, showed increased expression compared to the control groups (Fig. 5g). Moreover, aquaporins (*VvAQP: VIT_03s0038g02520, VIT_13s0019g00330, VIT_06s0004g02850* and *VIT_08s0007g04780*), which are participated in the enlarge and soften of berry, were also up-regulated post-BRs application (Fig. 5j).

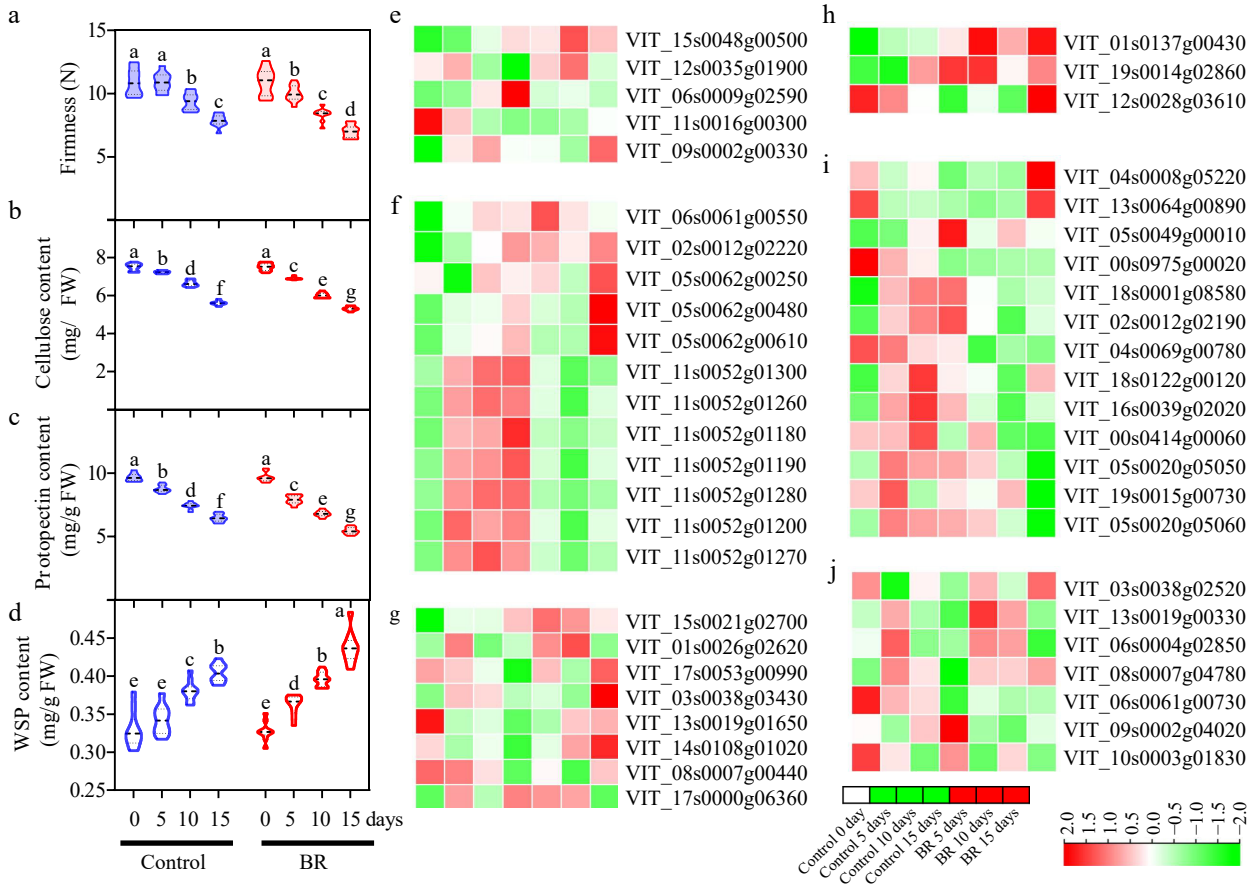

Fig. 5 The effect of exogenous 2,4-epibrassinolide (EBR) treatment on the cell wall components and the expression of genes related to the degrading enzymes of the cell wall. (a) Firmness, (b) cellulose, (c) protopectin, and (d) water-soluble pectin (WSP) content after EBR treatment. Data are expressed as mean ± standard from three biological replicates. Different letters indicate significant differences ($p < 0.05$) between two different treatments at each sampling point.

BRs application alters the transcription levels of multiple transcription factor families

BRs treatment had a strong effect on the expression of multiple transcription factor families, including *bHLH*, *bZIP*, *ERF*, *MYB*, *NAC*, and *WRKY*. Specifically, two *bHLHs*, four *bZIPs,* and two *VvMYBs* showed increased expression, whereas four *bHLHs*, three *bZIPs,* and five *VvMYBs* exhibited decreased expression in response to BRs treatment (Supplementary Table S6). Among them, the expression of *bHLH*, *VvbHLH013* (*VIT_11s0052g00100*), was markedly inhibited at two stages of BRs treatment (Supplementary Table S6). Moreover, four *VvMYB* members (*VIT_03s0180g00210*, *VIT_18s0001g11170*, *VIT_18s0001g09850* and *VIT_07s0129g01050*) belonging to the S22 subfamily also demonstrated down-regulated expression (Supplementary Table S6), indicating their potential role as negative regulators in BRs signaling during berry ripening. Furthermore, total a total of 10 *VvNACs* showed contrasting expression patterns under BRs treatment. Among them, two *VvNACs* (*VIT_02s0012g01040* and *VIT_01s0146g00280*) displayed increased expression at two stages, whereas three *VvNACs* (*VIT_15s0048g02280*, *VIT_17s0000g00770* and *VIT_14s0068g01490*) exhibited decreased expression at two stages responding to BRs application (Supplementary Table S6).

Interestingly, only one *VvERF* gene (*VIT_05s0049g00510*) displayed remarkably accumulated transcription levels on grape berry, and it was notably induced at 5 d post EBR treatment (Supplementary Table S6). However, eight *VvERF* genes (*VIT_12s0028g03270*, *VIT_02s0025g04460*, *VIT_16s0100g00380*, *VIT_18s0001g03240*, *VIT_11s0016g03350*, *VIT_03s0063g00460*, *VIT_11s0016g00670* and *VIT_16s0013g00990*) had decreased expression at all time points (Supplementary Table S6). Additionally, six *VvERF* genes also exhibited down-regulated transcription levels at one or two time points following BRs application (Supplementary Table S6). Similarly, only one *VvWRKY* gene (*VIT_12s0059g00880*) exhibited up-regulated expression, whereas three *VvWRKYs* (*VIT_09s0018g00240*, *VIT_04s0008g05760,* and *VIT_02s0025g01280*) displayed down-regulated transcription level in all three-time points after BRs application (Supplementary Table S6). Seven and five *VvWRKYs* demonstrated down-regulated transcription levels in the first two phases, respectively (Supplementary Table S6). The results indicated that *VvERFs* and *VvWRKYs* might play negative regulatory roles in BRs-mediated berry ripening.

qRT-PCR validation of DEGs based on RNA-seq

Twelve DEGs were chosen for qRT-PCR identification between control and BR-treated berries to confirm the precision and credibility of sequencing results. Five genes (*VvNCED, VvTAR1, VvCTR, VvPME, VvEXP*) exhibited up-regulation due to EBR treatment. Seven genes (*VvROT3, VvPRP1, VvERF058, VvLRR, VvCabBP, VvPUP and VvUbox*) were down-regulated by BRs treatment (Supplementary Table S7). Expression verification revealed that although the fold-changes obtained by qRT-PCR and RNA-Seq did not align precisely, the overall expression profiles between the two techniques were largely congruent to each other (Fig. 6), implying that the RNA-Seq data are reliable and reproducible.

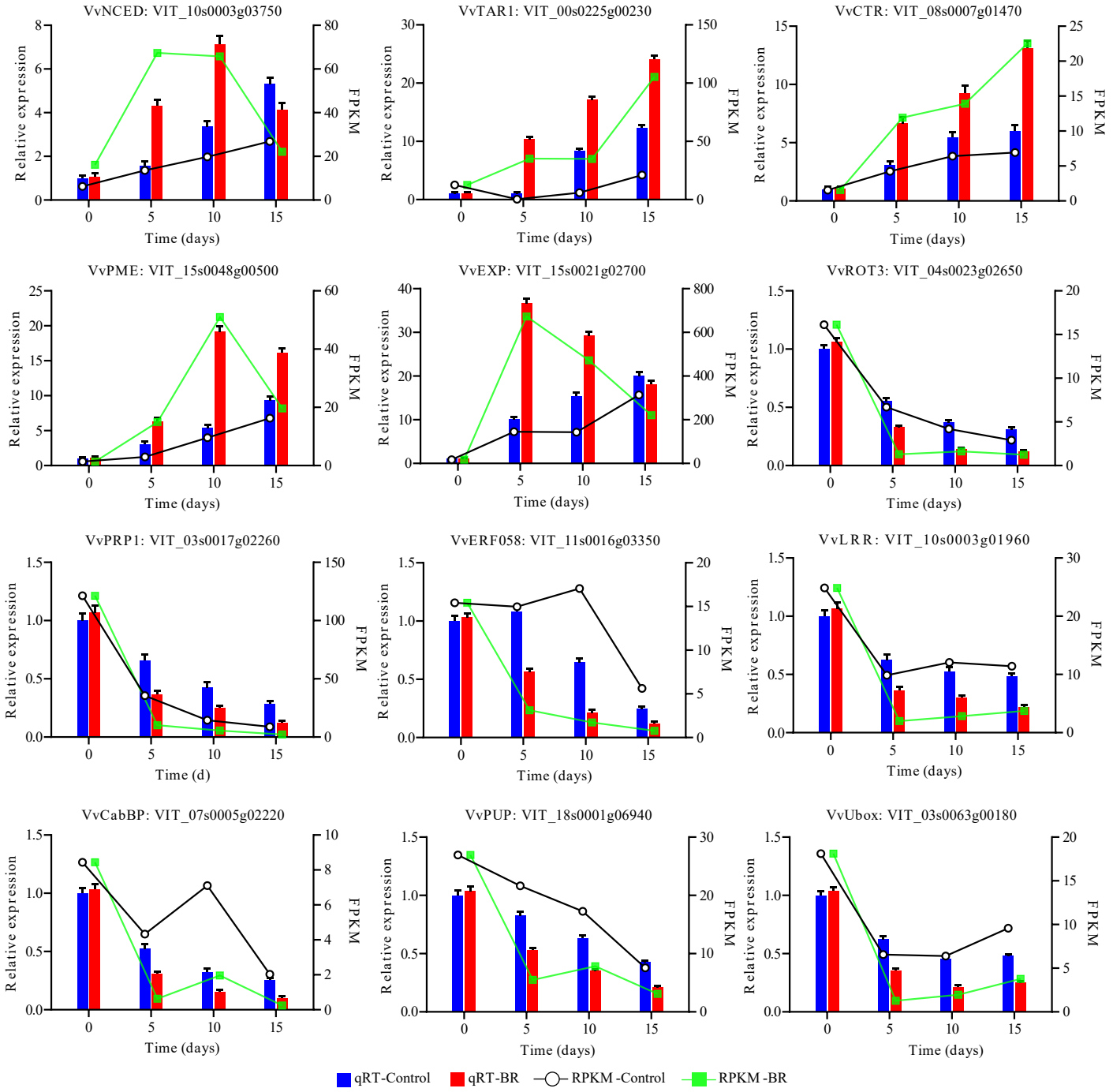

Fig. 6 Validation of 12 differentially expressed genes by qRT-PCR under EBR treatment in grapevine berry.

Transient overexpression in *VvDWF4* promotes anthocyanin accumulation and fruit softening in strawberry fruit

Due to *VvDWF4* being regulated in all three experimental stages, *VvDWF4*, a BR biosynthesis gene, was selected to confirm the significance of BRs during berry ripening by a strawberry transient expression system. Overexpression of *VvDWF4* significantly promoted fruit coloration and anthocyanin accumulation (Fig. 7a, b), 6 d post-infiltration with *VvDWF4,* fruits began to exhibit a reddening color and became almost entirely red within 10 d post-infiltration, while the control fruits reached approximately intermediate red stage within the same period (Fig. 7a). The concentrations of total anthocyanin in *VvDWF4*-overexpression fruits were up to 2.4-times higher on the 10th day compared with the control fruit (Fig. 7b). Transient overexpression of *VvDWF4* into strawberry fruit significantly enhanced the BRs content at 5 and 10 d of treatment (Fig. 7c), indicating an important role of *VvDWF4* in BRs-induced anthocyanin accumulation. Furthermore, transient overexpression of *VvDWF4* in strawberry increased the expression of both BRs biosynthetic genes (*FaBR6OX1 and FaCPD*) and the BRs receptor (*FaBRI1*) (Fig. 7d–f). Additionally, transient overexpression of *VvDWF4* also promoted fruit softening (Fig. 7g). *VvDWF4*-overexpression fruit exhibited a significant increase in water-soluble pectin content and concurrent reduction in protopectin content when compared to the control fruit (Fig. 7h, i), resulting in rapid softening of the fruit.

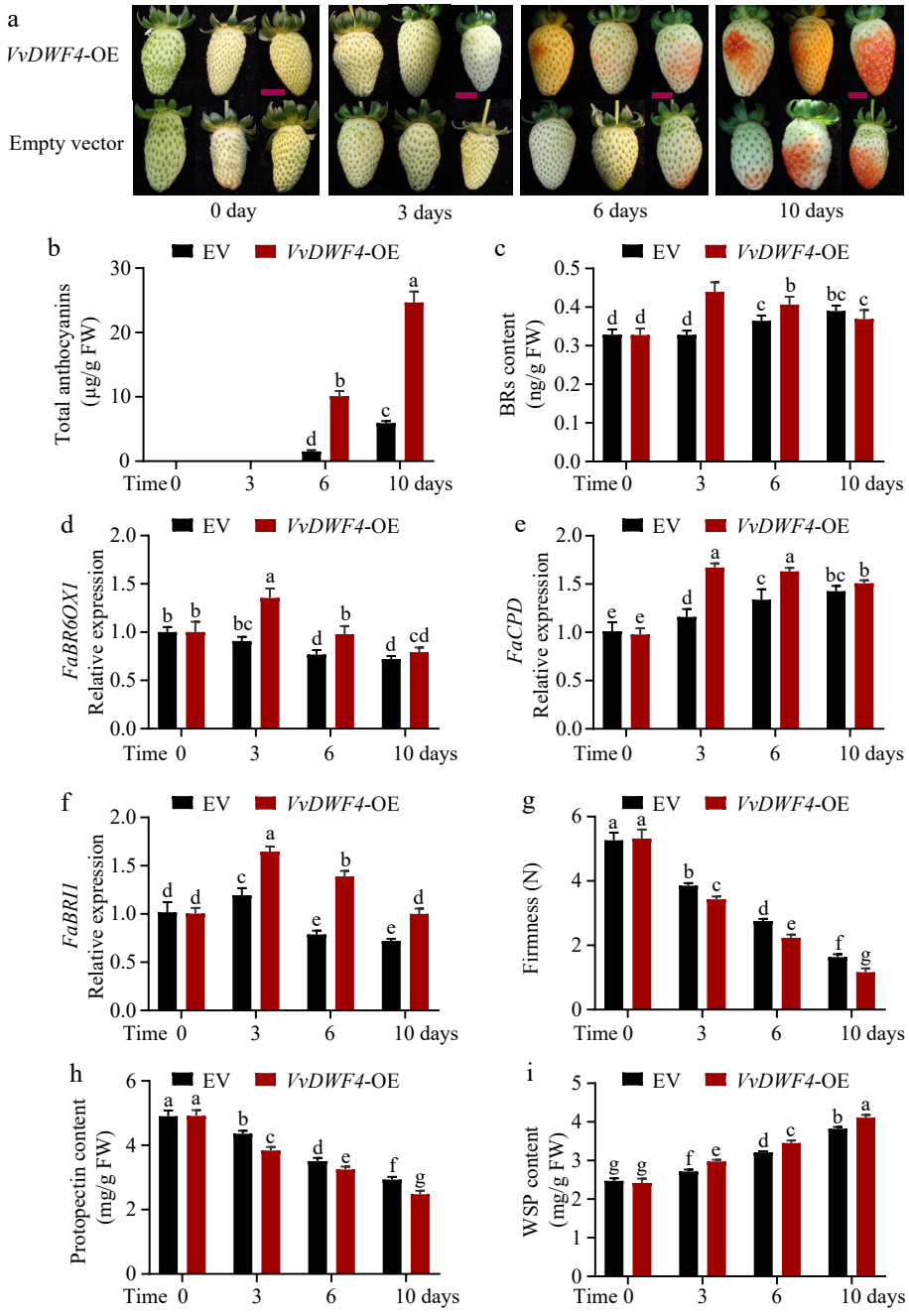

Fig. 7 Effects of transient overexpression of *VvDWARF4* (*VvDWF4*) in strawberry fruits. (a) The phenotype of strawberry fruits after transient overexpression *VvDWF4*. The red bar indicates 5 cm. (b) Anthocyanin contents in grape shin. FW, fresh weight; EV, empty vector. (c) BRs content. The relative expression of brassinosteroid-6-oxidase (*BR6OX1*) (d), cytochrome P450 90A1 (*CPD*) (e), and BRASSINOSTEROID-INSENSITIVE 1 (*BRI1*) (f), respectively. (g) Firmness, (h) Protopectin, and (i) water-soluble pectin (WSP), content after transient overexpression *VvDWF4*. Data are expressed as mean ± standard from three biological replicate analysis, and different letters indicate significant differences ($p < 0.05$) between two different treatments at each sample.

Discussion

The exogenous application of plant hormones is a crucial method to enhance the quality attributes of grape berry, such as their size, color, flavor, and sensory traits[28]. BRs, which is known as the environmental-friendly hormone, have been extensively used to improve yield and confer resilience in responding to a range of stress factors[19]. BRs are implicated in the development and ripening process of both climacteric (tomato and mango) and non-climacteric (strawberry and grape) fruit[21,29–31]. Similarly, current findings demonstrate that epibrassinolide (EBR) treatment increased endogenous BR contents and promoted grape berry ripening (Fig. 1). However, the molecular mechanism of BRs-mediated berry ripening is not perfectly clear in grape. Therefore, we employed a combination of physiological and transcriptomic analyses to delve into the underlying biological processes and identify potential candidate genes involved in BR-mediated berry development and ripening.

BRs homeostasis was maintained via a feedback regulation during berry ripening in grape

It is well-established that endogenous BR contents undergo a significant increase during the onset of the berry ripening process[3].

During this research, the BR contents notably increased at the initial stage of EBR treatment, leading to accelerated grape berry ripening (Fig. 1b), indicating that BRs may serve as potent elicitors during grape berry ripening. As previously reported, BR homeostasis is primarily regulated by intricate feedback transcriptional regulation at various steps in the BR biosynthetic routine[32]. For example, four BRs biosynthetic genes in *Arabidopsis*, including *AtDWF4*, *AtCPD*, *AtBR6ox1,* and *AtROT3*, exhibited decreased expression in response to exogenous BL treatment but increased expression when subjected to the BR synthesis inhibitor BRZ[7,8]. Correspondingly, *VvBR6OX1* and *VvDWF1*, two BR biosynthesis-related genes, also showed significant down-regulated transcription level responding to exogenous EBR application in grapes. Furthermore, transient overexpression in *VvDWF4* from strawberry fruit significantly improved the accumulation of BR contents through up-regulating transcription level of BRs biosynthetic genes and positive signaling gene BRI1 (Fig. 7), indicating a positive correlation between fruit ripening and transcription level of BR biosynthetic genes, including *GhDWF4* in upland cotton and *SlCYP90B3* (*SlDWF4*) of tomato[33]. All the results illustrated BR homeostasis is maintained by the feedback regulatory expression in multiple genes associated with BR biosynthesis in grape.

BRs promote ripening through the coordination of multiple hormone cross-talk networks

Different plant growth regulators, like ethylene, ABA, and auxin, have participated in the regulation network of berry ripening of grapes[22,34]. For example, BRs application have been demonstrated to improve ethylene accumulation and fruit ripening of tomato and banana by amplifying the transcription levels of *ACS* and *ACO* genes[35]. Herein, EBR application down-regulated transcription level of two negative regulators of ethylene signaling, *VvERS1* (*VIT_07s0005g00850*) and *VvETR2* (*VIT_05s0049g00090*), whereas up-regulated the expression of the positive regulator of ethylene signaling, *VvERF1* (*VIT_05s0049g00510*), suggesting that EBR application promotes ethylene signal transduction during berry ripening in grape. Similarly, BRs initiate a cascade of events leading to ripening by increasing ethylene levels in tomatoes[29].

BRs and ABA often exhibit antagonistic interactions in many plants' growth and stress response processes, including seed germination and dormancy[36], primary root development[37], and stomatal conductance[38]. For instance, high accumulation levels of BRs have appeared to suppress ABA-induced stomata closure by inhibiting ABA biosynthesis[38]. However, EBR treatment has been shown to increase ABA content and induce the transcription of ABA biosynthesis (*VvNCED1, VvNCED2, and VvZEP*) and signaling-related genes (*VvSnRK2.6, VvPP2C4, VvABF1, and VvABF2*) under water stress[39]. The current findings also reveal that EBR treatment activates ABA biosynthesis (*VvNCED*: *VIT_10s0003g03750*) and signaling-related genes (*VvPYR/PYL*: *VIT_02s0012g01270*, *VIT_15s0046g01050*, and *VvABF1: VIT_12s0055g00420*) while reducing the expression of 9 *VvPP2Cs*, which function as negative regulators within ABA signaling pathway (Fig. 4, Supplementary Table S4). Collectively, BR might exert a positive influence on ABA signaling during berry ripening, suggesting a more complex crosstalk mechanism between BR and ABA throughout the grape berry ripening process.

The crosstalk and interplay between BRs and auxin are complex and extensive, crucial for plant development. BRs and auxin work synergistically to regulate hypocotyl elongation and lateral root development[40]. BRs are also believed to be essential for fruit sets, reminiscent of auxin application. Interestingly, while exogenous BRs application promotes ripening[3], while auxin delays ripening[34]. Most auxin signaling-related genes, including three *VvIAAs*, seven *VvARFs,* and three *VvSAUR,* were significantly inhibited in response to EBR treatment (Fig. 4, Supplementary Table S4), indicating that BRs and auxin might exert opposing effects during the grape ripening process.

BRs accelerate berry color and softening by mediating anthocyanin- and cell wall- associated genes

In the current study, exogenous EBR application significantly enhanced berry coloration and anthocyanin concentration in grape berries (Fig. 1). Several researches have also indicated that BRs are involved in the development and ripening processes of different fruit crops, including tomato[29], mango[30], strawberry[31], persimmon[41], and jujube[42]. For example, EBR treatment was shown to regulate secondary metabolism by promoting the gene expression and enzyme activity of PAL in tomato[43]. Several studies in grapes have indicated that BRs treatment not only increases the activities of PAL and UFGT enzymes[19] but also stimulates transcript level of anthocyanin biosynthetic genes (*VvCHI1, VvCHS2, VvCHS3, VvDFR, VvUFGT*)[20]. The results depict that almost all anthocyanin structural and constitute genes were up-regulated responding to BR application in the first two stages, except for *VvDFR* (Fig. 3). These findings strongly indicated that exogenous BRs application activates the accumulation of anthocyanin through handling the transcription level of anthocyanin biosynthetic genes in grape.

Fruit texture and softening are critical factors for fruit shelf life and storage, primarily attributed to cell wall disassembly during ripening[44]. BRs have been implicated in berry ripening and softening. For example, BR treatment has been shown to accelerate ethylene production, enhance respiration rate, facilitate fruit coloring, and improve the development and softening of the fruit during the ripening process of mango[30]. In tomatoes, overexpressing *SlCYP90B3* which is known as an important enzyme involved in BR biosynthesis, elevated the levels of bioactive BRs, promoting the ripening process of various fruits, including accelerated softening, increased soluble sugar, and enhanced flavor volatile concentrations[33].

Fruit softening is tightly linked to the degradation of cell-wall-related constituents, such as pectin and cellulose. In this study, the application of EBR significantly increased WSP content while reducing protopectin and cellulose contents, leading to berry softening in grapevine (Fig. 5), which is in line with recent studies in persimmon fruit[41]. In addition, transient overexpression of *VvDWF4* in strawberry fruit also enhanced fruit softening by elevating WSP content and decreasing protopectin levels (Fig. 7). The process of cell wall degradation involves the role of multiple degrading enzymes of cell-wall. In persimmon, EBR application accelerated fruit softening by up-regulating the expression of genes related to cell-wall degrading pathway, including *DkPG1*, *DkPL1*, *DkPE2*, and *DkEGase1*, and increasing the activities of wall-degrading enzyme, including polygalacturonase, pectate-lyase, and endo-1,4-beta-glucanase, ultimately leading to fruit softening[41]. Correspondingly, various gene families encoding cell wall-degrading enzymes, including *VvPME*, *VvXET*, *VvCEL*, *VvEXP*, and *VvAQP*, were extensively regulated by BRs treatment (Fig. 5). These findings strongly support the positive association between BRs and grapevine berry ripening, either by impacting the genes about cell-wall degrading enzymes or through crosstalk network with other hormones.

As a non-toxic and ecologically friendly plant hormone, BRs have been shown to promote the growth, yield, and quality, stress tolerance of plants, and it is also effective at very low doses and degrade easily[45–48]. For example, BRs-treated mangoes outperformed the control group in terms of quality, including fruit weight, yield, the content of total soluble solids, total sugar, titratable acid, and

β-carotene[45–48]. In addition, the application of BRs can reduce the dependence on chemical fertilizers and pesticides, thereby reducing environmental pollution[49,50].

The high cost of BRs may limit their widespread use in the world. However, in order to reduce the cost of BRs synthesis and improve the stability when applied, many types of BRs analogs that were commercially synthesized have been developed to make them more affordable[49,50]. These synthetic analogs are used in many countries to improve the performance and yield of agricultural and horticultural crops[49,50].

In summary, the application of BRs in large-scale industrial production has potential, especially in improving crop yield and quality. However, cost and application technology are key factors to consider during implementation. The cost problem can be partially solved by using more cost-effective synthetic analogs, while the precise application of the technology requires further research and development.

Conclusions

The findings of this study provide compelling evidence that the exogenous BRs positively regulate numerous ripening processes, including sugar and anthocyanin accumulation, color development, and berry softening. Transcriptome analysis has unveiled the intricate crosstalk network among BRs and distinct plant hormones, including ethylene, abscisic acid, auxin, and cytokinin, in elevating the ripening process from grapes. The BR-induced color development and anthocyanin accumulation are positively linked with the up-regulation of anthocyanin biosynthetic genes. Moreover, epibrassinolide (EBR) treatment elevates WSP content while reducing protopectin and cellulose levels, ultimately leading to berry softening by modulating the genes responsible for cell wall-degrading enzymes. These findings offer valuable insights into the potential benefits of utilizing BRs to enhance the visual and nutritional qualities of grape berries without compromising yield.

Author contributions

The authors confirm contribution to the paper as follows: Project administration: Zhu X, Leng X, Gong D; formal analysis, data curation: Ren Y, Xu X, Ma Y; resources: Zhao P, Sun X, Zhang L, Zhang Z, Cui Z, Li Z; writing-review & editing: Zhu X, Leng X, Haider M; funding acquisition: Zhu X, Leng X; validation: Ren Y, Xu X, Ma Y; visualization: Liu Y, Zhu X, Leng X; investigation: Liu Y, Ji X; methodology: Liu Y, You R, Ji X; writing-original draft: Liu Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Kuhn N, Guan L, Dai ZW, Wu BH, Lauvergeat V, et al. 2014. Berry ripening: recently heard through the grapevine. *Journal of Experimental Botany* 65:4543–59
2. Li J, Quan Y, Wang L, Wang S. 2023. Brassinosteroid promotes grape berry quality-focus on physicochemical qualities and their coordination with enzymatic and molecular processes: a review. *International Journal of Molecular Science* 24:445
3. Symons GM, Davies C, Shavrukov Y, Dry IB, Reid JB, et al. 2006. Grapes on steroids. Brassinosteroids are involved in grape berry ripening. *Plant Physiology* 140:150–58
4. Clouse SD, Sasse JM. 1998. Brassinosteroids: essential regulators of plant growth and development. *Annual Review of Plant Physiology and Plant Molecular Biology* 49:427–51
5. Wei Z, Li J. 2016. Brassinosteroids regulate root growth, development, and symbiosis. *Molecular Plant* 9:86–100
6. Wei Z, Li J. 2020. Regulation of brassinosteroid homeostasis in higher plants. *Frontiers in Plant Science* 11:583622
7. Kour J, Kohli SK, Khanna K, Bakshi P, Sharma P, Singh AD, et al. 2021. Brassinosteroid signaling, crosstalk and, physiological functions in plants under heavy metal stress. *Frontiers in Plant Science* 12:608061
8. Zhao B, Li J. 2012. Regulation of brassinosteroid biosynthesis and inactivation. *Journal of Integrative Plant Biology* 54:746–59
9. Choe S, Dilkes BP, Fujioka S, Takatsuto S, Sakurai A, et al. 1998. The *DWF4* gene of *Arabidopsis* a cytochrome P450 that mediates multiple 22 α-hydroxylation steps in brassinosteroid biosynthesis. *The Plant Cell* 10:231–43
10. Azpiroz R, Wu Y, Locascio JC, Feldmann KA. 1998. An *Arabidopsis* brassinosteroid-dependent mutant is blocked in cell elongation. *The Plant Cell* 10:219–30
11. Ma Y, Xue H, Zhang L, Zhang F, Ou C, et al. 2016. Involvement of auxin and brassinosteroid in dwarfism of autotetraploid apple (*Malus*× *domestica*). *Scientific Reports* 6:26719
12. Li J, Chory J. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90:929–38
13. Noguchi T, Fujioka S, Choe S, Takatsuto S, Yoshida S, et al. 1999. Brassinosteroid insensitive *dwarf* mutants of *Arabidopsis* accumulate brassinosteroids. *Plant Physiology* 121:743–52
14. Clouse SD, Langford M, McMorris TC. 1996. A brassinosteroid-Insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. *Plant Physiology* 111:671–78
15. Wang ZY, Nakano T, Gendron J, He J, Chen M, et al. 2002. Nuclear-localized *BZR1* mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Developmental Cell* 2:505–13

16. Yin Y, Wang ZY, Mora-Garcia S, Li J, Yoshida S, et al. 2002. *BES1* accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell* 109:181−91
17. Yu X, Li L, Zola J, Aluru M, Ye H, et al. 2011. A brassinosteroid transcriptional network revealed by genome-wide identification of *BESI* target genes in *Arabidopsis thaliana*. *The Plant Journal* 65:634−46
18. Liu L, Liu H, Li S, Zhang X, Zhang M, et al. 2016. Regulation of *BZR1* in fruit ripening revealed by iTRAQ proteomics analysis. *Scientific Reports* 6:33635
19. Xi ZM, Zhang ZW, Huo SS, Luan LY, Gao X, et al. 2013. Regulating the secondary metabolism in grape berry using exogenous 24-epibrassinolide for enhanced phenolics content and antioxidant capacity. *Food Chemistry* 141:3056−65
20. Zhou Y, Yuan C, Ruan S, Zhang Z, Meng J, et al. 2018. Exogenous 24-epibrassinolide interacts with light to regulate anthocyanin and proanthocyanidin biosynthesis in Cabernet Sauvignon (*Vitis vinifera* L.). *Molecules* 23:93
21. Li J, Javed HU, Wu Z, Wang L, Han J, et al. 2022. Improving berry quality and antioxidant ability in 'Ruidu Hongyu' grapevine through preharvest exogenous 2,4-epibrassinolide, jasmonic acid and their signaling inhibitors by regulating endogenous phytohormones. *Frontiers in Plant Science* 13:1035022
22. Wang P, Yu A, Ji X, Mu Q, Salman Haider M, et al. 2022. Transcriptome and metabolite integrated analysis reveals that exogenous ethylene controls berry ripening processes in grapevine. *Food Research International* 155:111084
23. Xu F, Xi ZM, Zhang H, Zhang CJ, Zhang ZW. 2015. Brassinosteroids are involved in controlling sugar unloading in *Vitis vinifera* 'Cabernet Sauvignon' berries during véraison. *Plant Physiology and Biochemistry* 94:197−208
24. Yang B, Yao H, Zhang J, Li Y, Ju Y, et al. 2020. Effect of regulated deficit irrigation on the content of soluble sugars, organic acids and endogenous hormones in Cabernet Sauvignon in the Ningxia region of China. *Food Chemistry* 312:126020
25. Zhang K, Liu Z, Guan L, Zheng T, Jiu S, et al. 2018. Changes of anthocyanin component biosynthesis in 'Summer Black' grape berries after the red flesh mutation occurred. *Journal of Agricultural and Food Chemistry* 66:9209−18
26. Zhang Z, Shi Y, Ma Y, Yang X, Yin X, et al. 2020. The strawberry transcription factor *FaRAV1* positively regulates anthocyanin accumulation by activation of *FaMYB10* and anthocyanin pathway genes. *Plant Biotechnology Journal* 18:2267−79
27. Cheng S, Wu T, Gao J, Han X, Huang W, et al. 2023. Color myth: anthocyanins reactions and enological approaches achieving their stabilization in the aging process of red wine. *Food Innovation and Advances* 2:255−71
28. Crupi P, Alba V, Masi G, Caputo AR, Tarricone L. 2019. Effect of two exogenous plant growth regulators on the color and quality parameters of seedless table grape berries. *Food Research International* 126:108667
29. Vidya Vardhini B, Rao SSR. 2002. Acceleration of ripening of tomato pericarp discs by brassinosteroids. *Phytochemistry* 61:843−47
30. Zaharah SS, Singh Z, Symons GM, Reid JB. 2012. Role of brassinosteroids, ethylene, abscisic acid, and indole-3-acetic acid in mango fruit ripening. *Journal of Plant Growth Regulation* 31:363−72
31. Chai YM, Zhang Q, Tian L, Li CL, Xing Y, et al. 2013. Brassinosteroid is involved in strawberry fruit ripening. *Plant Growth Regulation* 69:63−69
32. Choudhary SP, Yu JQ, Yamaguchi-Shinozaki K, Shinozaki K, Tran LSP. 2012. Benefits of brassinosteroid crosstalk. *Trends in Plant Science* 10:594−605
33. Hu S, Liu L, Li S, Shao Z, Meng F, et al. 2020. Regulation of fruit ripening by the brassinosteroid biosynthetic gene *SlCYP90B3* via an ethylene-dependent pathway in tomato. *Horticulture Research* 7:163
34. Ziliotto F, Corso M, Rizzini FM, Rasori A, Botton A, et al. 2012. Grape berry ripening delay induced by a pre-véraison NAA treatment is paralleled by a shift in the expression pattern of auxin- and ethylene-related genes. *BMC Plant Biology* 12:185
35. Guo YF, Shan W, Liang SM, Wu CJ, Wei W, et al. 2019. *MaBZR1/2* act as transcriptional repressors of ethylene biosynthetic genes in banana fruit. *Physiologia Plantarum* 165:555−68
36. Zhang S, Cai Z, Wang X. 2009. The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. *Proceedings of the National Academy of Sciences of the United States of America* 106:4543−48
37. Yang X, Bai Y, Shang J, Xin R, Tang W. 2016. The antagonistic regulation of abscisic acid-inhibited root growth by brassinosteroids is partially mediated via direct suppression of *abscisic acid insensitive 5* expression by *brassinazole resistant 1*. *Plant Cell and Environment* 39:1994−2003
38. Ha YM, Shang Y, Yang D, Nam KH. 2018. Brassinosteroid reduces ABA accumulation leading to the inhibition of ABA-induced stomatal closure. *Biochemical and Biophysical Research Communications* 504:143−48
39. Wang YT, Chen ZY, Jiang Y, Duan BB, Xi ZM. 2019. Involvement of ABA and antioxidant system in brassinosteroid-induced water stress tolerance of grapevine (*Vitis vinifera* L.). *Scientia Horticulturae* 256:108596
40. Bao F, Shen J, Brady SR, Muday GK, Asami T, et al. 2004. Brassinosteroids interact with auxin to promote lateral root development in *Arabidopsis*. *Plant Physiology* 134:1624−31
41. He Y, Li J, Ban Q, Han S, Rao J. 2018. Role of brassinosteroids in Persimmon (*Diospyros kaki* L.) fruit ripening. *Journal of Agricultural and Food Chemistry* 66:2637−44
42. Ban Z, Niu C, Li L, Gao Y, Liu L, et al. 2024. Exogenous brassinolides and calcium chloride synergically maintain quality attributes of jujube fruit (*Ziziphus jujuba* Mill.). *Postharvest Biology and Technology* 216:113039
43. Ahammed GJ, Zhou YH, Xia XJ, Mao WH, Shi K, et al. 2013. Brassinosteroid regulates secondary metabolism in tomato towards enhanced tolerance to phenanthrene. *Biologia Plantarum* 57:154−58
44. Shao X, Liu F, Shen Q, He W, Jia B, et al. 2024. Transcriptomics and metabolomics reveal major quality regulations during melon fruit development and ripening. *Food Innovation and Advances* 3:144−54
45. Percio F, Rubio L, Amorim-Silva V, Botella MA. 2025. Crucial roles of brassinosteroids in cell wall composition and structure across species: new insights and biotechnological applications. *Plant, Cell & Environment* 65:1495−99
46. Poppenberger B, Russinova E, Savaldi-Goldstein S. 2024. Brassinosteroids in Focus. *Plant Cell Physiology* 65:1495−99
47. Manghwar H, Hussain A, Ali Q, Liu F. 2022. Brassinosteroids (BRs) role in plant development and coping with different stresses. *International Journal of Molecular Sciences* 23:1012
48. Nolan TM, Vukašinović N, Liu D, Russinova E, Yin Y. 2020. Brassinosteroids: multidimensional regulators of plant growth, development, and stress responses. *The Plant Cell* 32:295−318
49. Ali, B. 2017. Practical applications of brassinosteroids in horticulture-some field perspectives. *Scientia Horticulturae* 225:15−21
50. Coll Y, Coll F, Amorós A, Pujol M. 2015. Brassinosteroids roles and applications: an up-date. *Biologia* 70:726−32