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Genetic and Hormonal Regulation of Sweet Cherry (*Prunus avium* L.) Maturity across Altitudinal Gradients

Diana Nacouzi and Walid El Kayal *

Faculty of Agricultural and Food Sciences, American University of Beirut, Riad El Solh, P.O. Box 11-0236, Beirut 1107-2020, Lebanon
* Correspondence: we21@aub.edu.lb

Abstract: Lebanon is recognized as a major producer of sweet cherries (*Prunus avium* L.) within the Mediterranean region. This non-climacteric fruit is grown at various altitudes, leading to considerable variation in maturity dates among cultivated varieties and altitudes and subsequently influencing harvest timing. The interaction between genotype and environment significantly affects fruit maturity dates and physicochemical attributes. Fruit maturation entails the regulated activity of numerous genes. In this study, we analyzed gene expression in the berries of six sweet cherry varieties ("Skeena", "Teliani", "Banni", "Feraouni", "Mkahal", and "Irani") cultivated at five locations, ranging from 1130 m to 2080 m above sea level, from May to July. This research focused on the genes potentially associated with auxin response factors, Abscisic acid receptors, ethylene receptors, gibberellin, and cytokinin regulations. Additionally, hormone analysis encompassing Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA) quantification was conducted on the same samples. The results revealed significant differences in gene expression concerning harvest dates, varieties, and locations. Abscisic acid and Salicylic acid exhibited higher concentrations in the tested fruits throughout the season. Benzyl Adenine had the lowest detected content in fruits. Data also revealed dynamic changes in phytohormones, especially ABA content, among varieties. When comparing phytohormones for different harvest dates in the same location, significant differences were observed. This work contributes to a deeper understanding of the role of plant hormones and their gene expression in the maturation of non-climacteric fruits.

Keywords: sweet cherries; gene expression; hormones; maturity

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1. Introduction

Sweet cherries are among the most widely consumed fruits globally, cherished for their distinct sensory characteristics and antioxidant properties. Ongoing efforts aim to enhance fruit quality, focusing on prolonging shelf life and augmenting fruit size and firmness, especially since this non-climacteric fruit is typically cold-stored post-harvest to prevent over-ripening before reaching national and international markets [1].

The timing of fruit maturity holds significant importance in crop production [2]. Fruit phenology encompasses phenotypic variations, primarily genetically controlled and profoundly influenced by the environment [3]. Plant hormones, known for their role in regulating plant growth and development, have emerged as potential enhancers of yield, fruit quality, and post-harvest traits [4]. The phytohormone family includes Abscisic acid (ABA), auxin (AX), brassinosteroids (BRs), cytokinins (CKs), ethylene, gibberellins (GAs), Salicylic acid (SA), strigolactones, and jasmonates (JAs) [5].

Research has outlined a three-stage growth pattern for sweet cherries, as follows: an initial phase of exponential growth expressed by cell division and expansion; a subsequent phase characterized by endocarp hardening and embryo development, initiating the accumulation of carbohydrates and anthocyanins; culminating in a third stage of rapid growth and cell expansion [6]. Being non-climacteric, the maturation process, fruit ripening,

and color development in sweet cherries are notably associated with Abscisic acid (ABA). Molecular and physiological events occurring during color initiation and development are attributed to the upregulation of genes related to the anthocyanin or ABA pathways [7]. The interaction between ABA and Indol Acetic Acid (IAA) at the onset of fruit ripening has been investigated [8]. In contrast, climacteric fruits like tomatoes and avocados experience an increase in ethylene production and respiratory activity at the onset of ripening [6]. Several studies have demonstrated that ABA application accelerates the ripening of many species by increasing anthocyanin content, sugar accumulation, and softening, while decreasing titratable acidity [9]. The effects of Gibberellic acid (GA3) on sweet cherry fruit quality have been documented. Fruits treated with GA3 exhibited increased size, berry weight, and firmness compared to untreated fruits. Furthermore, the pre-harvest application of GA3 delayed fruit maturity, enhanced cold storage quality and shelf life, and reduced pedicel browning [10]. The impact of Benzyl Adenine (BA) on the post-harvest quality of sweet cherries has also been investigated. Research indicates that the application of Benzyladenine and Gibberellin increased fruit weight and delayed sweet cherry maturity. Fruits treated with BA and BA+GA 4+7 were significantly heavier and larger compared to the untreated control [10]. Cytokinins (CKs) are phytohormones that are crucially involved in various physiological and morphological processes in plants, including seed germination, apical dominance, shoot branching, flower and fruit development, productivity, increased stress tolerance, and overall plant morphology [11]. Pre-harvest treatments with Salicylic acid (SA) and acetylsalicylic acid (ASA) on sweet cherry fruits have been shown to delay the ripening process by enhancing the levels and activity of antioxidant compounds and enzymes [12].

Despite the potential use of phytohormones such as gibberellins, auxins, and cytokinins to enhance sweet cherry fruit quality in the agricultural sector, knowledge regarding their impact on fruit maturity remains limited. This study aims to advance our understanding of the genetic expression of fruit maturity genes and their interaction with the environment throughout the harvesting season for cherry fruits grown in Lebanon. The results intend to complement phenotypic data collected from previous physicochemical analyses, focusing on essential horticultural traits such as fruit size, berry weight, sugar content, fruit firmness, dry matter, and acidity [1]. Additionally, this study examines hormone profiles, including measurements of Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA) concentrations in Lebanese cherry orchards, correlating these with variations in altitudes during the growth and ripening of this fruit.

2. Materials and Methods

Traditional Lebanese cherry varieties were handpicked between May and July from different Lebanese orchard locations and altitudes for fruit maturity analysis. These locations included Arsal (2080 m), Qousaya (1130 m), Kaa El Reem (low altitude 1280 m and high altitude 1600 m), and Barqa (1930 m) (Supplementary Figure S1). The main evaluated cherry varieties were “Feraouni”, “Banni”, “Irani”, “Mkahal”, “Teliani”, and the recently introduced “Skeena” variety.

Sweet cherry fruit maturity was determined using a combination of factors due to the variability of orchards and fruit ripening on the trees, in addition to the unpredictability of ripening environments. On this basis, fruits were considered close to maturity based on their physiochemical characteristics such as caliber, berry weight, berry color, total soluble solids content, titratable acidity, fruit dry matter, and fruit firmness. Each sweet cherry variety is defined by its specific physiochemical characteristics that are actively evolving over fruit maturation in pre-harvest stages.

Sweet cherries are generally affected by altitude differences and the ripening process can vary in length between a few days and a few weeks, according to the varieties and locations of the fruit. Multiple harvests were conducted to target the semi-ripe and ripe stages to study the evolution of fruit ripening over this period and to understand the

interaction between gene expression and hormonal regulation. Moreover, multiple harvests were conducted because growers cannot set a fixed harvesting date, due to the high interplay between fruit maturity and environmental conditions.

The physicochemical characteristics of these fruits were presented per variety from all locations where this variety is planted during its ripening season in a previous study by [1]. The study described the variation of fruit diameter DIAM (mm), berry weight (g), dry matter DM (%), titratable acidity TA (g/L), and total soluble solids TSS (°Brix) in cherry varieties between different locations over the harvesting season. Supplementary Table S1 describes the maturity stages of these samples.

A total of 40 samples were used in this study. Every sample was collected from 30 to 40 trees representing every orchard. In total, 30 fruits were collected from every tree. The orchard was split into rows, and fruits were harvested from each row to ensure the representativeness of the experiment; then, samples were sent to the laboratory on the same day for gene expression and hormone quantification analysis.

2.1. RNA Extraction and Quantitative Real-Time PCR Analysis

A total of 40 samples of frozen cherry berries, collected from five different locations and six different varieties (Supplementary Tables S2 and S3), were ground into powder using liquid nitrogen throughout the harvesting season. Total RNA extraction followed the procedure outlined in a previous study [13]. Quantitative reverse transcription PCR (qRT-PCR) was then conducted for a selection of 12 genes, carefully chosen to encompass a range of biological functions related to hormones during the ripening process and various developmental stages (Supplementary Table S2). Gene-specific primers were designed using Primer Express (v3.0, Applied Biosystems, Carlsbad, CA, USA; Supplementary Table S4. Two micrograms of total RNA were treated with DNaseI (Bio-Rad, Hercules, CA, USA) prior to cDNA synthesis using an iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). PCR reactions were performed in a 10 µL solution, containing SYBR Green master mix (0.2 mM dNTPs, 0.3 U Platinum Taq Polymerase (Invitrogen (Waltham, MA, USA)), 0.25× SYBR Green, 0.1× ROX, 20 ng of cDNA, and 300 nM of each primer. Three biological and three technical replicates for each reaction were analyzed using a CFX96 Real-Time PCR Detection System (BioRad, Mississauga, ON, Canada) with a first step of 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melting curves were generated using the following program: 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s. The expression of each gene was normalized to that of two reference genes (Actin 7 and Histone 3) and was quantified using the $2^{-\Delta\Delta Ct}$ method [14]. The results of the target genes' relative transcript abundance are presented as a mean value of the three assay replicates, compared with the mean of the three control values.

2.2. Detection and Quantitation of Phytohormones Using UPLC/MS

A total of 300 mg of whole cherry fruits, excluding the seeds, using the same fruit sampling method previously described, were then suspended in 0.5 mL of extraction solvent, which was composed of 75% methanol (MS Grade, Fisher Scientific, Mississauga, ON, Canada; MeOH) and 5% formic acid (MS Grade, Fisher Scientific, Canada) in Milli-Q water. Samples were then held at −20 °C for 1 h, spun down (15 min, 4 °C, 14,000 rpm), and the supernatant was removed. A second extraction was performed on the same sample using similar conditions as described above and the supernatants were pooled. The pooled supernatant was then evaporated to dryness using nitrogen gas in a fume hood. The dried samples were reconstituted using 200 µL of buffer solution (formic acid: acetonitrile = 95:5) and were then filtered through a 0.22 µM centrifuge filter (Millipore; 1 min, 13,000 rpm). The filtered supernatant was then transferred to a 96-well collection plate and centrifuged (15 min, 4 °C, 14,000 rpm), before 100 µL of supernatant was transferred to a polypropylene vial (300 µL, 12 × 32 mm). All standards were of analytical grade and were purchased from Sigma Aldrich, Canada. Phytohormones were separated using reverse phase liquid chromatography (ultra-performance liquid chromatography system (UPLC); LC-40D MS,

Shimadzu, Kyoto, Japan) via the injection of a 5 µL aliquot of sample onto a Shim-pack Scepter LC column (2.1 × 50 mm, 1.9 µm; Mandel Scientific Company, Guelph, ON, Canada). Metabolites were separated with a gradient of solvents A (0.1% formic acid) and B (100% methanol), with initial conditions at 95% A (5% B) that increased to 5% A (95% B) over 4 min, using a curve of 0. The column temperature was 40 °C and the flow rate was 0.2 mL/min. Metabolite peaks were identified by comparison to standards and were quantified using a standard curve generated using a similar separation method and gradient conditions. Phytohormones were detected using a single quadrupole mass spectrometer (LCMS 2020, Shimadzu, Kyoto, Japan) in single ion recording mode (SIR). Phytohormones such as SA (137 m/z) and GA3 (345 m/z) were detected in negative mode, whereas Zeatin (220 m/z), ABA (265 m/z), and BA (226 m/z) were detected in positive mode. In all cases, the probe temperature was set to 250 °C with a gain of 5; the capillary voltage (positive and negative) was set to 0.5 kV. Instrument limits of detection were 1.52, 1.52, 6.1, 0.925, and 0.308 pg/mL for SA, Zeatin, GA3, ABA, and BA, respectively, and method detection limits were found to be 5.06 pg/g, 5.06 pg/g, 20.34 pg/g, 1.27 pg/g, and 0.308 pg/g. The linear range for each analyte was 5.94–6.25 µg/mL, 5.94–6.25 µg/mL, 24.4–25 µg/mL, 1.52–1.56 µg/mL, and 0.308–1.56 µg/mL for SA, ABA, GA3, Zeatin, and BA, respectively.

2.3. Statistical Analysis

A statistical comparison of ΔCt values for every sample was performed using a one-way ANOVA parametric test followed by a Student–Newman–Keuls (SNK) post hoc test to compare relative gene expression and hormone quantification among samples of the same variety from two or more orchard locations and altitudinal gradients at a particular collection date. The assumptions for ANOVA were that the ΔCt groups had Gaussian distribution and equal variance. However, when these assumptions were not valid, as in many real-time PCR experiments, as a non-parametric alternative to ANOVA, the Kruskal–Wallis test was used, followed by Dunn's test. Differences between samples were considered statistically significant at a p-value < 0.05 using RStudio (R 4.3.0) statistical software. The potential genes implicated in fruit maturity were compared in terms of their relative expression and hormone quantification for the same variety at different locations harvested on the same date.

In order to identify patterns or trends in gene expression across the chosen genes and time points that could provide insights into how these genes are regulated during the ripening process in a non-climacteric fruit such as sweet cherries growing at varying altitudes, qRT-PCR was conducted for a set of 12 genes, meticulously selected to cover a spectrum of hormone categories. These genes are Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9).

The expression of these 12 genes involved in fruit maturity was studied, as follows: the Feraouni cherry variety was evaluated between 31 May 2021 and 5 July 2021; the Skeena cherry variety was evaluated between 21 June 2021 and 28 June 2021; the Irani cherry variety was evaluated between 31 May 2021 and 28 June 2021; the Mkahal cherry variety was evaluated between 3 June 2021 and 5 July 2021; the Banni cherry variety was evaluated during the period between 21 June 2021 and 5 July; and the Teliani cherry variety was evaluated between 31 May 2021 and 10 June 2021.

A Principal Component Analysis (PCA) was applied on the hormones involved in fruit ripening and maturity to determine the most important ones, to observe clustering behaviors among the tested varieties, and to understand their interaction with the different harvested locations.

3. Results

3.1. Gene Expression

Figure 1 illustrates the transcript levels of the Feraouni cherry variety, evaluated between 31 May and 5 July, for the expression of the 12 genes involved in hormone biosynthesis.

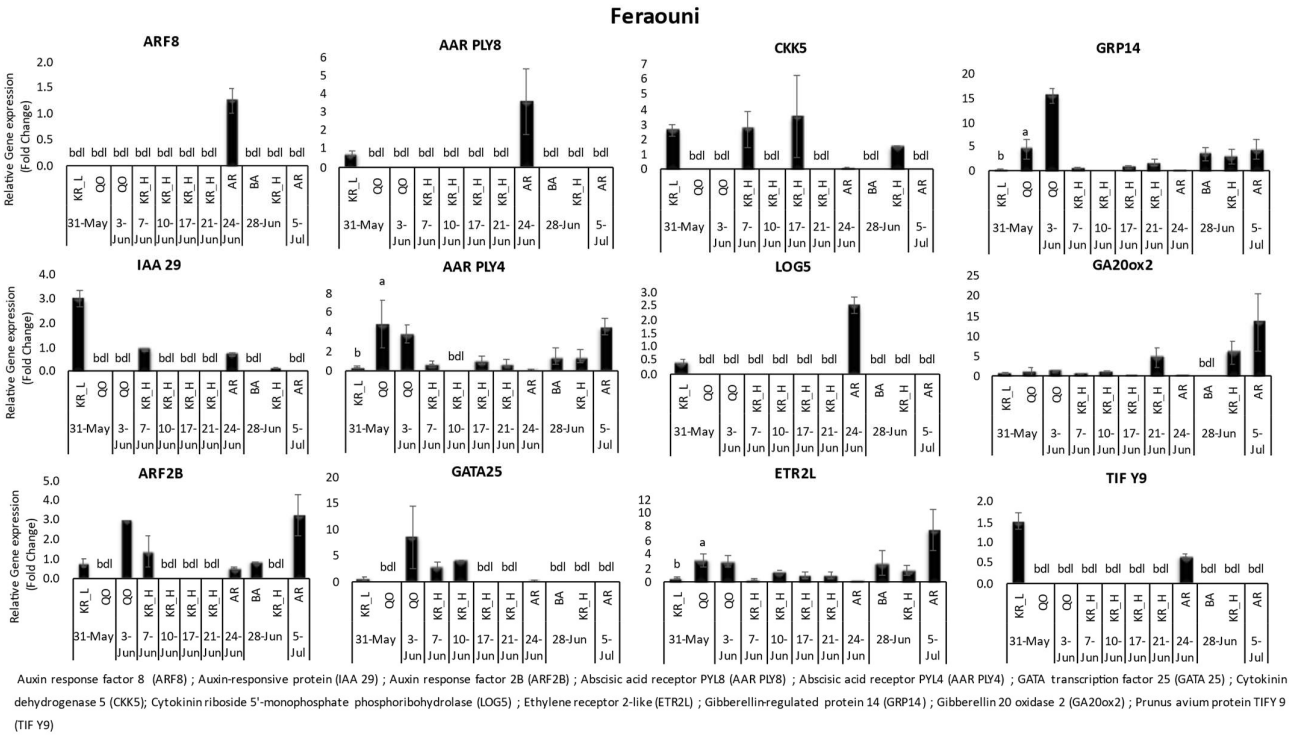


Figure 1. Expression profiles of Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9) of the Feraouni cherry variety harvested between 31 May 2021 and 5 July 2021 from Kaa El Reem (low) (KR_L), Qoussaya (QO), Kaa El Reem (high) (KR_H), Arsal (AR), and Barqa (BA). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (fold change). Values followed by the same letter are not significantly different at the $p \leq 0.05$ level. Variables were considered significant at the <0.05 risk level. "bdl" means below detectable level.

The expression levels of the Feraouni variety exhibited significant differences in the Kaa El Reel (low) and Qoussaya locations during the early season for the genes GRP14, AAR PLY4, and ETR2L, as depicted in Figure 1. However, there were no significant variations in the expression of the remaining genes for the same harvested date between June and July. Conversely, ARF8, AAR PLY8, LOG5, and TIF Y9 showed poor expression levels throughout the season, except for on 24 June. The gene expression levels exhibited intriguing fluctuations in various regions over specific dates. Notably, ETR2L expression in Arsal soared remarkably, showing a staggering 7.51-fold increase on 5 July. In Qoussaya, on 3 June, the GRP14 transcript level surged impressively, marking a substantial 15.51-fold increase. Similarly, the AAR PLY4 expression in Qoussaya showcased a notable upswing, multiplying by 4.84-fold on 31 May. Moving to Kaa El Reem's high-altitude area, CKK5 expression experienced a significant 3.52-fold increase on 17 June. Shifting the focus back to Arsal, GA20ox2 expression exhibited a remarkable boost, spiking by an impressive 13.36-fold on 5 July. Likewise, ARF2B expression in Arsal also showed an upward trend, scaling up by 3.2-fold on the same date. On 24 June, ARF8 expression in Arsal saw a modest 1.25-

fold increase. Transitioning to the low-altitude region of Kaa El Reem, IAA 29 expression increased notably by 2.98-fold on 31 May. Furthermore, AAR PLY8 expression in Arsal witnessed a substantial surge of 3.58-fold on 24 June. In Qoussaya, GATA25 expression showed a significant augmentation, with an 8.44-fold increase on 3 June. Adding to the complexity, LOG5 expression in Arsal also experienced a notable surge, peaking at 2.52-fold on 24 June. Lastly, TIF Y9 expression in Kaa El Reem's low-altitude region moderately rose by 1.53-fold on 31 May. These shifts in gene expressions depict a dynamic biological landscape within the mentioned regions and dates.

The gene expression profiles for twelve genes (ETR2L, CKK5, ARF8, GATA25, GRP14, GA20ox2, IAA 29, LOG5, AAR PLY4, ARF2B, AAR PLY8, and TIF Y9) in the Banni cherry variety were evaluated during the period between 21 June 2021 and 5 July.

The samples for the Banni variety were harvested from two different locations—Kaa El Reem (high altitude—KR_H) and Arsal (AR) (Figure 2).

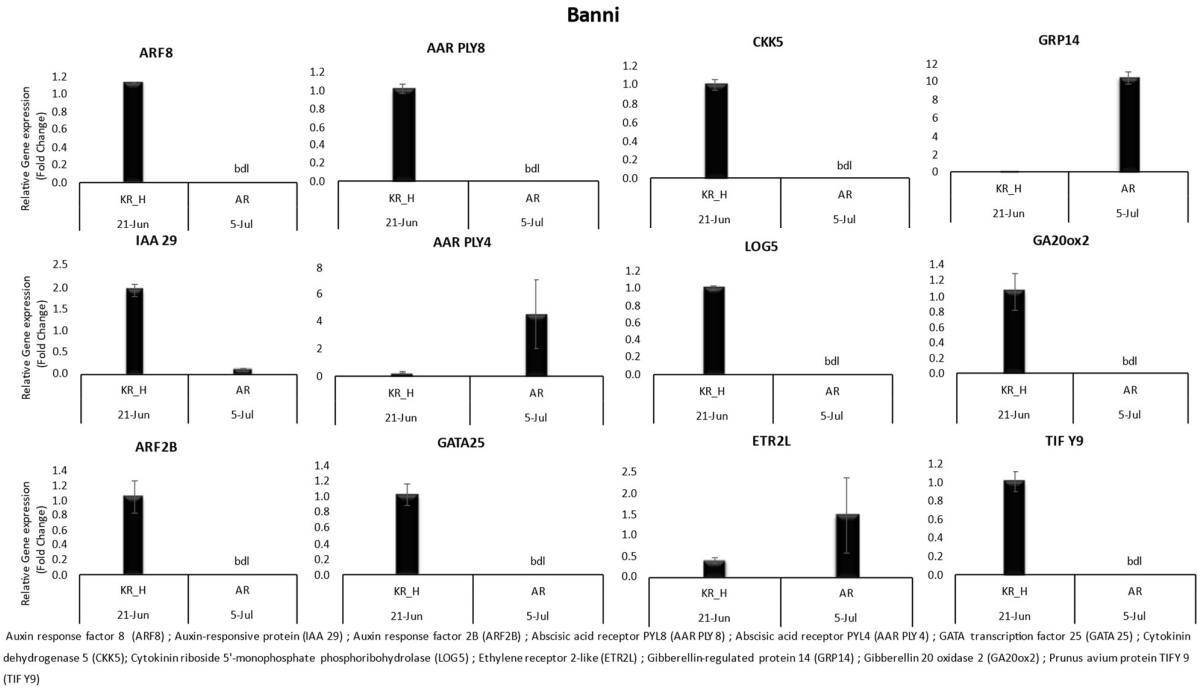


Figure 2. Expression profiles of Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9) of the Banni cherry variety harvested between 21 June 2021 and 5 July 2021 from the Kaa El Reem (high) (KR_H) and Arsal (AR) locations. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (fold change). "bdl" means below detectable level.

On 21 June 2021, a discernible modulation in gene expression levels was noted. CKK5 exhibited a marginal yet measurable 1-fold increment, indicative of a subtle regulatory influence. ARF8, ARF2B, AAR PLY8, and GA20ox2 expression saw a slight but notable elevation of 1.05-fold, 1.04-fold, 1.02-fold, and 1.14-fold increase, respectively, suggesting a regulatory response indicating a nuanced regulatory role. In contrast, IAA 29 exhibited a substantial surge, escalating significantly by 1.92-fold, suggesting a notable influence on the biological processes. Moreover, LOG5, GATA25, and TIF Y9 also manifested a marginal augmentation of 1.02-fold, signifying a subtle regulatory effect, possibly contributing to the genetic regulatory dynamics.

Expression of the Irani variety was significantly different between the Kaa El Reem (low) and Qoussaya locations during early season for the ARF8, IAA29, ARF2B, and ETR2L genes (Figure 3).

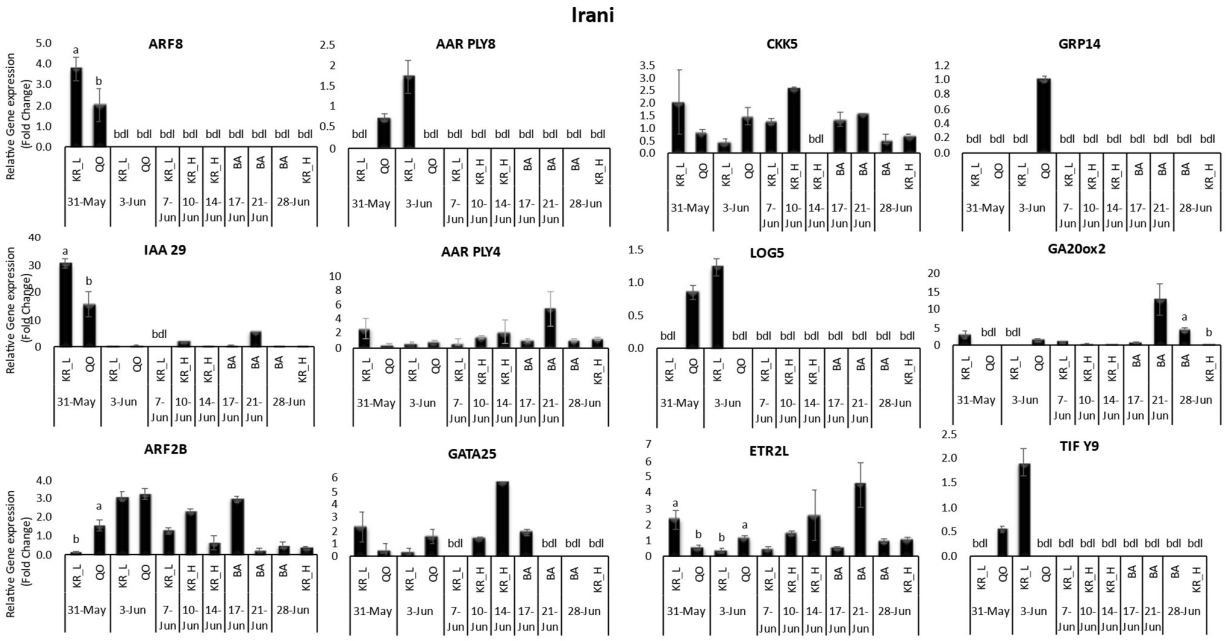
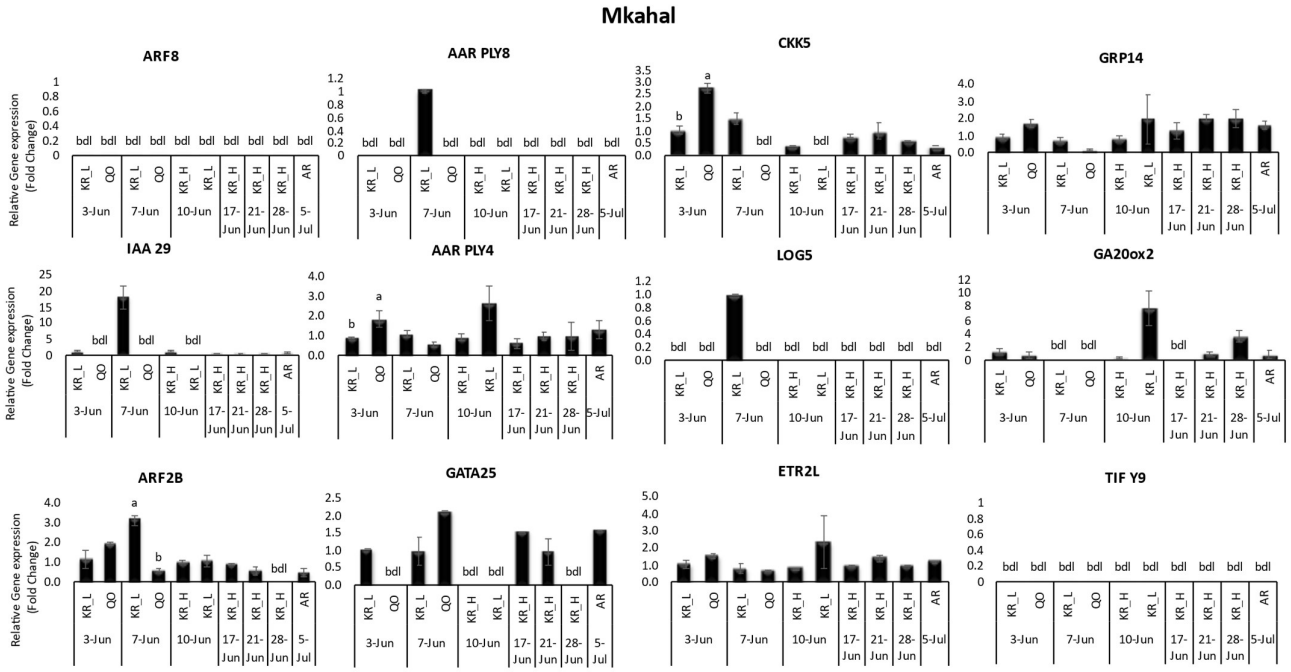


Figure 3. Expression profiles of Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9) of the Irani cherry variety harvested between 31 May 2021 and 28 June 2021 from Kaa El Reem (low) (KR_L), Qoussaya (QO), Kaa El Reem (high) (KR_H), and Barqa (BA). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (fold change). Values followed by the same letter are not significantly different at the $p \leq 0.05$ level. Variables were considered significant at the <0.05 risk level. "bdl" means below detectable level.

There were no significant variations in the expression of the remaining genes for the same harvest date between June and July, except for GA20ox2 in Barqa and Qaa El Reem (high) on 28 June 2021.

GA20ox2 showed an increased expression with harvest date. Overall, the RT-qPCR data showed a different expression pattern of the studied genes over the harvest season among locations. On 21 June, ETR2L expression was notably upregulated in Baqa, demonstrating a 4.46-fold increase. The transcript level of GRP14 was markedly increased on 3 June 2021, demonstrating a 1-fold increase in Qoussaya. The expression pattern of AAR PLY4 showed a 5.42-fold increase in Barqa on 21 June. The transcript abundance of CKK5 was markedly increased by 2.59-fold in Kaa El Reem high altitude on 10 June 2021. GA20ox2 was notably expressed in Barqa on 21 June, with a 12.76-fold increase. Noticeably, ARF2B was expressed in Qoussaya with a 3.29-fold increase on 3 June 2021. In particular, ARF8 was upregulated, showing an approximate 3.76-fold increase, and IAA 29 was upregulated, demonstrating a 30.32-fold increase, both in Kaa El Reem low altitude on 31 May 2021. Furthermore, GATA25 was notably expressed in Kaa El Reem high altitude, with a 5.67-fold increase on 14 June 2021. LOG5 and TIF Y9 were both particularly upregulated in Kaa El Reem low altitude on 3 June 2021 with 1.23- and 1.91-fold increases, respectively.

Expression of the Mkahal variety was significantly different between the Kaa El Reel (low) and Qoussaya locations during the early season for the CKK5 and AAR PLY4 genes (Figure 4). Expression of the ARF2B gene was significantly different in the mid-season between Kaa El Reem (low) and Qousaya. There were no significant variations in the expression of the remaining genes between June and July. ARF8, IAA29, AAR PLY8, LOG5, and TIF Y9 were poorly expressed over the season, except for 7 June 2021 for AARPLY8, IAA29, and LOG5 in Kaa El Reem (low). On 21 June, ETR2L expression was notably upregulated in Kaa El Reem low altitude, showing a 2.29-fold increase. The transcript level of GRP14 was markedly increased on 10 June 2021 by 1.95-fold in Kaa El Reem low altitude. The expression pattern of AAR PLY4 showed a 2.63-fold increase in Kaa El Reem low altitude on 10 June. The transcript abundance of CKK5 showed a marked 2.73-fold increase in Qoussaya on 3 June 2021. GA20ox2 was notably expressed in Kaa El Reem low altitude on 10 June, with a 7.75-fold increase. Noticeably, ARF2B was expressed in Kaa El Reem low altitude, with a 3.11-fold increase on 7 June 2021. In particular, ARF8 and TIFY9 were below detectable levels on all harvest dates and IAA 29 was upregulated, showing a 18.03-fold increase, in Kaa El Reem low altitude on 7 June 2021. Furthermore, GATA25 was notably expressed in Qoussaya, with a 2.1-fold increase on 7 June 2021. AAR PLY8 and LOG5 were both particularly upregulated in Kaa El Reem low altitude on 7 June 2021, both demonstrating a 1- fold increase.



Auxin response factor 8 (ARF8) ; Auxin-responsive protein (IAA 29) ; Auxin response factor 2B (ARF2B) ; Abscisic acid receptor PYL8 (AAR PLY8) ; Abscisic acid receptor PYL4 (AAR PLY4) ; GATA transcription factor 25 (GATA 25) ; Cytokinin dehydrogenase 5 (CKK5); Cytokinin riboside 5'-monophosphate phosphoribohydrolase (LOG5) ; Ethylene receptor 2-like (ETR2L) ; Gibberellin-regulated protein 14 (GRP14) ; Gibberellin 20 oxidase 2 (GA20ox2) ; Prunus avium protein TIFY 9 (TIF Y9)

Figure 4. Expression profiles of Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9) of the Mkahal cherry variety harvested between 3 June 2021 and 5 July 2021 from Kaa El Reem (low) (KR_L), Qoussaya (QO), Kaa El Reem (high) (KR_H), and Arsal (AR). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (fold change). Values followed by the same letter are not significantly different at the $p \leq 0.05$ level. Variables were considered significant at the <0.05 risk level. "bdl" means below detectable level.

Regarding the Skeena cherry variety, AAR PLY4, GA20ox2, and ETR2L had an increased gene expression in relation to the harvest season, whereas the expression of AAR PLY8, CKK5, LOG5, IAA29, ARF2B, and TIF Y9 had a decreased expression in relation to the harvest date (Figure 5). Overall, the RT-qPCR data showed a different expression pattern of the studied genes over the harvest season. On 28 June, ETR2L expression was notably upregulated in Kaa El Reem high altitude, demonstrating a 1.61-fold increase. The transcript level of GRP14 and ARF8 were below detectable levels on the three harvest dates. AAR PLY4 was markedly increased on 28 June 2021 by 1.88-fold in Kaa El Reem high altitude. On 21 June, the expression pattern of AAR PLY8, CKK5, LOG5, IAA29, ARF2B, and TIF Y9 showed increases in Kaa El Reem high altitude of 1.43-, 2.2-, 5.3-, 1-, 1-, and 1-fold, respectively. GA20ox2 was notably expressed in Kaa El Reem high altitude on 28 June, with a 2.4-fold increase. GATA25 was notably expressed in Kaa El Reem high altitude, showing a 3.68-fold increase on 24 June 2021.

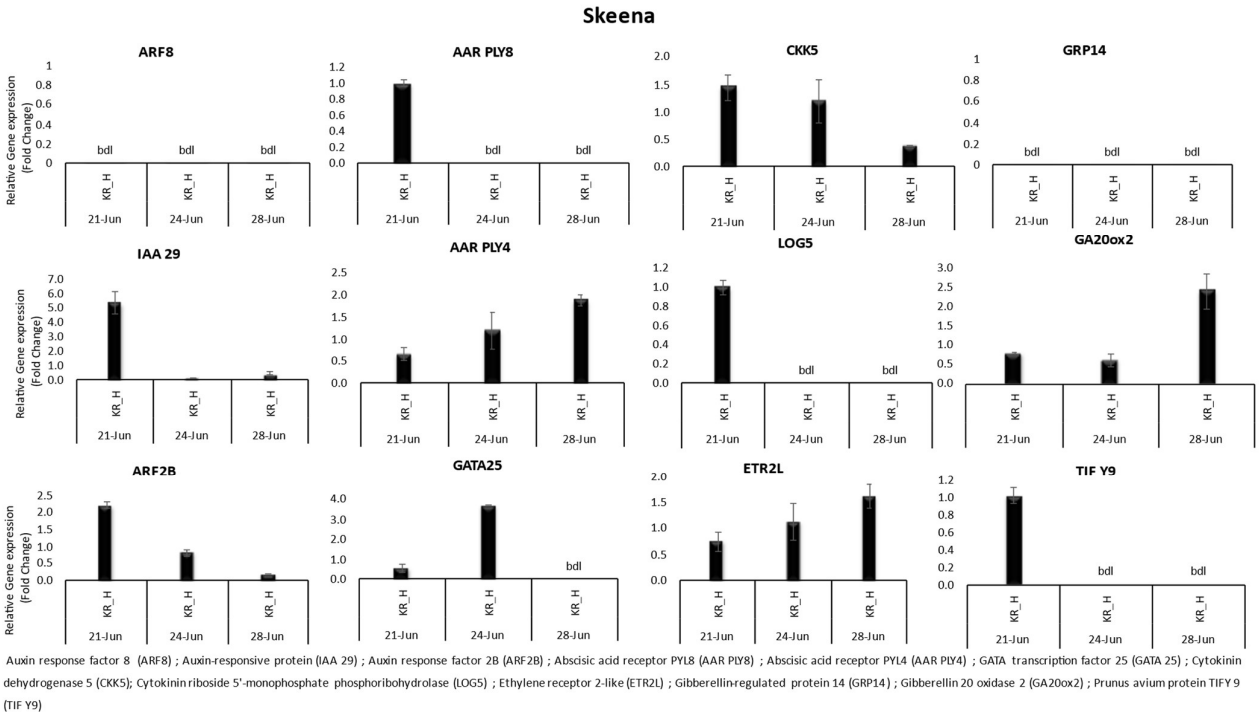


Figure 5. Expression profiles of Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9) of the Skeena cherry variety harvested between 21 June 2021 and 28 June 2021 from Kaa El Reem (high) location. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (fold change). "bdl" means below detectable level.

Regarding the Teliani cherry variety, CKK5, AAR PLY4, ARF2B, and ETR2L showed an increased expression in the mid-harvest season in Barqa. ARF8, AAR PLY8, GRP14, LOG5, GA20ox2, and TIF Y9 were poorly expressed over the season (Figure 6). On 3 June, CKK5, AAR PLY4, ARF2B, and ETR2L expression was notably upregulated in Barqa, showing 3.15-, 1.86-, 2.51-, and 3.87-fold increases, respectively. The transcript level of IAA 29 and GATA25 was markedly increased on 10 June 2021, with a 1.5- and 1-fold increase in Arsal, respectively.

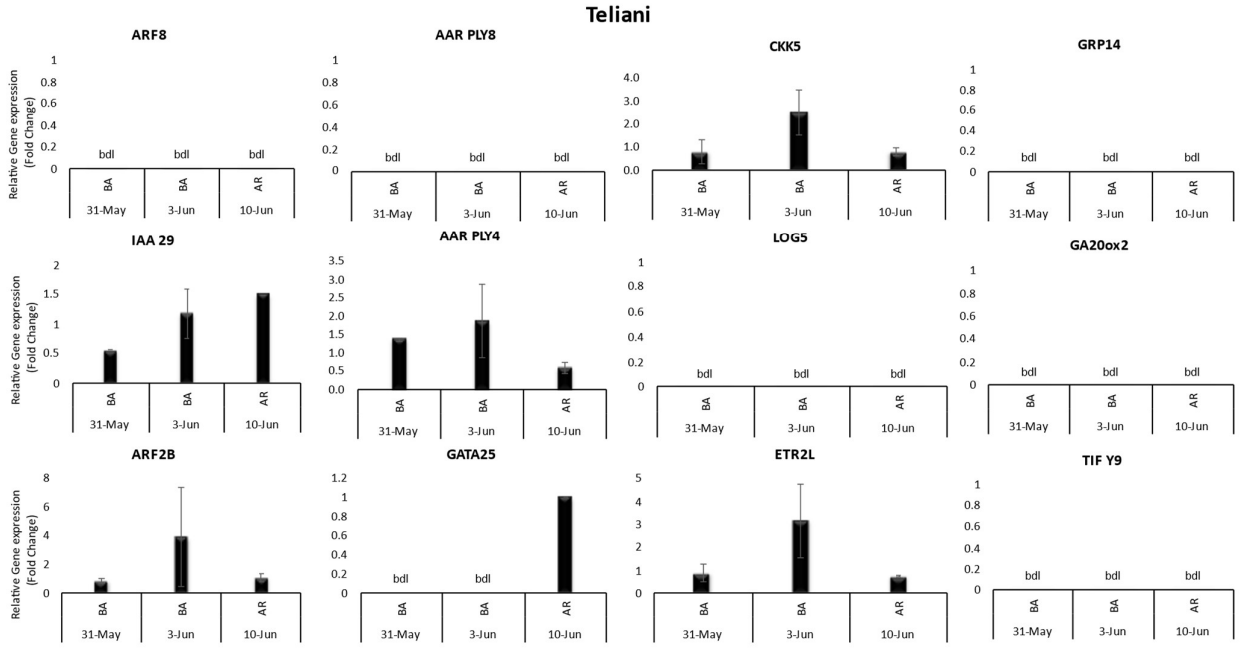


Figure 6. Expression profiles of Auxin response factor 8 (ARF8), Auxin-responsive protein (IAA 29), Auxin response factor 2B (ARF2B), Abscisic acid receptor PYL8 (AAR PLY8), Abscisic acid receptor PYL4 (AAR PLY4), GATA transcription factor 25 (GATA25), Cytokinin dehydrogenase 5 (CKK5), Cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG5), Ethylene receptor 2-like (ETR2L), Gibberellin-regulated protein 14 (GRP14), Gibberellin 20 oxidase 2 (GA20ox2), and *Prunus avium* protein TIFY 9 (TIF Y9) of the Teliani cherry variety harvested between 31 May 2021 and 10 June 2021 from Barqa (BA) and Arsal (AR). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (fold change). "bdl" means below detectable level.

3.2. Hormone Quantification

Table 1 describes the Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA) concentrations (ng/g DW) in the Banni (BA), Feraouni (FE), Irani (IR), Mkahal (MK), Skeena (SK), and Teliani (TE) cherry varieties harvested from the Arsal (AR), Barqa (BA), Kaa El Reem (high) (KR_H), Kaa El Reem (low) (KR_L), and Qousaya (QO) locations.

Banni (BA) cherry samples were harvested from Arsal on 5 July 2021 and Kaa El Reem (high) on 21 June 2021. The Benzyl Adenine (BA), Zeatin, Gibberellic acid (GA3), and Abscisic acid (ABA) contents in the Arsal (AR) samples were 0.06, 4.11, 2.24, and 18.11 ng/g DW, respectively, whereas in the Kaa El Reem (low) samples there was a lower content (0.04, 1.85, 0.91, and 17.78 ng/g DW, respectively). Only the Salicylic acid (SA) content was slightly lower in Arsal (AR) (5.88 ng/g DW), compared to Kaa El Reem (high) (6.39 ng/g DW).

Feraouni (FE) cherry samples were harvested from Arsal (AR), Barqa (BA), Kaa El Reem (high) (KR_H), Kaa El Reem (low) (KR_L), and Qoussaya (QO) between 31 May 2021 and 5 July 2021. In Arsal, there was no significant difference in Benzyl Adenine content between 24 June 2021 (0.08 ng/g DW) and 5 July 2021 (0.06 ng/g DW), but it was observed that significantly higher Zeatin, SA, GA3, and ABA levels were recorded on 24 June 2021 (4.58, 9.27, 1.73, and 9.01 ng/g DW, respectively) compared to 5 July 2021 (2.07, 5.43, 0.79, and 1.85 ng/g DW, respectively).

In Kaa El Reem (high), levels of Benzyl Adenine were not detectable on 7 June 2021 and 10 June 2021 and there was no significant difference for the other dates, as concerns Benzyl Adenine content. The Zeatin, SA, and ABA contents tended to be greater on the early dates in June (3.98, 19.01, and 18.13 ng/g DW, respectively) compared to the later

dates (1.72, 6.12, and 2.63 ng/g DW, respectively), with significant differences. The GA3 levels had their lowest content around mid-June (0.85 ng/g DW).

Table 1. Shows the Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA) concentrations (ng/g DW) in the Banni (BA), Feraouni (FE), Irani (IR), Mkahal (MK), Skeena (SK), and Teliani (TE) cherry varieties harvested from the Arsal (AR), Barqa (BA), Kaa El Reem (high) (KR_H), Kaa El Reem (low) (KR_L), and Qousaya (QO) locations between 31 May 2021 and 5 July 2021. While we compared phytohormones of the same variety on different dates for the same location, means with the same letter are not significantly different from each other ($p > 0.05$).

Variety	Location		Date of Harvest	Benzyl Adenine BA (ng/g DW)	Zeatin (ng/g DW)	Salicylic Acid SA (ng/g DW)	Gibberellic Acid GA3 (ng/g DW)	Abscisic Acid ABA (ng/g DW)
Banni (BA)	Arsal	AR	5 July	0.06 ± 0.02	4.11 ± 0.07	5.88 ± 0.18	2.24 ± 0.07	18.11 ± 0.24
	Kaa El Reem (high altitude)	KR_H	21 June	0.04 ± 0	1.85 ± 0.02	6.39 ± 0.35	0.91 ± 0.18	17.78 ± 0.33
Feraouni (FE)	Arsal	AR	24 June	0.08 ± 0	4.58 ± 0.08 a	9.27 ± 0.28 a	1.73 ± 0.17 a	9.01 ± 0.27 a
			5 July	0.06 ± 0	2.07 ± 0 b	5.43 ± 0.55 b	0.79 ± 0.05 b	1.85 ± 0.12 b
	Barqa	BA	28 June	0.1 ± 0	2.34 ± 0.09	9.36 ± 0.03	2.18 ± 0.13	5.52 ± 0.08
	Kaa El Reem (high altitude)	KR_H	7 June	ND	3.98 ± 0.06 a	19.01 ± 0.32 a	1.84 ± 0.16 ab	18.13 ± 0.25 a
			10 June	ND	3.74 ± 0.03 a	7.45 ± 0.16 b	1.78 ± 0.04 ab	18.93 ± 0.11 a
			17 June	0.06 ± 0.01	1.3 ± 0.12 c	5.52 ± 0.51 c	0.85 ± 0.05 b	2.91 ± 0.14 b
			21 June	0.11 ± 0	2.1 ± 0.07 b	5.4 ± 0.26 c	2.14 ± 0.06 a	4.12 ± 0.41 b
			28 June	0.18 ± 0.02	1.72 ± 0.02 b	6.12 ± 0.08 c	1.23 ± 0.46 ab	2.63 ± 0.29 b
	Kaa El Reem (low altitude)	KR_L	31 May	0.07 ± 0.01	3.54 ± 0.12	13.42 ± 0.92	1.12 ± 0.02	3.52 ± 0.04
	Qoussaya	QO	31 May	0.19 ± 0.03	3.19 ± 0.51	3.92 ± 0.62	0.98 ± 0.08	5.98 ± 1.25
			3 June	0.08 ± 0.02	3.59 ± 0.03	3.48 ± 0.15	0.82 ± 0.05	4.35 ± 0.06
Irani (IR)	Barqa	BA	17 June	0.26 ± 0.02	1.9 ± 0.04 a	3.78 ± 0.05 b	1.73 ± 0.05	25.88 ± 0.16 a
			21 June	0.21 ± 0	2.31 ± 0.13 a	11.19 ± 0.02 a	1.14 ± 0.04	9.94 ± 0.14 b
			28 June	ND	1.42 ± 0.04 b	10.99 ± 1.04 a	2.85 ± 0.5	1.87 ± 0.15 c
	Kaa El Reem (high altitude)	KR_H	10 June	0.06 ± 0	1.26 ± 0.03 b	1.5 ± 0.15 b	0.71 ± 0.03	2.89 ± 0.04 b
			14 June	0.15 ± 0	2.42 ± 0.09 a	3.52 ± 0.04 a	1.2 ± 0.26	11.57 ± 0.16 a
			28 June	0.16 ± 0.02	1.05 ± 0.01 b	2.37 ± 0.34 ab	1.57 ± 0.02	1.16 ± 0.04 c
	Kaa El Reem (low altitude)	KR_L	31 May	0.24 ± 0 a	2.3 ± 0.01 c	1.85 ± 0.81	1.33 ± 0.11	5.48 ± 0.35 b
			3 June	0.27 ± 0.02 a	4.78b± 0.1 a	1.61 ± 0.28	1.09 ± 0.08	6.8 ± 0.17 b
			7 June	0.15 ± 0.01 b	4.05 ± 0.15 b	4.32 ± 0.82	1.32 ± 0.04	12.9 ± 0.13 a
	Qoussaya	QO	31 May	0.19 ± 0.01	5.59 ± 0.07 a	3.1 ± 0.33	1.55 ± 0.05	14.17 ± 0.1 a
			3 June	0.02 ± 0	2.73 ± 0.09 b	3.86 ± 0.03	1.27 ± 0.07	3.35 ± 0.21 b
Mkahal (MK)	Arsal	AR	5 July	0.07 ± 0.01	4.49 ± 0.01	4.97 ± 0.14	1.63 ± 0.15	17.5 ± 0.02
	Kaa El Reem (high altitude)	KR_H	10 June	0.11 ± 0	7.44 ± 0.15 a	17.28 ± 1.14 a	2.01 ± 0.33	51.14 ± 0.25 a
			17 June	0.15 ± 0	7.71 ± 0.03 a	7.87 ± 0.06 b	2.16 ± 0.58	27.04 ± 0.04 b
			21 June	ND	5.74 ± 0.02 b	15.6 ± 0.81 a	1.7 ± 0.17	7.62 ± 0.37 c
			28 June	ND	3.78 ± 0.09 c	6.32 ± 0.49 b	1.75 ± 0.03	3.71 ± 0.04 d
	Kaa El Reem (low altitude)	KR_L	3 June	0.05 ± 0.02	4.59 ± 0.06 a	10.47 ± 0.16	1.92 ± 0.11	19.69 ± 0.86 a
			7 June	ND	5.03 ± 0.13 a	8.05 ± 0.24	1.44 ± 0.34	15.39 ± 0.22 b
			10 June	ND	3.21 ± 0.01 b	8.28 ± 1	2.14 ± 0.2	4.36 ± 0.02 c
	Qoussaya	QO	3 June	0.13 ± 0.04	5.1 ± 0	4.59 ± 0.13	1.21 ± 0.07	12.24 ± 0.44
			7 June	ND	5.96 ± 0.68	5.49 ± 0.72	1.82 ± 0.18	17.62 ± 2.16
Skeena (SK)	Kaa El Reem (high altitude)	KR_H	21 June	0.2 ± 0.02	2.82 ± 0.04 a	2.77 ± 0.24 a	2.15 ± 0.21	3.46 ± 0.29 ab
			24 June	0.27± 0	2.49 ± 0.05 b	3.01 ± 0.17 a	2.61 ± 0.15	3.66 ± 0.06 a
			28 June	0.3 ± 0.05	2.05 ± 0.04 c	1.2 ± 0.12 b	2.01 ± 0.14	2.06 ± 0.34 b
Teliani (TE)	Arsal	AR	10 June	1.17 ± 0.01	1.48 ± 0.01	2.31 ± 0.46	1.94 ± 0.01	18.96 ± 0.14
	Barqa	BA	31 May	0.34 ± 0.02	1.72 ± 0.03 a	3.32 ± 0.31	1.47 ± 0.08 a	8.97 ± 0.2 a
			3 June	0.28 ± 0.01	1.3 ± 0.03 b	2.73 ± 0.24	0.31 ± 0.05 b	6.03 ± 0.24 b

In Qoussaya, there was no significant difference for all phytohormones, Benzyladenine (BA), Zeatin, Salicylic Acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA), between

31 May 2021 (0.19, 3.19, 3.92, 0.98, and 5.98 ng/g DW, respectively) and 3 June 2021 (0.08, 3.59, 3.48, 0.82, and 4.35 ng/g DW, respectively).

Irani (IR) cherry samples were harvested from Barqa (BA), Kaa El Reem (high) (KR_H), Kaa El Reem (low) (KR_L), and Qoussaya (QO) between 31 May 2021 and 28 June 2021.

In Barqa, Benzyl Adenine levels were only detectable on 17 June 2021 (0.26 ng/g DW) and 21 June 2021 (0.21 ng/g DW), with no significant difference. A significantly higher Zeatin content was observed on 17 (1.9 ng/g DW) and 21 June (2.31 ng/g DW), compared to 28 June 2021 (1.42 ng/g DW). A significantly higher SA content was observed on 21 June 2021 (11.19 ng/g DW) and 28 June 2021 (10.99 ng/g DW), compared to 17 June 2021 (3.78 ng/g DW). No significant differences were observed for the GA3 content. As for ABA, a higher content was observed on 17 June 2021 (25.88 ng/g DW), compared to 21 June 2021 (9.94 ng/g DW) and 28 June 2021 (1.87 ng/g DW), with significant differences.

In Kaa El Reem (high), no significant difference between 10 June, 14 June, and 28 June 2021 was observed for Benzyl Adenine (0.06, 0.15, and 0.16 ng/g DW, respectively) and GA3 (0.71, 1.2, and 1.57 ng/g DW, respectively). Zeatin, SA, and ABA content were significantly higher on 14 June 2021 (2.42, 3.52, and 11.57 ng/g DW, respectively), compared to 10 June (1.26, 1.5, and 2.89 ng/g DW, respectively) and 28 June 2021 (1.05, 2.37, and 1.16 ng/g DW, respectively).

In Kaa El Reem (low), a higher Benzyl Adenine content was observed on 31 May 2021 (0.24 ng/g DW) and 3 June 2021 (0.27 ng/g DW), compared to 7 June 2021 (0.15 ng/g DW). A significantly higher Zeatin content was observed on 3 June 2021 (4.78 ng/g DW), compared to 31 May 2021 (2.3 ng/g DW) and 7 June 2021 (4.05 ng/g DW). No significant difference was observed between the dates for SA and GA3. ABA content was significantly greater on 7 June 2021 (12.9 ng/g DW), compared to 31 May (5.48 ng/g DW) and 3 June 2021 (6.8 ng/g DW).

In Qoussaya, the Benzyl Adenine, SA, and GA3 contents did not differ significantly between 31 May 2021 (0.19, 3.1, and 1.55 ng/g DW, respectively) and 3 June 2021 (0.02, 3.86, and 1.27 ng/g DW, respectively). Zeatin and ABA content were significantly greater on 31 May 2021 (5.59 and 14.17 ng/g DW, respectively) compared to 3 June 2021 (2.73 and 3.35 ng/g DW, respectively).

Mkahal (MK) cherry samples were harvested from Arsal (AR), Kaa El Reem (high) (KR_H), Kaa El Reem (low) (KR_L), and Qoussaya (QO) between 3 June 2021 and 5 July 2021. In Kaa El Reem (high), Benzyl Adenine levels were not detectable on 21 and 28 June 2021. Significantly higher Zeatin, SA, and ABA levels were detected on 10 June 2021 (7.44, 17.28, and 51.14 ng/g DW, respectively), compared to later dates. No significant difference was observed for GA3 between the different dates.

In Kaa El Reem (low), Benzyl Adenine levels were not detected on 7 and 10 June 2021. Significantly higher Zeatin and ABA levels were observed on 3 June 2021 (4.59 and 19.69 ng/g DW, respectively) compared to 10 June 2021 (3.21 and 4.36 ng/g DW, respectively). No significant differences for the SA and GA3 levels were observed between these dates.

In Qoussaya, there was no detectable level of Benzyl Adenine on 7 June 2021. There was no significant difference for Zeatin, SA, GA3, and ABA levels between 3 June 2021 (5.1, 4.59, 1.21, and 12.24 ng/g DW, respectively) and 7 June 2021 (5.96, 5.49, 1.82, and 17.62 ng/g DW, respectively).

Skeena (SK) cherry samples were harvested from Kaa El Reem (high) (KR_H) between 21 June 2021 and 28 June 2021. There was no significant difference between 21, 24, and 28 June 2021 for Benzyl Adenine (0.2, 0.27, and 0.3 ng/g DW, respectively) and GA3 content (2.15, 2.61, and 2.01 ng/g DW, respectively). Zeatin, SA, and ABA levels were higher on 21 June 2021 (2.82, 2.77, and 3.46 ng/g DW, respectively) compared to 28 June 2021 (2.05, 1.2, and 2.06 ng/g DW, respectively).

Teliani (TE) cherry samples were harvested from Arsal (AR) and Barqa (BA) between 31 May 2021 and 3 June 2021. Between 31 May 2021 and 3 June 2021, there was no significant difference for Benzyl Adenine (0.34 and 0.28 ng/g DW, respectively) and SA content (3.32

and 2.73 ng/g DW, respectively). Zeatin, GA3, and ABA levels were significantly greater on 31 May 2021 (1.72, 1.47, and 8.97 ng/g DW, respectively) compared to 3 June 2021 (1.3, 0.31, and 6.03 ng/g DW, respectively).

The average hormone quantification in the six evaluated sweet cherry varieties is described in Supplementary Table S5, and the graphs of phytohormones contents (ng/g DW) in sweet cherry fruits harvested at several ripening stages in different locations is described in Supplementary Figure S2. The Genotype x Location interaction is described in Supplementary Table S6.

Figure 7A describes the PCA biplot in the harvested locations of Arsal, Kaa El Reem (Low), Kaa El Reem (High), Qoussaya, and Barqa, along with the sweet cherry varieties of Feraouni, Irani, Teliani, Mkahal, Banni, and Skeena for the studied hormones (Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA)).

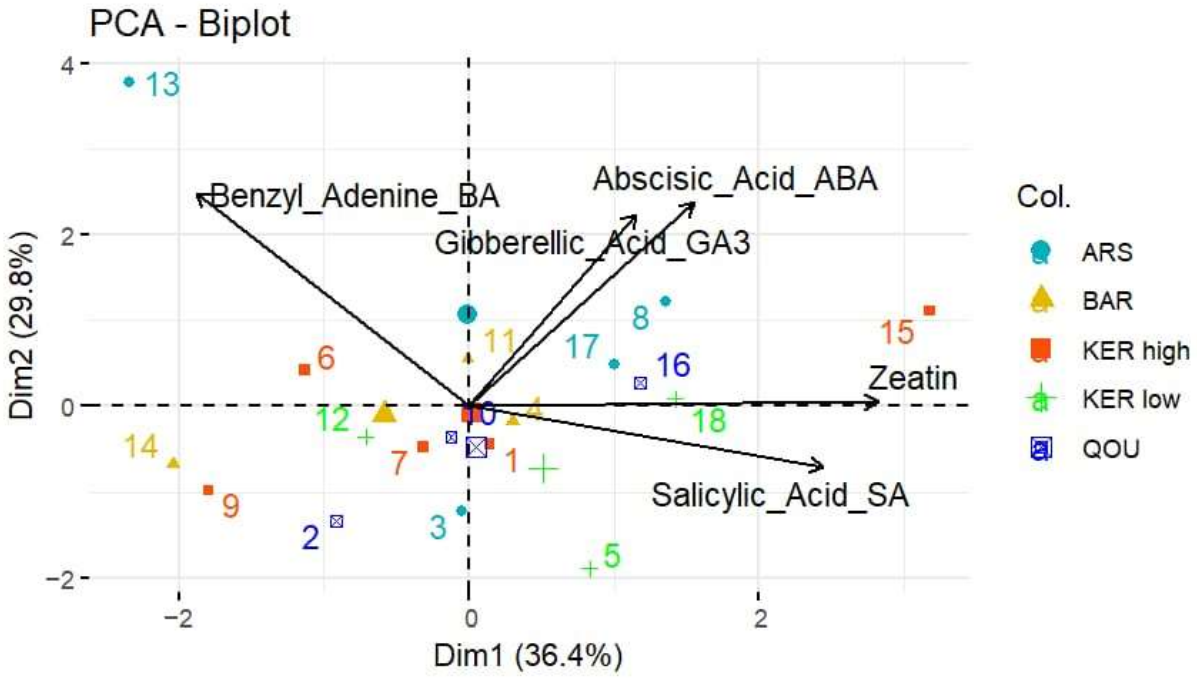
The first principal component was positively correlated with Zeatin and Salicylic Acid (SA). The highest variable contribution on the second principal component was positively correlated with Benzyl Adenine (BA), Abscisic acid (ABA), and Gibberellic acid (GA3).

The PCA biplot allowed the detection of classes or groups of individuals associated with phytohormones, as per Figure 7B.

Cluster 1: high Zeatin and Abscisic acid content for the Mkahal variety in all locations and the Banni variety harvested from Arsal.

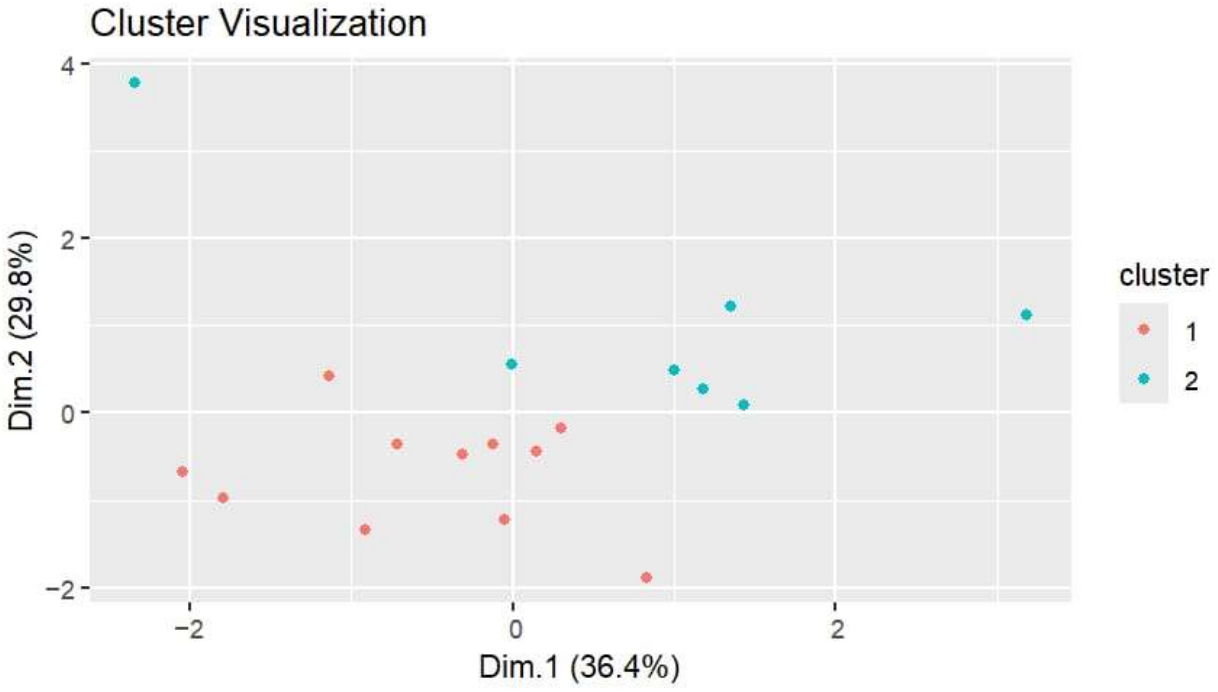
Cluster 2: high Benzyl Adenine content mainly for the Teliani cherry variety harvested from the Arsal location. Low Zeatin and Abscisic acid content for the Feraouni, Skeena, and Irani varieties harvested from all locations, as well as Banni harvested from Arsal, in addition to Teliani harvested from Barqa.

The correlation between hormones was also revealed; a slight positive correlation was observed between Zeatin and Salicylic acid (SA) and between Abscisic acid (ABA) and Gibberellic acid (GA3).



(**A**)

Figure 7. *Cont.*



(**B**)

Figure 7. Principal Component Analysis (PCA) biplot (**A**) showing the relationships among the tested hormones (Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA)), locations (Arsal, Qousaya, Kaa El Reem (low altitude and high altitude), and Barqa, and sweet cherry cultivars ("Feraouni" 1–5, "Banni" 7–8, "Irani" 9–12, "Mkahal" 15–18, "Teliani" 13–14, and "Skeena" 6, as well as a factor map (**B**) showing the clustering of samples.

4. Discussion

Fruit ripening is a complex biological process regulated by various hormones, including ethylene, auxins, Abscisic acid (ABA), and gibberellins (GAs), among others. While climacteric fruits exhibit a characteristic ethylene-dependent ripening process, non-climacteric fruits, such as cherries, do not rely on ethylene and follow a different regulatory mechanism. Understanding the relationship between hormone gene expression and hormone quantification in non-climacteric fruits like cherries is critical for improving fruit quality, shelf life, and overall marketability. This study explores the current knowledge of the correlation between hormone gene expression and hormone quantification in non-climacteric fruits, with a specific focus on cherries, while also considering the impact of altitude on these processes. Non-climacteric fruits, like cherries, primarily rely on hormonal regulation to control their growth and ripening. The expression of the genes involved in hormone biosynthesis, perception, and signal transduction pathways plays a pivotal role in this process. In cherries, the key genes associated with hormone regulation include those responsible for ABA biosynthesis and signaling, auxin metabolism, and GA biosynthesis.

Abscisic Acid (ABA): ABA is known to have a significant influence on the maturation and quality of non-climacteric fruits. In cherries, ABA-related genes, such as NCED (9-cis-epoxycarotenoid dioxygenase), play a crucial role in the biosynthesis of ABA. The expression of these genes increases during fruit development and maturation, leading to higher ABA levels in ripe cherries. Studies have shown a positive correlation between the expression of ABA biosynthesis genes and ABA quantification in cherries. In this study, ABA was expressed by the AAR PLY4 and AAR PLY8 genes, which showed different expression patterns according to harvest date and variety. In the Banni, Irani, Mkahal, and Skeena varieties, AAR PLY8 was more expressed in early harvest dates and AAR PLY4 was more expressed in later harvest dates. Banni and Mkahal had the highest ABA contents (17.94 (ng/g DW) and 17.63 (ng/g DW), respectively), on average over

the season, regardless of location. In this study, ABA concentrations were higher in all varieties, compared to the other evaluated hormones. The average concentrations ranged between 3.06 (ng/g DW) in the Skeena variety and 17.94 (ng/g DW) in the Banni variety. Researchers found that ABA levels detected in sweet cherries were higher in the immature stages and decreased with fruit ripening [15]., whereas other researchers concluded that ABA stimulates fruit ripening and impacts quality parameters in the pre-harvest stages [16]. This trend was observed in some varieties like Feraouni (Kaa El Reem high), Irani (Barqa), Mkahal (Kaa El Reem high, Kaa El Reem low), Skeena (Kaa El Reem high), and Teliani (Barqa).

Auxins: Although auxins are primarily associated with cell elongation and division, they also contribute to the regulation of non-climacteric fruit development. The genes responsible for auxin biosynthesis, transport, and perception are expressed during cherry fruit development. A correlation exists between the expression of these genes and the levels of auxins in cherries. In this study, three Auxin-related genes (ARF 8, IAA 29, and ARF 2B) were expressed in the tested samples. ARF8 was poorly expressed, except in the Irani variety on 31 May 2021 and in the Feraouni variety on 24 June 2021. As for IAA 29, it had a higher expression on the early sampling dates for Banni, Irani, Mkahal, Skeena, and Feraouni. ARF 2B was more expressed in Irani and Mkahal, compared to the other varieties.

Gibberellins (GAs): GAs are another class of hormones that influence fruit growth. In cherries, genes involved in GA biosynthesis, such as GA20ox and GA3ox, are expressed at different stages of fruit development. A higher gene expression corresponds to increased GA levels, which promote cell expansion and fruit enlargement. In this study, the transcript levels of GRP14 and GA20ox2 were expressed in Banni, Irani, Mkahal, and Feraouni, but were not expressed in Teliani, which had early sampling dates compared to the other varieties. In this study, GA3 quantification was considered low compared to other hormones, with some slight differences between varieties. The average GA3 values ranged from 1.24 (ng/g DW) in the Teliani variety to 2.25 (ng/g DW) in the Skeena variety.

Altitude can significantly impact the growth and development of climacteric and non-climacteric fruits, including cherries [17]. As the altitude increases, several environmental factors change, including temperature, light intensity, and atmospheric pressure, which can affect hormone gene expression and hormone quantification.

Cherries, as a model non-climacteric fruit, provide valuable insights into the regulatory mechanisms that govern fruit development and ripening. Altitude, with its associated changes in temperature, light intensity, and atmospheric pressure, can influence hormone gene expression and hormone quantification in cherries, adding an additional layer of complexity to the understanding of fruit development at different altitudes. Continued research in this field holds promise for improving fruit quality, enhancing post-harvest preservation, and advancing our understanding of non-climacteric fruit biology under varying environmental conditions.

Cytokinin: In different plant species, plant hormones, such as Cytokinin, have been successfully used to convert male flowers to female flowers [18].. Benzyl adenine is known as a natural cytokinin that stimulates cell division and influences fruit size and weight. BA is used, as well as an effective thinning compound, for many crops like apples [19]. The pre-harvest treatment of Benzyl adenine combined with gibberellin in sweet cherries resulted in larger and firmer fruits [20]. Benzyl Adenine is considered the main CK used in the micropropagation of horticultural fruits crops, through organogenesis and somatic embryogenesis. In mass propagation through organogenesis, other commonly known CKs such as Zeatin are used [21].

In this study, it was demonstrated that Benzyl Adenine concentrations in sweet cherries were different according to the varieties and quantification of this hormone, which was relatively low compared to other hormones. The highest concentrations were detected in the Teliani variety (0.6 (ng/g DW)), whereas the lowest concentrations were detected in the Banni variety (0.05). CKK5 and LOG5 were the studied genes for Cytokinin expression in

this study. CKK5 showed higher gene expression for all varieties, compared to LOG5. As for Zetain, its quantification was greater than BA, with average values ranging between 1.5 (ng/g DW) in the Teliani and 5.31 (ng/g DW) in the Mkahal variety.

Salicylic acid (SA): Researchers explored the potential of pre-harvest treatments with SA or ASA and their importance as promising tools to increase sweet cherry fruit quality and health benefits [22]. Post-harvest treatments with Salicylic acid (SA), acetylsalicylic acid (ASA), or oxalic acid (OA) could be innovative tools to extend the storability of sweet cherry with a higher content of bioactive compounds and antioxidant activity, as compared with control fruits [23]. Researchers proved that a Salicylic acid treatment combined with ultrasound treatment has the potential to decrease cherry fruit decay and enhance its shelf life [24]. In the current experimental study, it was demonstrated that SA concentrations varied according to sweet cherry varieties and its average ranged between 2.33 (ng/g DW) in Skeena and 8.89 (ng/g DW) in Mkahal. Quantification of this hormone was important compared to other hormones.

According to researchers, quantification of the hormone levels and determination of their ratios can reveal different plant strategies to cope with the stress [25]. (Dobrev and Vankova, 2012) and the relationship between hormone levels and expression patterns of related genes.

ABA: Since sweet cherries are non-climacteric fruits, ripening is promoted by ABA. In this study, ABA was expressed by the AAR PLY4 and AAR PLY8 genes. It is noteworthy that the Abscisic acid and Salicylic acid levels increased in cherry varieties, compared with GA3, BA, and Zeatin concentrations. In the Feraouni variety, ABA levels increased sharply on 7 June 2021 and 10 June 2021 in Kaa El Reem high altitude and on 24 June 2021 in the Arsal location. In contrast, the expression profiles of AARR PLY4 did not show a significant increase in fold change on these dates; however, the expression profile of AAR PLY8 on 24 June 2021 witnessed a remarkable increase of 3.58-fold. By observing the expression patterns in the Banni variety, AAR PLY8 and AAR PLY4 were both upregulated in Arsal on 21 June 2021 and Kaa El Reem High altitude on 5 July 2021, respectively, and in parallel with the high ABA contents detected for the same samples. In the Irani variety, ABA levels reached a maximum level of 25.87 ng/g DW in Barqa on 17 June 2021; moreover, the expression profiles of AARR PLY4 showed an increase of 1.05-fold for this date, but were below detectable levels for the AAR PLY8 gene. The Mkahal variety displayed high levels of ABA in early, mid, and late harvest dates, with intermediate to high expression levels for the AA PLY4 gene, but only one upregulation for one harvest date for the AAR PLY8 gene. While ABA levels in the Skeena variety were lower than those observed in other varieties, an increasing upregulation of AAR LPY4 with harvest date was exhibited. However, AAR PLY8 was only upregulated on early harvest dates and was below detectable levels for the mid and late harvest dates. ABA levels reached a maximum of 18.95 in the Teliani variety for a late harvest date. A 0.6-fold upregulation was observed for the same date in the AAR PLY4 gene, but AAR PLY8 was below detectable levels for all harvest dates.

Gibberellins (GA3): In this study, the genes involved in GA biosynthesis were GA20ox and GRP14. In the Feraouni variety, levels of GA3 were much lower and more stable than those of ABA, with maximum levels of 2.18 ng/g DW recorded on 28 June 2021 in Barqa. Regarding the GA3 biosynthetic pathway, neither GA20ox expression nor GRP14 expression levels rose on the mentioned date. These genes were upregulated in the early season and late season harvests.

Auxin: Three Auxin-related genes (ARF 8, IAA 29, and ARF 2B) were expressed in the tested samples. In the Feraouni variety, some genes coding for auxin-respone factor 8, 2B, and proteins were upregulated in the early harvest dates and were below detectable levels in the mid-season harvest dates.

Cytokinin: In the Feraouni variety, many genes coding for Cytokinin dehydrogenase 5 and Cytokinin riboside 5′-monophosphate phosphoribohydrolase were upregulated. The expression patterns of CKK5 and LOG5 were observed in this study. Levels of Zeatin were higher than those of Benzyl Adenine, with maximum levels of 4.58 ng/g DW recorded on

24 June 2021 in Arsal. Interestingly, the expression pattern of the LOG5 gene recorded an important upregulation for the same date.

Ethylene: In addition to observing the expression patterns of the genes associated with Benzyl Adenine, Zeatin, Gibberellic acid, and Abscisic acid, it was of interest to specifically examine the expression of the genes involved in ethylene response, to better understand how these responses may vary at the cultivar level and according to harvest date and location. Genes coding for Ethylene receptor 2-like were observed. The expression patterns of Ethylene receptor 2-like gene (E2RL) were upregulated in all studied cherry varieties for all harvest dates. Noticeably, the Feraouni and Irani varieties displayed the highest elevated expression. In the Skeena variety, the Ethylene response genes were increasingly upregulated over the harvest season; conversely, the expression of Auxin related genes like ARF8, ARF 2B, and IAA 29 were below detectable levels or showing a decreasing upregulation with harvest season. This is consistent with previous work demonstrating that the response to auxin and/or reduction in auxin transport was inhibited in the presence of exogenously applied ethylene.

It is noteworthy that the phytohormone levels in cherry samples were influenced by altitude. Feraouni fruits harvested on 31 May showed that Benzyl Adenine (BA) and Abscisic acid (ABA) increased in Qoussaya, while Salicylic acid (SA) and Gibberellic acid (GA3) increased in Kaa El Reem at low altitude. Zeatin content remained relatively stable. Similarly, Feraouni samples harvested on June 28th from Barqa and Kaa El Reem at high altitudes exhibited varying phytohormone concentrations due to different growth conditions. BA increased in Kaa El Reem at high altitude, whereas Zeatin, SA, GA3, and ABA increased in Barqa. For Irani samples harvested on 31 May, Zeatin, SA, GA3, and ABA increased in Qoussaya, and only BA increased in Kaa El Reem at low altitude. On 28 June, similar to Feraouni samples, those from Barqa exhibited increased Zeatin, SA, GA3, and ABA, compared to those from Kaa El Reem at high altitude. In Mkahal cherry samples harvested on 10 June, Kaa El Reem at high altitude led to increased concentrations of BA, Zeatin, SA, and ABA compared to samples from Kaa El Reem at low altitude. Similarly, for the same variety harvested on 3 June, BA increased in Qoussaya, while SA, GA3, and ABA concentrations increased under Kaa El Reem at low altitude, with Zeatin levels remaining relatively consistent.

A previous study investigated the effect of altitude on apple fruit physiology, demonstrating that peel tissues at high altitudes exhibited significantly reduced metabolites such as fumarate, malate, and glutamic acid, compared to samples from low altitudes. Additionally, the study highlighted that phenolic compounds like chlorogenic acid were stimulated by high altitudes, underscoring the environmental influence on phenolic metabolites [26].

5. Conclusions

In this study, Benzyl Adenine (BA), Zeatin, Salicylic acid (SA), Gibberellic acid (GA3), and Abscisic acid (ABA) concentrations changed significantly among varieties and harvested locations. Comparing phytohormones of the same variety on different dates for the same location revealed important differences, emphasizing the effect of hormonal regulations on fruit ripening. Abscisic acid and Salicylic acid levels were higher in cherry varieties compared with GA3, BA, and Zeatin concentrations. On the other hand, the effect of altitude was revealed when comparing gene expression of the same variety and date under different altitudes, especially for the Irani variety (ARF8, IAA29, ARF2B, GA20ox2, and ETR2L genes), the Mkahal variety (CKK5, AAR PLY4, and ARF2B genes), and the Feraouni variety (GRP14, AAR PLY4, and ETR2L genes).

The highest quantified phytohormones in cherry fruits were ABA and SA, whereas the lowest quantified phytohormones were BA and GA3.

Our results show several patterns of variation in phytohormone levels in developing cherry fruits and deliver essential phytohormone data that are useful for understanding cherry fruit physiology and improving the physiochemical characteristics and post-harvest traits of traditional and new cultivars.

The mechanism of fruit ripening in sweet cherries has been investigated only to a limited extent. Phytohormones that regulate the major maturation processes should be quantified and genes encoding key hormones should be characterized. Such studies will provide useful information that can be used to further improve the yield of sweet cherries via genetic engineering and breeding programs. Phytohormones have a wide-ranging action on plant development; hence, they can be used for extending the shelf life of sweet cherries during storage. Further research is, however, needed to better understand the mechanisms underlying the regulation of fruit ripening regarding gene expression and phytohormones, according to different varieties.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10040408/s1, Table S1: Average fruit physicochemical characteristics at different maturity stages in the 6 evaluated sweet cherry varieties, Table S2: Harvested cherry varieties with their corresponding dates and locations, Table S3: Description of harvested varieties, location, altitudes and maturity stages of samples, Table S4: List of primers used for quantitative real-time PCR, Table S5: Average hormone quantification in the 6 evaluated sweet cherry varieties, Table S6: Genotype x Location interaction for evaluated hormones, Figure S1: Map of Lebanon describing harvested locations, Figure S2: Phytohormones contents (ng/g DW) in sweet cherry fruits harvested at several ripening stages in different locations.

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