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# Comparative Analysis of Gene Expression between Early Maturation Mutant 'Beni Shogun' and 'Fuji' Cultivars during Fruit Development and Ripening

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**Abstract:** We aimed to compare the maturation and ripening characteristics of two apple cultivars, early maturation mutant 'Beni Shogun' and 'Fuji'. The study revealed that both cultivars reached full bloom on the same day, but 'Beni Shogun' matured earlier than 'Fuji'. In addition, differences were observed in fruit size, length, width, sweetness, acidity, and ethylene production levels. The study also examined the expression patterns of genes involved in ethylene biosynthesis and signal transduction, as well as those involved in auxin signal transduction and transcriptional regulation, to investigate the putative molecular mechanism behind the distinct fruit development, maturation, and ripening. The expression of the *MdACO1* gene showed a sharp increase after the maturation date, whereas the expression of the *MdACO7* gene was higher in the early and middle stages of fruit development. The clustering analysis provided insights into the correlation between the phenotypic traits and expression levels of the key genes. They were categorized into three clusters, and the third cluster consisted of six phenotypes, including fruit size, length, width, sweetness, starch content, and ethylene production, as well as the one gene *MdACO1*. These findings suggest that 'Beni Shogun' and 'Fuji' have distinct fruit development and ripening behaviors, with 'Beni Shogun' maturing earlier than 'Fuji'.

**Keywords:** apple; Fuji; Beni Shogun; fruit development; maturation; ripening; gene expression



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## 1. Introduction

Somatic mutation is a phenomenon that occurs in plants, causing genetic diversity. However, because it is a process that does not involve reproduction, it cannot be passed on to the next generation [1]. In the case of fruit crops such as apples, grapes, citrus, peaches, plums, pears, and persimmons, they are commonly found in the form of bud sports [2]. Bud sports are discovered by breeders when they exhibit noticeable differences from the original cultivar in fruit color [3–5], size [6,7], shape [8,9], and maturation time [10–13], and can be developed into new cultivars through vegetative propagation. The 'Fuji' apple is a widely grown and consumed cultivar with notable commercial importance attributed to its distinct characteristics, such as its crisp texture, sweet flavor, and extended shelf life. Given its significance, studies have been conducted on the cultivar, resulting in the discovery of 91 somatic mutations in 'Fuji' [14].

'Beni Shogun' is a bud mutation that occurred in 'Yataka', a sport of 'Fuji' [15,16]. Its full bloom date is similar to 'Fuji', but it is known to mature about three weeks earlier than 'Fuji' [15], and its color is described as blushed [16]. Because fruit maturation is a complicated process that involves the interaction of multiple genes responsible for fruit development pathways, it poses a significant challenge for research. The current state of

research on the reasons behind the earlier maturation of 'Beni Shogun' compared to 'Fuji' and related factors is still limited. Various approaches have been attempted to uncover the molecular mechanisms of somatic mutation in bud sports related to fruit maturation and ripening, as follows: One such approach involves using PCR-based DNA fingerprinting techniques [17–19] to identify differences between the original cultivar and the bud sport, as well as to detect genomic DNA differences [20,21], including SNPs and insertions/deletions. Another approach involves comparing differences in the expression levels of key genes during fruit development [12,13,22].

Hormones are key regulators involved in the major fruit development processes [23–25]. In particular, in climacteric fruits such as apples, ethylene plays a very important role in transitioning from fruit maturation to the ripening stage [26,27]. The ethylene biosynthesis pathway involves several enzymes, including 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1) and serine/threonine-protein kinase CTR1 [27,28]. The downstream effects of ethylene are mediated by transcription factors such as ETHYLENE INSENSITIVE 3-like 1 protein (EIL1) [29] and ethylene-responsive transcription factor RAP2-3-like (ERF1) [30,31]. Understanding the regulation of ethylene in apple fruit maturation and ripening is essential for improving fruit quality and post-harvest storage.

Another hormone, auxin, is widely recognized as a key regulator of fruit growth and maturation [23,28]. In particular, auxin also plays a role in fruit maturation, with a decrease in auxin levels being a prerequisite for this process in many fruit crops [32–34]. The interplay between auxin and other hormones, such as ethylene, is also crucial for fruit development [28]. In addition, transcription factors such as NAC [35] and MADS [36,37] are known to have a significant role in fruit development and maturation.

Based on these significant roles of hormones and transcription factors in fruit development and maturation, we selected ethylene-related genes, auxin-related genes, and transcription factors for expression profiling in our study. Ethylene is a key regulator of climacteric fruit ripening, and we focused on the genes involved in ethylene biosynthesis, perception, and downstream signaling, including ACO1, CTR1, EIL1, and ERF1 [26–31]. Auxin is also an important hormone for fruit growth and maturation, and we chose to investigate the expression levels of auxin-related genes, which are involved in auxin biosynthesis, transport, and signaling [32–34]. Furthermore, we included transcription factors, such as NAC and MADS, which are known to regulate fruit development and maturation [35–37]. By analyzing the expression levels of these key genes during fruit development and ripening, we aimed to elucidate the molecular mechanisms underlying the earlier maturation of 'Beni Shogun' compared to 'Fuji' and provide valuable insights for improving fruit quality and post-harvest storage.

## 2. Materials and Methods

### *2.1. Plant Materials*

'Beni Shogun' and 'Fuji' apples were planted in an orchard belonging to the Apple Research Institute of the Rural Development Administration in Gunwi, Korea (N36°17′, E128°28′). For the experiment, a total of nine 'Beni shogun' and nine 'Fuji' trees were selected. These trees were all 10 years old and grown on M9 rootstock with a slender spindle bush form. The distance between the plants was 2 m, and the inter-row spacing was 4 m. Although the two cultivars had the same full bloom date (23 April 2018), the final harvest time of 'Beni Shogun' was more than a month earlier compared with that of the 'Fuji' apple. The weather conditions during the experiment from April to October were as follows: Average temperature (°C) for April, 13.2; May, 18.1; June, 22.3; July, 27.4; August, 27.1; September, 19.1; and October, 10.9. Cumulative precipitation (mm) for April, 119; May, 138.5; June, 101.5; July, 166.5; August, 240.5; September, 102.5; and October, 160. Due to the difference in harvest time, sampling was conducted at 10 points for 'Fuji' and 7 points for 'Beni Shogun' (Figure 1).

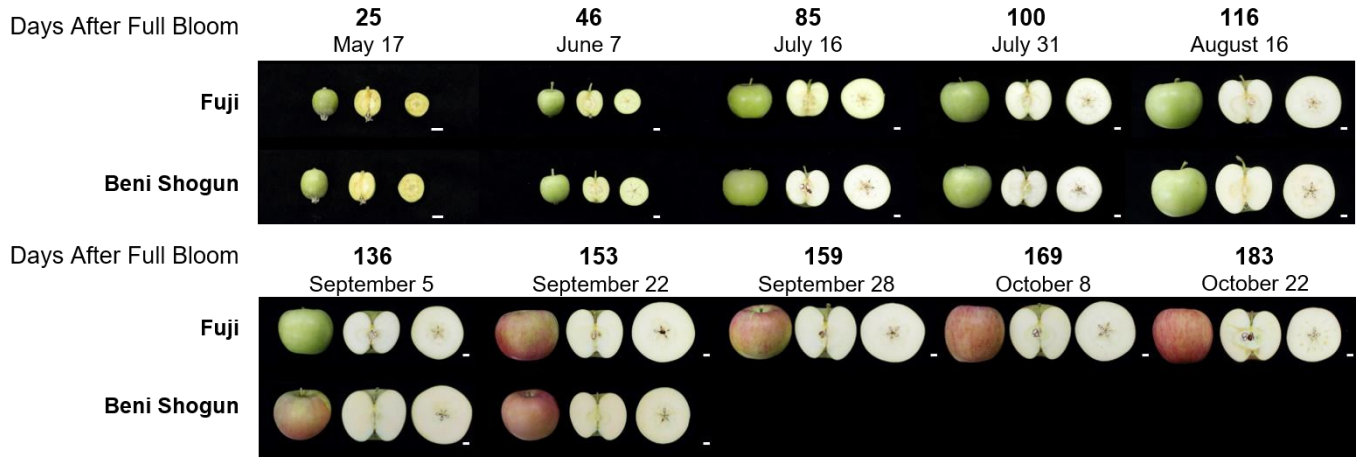


**Figure 1.** Surface and cross-sectional images of fruit at ten developmental stages. 'Fuji' and 'Beni Shogun' apples were harvested, periodically after full bloom (DAFB), at days 25, 46, 85, 100, 116, 136, 153, 159, 169, and 183 from trees grown at the Apple Research Institute in Gunwi, Republic of Korea. The full bloom date was 23 April 2018. The last developmental stage is the commercial harvest period for each cultivar. Bars represent 1 cm.

At each sampling point, a minimum of nine biological replicates were obtained from three trees for each cultivar for phenotype measurements and three replicates for gene expression comparisons. The fruit was divided into wedges at random, and the peels and cores were removed. The fruit flesh was immediately frozen using liquid nitrogen and stored at −80 °C until RNA extraction. Prior to RNA extraction, the samples were pulverized using a 6870 Freezer/Mill (SPEX SamplePrep., Metuchen, NJ, USA).

### *2.2. Fruit Quality Analysis and Internal Ethylene Measurement*

Eight traits were measured, including fruit weight, fruit height, fruit width, soluble solids content (SSC), starch–sugar interconversion, titratable acidity (TA), firmness, and internal ethylene concentration (IEC). We randomly selected nine fruits to observe their characteristics at different developmental stages. The weight of the fruits was determined using a scale (CPA 4202S, Sartorius, Germany). The length and width of the fruits were measured using digital 500-197-20/30 200mm/ 8" Absolute Digital Digimatic Vernier calipers (Mitutoyo, Kawasaki, Kanagawa, Japan) at the shoulder and equatorial axis, respectively. The SSC was measured using a PAL-1 digital refractometer (Atago, Minato-ku, Tokyo, Japan). The interconversion between starch and sugar was measured using a 5% potassium iodide (KI) solution and 1% iodide and determined using the Cornell starch–iodine index [38], where a score of 1 indicates 100% starch and a score of 8 indicates 0% starch. The juice of the pulp was squeezed, and 1 mL was used for the TA measurement. TA was determined by titrating 5 mL of juice with 0.1 N NaOH. Firmness was measured on 3 sides of each fruit by penetration with a digital fruit firmness tester (FT327, EFFEGI, Alfonsine, Italy) using an 11 mm diameter plunger. Internal ethylene concentration (IEC) was measured at each stage by using gas chromatography (Agilent 7820A, Agilent Technologies, Santa Clara, CA, USA). We inserted a 1 mL syringe into the calyx area and collected 1 mL of gas from inside the core. The collected gas was injected into the inlet and analyzed by the instrument.

### *2.3. RNA Extraction*

RNA was extracted from the ground fruit cortex powder using the cetyltrimethylammonium bromide (CTAB) method [39]. Initially, a tube containing 2 g of powder was mixed with 20 mL of extraction buffer. After that, 12 mL of chloroform: isoamyl alcohol (24:1) (C:I) was added, and the mixture was separated into phases by centrifugation (12,000 RPM, 30 min, 4 °C). Then, an equal volume of C:I was added to the supernatant, and the mixture

was centrifuged again (12,000 RPM, 10 min, 4 °C). The supernatant was collected and mixed with an equal volume of C:I and then re-centrifuged. To the resulting supernatant, 5 mL of 8 M lithium chloride (LiCl) was added, and the samples were incubated overnight. After centrifugation and discarding the supernatant, the pellet was dissolved in sodium dodecyl sulfate–Tris-HCl–EDTA (SSTE) buffer and extracted once again with an equal volume of C:I. The pellet was washed with EtOH, dried, and dissolved in Tris-EDTA (TE) buffer. RNA quality and quantity were evaluated using spectrophotometry at 240 nm and electrophoresis with a 0.8% agarose gel.

### *2.4. Reverse Transcription*

A QuantiTect Reverse Transcription Kit (QIAGEN, Germany) was used for reverse transcription. The reverse transcription reaction involved incubation at 42 °C for 10 min and 4 °C for 5 min to eliminate gDNA, followed by 42 °C for 20 min and 95 °C for 3 min to synthesize complementary DNA (cDNA). The resulting cDNA was stored at −20 °C.

### *2.5. Quantitative Real-Time PCR*

Quantitative real-time PCR (Rotor-Gene Q, QIAGEN, Germany) was conducted in triplicate using 50 ng of cDNA as a template in a 20 µL reaction mixture containing 2X qPCRBIO SyGreen Blue Mix Lo-ROX. The reaction involved an initial denaturation at 95 °C for 5 min, followed by 40 cycles of reaction at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. Genes associated with fruit maturation and ripening were selected, and *ACTIN* [40] was used as an internal standard. Primer pairs were designed using the Primer-BLAST program of the National Center for Biotechnology Information. In this experiment, qPCR results were validated using Rotor-Gene Q Series Software 2.3.1. The successful amplification was confirmed only in the sample, without amplification in the negative control. The specificity of the qPCR reaction was also checked by examining the melt curve. Only primers that passed these tests were used in this experiment. The gene information and primer sequences are shown in Table 1.

**Table 1.** Gene information and primer pairs related to fruit maturation and used in qRT-PCR.

| Gene Name | Gene ID | Ch. | Forward Primer (5′-3′) | Reverse Primer (5′-3′) | Description |
|---|---|---|---|---|---|
| | Ethylene synthesis and signal transduction | | | | |
| *MdACO1* | MDP0000195885 | 10 | CAGAATGTCGA-TAGCCTCGT | GCAGTCCAG-AATACAGCTTC | 1-aminocyclopropane-1-carboxylate oxidase 1 |
| *MdACO7* | MDP0000200896 | 15 | GGTGGAAAG-GGTCCTTCTGT | CACCAGCAT-CAGTGTGCTCT | 1-aminocyclopropane-1-carboxylate oxidase 1 |
| *MdCTR1* | MDP0000230308 | 12 | ACAAGATTTT-CATGCCGAAC | TATGGACAA-GTTTGGAGGCT | Serine/threonine-protein kinase CTR1 |
| *MdEIL1* | MDP0000423881 | 15 | GTTCGATGC-TTCGGGACTT | ACCTGACT-GGTTCACTGGTTG | ETHYLENE INSENSITIVE 3-like 1 protein |
| *MdERF1* | MDP0000128979 | 13 | TCAGATCTTGA-CACCATCTCT | CACTTGTCAC-TACTTTGGTGATAG | Ethylene-responsive transcription factor RAP2-3-like |
| | Auxin signal transduction | | | | |
| *MdARF1* | MDP0000194603 | 7 | AAAGATTGG-TTGCTGGTGAC | TCACTGACGA-GGGTATCTGA | ADP-ribosylation factor 1 |
| *MdIAA11* | MDP0000164095 | 9 | TTCGTAAG-TGCAGTTCCTCC | TCCATCTCA-GTGGCCATATCT | Auxin-responsive protein IAA26-like |

**Table 1.** *Cont.*

| Gene Name | Gene ID | Ch. | Forward Primer (5′-3′) | Reverse Primer (5′-3′) | Description |
|---|---|---|---|---|---|
| | Transcription factors | | | | |
| *MdNAC5* | MDP0000868419 | 3 | CATGCAGTT-CTGGGGTCACT | TCAAGCGCT-AAATGATACGTGC | NAC domain-containing protein 18 |
| *MdNAC3* | MDP0000173636 | 5 | GACCACTA-GGAGATGGGGTT | ACGTTACCC-GTATATCGTTGCT | NAC transcription factor 25-like |
| *MdMADS8* | MDP0000366022 | 17 | GCAAAGGAA-CTTGAGAGCAGC | AATGGACCC-AAGTCCTCACC | Developmental protein SEPALLATA 1 |
| *MdMADS7* | MDP0000326390 | 14 | AACCTACCA-GCCAACGAGAC | CCTTTGTGTT-CAGGTGGGAC | MADS-box protein CMB1-like |
| | Housekeeping gene | | | | |
| *MdACTIN* | MDP0000170174 | 7 | GGCTCTATTC-CAACCATCCA | TAGAAGCAGT-GCCACCACAC | Actin-related protein 7 |

Relative expression levels were determined using the delta–delta Ct relative quantitation method.

### 2.6. Statistical Analysis

Significant differences between samples were examined by using Student's *t*-test ($p < 0.05$: *, $p < 0.01$: **). Statistical analyses were performed using SAS 9.4 software.

## 3. Results

### 3.1. Fruit Quality Analysis during Maturation of 'Beni Shogun' and 'Fuji'

Both 'Fuji' and 'Beni Shogun' reached full bloom on April 23, 2018. The maturation of fruits was determined based on the Cornell starch index [38], and 'Beni Shogun' reached the index of 4 at 116 days after full bloom (DAFB), while 'Fuji' reached it at 153 DAFB. After this point, the fruits were considered to be in the ripening stage (Figure 2a).

'Beni Shogun' fruits reached the starch index of 8 on 22 September, while 'Fuji' fruits reached the Cornell starch index of 8 on 22 October, which was 183 DAFB. The fruit weight, length, and width of both 'Fuji' and 'Beni Shogun' grew similarly until 153 DAFB. Afterward, the size of the 'Fuji' fruit grew very slowly, increasing by only about 18 g from 237 g to 255 g over a period of 30 days (Figure 2b–d).

In terms of acidity, 'Beni Shogun' was generally lower than 'Fuji' at all stages, but there was no significant difference between the two ('Beni Shogun', 0.51%; 'Fuji', 0.53%; $p = 0.26$) at the maturation stage ('Beni Shogun', 116 DAFB; 'Fuji', 153 DAFB) (Figure 2e). As for sweetness measured by SSC, both showed a similar trend in increase until 153 DAFB, but 'Fuji' had a higher sugar content ('Beni Shogun', 10.1%; 'Fuji', 11.9%; $p < 0.0001$) compared to 'Beni Shogun' at the maturation stage (Figure 2f). The firmness of 'Beni Shogun' was lower than 'Fuji' at all stages, even at the maturation stage ('Beni Shogun', 64 N; 'Fuji', 70 N; $p < 0.0001$) (Figure 2g). When comparing the ethylene production levels, a statistically significant difference was observed at the maturation stage ('Beni Shogun', 0.7 µL/L; 'Fuji', 0.5 µL/L; $p = 0.026$), and this difference became greater at the last sampling point ('Beni Shogun', 4.2 µL/L; 'Fuji', 2.2 µL/L; $p = 0.0003$) (Figure 2h).

### 3.2. Comparison of Gene Expression Associated with Ethylene

The ripening behaviors of 'Beni Shogun' and 'Fuji' apples were investigated at the molecular level by comparing the expression levels of genes involved in ethylene biosynthesis. 'Beni Shogun' showed continued fruit growth and increased ethylene production even during the ripening stage (136~153 DAFB), while 'Fuji' exhibited limited fruit growth and lower ethylene production compared to 'Beni Shogun' during the ripening stage (159~183 DAFB) (Figure 2a–c,h).

The ethylene biosynthesis genes, *MdACO1* and *MdACO7*, showed distinct and contrasting patterns from each other. In *MdACO1*, the expression levels were low in the early and middle stages of fruit development for both apple varieties but sharply increased after

the maturation date of each variety, with much higher expression levels in 'Beni Shogun' compared to 'Fuji' (Figure 3a).

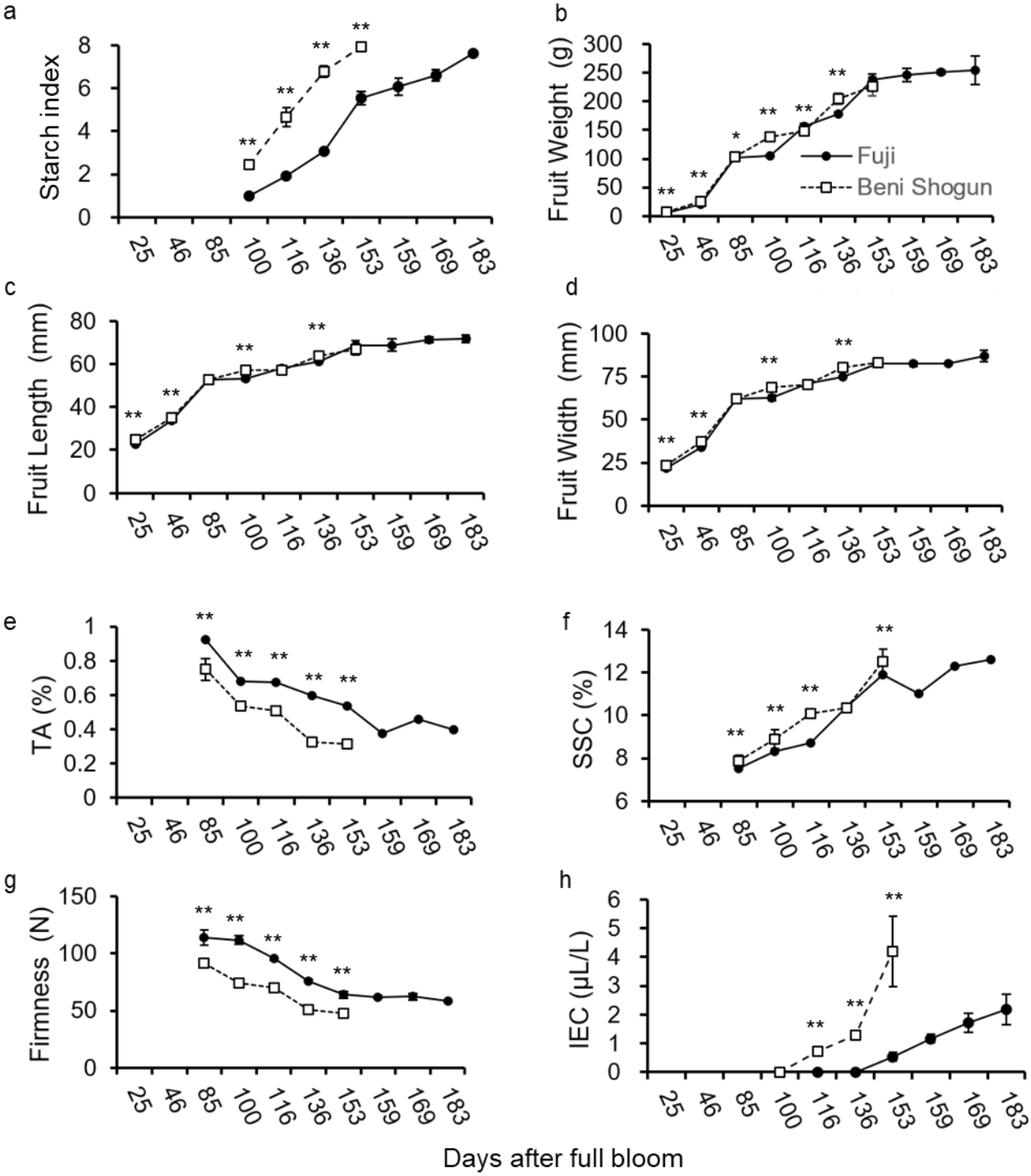


**Figure 2.** Fruit quality changes in 'Fuji' and 'Beni Shogun' during developmental stages. Starch–iodine index (**a**), fruit weight (**b**), length (**c**), width (**d**), titratable acidity (TA) (**e**), soluble solids content (SSC) (**f**), firmness (**g**), and internal ethylene concentration (IEC) (**h**) were measured until maturity. The starch–iodine index values are based on the Cornell index chart. Error bars indicate the ±SE ($n$ = 9), where the IEC and starch–iodine index were $n$ = 15. Single stars (*) indicate statistical significance at $p < 0.05$, while double stars (**) indicate significance at $p < 0.01$.

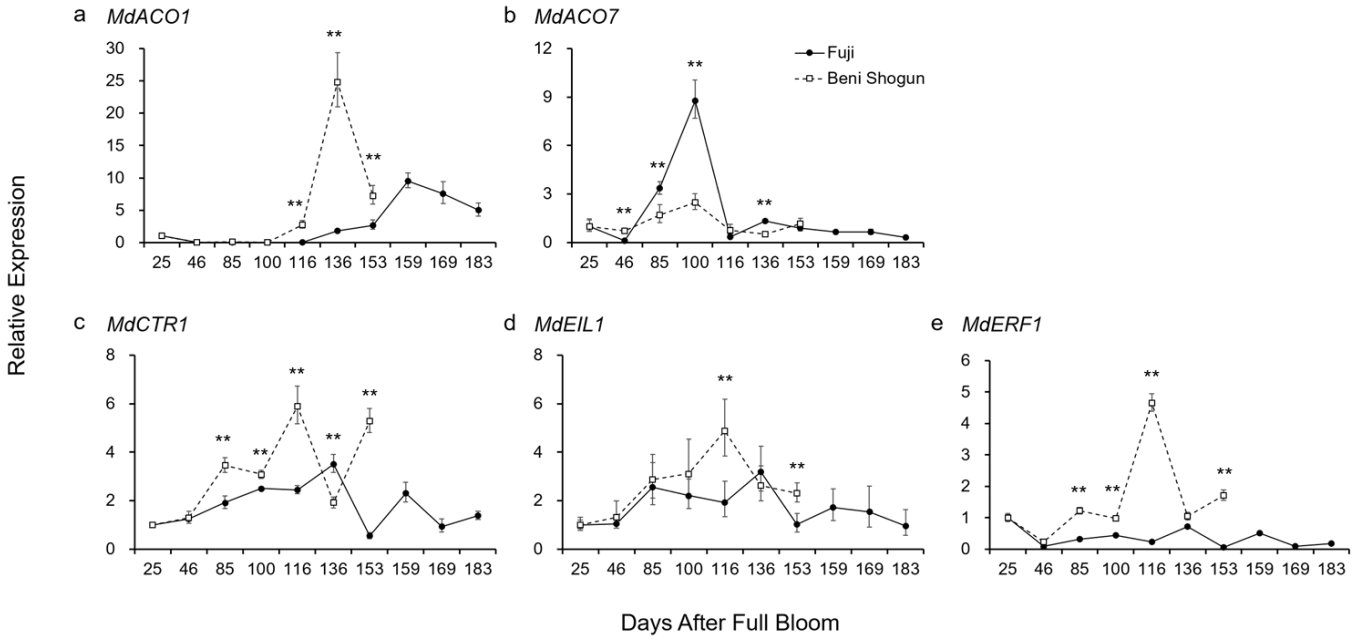


**Figure 3.** Relative gene expression patterns related to ethylene in 'Beni Shogun' and 'Fuji' during fruit development. Genes are grouped as ethylene synthesis (**a**,**b**) (*MdACO1* and *MdACO7*) and signal transduction (**c**–**e**) (*MdCTR1*, *MdEIL1*, and *MdERF1*) genes. All genes were analyzed by using qRT-PCR and normalized relative to the expression reference gene, *ACTIN*. Relative expression levels were normalized to 25 DAFB values. Error bars indicate the ±SE ($n$ = 3). Double stars (**) indicate significance at $p < 0.01$.

On the other hand, *MdACO7* showed higher expression levels in the early and middle stages of fruit development and lower levels after the maturation date, with much higher expression in 'Fuji' compared to 'Beni Shogun' (Figure 3b). Genes associated with ethylene signal transduction reached their highest expression levels in 'Beni Shogun' at 116 DAFB, whereas in 'Fuji', they peaked at 136 DAFB, with higher expression levels in 'Beni Shogun' at the peak (Figure 3c–e). The difference in expression levels of ethylene-biosynthesis-related genes between the two apple varieties suggests that there are variations in the molecular pathways responsible for ethylene production, which may explain the differences in ripening behavior.

### *3.3. Comparison of Gene Expression Linked to Auxin and Developmental Regulation*

Ethylene is a significant factor in the regulation of ripening, although maturity is not entirely dependent on ethylene alone. *MdARF1* is a gene involved in auxin signal transduction, and its expression levels in 'Beni Shogun', which suppresses auxin production, continuously increased from the early stages of fruit development to the final sampling period, except at 136 DAFB (Figure 4a).

In contrast, the expression levels of *MdARF1* in 'Fuji' showed a more monotonic change and peaked at 100 DAFB, which is lower than that of 'Beni Shogun' (Figure 4a). Similarly involved in auxin signal transduction, the gene, *MdIAA11*, which promotes auxin production, showed a decreasing trend after peaking at 46 DAFB in 'Beni Shogun', whereas in 'Fuji', it peaked at 100 DAFB and then decreased (Figure 4b).

An analysis of the expression levels of transcription factor genes, *MdNAC3*, *MdNAC5*, *MdMADS7*, and *MdMADS8*, which regulate fruit development and ripening, was also conducted. Both *MdNAC3* and *MdNAC5* showed a continuous increase in expression levels from the early stage of fruit development, with *MdNAC3* reaching its peak at 116 DAFB and being higher in 'Beni Shogun', while *MdNAC5* reached its peak at 136 DAFB and was higher in 'Fuji' (Figure 4c,d). As for the expression levels of *MdMADS7* and *MdMADS8*,

'Beni Shogun' reached its highest level at 116 DAFB, while 'Fuji' reached its highest level at 136 DAFB and sharply decreased at 153 DAFB (Figure 4e,f). Before that stage, 'Beni Shogun' tended to show higher expression levels than 'Fuji'.

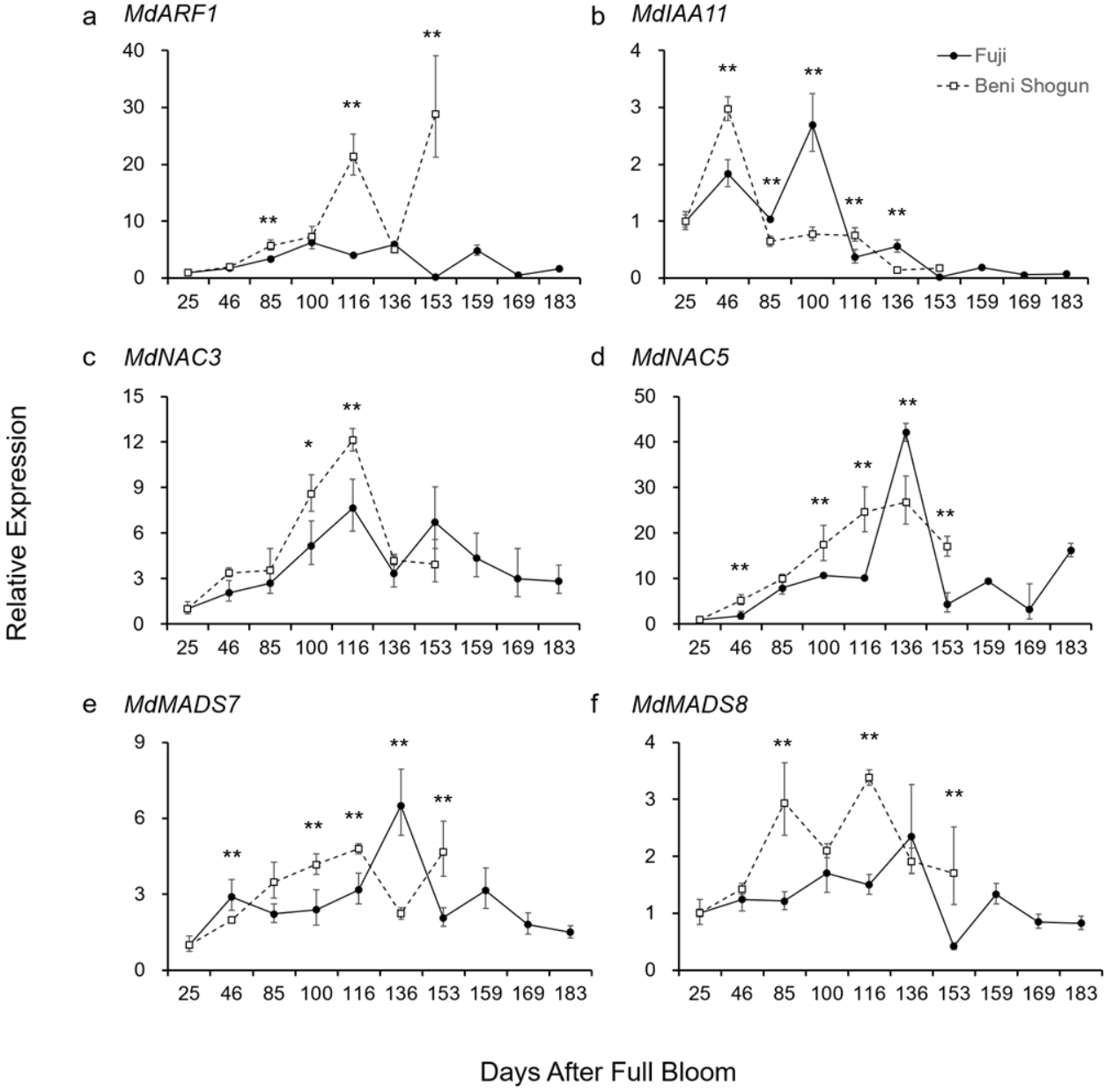


**Figure 4.** Relative gene expression patterns related to auxin and developmental regulation of 'Beni Shogun' and 'Fuji'. Genes are grouped as auxin signal transduction (**a**,**b**) and developmental regulation (**c**–**f**) genes. All genes were analyzed by using qRT-PCR and normalized relative to the expression reference gene, *ACTIN*. Relative expression levels were normalized to 25 DAFB values. Error bars indicate the ±SE ($n$ = 3). Single stars (*) indicate statistical significance at $p < 0.05$, while double stars (**) indicate significance at $p < 0.01$.

### *3.4. Clustering Analysis of Phenotyping Trait and Expression Data*

When analyzing the phenotypes and expression patterns of key hormone and fruit development and ripening-related transcription factor genes observed in 'Beni Shogun' and 'Fuji' separately, it is difficult to determine the correlation between them. Therefore, clustering analysis was conducted to discover the hidden relationships between them. As a result, three major clusters were identified: C1, consisting of two genes and two phenotypes,

totaling four; C2, consisting of only eight genes; and C3, consisting of six phenotypes and one gene, totaling seven (Figure 5).

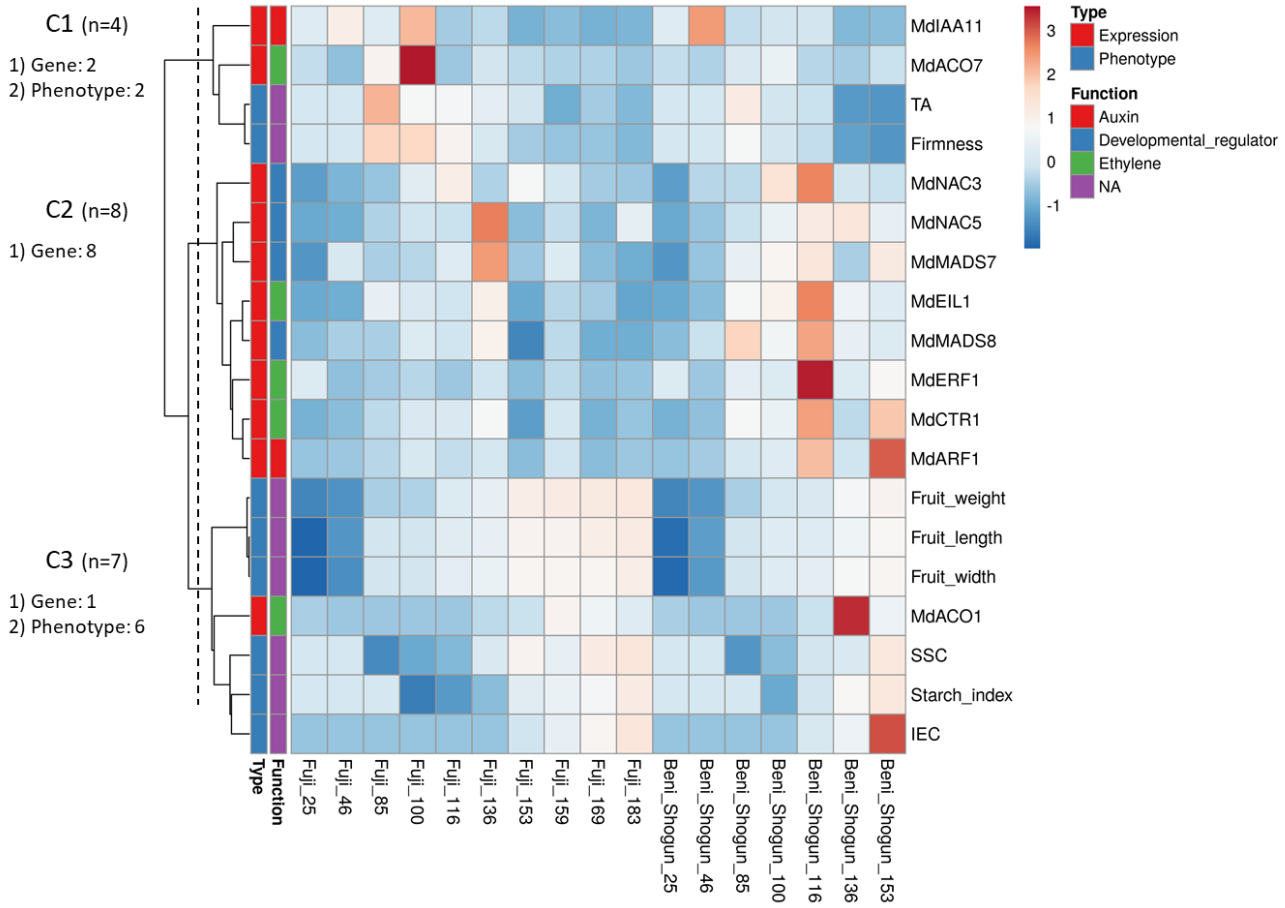


**Figure 5.** Hierarchical clustering heatmap analysis of 8 phenotypes and 11 genes related to fruit development, maturation, and ripening between 'Beni Shogun' and 'Fuji'. They are divided into 3 clusters (C1 to C3). Unit variance scaling is applied to rows.

In C1, *MdACO7* and *MdIAA11* were clustered with fruit acidity and firmness, and they both showed higher levels at 100 DAFB in 'Fuji' compared to the other stages. Regarding the eight genes belonging to C2, *MdNAC5* and *MdMADS7* showed relatively high values at 136 DAFB in 'Fuji', which is right before the maturation stage of 'Fuji', while the other six genes (*MdNAC3*, *MdEIL1*, *MdMADS8*, *MdERF1*, *MdCTR1*, and *MdARF1*) showed high values at 116 DAFB in 'Beni Shogun', which is the maturation stage of 'Beni Shogun'. Such data imply that these genes play a role in fruit maturation. Lastly, C3 included six phenotypes related to fruit size, length, width, sweetness (SSC), starch content, and ethylene production (IEC), along with one gene, *MdACO1*. This suggests that the *MdACO1* gene may have a diverse role in overall fruit quality.

## 4. Discussion

### *4.1. Fruit Quality and Ripening Behavior Difference between Two Cultivars*

The present study aimed to compare the fruit development, maturation, and ripening characteristics of two apple cultivars, 'Beni Shogun' and 'Fuji'. The results show that both cultivars reached full bloom on the same day in 2018, but 'Beni Shogun' matured earlier than 'Fuji', reaching the Cornell starch index of 4 at 116 DAFB, while 'Fuji' reached it at 153 DAFB (Figure 2a). This early maturation in 'Beni Shogun' is consistent with previous results [15]. Moreover, the fruit weight, length, and width of both cultivars grew similarly until 153 DAFB, but 'Fuji' grew very slowly afterward (Figure 2b–d). Although there

were no statistically significant differences in acidity at the maturation stage (Figure 2e), significant differences were observed in other fruit quality parameters such as SSC, firmness, and IEC, and these differences were observed throughout the entire fruit development process (Figure 2f–h). These findings suggest that 'Beni Shogun' and 'Fuji' have distinct fruit development and ripening behaviors, with 'Beni Shogun' maturing earlier than 'Fuji'. This information could be useful for growers to optimize the harvest time for 'Beni Shogun' and 'Fuji'. Moreover, the differences in acidity, sugar content, and firmness at different stages of fruit development could help inform the processing and storage of apples for different purposes.

### *4.2. Putative Molecular Mechanism behind Distinct Fruit Development, Maturation, and Ripening*

The regulation of fruit development and ripening is a complex process involving multiple signaling pathways [29–31], hormones [23–25], and transcription factors [35–37]. The results of our study show that the expression patterns of genes involved in ethylene biosynthesis and signal transduction (Figure 3), as well as those involved in auxin signal transduction and transcriptional regulation (Figure 4), differ between the two apple varieties, 'Beni Shogun' and 'Fuji'.

Our analysis of the ethylene biosynthesis genes, *MdACO1* and *MdACO7*, revealed distinct and contrasting patterns from each other in both varieties (Figure 3a,b). The higher expression levels of *MdACO1* in 'Beni Shogun' compared to 'Fuji' after maturation suggest that 'Beni Shogun' produces more ethylene than 'Fuji', which could explain the difference in ethylene production (Figure 2h). Although ethylene is an important regulator of fruit ripening, it is not the only factor involved. In fact, other factors such as auxin have been found to play a significant role in fruit maturation [23,28]. Specifically, a decrease in auxin levels has been shown to be a prerequisite for the maturation process in diverse fruit crops [32–34]. For example, according to Ireland et al. (2013) and Schaffer et al. (2013), the initiation of ripening was postponed and high levels of auxin were maintained during the maturation period in transgenic apples that inhibited the ripening inhibitor (rin)-like MADS-box gene [32,33]. The interplay between auxin and other hormones, such as ethylene, is also crucial for fruit development. It was discovered that the regulation of auxin transport and homeostasis affects the ripening time in apples by potentially activating the ethylene biosynthesis pathway [41]. When the *MdARF* gene was overexpressed or treated with auxin, ethylene production increased in apples, indicating the effect of auxin on ethylene production in apples [42]. Our analysis of the expression levels of *MdARF1* and *MdIAA11*, genes involved in auxin signal transduction, showed that their expression patterns also differed between the two varieties. In 'Beni Shogun', the expression of *MdARF1* continuously increased from the early stages of fruit development, while *MdIAA11* decreased after peaking at 46 DAFB (Figure 4a,b). In contrast, in 'Fuji', both genes showed a more monotonic change, peaking at 100 DAFB for *MdARF1* and *MdIAA11*. These results suggest that the regulation of auxin signaling pathways may also contribute to the differences in ripening behavior between the two varieties.

We also analyzed the expression levels of the transcription factor genes, *MdNAC3*, *MdNAC5*, *MdMADS7*, and *MdMADS8*, which regulate fruit development and ripening [35–37]. The higher expression levels of *MdNAC3* in 'Beni Shogun' and *MdNAC5* in 'Fuji' suggest that these transcription factors may play a role in the differential regulation of fruit ripening between the two varieties (Figure 4c,d). Additionally, the higher expression levels of *MdMADS7* and *MdMADS8* in 'Beni Shogun' at 116 DAFB may contribute to the earlier and faster ripening of this variety compared to 'Fuji' (Figure 4e,f).

### *4.3. Clustering Analysis Reveals Hidden Relationships between Gene Expression Patterns and Fruit Phenotypes in Two Apple Varieties*

The results of the clustering analysis provide insights into the correlation between the phenotypic traits and expression levels of the key hormone and fruit development and ripening-related genes in 'Beni Shogun' and 'Fuji' apples. The three major clusters

identified suggest that the genes and traits within each cluster may play a role in the same developmental or ripening process.

Cluster C1, consisting of *MdACO7* and *MdIAA11* along with fruit acidity and firmness, showed high values in 'Fuji' at 85 or 100 DAFB (Figure 5). This suggests that the expression levels of these genes may be associated with fruit quality in 'Fuji' apples. The eight genes in cluster C2, including *MdNAC5* and *MdMADS7*, showed high expression levels in 'Fuji' just before the maturation stage, indicating that these genes may be involved in the regulation of fruit maturation in 'Fuji' apples. The six genes in C2, including *MdNAC3*, *MdEIL1*, *MdMADS8*, *MdERF1*, *MdCTR1*, and *MdARF1*, had high expression levels at the maturation stage of 'Beni Shogun', indicating their role in the regulation of maturation in this variety. Cluster C3 included six phenotypic traits related to fruit size, length, width, sweetness, starch content, and ethylene production, as well as one gene, *MdACO1*. This suggests that *MdACO1* may play a diverse role in overall fruit quality [23–25].

Overall, the clustering analysis provides a more comprehensive understanding of the relationships between gene expression patterns and phenotypic traits in these two apple varieties. These findings could be useful for further studies on apple development and ripening, as well as for developing strategies for apple breeding and genetic modification to enhance fruit quality. We anticipate further studies to be conducted to identify the co-expressed genes with those identified in this study, and to investigate their functions through over-expression or knockdown experiments. Through these additional experiments, it is anticipated that a higher level of understanding of the molecular differences between the commercially valuable 'Fuji' apple and its bud mutation 'Beni Shogun' will be possible.

### *4.4. A Working Model for How 'Beni Shogun' and 'Fuji' Showed Different Fruit Development, Maturation, and Ripening Behaviors*

Based on the results and discussions presented above, the following working model can be suggested for the regulation of fruit development, maturation, and ripening in 'Beni Shogun' and 'Fuji' apples:

(1) Multiple signaling pathways, hormones, and transcription factors regulate fruit development and ripening in apples.
(2) Ethylene biosynthesis and signal transduction play an essential role in the ripening process of both 'Beni Shogun' and 'Fuji' apples.
(3) The higher expression of *MdACO1* in 'Beni Shogun' suggests that this variety produces more ethylene than 'Fuji', which could explain the difference in ethylene production between the two varieties.
(4) Auxin also plays a significant role in fruit maturation, and its interplay with ethylene is crucial for fruit development.
(5) The regulation of auxin transport and homeostasis-related genes including *MdARF1* and *MdIAA11* affects the ripening time in apples by potentially activating the ethylene biosynthesis pathway.
(6) The higher expression levels of *MdNAC3*, *MdNAC5*, *MdMADS7*, and *MdMADS8* in 'Beni Shogun' and *MdNAC5* in 'Fuji' suggest that these transcription factors may play a role in the differential regulation of fruit ripening between the two varieties.

## 5. Conclusions

Based on the findings of our study, it is clear that the regulation of fruit development and ripening in apples is a complex process that involves multiple signaling pathways, hormones, and transcription factors. Ethylene biosynthesis and signal transduction are essential for the ripening process in both 'Beni Shogun' and 'Fuji' apples. Additionally, auxin has a significant role in fruit maturation, and its interplay with ethylene is crucial for fruit development. The regulation of auxin transport and homeostasis-related genes including *MdARF1* and *MdIAA11* affects the ripening time in apples by potentially activating the ethylene biosynthesis pathway. Finally, the higher expression levels of *MdNAC3*,

*MdNAC5*, *MdMADS7*, and *MdMADS8* in 'Beni Shogun' and *MdNAC5* in 'Fuji' suggest that these transcription factors may play a role in the differential regulation of fruit ripening between the two varieties. These findings provide molecular insights into the differences in fruit qualities and ripening behaviors of 'Beni Shogun' and 'Fuji' apples, which could have implications for apple breeding and cultivation practices. However, further studies are needed to determine how these differences can be exploited to improve the quality and shelf-life of apples.

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