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Bilberry metabolomic and proteomic profiling during fruit ripening reveals key dynamics affecting fruit quality

Nga Nguyen[1] | Ulrich Bergmann[2] | Laura Jaakola[3,4] | Hely Häggman[1] | Soile Jokipii-Lukkari[1] | Katalin Toth[1,5]

[1]Department of Ecology and Genetics, University of Oulu, Oulu, Finland

[2]Biocenter Oulu, University of Oulu, Oulu, Finland

[3]Climate laboratory Holt, Department of Arctic and Marine Biology, UiT the Arctic University of Norway, Tromsø, Norway

[4]Division of Food Production and Society, Norwegian Institute of Bioeconomy Research (NIBIO), Ås, Norway

[5]Inari Agriculture Nv, Ghent, Belgium

Correspondence
Soile Jokipii-Lukkari,
Email: soile.jokipii-lukkari@oulu.fi

Katalin Toth,
Email: katalintoth2005@gmail.com

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Abstract

Bilberry (*Vaccinium myrtillus* L.) is a wild berry species that is prevalent in northern Europe. It is renowned and well-documented for its nutritional and bioactive properties, especially due to its anthocyanin content. However, an overview of biological systems governing changes in other crucial quality traits, such as size, firmness, and flavours, has received less attention. In the present study, we investigated detailed metabolomic and proteomic profiles at four different ripening stages of bilberry to provide a comprehensive understanding of overall quality during fruit ripening. By integrating omics datasets, we revealed a novel global regulatory network of plant hormones and physiological processes occurring during bilberry ripening. Key physiological processes, such as energy and primary metabolism, strongly correlate with elevated levels of gibberellic acids, jasmonic acid, and salicylic acid in unripe fruits. In contrast, as the fruit ripened, processes including flavour formation, cell wall modification, seed storage, and secondary metabolism became more prominent, and these were associated with increased abscisic acid levels. An indication of the increase in ethylene biosynthesis was detected during bilberry development, raising questions about the classification of non-climacteric and climacteric fruits. Our findings extend the current knowledge on the physiological and biochemical processes occurring during fruit ripening, which can serve as a baseline for studies on both wild and commercially grown berry species. Furthermore, our data may facilitate the optimization of storage conditions and breeding programs, as well as the future exploration of beneficial compounds in berries for new applications in food, cosmetics, and medicines.

1 | INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.), also known as European blueberry, is an economically important wild-harvested berry, notably in Northern Europe, due to its significant abundance of anthocyanins and various bioactive compounds (Pires et al., 2020). With a long history of traditional medicinal use and widespread industrial application, bilberries are now increasingly recognized as one of the most demanded wild fruits while also being a sustainable product (Pires et al., 2021). The taste and nutritional value of bilberry fruits generally result from the intricate processes of fruit ripening involving a combination of changes in morphology, physiology, and biochemistry. However, a full understanding of the developmental and ripening process of bilberry remains incomplete, representing a crucial frontier for enhancing fruit quality in post-harvest storage management and breeding program innovations.

Fruit development initiates following successful pollination and fertilization and continues with cell division and expansion. The growth process demands a high energy source from different metabolic pathways processing carbohydrates, amino acids, and nucleic acids (Bianco et al., 2009). Several studies on bilberry fruit ripening have detected high abundances of proanthocyanidins (PAs), carotenoids, and triterpenoids in unripe fruits that subsequently decrease during ripening (Karppinen et al., 2016; Suvanto et al., 2020; Trivedi et al., 2021). The ripening process is marked by colour changes attributed to chlorophyll degradation and pigment accumulation. The blue pigment of bilberries results from anthocyanin accumulation in both the skin and flesh of the fruit. The role of anthocyanins in bilberry fruit colouration has been studied intensively and is well understood (Jaakola et al., 2002, 2010; Dare et al., 2022). By contrast, few studies have been conducted on the changes related to bilberry flavour, which is also one of the most important fruit quality traits. Fruit flavour perception, including sweetness and aroma, is determined by changes in sugar and organic acid ratios and the accumulation of volatile compounds during ripening (Klee, 2010). Recent studies on bilberry ripening have shown that ripe berries have the highest content of total soluble sugars, with fructose and glucose being the predominant sugars (Dare et al., 2022; Samkumar et al., 2022). Additionally, Sater et al. (2020) reported that bilberry contains more individual volatile compounds than other berries. A comprehensive knowledge of flavour metabolomics and other physiological processes (e.g., cell wall modification and seed maturation) during bilberry ripening could provide valuable insights into the improvement of flavour traits and other characteristics of ripe fruits.

The regulation of fruit development and ripening by plant hormones is well described. Auxin and gibberellin (GA) play essential roles in fruit set and development, while ethylene and abscisic acid (ABA) are major regulators of the ripening process (Fenn and Giovannoni, 2021). Depending on whether a burst of respiration rate and ethylene production at the onset of fruit ripening occurs or not, fruits are classified as climacteric (e.g., tomato, apple, and banana) and non-climacteric (e.g., strawberries, grapes, and cherries), respectively (Paul et al., 2012). Bilberry has traditionally been categorized as a non-climacteric fruit, in which the ripening process is regulated by ABA. A previous study showed that ABA levels increased during bilberry ripening, corresponding with the expression of the key gene, 9-cis-epoxycarotenoid dioxygenase (*NCED*), involved in ABA biosynthesis (Karppinen et al., 2013). A similar observation was reported in the ripening of non-climacteric strawberry (Jia et al., 2011; Kim et al., 2019). ABA was also shown to regulate anthocyanin biosynthesis and cell wall modification (Karppinen et al., 2018; Samkumar et al., 2021). Besides ABA, our previous study suggested that several hormone-related transcription factors, including ABA insensitive, ethylene-responsive transcription factors, and auxin-responsive factor (ARF), may be involved in the ripening process of bilberry (Nguyen et al., 2018). ARF and indole-3-acetic acid were reported to regulate the accumulation and transport of auxin, causing its content to decrease during the ripening of blueberry (*Vaccinium corymbosum*) (Liu et al., 2022). Many studies have focused on analyzing the exogenous or endogenous effects of plant hormones on fruit ripening (Jia et al., 2016; Coelho et al., 2019; Li et al., 2019b; Fresno and Munné-Bosch, 2021; Yang et al., 2023). For example, endogenous GA was demonstrated to have a role in the development of strawberry receptacles (Csukasi et al., 2011). Jasmonate (JA) and salicylic acid (SA) concentrations were also analyzed in strawberry fruit ripening (Kim et al., 2019). However, to our knowledge, there is very little information on the dynamics of these hormones in bilberry ripening.

The aim of this study was to create a holistic understanding of bilberry fruit development and ripening. Therefore, we applied untargeted metabolomic and proteomic approaches to obtain new knowledge of the whole process of bilberry ripening. Integrative analysis of metabolomic and proteomic datasets was conducted to investigate the key regulatory networks associated with all physiological processes occurring during fruit development and ripening, focusing on the role of plant hormones, including ABA, GA, JA, and SA. The expressions of hormone-related genes over four different ripening stages of bilberry were analyzed based on our previous transcriptome dataset (Nguyen et al., 2018; Wu et al., 2022). The study provides new knowledge on the key factors affecting the ripening process of bilberries, which is beneficial information for improving fruit quality considering other wild and cultivated berries.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Wild bilberry fruits (*V. myrtillus* L.) from the natural forest in Oulu, Finland (65°03′37.0″N 25°28′30.4″E) were used in this study. Berries from four stages (S2 - small green fruits, S3 - large green fruits, S4 - purple fruits, S5 – ripe, blue fruits) (Figure 1A) were collected during summer. The fruits were frozen immediately in liquid nitrogen and stored at −80°C until further analyses.

2.2 | Metabolite extraction

The metabolite extraction was modified from the method described by Salem et al. (2016). Three biological replicates for each ripening stage were used in the analysis. First, berries were freeze-dried at −51°C for two days, then ground into a fine powder in liquid nitrogen. Freeze-dried berry powder (100 mg) was extracted with 1 mL of methyl tert-butyl ether/methanol (3:1 v/v). The mixture was vortexed for 30 s three times, incubated at 4°C for 45 min, and then sonicated for 15 min. Next, 650 μL of water/methanol (3:1 v/v) were added to each tube and vortexed for 30 s three times, followed by centrifugation at 20,000 *g* for 5 min at 4°C. After centrifugation, 500 μL of the upper phase containing nonpolar compounds and 400 μL of the lower phase containing polar compounds were transferred to new tubes and dried at room temperature (RT). The dried polar and nonpolar samples were resuspended in 100 μL of water/methanol (1:1 v/v) and acetonitrile/isopropanol (7:3 v/v), respectively, for ultra-performance liquid

chromatography coupled to tandem mass spectrometry (UPLC–MS^2) analysis.

2.3 | Metabolite analysis

A Waters Acquity UPLC system (Waters) coupled to a Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific) was used to analyse polar and nonpolar metabolites. The metabolites were separated with an ACQUITY™ PREMIER HSS T3 1.8 µm column (dimension 100 × 2.1 mm, Waters). The sample injection volume was 5 µL, and the flow rate was 0.4 mL min^{-1}. Buffers and gradient programs for polar and nonpolar metabolite analyses are described in Table S1. The UPLC-MS^2 run was performed in three technical replicates for each biological sample.

Mass spectra were acquired in both positive and negative ionization modes and data-dependent scan mode, with a mass range of 135–1,200 m/z, NCE stepped 20–40-60. Raw MS^2 data was processed using Compound Discoverer (CD) v3.1 (Thermo Fisher Scientific) with minimum peak intensity at 1,000,000 and mass tolerance at 5 ppm. The mass identification was searched against mass libraries and databases provided by CD software, including Chemspider, mzVault, and mzcloud. The high confidence of identified metabolites was filtered with retention time ≥0.9 min, best match of mzVault and mzCloud ≥60%. Relative abundance was represented by normalized peak areas in UPLC-MS.

2.4 | Protein extraction, trypsin digestion, acetone precipitation and C18 tip treatment

Proteins analyzed in this study were extracted from the pellet generated during metabolite extraction, according to Salem et al. (2016). Briefly, the remaining pellets were washed with methanol and centrifuged at 20,000 *g* for 10 min at 4°C, four times. The washed pellets were dried at RT and extracted with 500 µL protein extraction buffer of 6 M urea, 2 M thiourea, 15 mM DTT, 2% CHAPS, 1% protease and phosphatase inhibitors. The samples were then sonicated for 10 min and incubated for 30 min at RT.

The protein concentration was determined using Roti®nanoquant (Roth) following the manufacturer's instructions. 100 µg of protein was digested with 500 ng $µl^{-1}$ of Trypsin (Thermo Fisher Scientific) at 37°C overnight. The digested proteins were precipitated once with 100% cold acetone (1:4 v/v) at −20°C for 2 h. Samples were centrifuged at 14,000 *g* for 30 min at 4°C. After removing the supernatant, proteins were precipitated once in 80% cold acetone at −20°C for 1 h, then centrifuged for 30 min at 4°C. The pellets were digested once with 500 ng $µl^{-1}$ of Trypsin overnight at 37°C. The resultant peptides were purified using 100 µL C18 Tips according to the manufacturer's instructions (Thermo Fisher Scientific). The eluted peptides were dried at RT and redissolved in 0.1% formic acid.

2.5 | Protein analysis

The peptide solutions (5 µL) were injected into a Thermo Easy-nLC coupled to Orbitrap Fusion™ LUMOS™ system (Thermo Fisher Scientific). Buffers and the gradient program for protein analysis are described in Table S1. The UPLC-MS^2 run was performed in three technical replicates for each biological sample.

A full scan for detecting peptides was set at 375–1,500 m/z in the Orbitrap at a resolution of 120,000. The peptides were detected in $ddMS^2$ mode with 30% HCD collision energy, and the first mass was fixed at 110 m/z. The obtained raw data was processed by Protein Discoverer v2.2.038 (Thermo Fisher Scientific). Parameters used for the analysis were as follows: enzyme was trypsin; two miss cleavage sites; mass tolerance of precursor ion was set at 10 ppm, and mass tolerance of fragment ions was set at 0.6 Da; acetylation for N-terminal modification, oxidation of methionine and deamidation of asparagine and glutamine for dynamic modifications, and carbamidomethyl for static modification.

The quality of proteins was ensured by removing contaminants, filtering with at least two peptides, and removing outliers. High-quality proteins were identified by searching against three-frame forward translated protein sequences from the bilberry transcriptome (Nguyen et al., 2018) and bilberry protein sequences obtained from a recent study (Wu et al., 2022) (https://www.vaccinium.org/bio_data/1085390). The functional classification of identified proteins was assigned to KOALA (KEGG Orthology And Links Annotation) using the BLASTKOALA tool (Kanehisa et al., 2016).

2.6 | Identification of hormone-related candidate genes and gene expression analysis by qRT-PCR

To identify genes involved in the biosynthesis of ABA, GA, JA, and SA, we searched for enzymes participating in these hormone pathways in the bilberry transcriptome dataset (Nguyen et al., 2018). The genes were searched for with reference protein sequences of other plant species (National Centre for Biotechnology Information - NCBI) using the BLASTX method, cutoff E-value <1e-5. Genes showing high sequence similarity with references were chosen for further evaluation of their expression levels over the four stages of bilberry using real-time quantitative reverse transcription PCR (qRT-PCR) (Table S2).

For gene expression analysis, total RNA from the same berry samples used for the metabolite and protein analyses was extracted using the method described by Jaakola et al. (2001). RNA was treated with Turbo™ DNase (Thermo Fisher Scientific) and then converted to cDNA using M-MLV Reverse Transcriptase (Promega) following the manufacturer's instructions. The qRT-PCR analysis was performed as described previously (Nguyen et al., 2018). Relative transcript levels were normalized based on a stable expression of reference genes, Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *SAND*. The analysis was performed in technical duplicates. Primer sequences used for qRT-PCR analysis are listed in Table S3.

2.7 | Statistical analysis

Principal component analysis (PCA) was conducted to examine the overall variance of all fruit samples. A heat map was done based on the PCA score, and hierarchical cluster analysis was performed using Canberra distance and average linkage agglomerative methods. Correlation analysis between metabolites and proteins was carried out using sparse partial least squares regression models, and the cutoff value for establishing a strong correlation was set to 0.7. The correlation network was performed and edited using Cytoscape v3.9.1. The analyses were carried out in R v4.2 with the *mixomics* package (Rohart et al., 2017). Datasets were scaled before analysis. Statistically significant differences in metabolites, proteins, and gene expression over the four different stages were analyzed using a one-way analysis of variance with an adjusted *p*-value of 5%. In the gene expression analysis, data were presented as mean ± standard error.

3 | RESULTS

3.1 | Changes in metabolite profiles during bilberry fruit development and ripening

To gain a better insight into metabolic changes during bilberry fruit ripening, berries from four different stages, i.e., S2, S3 (representing the development process), S4 and S5 (representing the ripening process) were collected for analyses (Figure 1A). A total of 1029 metabolites were putatively annotated and grouped into 11 classes, i.e., terpenoids, polyketides, phenylpropanoids, organic acids, nucleic acids and derivatives, lipids, flavonoids, carbohydrates, amino acids and derivatives, alkaloids, and others (Table S4). Based on the sum of the relative abundance of each class, we observed changes in the metabolite profiles throughout the development and ripening processes (Figure 1A). Primary metabolites were found to be most abundant in unripe berries (including lipids, nucleic acids, amino acids,

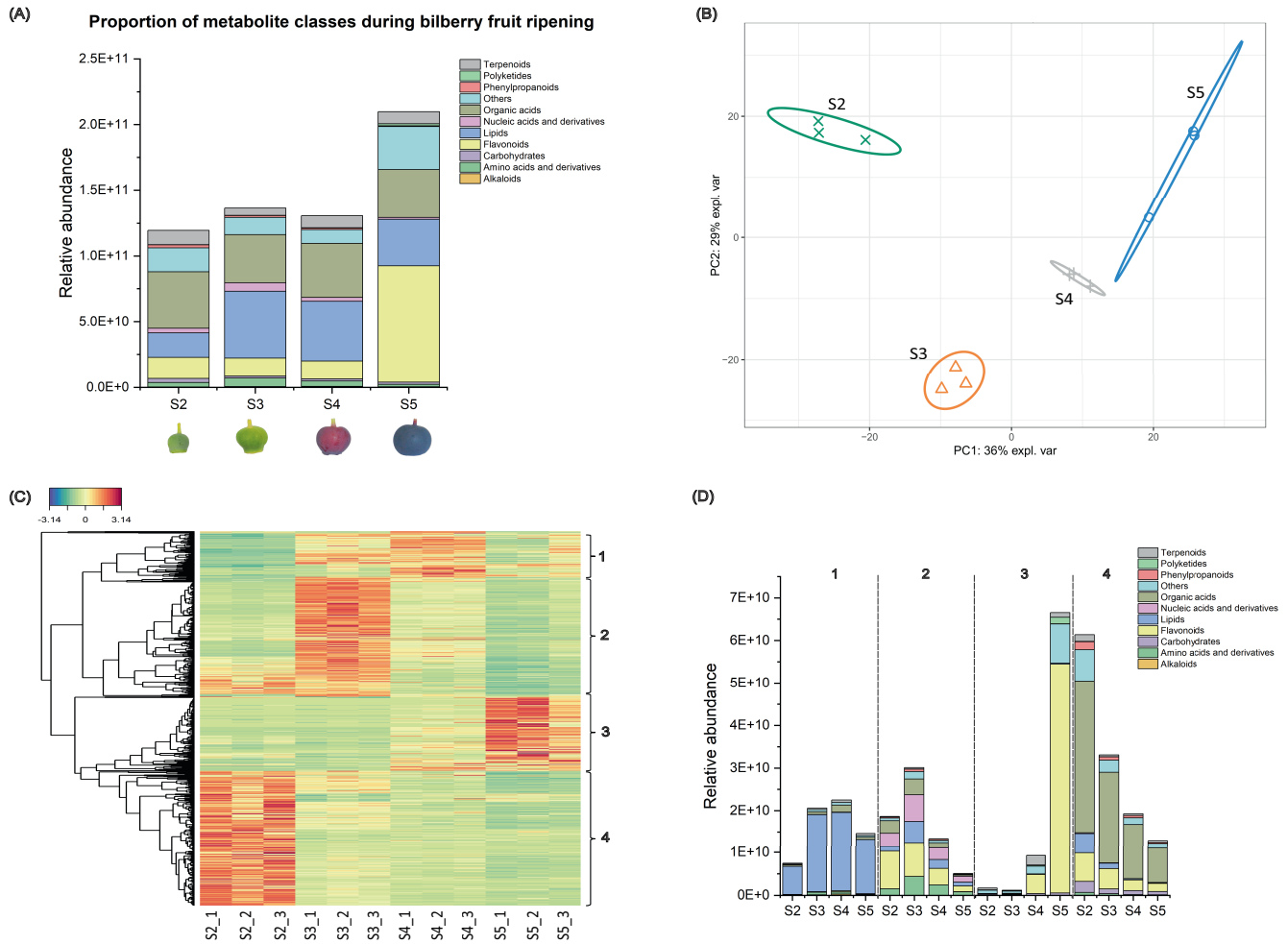

FIGURE 1 Metabolite profiles of bilberry development and ripening. (A) Proportion of metabolite classes in bilberry during the fruit development and ripening process. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit. (B) PCA of all identified metabolites of the four ripening stages. (C) Hierarchical clustering analysis and heat map of significantly different metabolites over the four stages. (D) Proportion of metabolite classes during fruit development and ripening in each group. Data represents relative abundance of each metabolite class and mean of three biological replicates.

organic acids, and carbohydrates), while ripe fruits were dominated by secondary metabolites such as phenolics (Figure 1A). PCA results of all identified metabolites demonstrated a clear separation of bilberry fruits in the developmental stages (S2 and S3) compared to fruits in the ripening stages (S4 and S5) (PC1). The metabolic profiles of S2 and S3 fruits could be clearly distinguished while ripening stage S4 and S5 fruits were only slightly separated (PC2) (Figure 1B).

Furthermore, 706 identified metabolites resulted in significant differences over the four stages, and the levels of which were clustered into four groups 1, 2, 3, 4, with 96, 216, 140, 254 metabolites, respectively (Figure 1C, Table S5). We observed that group 1 exhibited an increase in metabolite abundance from S2 to S3 and peaked at S4 (Figure 1D). Lipids were the dominant class in this group with the highest content of oleic acid, followed by octadecanoids, linoleic acid derivative, and palmitic acid. Caffeic acid was found to be the second most abundant metabolite in this group, its amount increased from S2 to S4 and then dramatically decreased in S5. Pavetannin B6 and a terpene glucoside were also highly accumulated in S4 (Figure 1D, Table S5).

In group 2, metabolites mainly accumulated during fruit development and decreased during ripening (Figure 1D). The results revealed various roles of these metabolites which can be divided into different classes including flavonoids (catechin), amino acids (tryptophan, phenylalanine), nucleic acids (adenosine, uridine, guanine, adenine), lipids (13(S)-HpOTrE, 12-oxo-phytodienoic acid, 9-HpODE), organic acids (benzoic acids, trans-cinnamic acid, ferulic acid, dihydrocaffeic acid), phenylpropanoids (lariciresinol 4-O-glucoside), and terpenoids (GA3) (Table S5).

Metabolites in group 3 notably increased in S5 and mostly belonged to the flavonoid class (Figure 1D, Table S5). Among them, cyanidin-3-O-beta-D-glucoside (anthocyanins) and tricin-5-O-β-D-glucoside (flavone) were the most abundant compounds, followed by flavonols (quercetin and quercetin glucosides, taxifolin, kaempferol-3-O-arabinoside, myricetin) and flavones (homoplantaginin, luteolin, diosmetin). Citric acid related to fruit flavour formation increased during ripening and reached the highest level at S5. Other phenolic acids, including gallic acid, vanillin, and gentisic acid, also increased from S2 to S5. We observed that sugar was accumulated during bilberry ripening, indicated by the highest abundance of glucose at S5 and the increase in the level of maltose, a product of starch breakdown, from S3 to S5 (Table S5, Figure S1). The amount of ABA significantly increased during fruit ripening, peaking at S4.

Group 4 was present in high abundance at S2, and it continuously decreased throughout the ripening process (Figure 1D). Organic acids were the main class accumulated in this group with the highest level of chlorogenic acid, followed by quinic acid, hydroxycinnamic acid and melilotoside, coumaric acid, and 4-methoxycinnamic acid (Figure 1D, Table S5). Flavonoids were the second most abundant class of group 4, including the highest level of PAs (epicatechin and epigallocatechin) and quercetin-3-O-glucuronide. Medium-chain fatty acids (dodecanoic acids, JA), phenylpropanoids (coumarins, lignans), methionine, and terpenoids (mono-, sesquiterpenoids, GA4) decreased from S2 to S5.

3.2 | Protein profiling during bilberry fruit development and ripening

In total, 1786 proteins were identified from all the fruit samples by searching against the bilberry databases (Nguyen et al., 2018; Wu et al., 2022) (Table S6), of which 1329, 1564, 1550, and 1458 proteins were found in S2, S3, S4, S5, respectively (Figure 2A). Among these, a high number of proteins (1078) were common to all the fruit samples (Figure 2B). However, the PCA of all proteins still revealed a separation between four different stages (Figure 2C).

We examined significant changes in the protein profiles during bilberry ripening that resulted in 1048 differentially expressed proteins over four stages clustered into three groups (Figure 2D, Table S7). Group 1 (359 proteins) and group 3 (444 proteins) were observed to have significant abundance at the developmental stages, S3 and S2, respectively. Group 2 indicated a high expression of 245 proteins at the ripening stages, S4 and S5 (Figure 2D). Moreover, the functional classification of these groups was annotated by the KEGG pathway database, which could provide an understanding of the processes occurring during fruit development and ripening. There were 255 proteins accounting for 71% of group 1, 144 proteins (58.5%) of group 2, and 258 proteins (58.1%) of group 3 assigned to 211, 162, and 190 KEGG pathways, respectively (Table S8). Groups 1 and 2 had the largest proportion of proteins related to metabolism, especially carbohydrate metabolism, indicating that the most essential activities for fruit growth and ripening are the formation, breakdown, and interconversion of carbohydrates (Figure 2E). The second largest KEGG pathway was genetic information processing that was highest present in group 3, indicating that transcription, translation, post-translation, replication, and repair are most active at S2 (Figure 2E). Proteins related to organismal systems and cellular processes were assigned more in group 3 than in groups 1 and 2, suggesting that activities at cellular and organismal levels are needed for the early developmental stages. There were more proteins related to the biosynthesis of other secondary metabolites in group 2, whereas amino acid and lipid metabolism-related proteins were higher in groups 1 and 3. The results were consistent with the accumulation of amino acids and lipids at the developmental stages and the accumulation of secondary metabolites (e.g., flavonoids) at the ripening stages in the metabolite analysis. The number of proteins related to energy metabolism was slightly higher in groups 1 and 3 compared to group 2, which could be explained by functional photosynthesis in the unripe berries (Figure 2E).

3.3 | Hormonal regulatory network controlling bilberry fruit ripening

To better understand the connections between plant hormones and the physiological processes during bilberry ripening, we examined the correlation between identified proteins and plant hormones, including ABA, GAs, JA, and SA (Table S5). Auxin and ethylene were excluded as they were not detected in the metabolite dataset; however, high

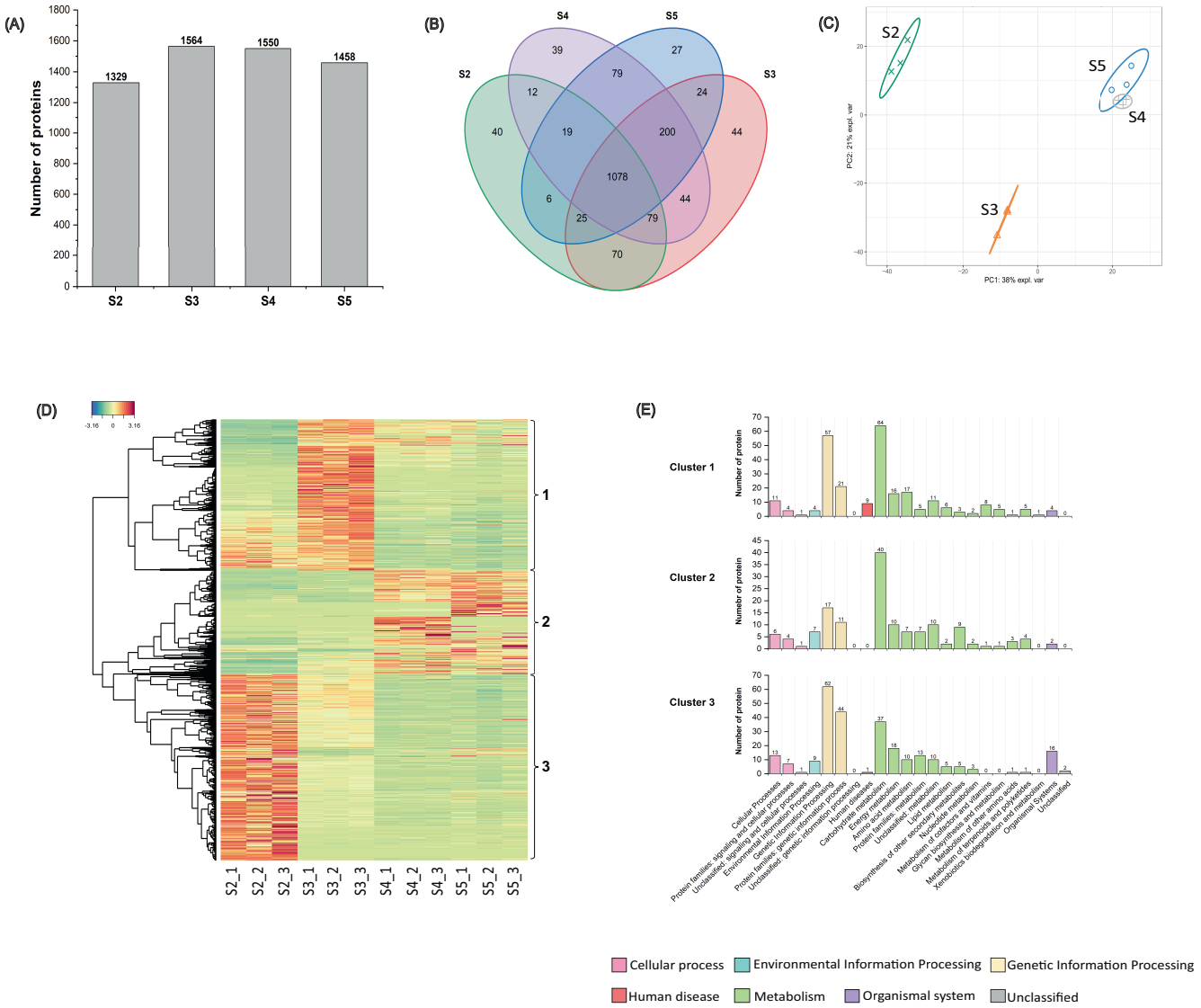

FIGURE 2 Proteomic profiles in bilberry fruits. (A) Total proteins identified in the four ripening stages. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit. (B) Venn diagram of all identified proteins in the four ripening stages. (C) PCA of 1078 identified proteins in the four ripening stages. (D) Hierarchical clustering analysis and heatmap of significantly different proteins over the four stages S2, S3, S4, and S5. (E) KEGG analysis for each protein cluster.

abundances of their precursors (i.e., tryptophan and methionine, respectively) were detected in the developmental stages (Table S5). Thus, the estimation of the dynamics of ethylene metabolism was made based on methionine metabolism.

The results showed that 628 proteins exhibited strong positive or negative correlations to changes in ABA, GA3, GA4, JA, and SA throughout fruit development and ripening (Figure 3A, Table S9). The proteins associated with ABA exhibited opposite correlations with other hormones. This is in line with the increase in accumulation of ABA at S4 (Figure 3B) in contrast with the higher levels of GA4, JA, and SA at S2 (Figures 3D-F) and GA3 at S3 (Figure 3C). Moreover, the correlation network revealed numerous connections between GA4, JA, and SA hormones, suggesting a collaborative role for these three hormones in regulating bilberry fruit growth.

A high number of proteins had a positive correlation with GAs, JA, and SA, indicating that most hormonally regulated physiological processes are mainly active during the developmental stages (Figure 3A, Table S9). The processes needed for the growing phase are related to nutritional preparation and energy metabolism, transcription, protein biosynthesis, cell proliferation, transport, and cell wall biogenesis/degradation. Several proteins related to ABA, GA, and auxin signalling pathways (e.g., chaperonin 20, bHLH164, and auxin binding proteins, respectively) were also found in the developmental stages. By contrast, there were fewer negative correlations with these hormones during the developmental stages, including processes related to gluconeogenesis, lignan biosynthesis, seed development, seed storage proteins, cell adhesion, and two proteins related to ABA and auxin signalling pathways, e.g., SAL1 phosphatase-like and

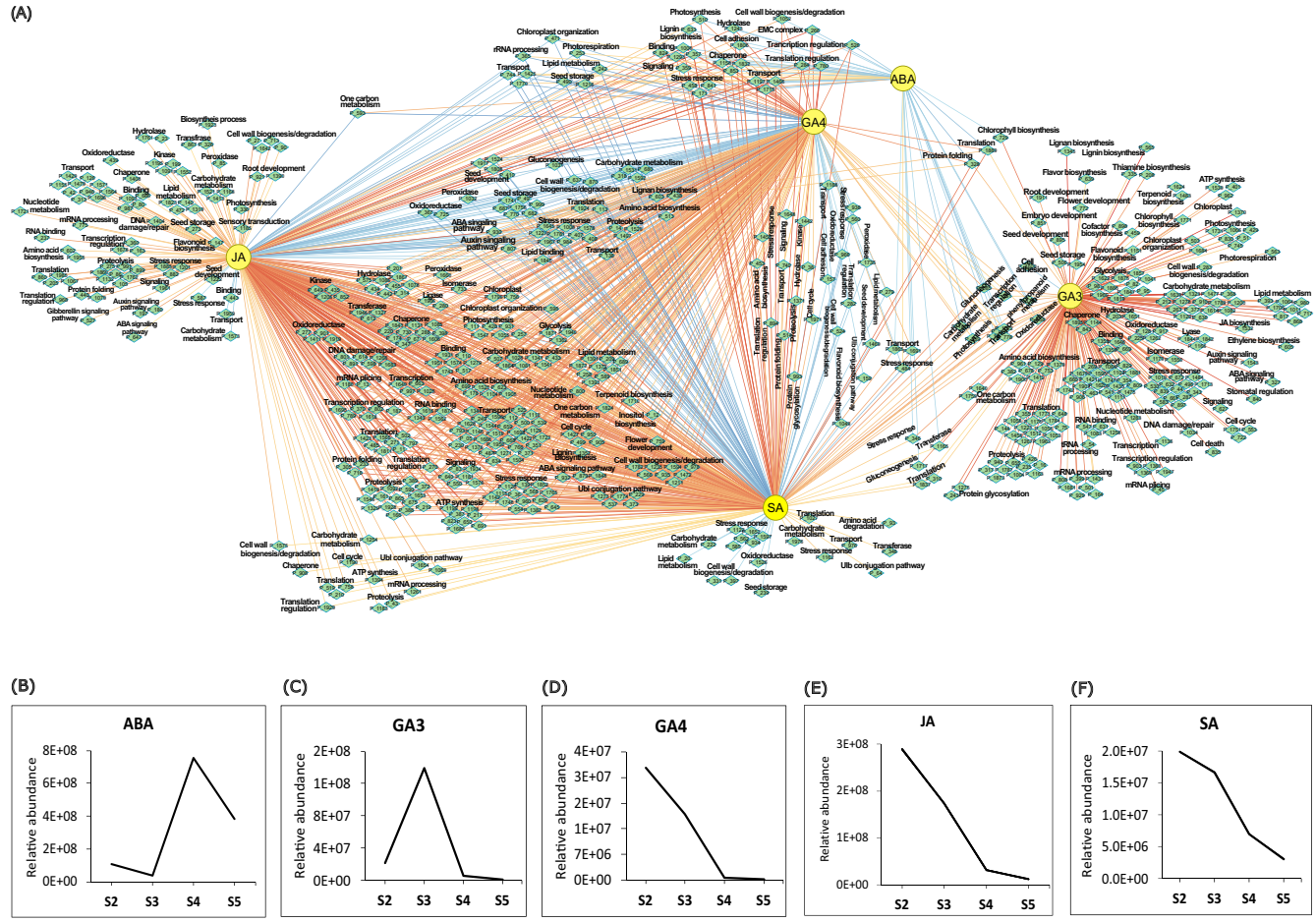

FIGURE 3 (A) Correlation network analysis of plant hormones (yellow circles) including abscisic acid (ABA), gibberellic acids (GA3, GA4), jasmonic acid (JA), and salicylic acid (SA) with significantly different proteins (green diamonds) during bilberry fruit development and ripening. The strong correlation coefficient was set to a threshold of 0.7. Blue edge indicates strong negative correlations (< −0.7), red edge indicates strong positive correlations (>0.7). Line charts represent the relative abundance of hormones (B) ABA, (C) GA3, (D) GA4, (E) JA, and (F) SA during bilberry development and ripening. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit.

patelin-5, respectively. This indicates that these activities may be involved in functions positively regulated by GAs, JA, and SA during the ripening process of bilberry.

3.4 | Biosynthesis of hormones during bilberry fruit development and ripening

A total of 20 genes involved in the biosynthetic pathways of ABA, GAs, JA, and SA were selected for further gene expression analysis to investigate the relationship between these hormones and bilberry fruit development and ripening (Table S2). We analyzed the expression of five genes involved in the ABA biosynthetic pathway, namely zeaxanthin epoxidase (*ZEP*), neoxanthin synthase (*NYS*, also called ABA deficient 4 - *ABA4*), *NCED*, short-chain dehydrogenase/reductase (*SDR*), and abscisic aldehyde oxidase (*AAO*) (Figure 4A, Table S2). The expression of *ZEP*, which encodes the enzyme responsible for the first step of the pathway converting zeaxanthin to violaxanthin, increased during the development process from S2 to S3 and decreased during the ripening process (Figure 4B). A similar expression trend was also observed in the late structural pathway genes, *SDR* and *AAO* (Figures 4E-F). By contrast, *NYS* and *NCED* showed significant upregulation in the ripening stages (Figures 4C-D). The expressions of the key genes *NYS* and *NCED* correspond to the increase in ABA level during fruit ripening (Figure 3B).

The biosynthetic pathway of GAs starts from converting common precursor Geranylgeranyl pyrophosphate to the intermediate ent-kaurene catalyzed by ent-kaurene synthase (KS) and ent-kaurene oxidase (KO) (Figure 5A). The expression of *KS* increased during fruit ripening and was sharply upregulated at S5 (Figure 5B), while *KO-like* was slightly downregulated at S4 (Figure 5C). However, no significant differences in the expression of these two genes during fruit ripening were observed. From the bilberry transcriptome, we only found two genes of the GA 2-oxidases (*GA2ox*) family involved in GA catabolism for regulating the concentration of bioactive GAs, named *Ga2ox8-like* and *Ga2ox1-like*. The expression of *Ga2ox8-like* was upregulated, and *Ga2ox1-like* was downregulated during the ripening process (Figures 5D-E). Moreover, from the transcriptome dataset, we found

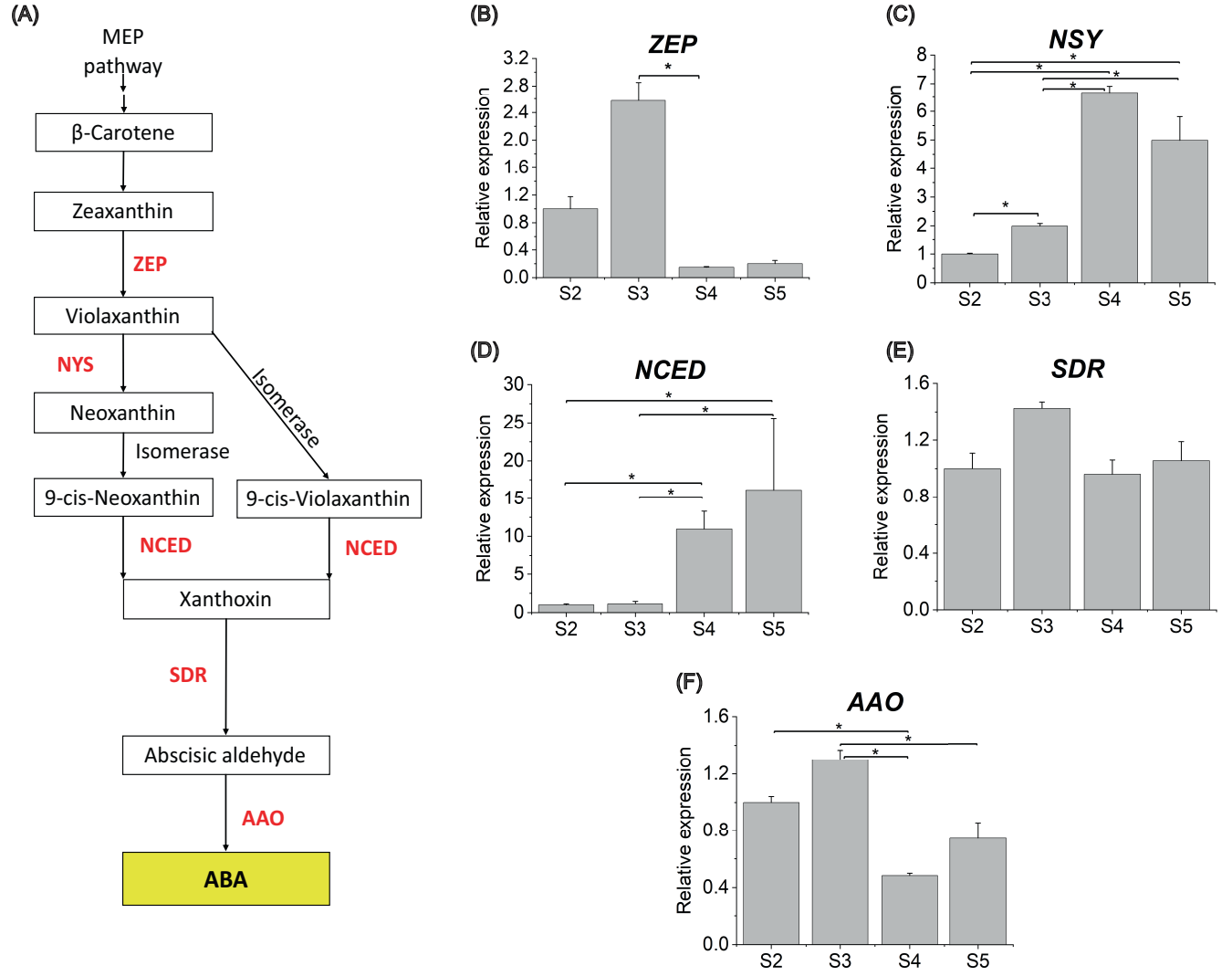

FIGURE 4 Abscisic acid (ABA) biosynthesis during the ripening process of bilberry. (A) Biosynthetic pathway of ABA. Yellow box indicates the compound detected in the metabolite analysis. Bar charts represent the relative expression of genes encoding enzymes (red text) involved in the biosynthesis of ABA during bilberry development and ripening: (B) *ZEP*: Zeaxanthin epoxidase, (C) *NYS*: neoxanthin synthase, (D) *NCED*: 9-cis-epoxycarotenoid dioxygenase, (E) *SDR*: short chain dehydrogenase/reductase, and (F) *AAO*: abscisic aldehyde oxidase. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit. Asterisks indicate significant differences among ripening stages at level $^*p < 0.05$ using one-way ANOVA.

two genes related to GA signalling, *GID1C-like* and gibberellin-regulated 1-like (*GR1-like*). The most prominent expression of *GR1-like* showed in S3 and then dropped thereafter (Figure 5F), correlating with the detection of two bioactive GAs (GA3 and GA4) in the developmental stages (S3 and S2, respectively) (Figures 3C-D).

In JA biosynthesis, we selected three genes carrying out the early steps of JA biosynthesis, namely linoleate 13 s-lipoxygenase 2 (*13-LOX2*), allene oxide cyclase (*AOC*), 12-oxophytodienoate reductase 3 (*OPR3*), and one gene involved in the biosynthesis of JA derivative jamonoyl isoleucine (JA-Ile), namely jasmonic acid-amido synthetase JAR1-like (*JAR1-like*) (Figure 6A, Table S2). The expression of the key genes *13-LOX2* and *OPR3* showed significant upregulation during fruit development (Figures 6B,D), which is consistent with the highest abundances of precursors α-linolenic acid (α-LA) and 12-oxophytodienoic acid at S3 (Figures 6F-G). *AOC* showed a slightly higher expression level at S2 and was relatively stable throughout the ripening process (Figure 6C). Even though we did not detect JA-Ile in the metabolite dataset, the *JAR1-like* gene was found to be significantly upregulated in S3 and then decreased in S4 (Figure 6E). Another JA derivative, cis-Jasmone, was detected with a peak at S4 (Figure 6H), however, the genes related to its biosynthesis have not been described and identified in our transcriptome dataset and therefore require further investigation.

SA is synthesized from chorismate via two different routes, i.e., the isochorismate pathway in chloroplast and the phenylalanine pathway in the cytosol (Figure 7A) (Lefevere et al., 2020). The expression of a gene encoding chorismate synthase (*CS*), responsible for the biosynthesis of a common precursor chorismate, highly increased at the onset of fruit ripening and decreased afterwards (Figure 7B). Compared to the accumulation of shikimic acid (Figure 7G), a precursor of

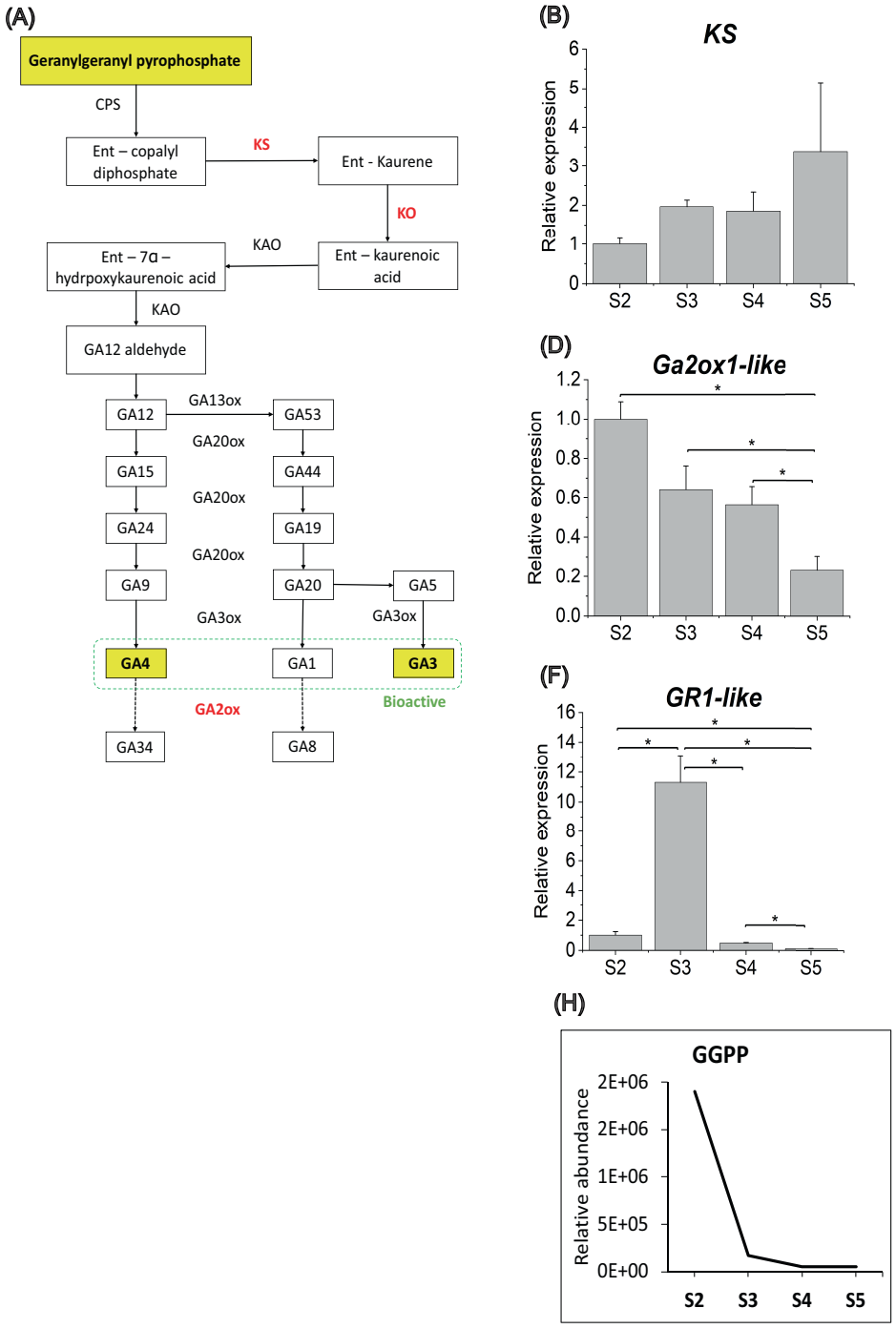

FIGURE 5 Gibberellin (GA) biosynthesis during the ripening process of bilberry. (A) Biosynthetic pathway of GA. Yellow boxes indicate the compounds detected in the metabolite analysis. Green box indicates bioactive forms of GAs. Dashed arrows indicate the GA catabolism pathway. Bar charts represent the relative expression of genes encoding enzymes (red text) involved in the biosynthesis of GAs during bilberry development and ripening: (B) *KS*: ent-kaurene synthase, (C) *KO*: ent-kaurene oxidase, (D) *GA2ox1-like*: GA 2 oxidase 1 like, (E) *GA2ox8-like*: GA 2 oxidase 8 like, (F) *GR1-like*: gibberellin-regulated 1 like, and (G) *GID1C-like*: gibberellin receptor GID1C. (H) Line chart represents the relative abundance of trans geranylgeranyl pyrophosphate (GGPP) during bilberry development and ripening. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit. CPS: *ent*-copalyl diphosphate synthase, KAO: ent-kaurenoic acid oxidase, GA13ox: GA 13 oxidase, GA20ox: GA 20 oxidase, GA3ox: GA 3 oxidase. Asterisks indicate significant differences among ripening stages at level $^*p < 0.05$ using one-way ANOVA.

chorismite, the expression trend of the *CS* gene was different during fruit development but similar during the ripening process. In the isochorismate pathway, enhanced pseudomonas susceptibility 1 gene (*EPS1*) encoding BAHD acyltransferase protein for synthesizing SA from isochorismate, was significantly upregulated at S3 and downregulated thereafter (Figure 7C). The expression of *EPS1* correlates with

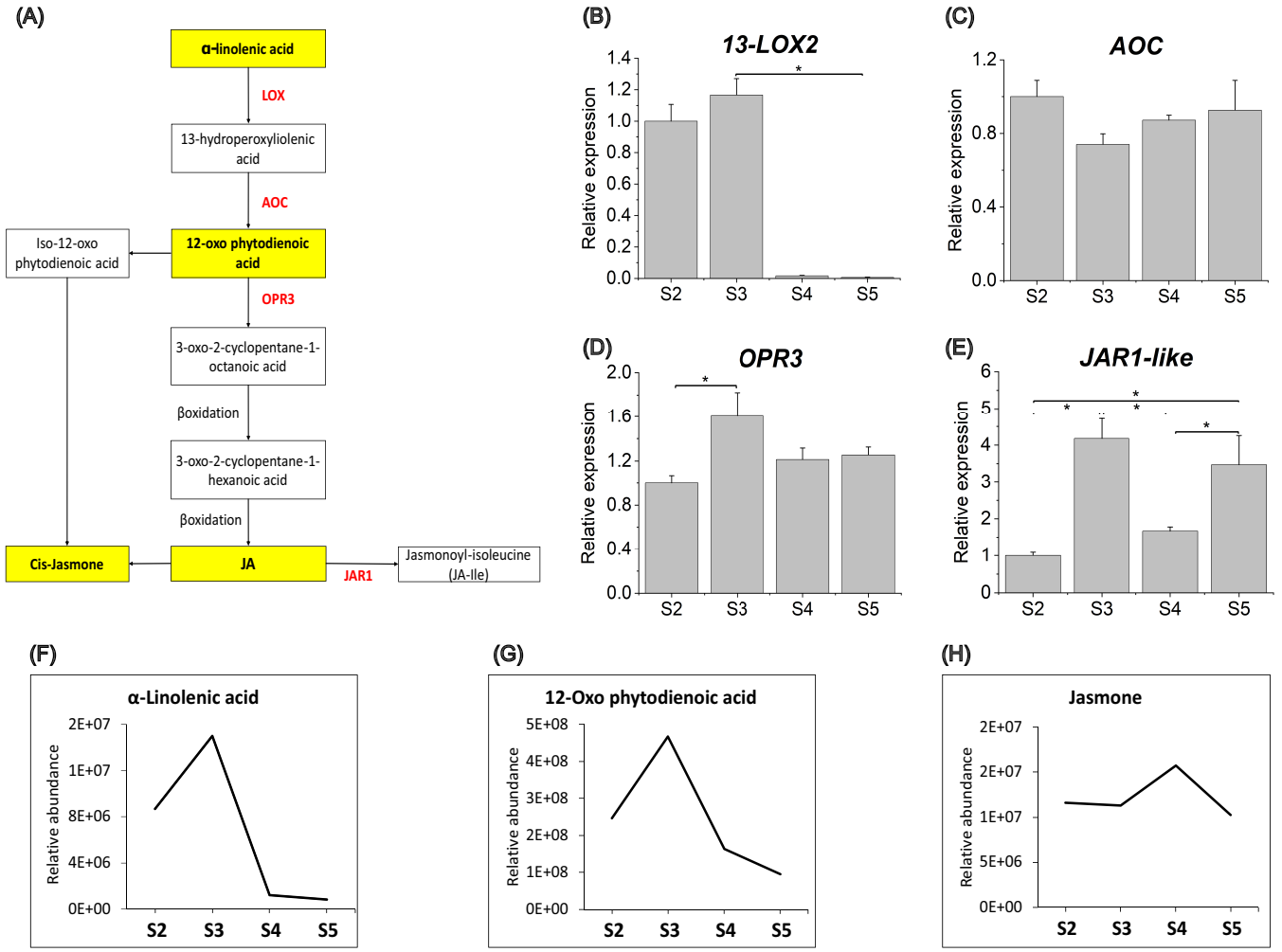

FIGURE 6 Jasmonic acid (JA) biosynthesis during the ripening process of bilberry. (A) Biosynthetic pathway of JA. Yellow boxes indicate the compounds detected in the metabolite analysis. Bar charts represent the relative expression of genes encoding enzymes (red text) involved in the biosynthetic pathway of JA during bilberry development and ripening: (B) *13-LOX2*: 13 s-lipoxygenase 2, (C) *AOC*: allene oxide cyclase, (D) *OPR3*: 12-oxophytodienoate reductase 3, and (E) *JAR1-like*: jasmonic acid-amido synthetase JAR1-like. Line charts represent the relative abundance of JAs and intermediates throughout bilberry fruit development and ripening: (F) α-linolenic acid, (G) 12-oxophytodienoic acid, and (H) Jasmone. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit. Asterisks indicate significant differences among ripening stages at level $^*p < 0.05$ using one-way ANOVA.

the level of SA from S3 to S5 (Figure 3F). In the phenylalanine pathway, a gene encoding phenylalanine ammonia-lyase (*PAL*) showed the highest peak at S4 (Figure 7D). However, not all the enzymes have been described for these pathways. We observed that the levels of benzoic acid, SA, and its derivative methyl salicylate (MeSA) decreased throughout fruit development and ripening (Figures 3F, 7J,K). Interestingly, we found genes in the transcriptome dataset encoding benzoate carboxyl methyltransferase (*BSMT-like*) and salicylic acid binding protein (*SABP2-like*) for conversion of SA to MeSA and vice versa, respectively, related to the SA homeostasis. These two genes showed similar expression patterns, which were relatively constant from S2 to S4 and were dramatically upregulated at S5 (Figures 7E-F).

Even though ethylene was not detected in the current study using the LC-MS analysis method due to its gaseous nature, a preliminary finding involved in ethylene biosynthetic pathway in bilberry (considered as a non-climacteric fruit) is reported by integrating metabolite, protein, and transcriptome datasets (Figure S2, Table S10). The abundances of precursor, methionine, and by-product, 5′-methylthioadenosine (MTA), decreased throughout fruit development and ripening, while the enzymes and their related transcripts, i.e., s-adenosyl-l-methionine synthase (SAMS), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and oxidase (ACO), showed different expressions during the ripening process (Figure S2).

4 | DISCUSSION

Our study provides comprehensive metabolome and proteome datasets of wild bilberry during the development and ripening process, which contribute to building database resources for future studies on wild and commercially grown berries (e.g., cultivated blueberries). Through data integration analysis, we revealed for the first time the

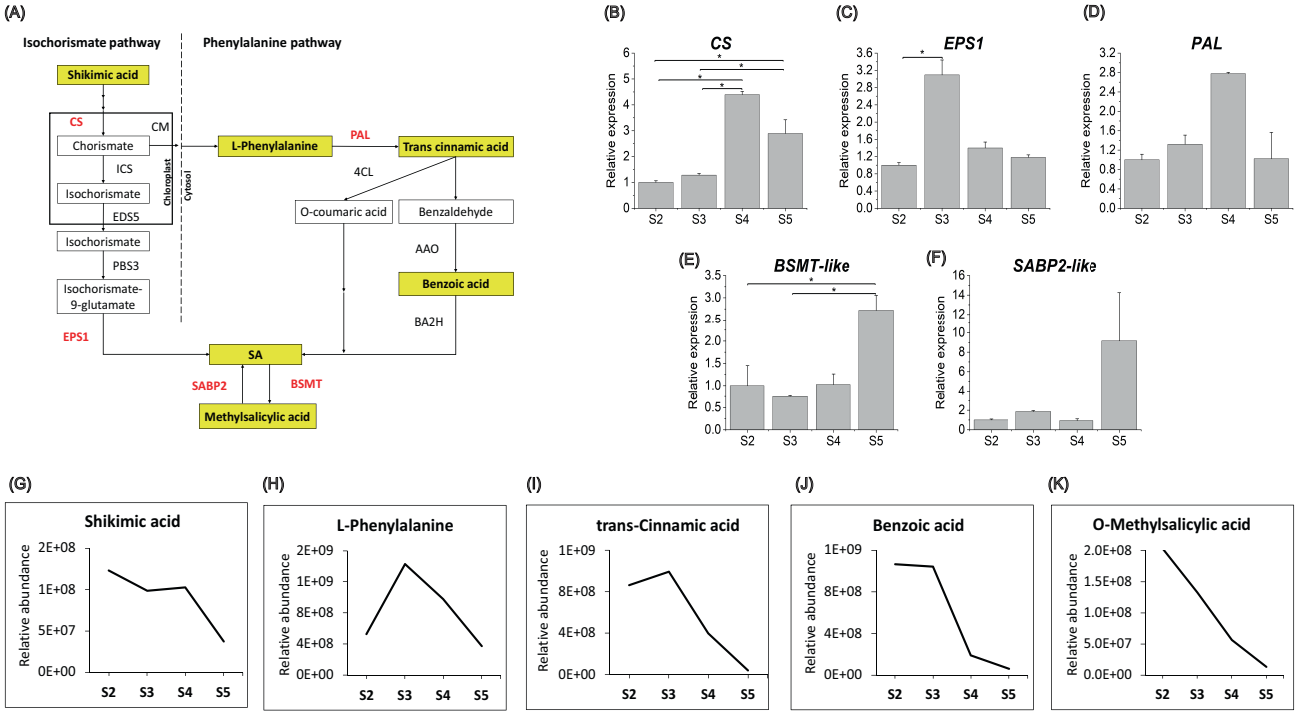

FIGURE 7 Salicylic acid (SA) biosynthesis during the ripening process of bilberry. (A) Biosynthetic pathway of SA. Yellow boxes indicate the compounds detected in the metabolite analysis. Bar charts represent the relative expression of genes encoding enzymes (red text) involved in the biosynthesis of SA during bilberry development and ripening: (B) *CS*: chorismate synthase, (C) *EPS1*: enhanced pseudomonas susceptibility 1, (D) *PAL*: phenylalanine ammonia-lyase, (E) *BSMT-like*: benzoate carboxyl methyltransferase, and (F) *SABP2-like*: salicylic acid binding protein. Line charts represent the relative abundance of JAs and intermediates throughout bilberry fruit development and ripening: (G) Shikimic acid, (H) L-phenylalanine, (I) trans-Cinnamic acid, (J) Benzoic acid, a (K) O-methylsalicylic acid. S2: small green fruit, S3: large green fruit, S4: purple ripening fruit, S5: dark blue ripe fruit. Asterisks indicate significant differences among ripening stages at level $^*p < 0.05$ using one-way ANOVA.

regulatory network of physiological processes associated with key hormones, i.e., ABA, GA, JA, and SA, which control the bilberry development and ripening process. In addition, we observed an increase in ethylene-related metabolism during developmental stages. Our findings serve as a valuable tool for identifying key requirements for fruit quality improvement in post-harvest storage and new breeding program strategies, such as aiming to enhance blue/red flesh and distinctive flavor. The data also opens opportunities to discover novel beneficial compounds in berry fruits with potential applications in foods, cosmetics, and pharmaceutical industries.

The datasets revealed significant differences in metabolite and protein profiles between and within bilberry developmental and ripening stages (S2 vs. S3 and S4 vs. S5) (Figures 1B, 2C), highlighting a clear metabolic shift from primary to secondary metabolites during ripening (Figures 1A, D). Indeed, primary metabolites serve as key precursors of various secondary metabolic pathways to fulfil adaptive features, including nutritional value and palatability characteristics, and plant defence system for ripe fruits and seed dispersal. For example, fruit volatiles are derived from free amino acids (e.g., phenylalanine, methionine, valine, leucine) and fatty acids (e.g., α-LA) (Pott et al., 2019). Methionine, tryptophan, and phenylalanine are known to be crucial for fruit ripening by participating in the biosynthesis of plant hormones, i.e., ethylene, auxin, and SA, respectively (Murch et al., 2000; Lefevere et al., 2020; Pattyn et al., 2021). Phenylalanine also serves as a general precursor of the central phenylpropanoid pathway (Singh et al., 2010; Dare et al., 2022). From the metabolite dataset, significant levels of several amino acids, including tryptophan, phenylalanine, methionine, and glutamate, were found in unripe berries, while tyrosine mostly accumulated at the onset of ripening (Table S5). The results are consistent with previous reports on bilberry (Dare et al., 2022) and other fruits (Diboun et al., 2015). Moreover, the significant level of phenylalanine in S3 may be related to the upregulation of the *PAL* gene encoding the first enzyme utilized in the pathway (Table S5, Figures 7D,H). This indicates that phenylalanine is an important primary compound in bilberry ripening, aligning with the fact that phenolics are the main metabolites in bilberry, with flavonoids being the most abundant in ripe fruits (Figure 1D) (Pires et al., 2020). We also observed a high level of 5'-S-Methyl-5'-thioadenosine (or 5'-methylthioadenosine – MTA), in S2 (Table S5). MTA is a by-product of ethylene synthesis (Figure S2) and serves as an intermediate of the methionine salvage pathway/Yang cycle for methionine recycling (Pattyn et al., 2021). This finding proposes the important role of methionine in the developmental stages and implies the biosynthesis of ethylene in bilberry.

The regulation of energy was also demonstrated for bilberry development. For example, adenosine, a crucial precursor of energy carriers, showed the highest level in S3 (Table S5), proposing the contribution to energy metabolism or possibly related to signalling

required for fruit growth (Pietrowska-Borek et al., 2020). We also observed that the primary processes occurring in the developmental stages are related to photosynthesis, ATP synthesis, and carbohydrate/lipid metabolism (Table S9). Currently, photosynthesis in unripe fruits and a decrease in its activity and chlorophyll contents during ripening are poorly understood. In tomato, photosynthetic activity has been considered essential for seed development (Lytovchenko et al., 2011). A recent review has discussed the function of fruit photosynthesis, such as involvement in photoassimilate production (e.g., sugar/starch) in the early developmental stages and in supplying carbon sources in the later stages (Garrido et al., 2023). Concerning other energy-rich compounds, lipid storage for ripe fruits was demonstrated by their high abundances found in the developmental process (S3) and in the onset of ripening (S4) (Table S5) in the present work. This corresponds to the highly active processes related to lipid metabolism observed in S3 (Figure 3A, Table S9). High mono- and polyunsaturated fatty acid levels detected in oil extracts from bilberry seeds (Gustinelli et al., 2018) indicate that lipid biosynthesis may be prioritized for seed development. Elucidating the processes of nutrient accumulation throughout fruit development and ripening could provide valuable insights into the factors governing the characteristics of ripe fruits, facilitating improvements in fruit quality.

During development, fruit size increase is determined by cell division and expansion (Karim et al., 2022). These activities are associated with the formation and organization of the cell wall. In this study, the proteins involved in cell wall biosynthesis, organization, and expansion exhibit the highest level in the developmental stages and significantly decrease thereafter, whereas enzymes associated with cell wall loosening increase in the ripening stages (Table S9). This explains the increase in fruit size and softening during bilberry ripening. Interestingly, we found significant decreases in the levels of lignin derivatives and its precursors, and proteins related to lignin biosynthesis throughout the development and ripening process (Tables S5, S9). Lignin is one of the important components of cell walls and plant fibers (Liu et al., 2018), and therefore, the fruit firmness could also be affected by the changes in the level of lignin and its related compounds.

The essential role of GA in promoting cell division and expansion has been well documented (Fenn and Giovannoni, 2021). A high content of endogenous GAs was previously found in unripe strawberries (Csukasi et al., 2011) and tomatoes (Li et al., 2019b). Moreover, the application of exogenous GA delays fruit ripening and softening by inhibiting cell wall degradation, aroma, and ethylene production in postharvest kiwifruit (Yang et al., 2023), and tomatoes (Li et al., 2019b). In our correlation analysis, we observed high levels of GAs in the developmental stages have a positive correlation with different physiological processes, including cell wall modification (Figures 3A,C-D). Additionally, GAs also exhibited a strong correlation with JA and SA during development (Figure 3A). Several studies have demonstrated that exogenous JA and SA delay the ripening process (Valero et al., 2011; Jia et al., 2016; Kumar et al., 2021). Our study detected that the levels of JA, SA, and their derivatives, as well as the expression of related genes in bilberry, declined during the ripening process (Figures 3E-F, 6, 7) similarly to those previously observed in grape (Coelho et al., 2019) and sweet cherry (Fresno and Munné-Bosch, 2021). These findings highlight the key roles and interplay of GAs, JA, and SA in regulating the developmental process of bilberry. Importantly, genes related to GA, JA, and SA homeostasis, which are crucial for maintaining the balance between endogenous hormones in the regulation of fruit ripening (Fenn and Giovannoni, 2021), were identified in this study, particularly genes *Ga2ox1-like* and *Ga2ox8-like* involved in GA catabolism (Figures 5D-E), *JAR1-like* related to synthesize JA-Ile (Figure 6E), and *BSMT-like* and *SABP2-like* controlling levels of SA and MeSA (Figures 7E-F).

The changes in fruit flavour and colour that distinguish between the development and ripening stages occur simultaneously with fruit softening. In this study, we provide knowledge on different flavour-related compounds in the developmental and ripening stages, which have not been extensively studied in bilberry. Particularly, quinic acid, courmarins, and benzoic acids represent key flavour compounds in young bilberry, whereas citric acid, gallic acid, vanillin, and ascorbic acid seem to mainly contribute to the flavouring of ripe bilberry (Table S5). According to the flavour profiles in our study and previous studies on bilberry (Suvanto et al., 2020; Dare et al., 2022), strawberry (Amil-Ruiz et al., 2011) and apricot (García-Gómez et al., 2020) the sour, bitter, and acrid taste of unripe berries could be related to the dominant levels of PAs and chlorogenic acid. In contrast, the sweetness of bilberry is determined by the content of fructose and glucose in ripe fruits (Dare et al., 2022; Samkumar et al., 2022), which is also corroborated in this study with the highest glucose level in S5 (Table S5). Regarding fruit colour change during the ripening process, noticeable metabolites in the ripe bilberry were flavonoids, with dominant levels of cyanidin-3-o-beta-D-glucoside and high abundances of flavones and flavonols (Table S5), in agreement with previous report (Dare et al., 2022).

The role of ABA in non-climacteric fruit ripening has been extensively studied with a focus on the ABA level and the upregulation of key biosynthetic gene, *NCED*, in the ripening stages, as demonstrated in studies on strawberry (Kim et al., 2019), wolfberry (Li et al., 2019a), and grape (Coelho et al., 2019), consistent with our results. Furthermore, ABA has also been shown to be involved in promoting anthocyanin pigment biosynthesis and inducing cell wall metabolism-related genes during fruit ripening (Pilati et al., 2017; Karppinen et al., 2018; Chung et al., 2019; Li et al., 2019a). Our observation in bilberry reveals the positive correlation between ABA level and flavonoids, sugars, and flavour compounds, as well as with processes related to cell wall modification and seed storage proteins (Figure 3A, Table S9). These results confirm the role of ABA in bilberry ripening and support the assumption of bilberry classification into non-climacteric fruits.

Recently, Watanabe et al. (2021) have pointed out controversy in the classification of blueberries as a non-climacteric or climacteric type because some of the earlier studies since 1967 have detected the ethylene peak during blueberry ripening (cf. Watanabe et al., 2021), whereas some have not but have detected the increase in ABA concentration instead (Zifkin et al., 2012; Chung et al., 2019). To our knowledge, an ethylene peak has not been detected during bilberry ripening; however, our datasets indicate an activation of ethylene biosynthesis during bilberry development (Figure S2). Therefore, a

similar question may be raised about the bilberry fruit type. Moreover, a new perspective on fruit classification has recently emerged based on starch metabolism during fruit ripening due to the occurrence of both climacteric and non-climacteric types within the same species, e.g., melons, Asian pears, and plums (Chervin, 2020). It has been proposed that starch synthesis begins at the onset of ripening of climacteric fruits, while starch breakdown is found throughout the developmental and ripening process of non-climacteric fruits. A similar pattern of starch metabolism in non-climacteric types was observed in bilberry fruit ripening (Figure S1, Table S4, Table S11). We suggest the need to revisit the classification of climacteric and non-climacteric fruits. If most of the fruits have activation of both ethylene and ABA biosynthesis during fruit development, the borderlines on the levels of these hormones at the onset of ripening and the dynamics of starch contents should be explored and determined to clarify the classification of climacteric and non-climacteric types. The new findings will provide insights into the mechanism controlling the ripening process of berry species.

AUTHOR CONTRIBUTIONS

KT and HH conceived the research idea. KT and NN planned and designed the experiment. UB supervised the UPLC-MS analysis and preprocessing of the data. NN performed the experiments, analyzed the data, and wrote the manuscript. KT, SJL, and LJ discussed the results and revised the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (https://www.ebi.ac.uk/pride/) partner repository with the dataset identifier PXD047290 and project DOI 10.6019/PXD047290.

ORCID

Nga Nguyen https://orcid.org/0000-0002-2332-0889
Ulrich Bergmann https://orcid.org/0000-0002-8684-9640
Laura Jaakola https://orcid.org/0000-0001-9379-0862
Hely Häggman https://orcid.org/0000-0001-7381-1750
Soile Jokipii-Lukkari https://orcid.org/0000-0002-8935-1469
Katalin Toth https://orcid.org/0000-0001-7422-2210

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SUPPORTING INFORMATION

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