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Cell Division Controls Final Fruit Size in Three Apple (*Malus x domestica*) Cultivars

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Abstract: Apple (*Malus x domestica*) fruit size is dependent on cell division and cell expansion, processes that are subsequently regulated by plant hormones such as auxins, gibberellins, and cytokinins. In this study, we investigated the role of cell division and cell expansion in apple growth and identified which of the two was more deterministic of final fruit size. Three cultivars of different sizes were selected, namely, "Twenty Ounce" (large-sized), "Royal Gala" (medium-sized), and "Crabapple" (small-sized). Gene expression and cell size analyses were conducted over the course of two consecutive seasons. The expression patterns of three classes of genes were markedly similar across all cultivars. Two cell division markers, namely *MdCDKB2;2* and *MdANT2*, were discovered to be correlatively expressed, as both displayed initially high expression levels, which gradually declined from the early to late stages of the growth time course. For cell expansion markers, *MdEXP3* was upregulated as the cells expanded, while *MdARF106* was expressed in both the cell division and expansion stages. Meanwhile, the ripening-related gene *MdACO1* was expectedly expressed only during the ending stages associated with ripening. Interestingly, the cell measurements taken regularly from each cultivar throughout the same experimental timespan showed that cell sizes were unaltered and remained constant from initial pollination at the zeroth Day After Pollination (DAP) to ripening at 120 Days After Full Bloom (DAFB).

Keywords: apple; fruit size; fruit development; cell division; cell expansion; ripening

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1. Introduction

Apple fruit size is dependent on its cell number and size, characteristics regulated by cell division and expansion rates [1,2]. Although the average-sized fruit has approximately 50 million cells, some grow larger by either increasing their cell numbers or enlarging their cells [3]. For commercially grown apples, fruit size is an important factor in determining the keeping quality of apples in storage [4]. All fruits stored together must be of a similar size as smaller apples have a shorter storage life than their bigger counterparts. Furthermore, as they are sold by weight, producing larger fruits will help growers gain maximum profit [3]. Therefore, research on the factors controlling final fruit size hold both economic and scientific interest.

Great variations in fruit size exist among apples. For instance, Crabapples are typically 5 cm in diameter, whereas other cultivars may produce fruit in excess of 10 cm [1,5]. Apart from cellular growth, harvest time is also a critical determinant of fruit size. Red Delicious are larger than Royal Gala at harvest as their delayed maturity means a longer time on the tree [3]. Enlargement strategies have also been introduced to improve fruit size. In fruit thinning, lateral flowers are removed either manually [1,6] or by utilising plant growth hormones, such as gibberellin, auxin, and cytokinin [7,8]. This increases the leaf:fruit ratio,

thus assisting the remaining flowers to grow larger fruits [1,9]. Unfortunately, genetics bears a strong influence and may render the thinning process unsuccessful in some cultivars: for instance, Crabapples will remain small despite cultivation under the same conditions as bigger cultivars [3]. Deeper knowledge of fruit development at a cellular and molecular level would therefore be valuable to improve techniques of fruit size manipulation in apple breeding.

Fruits are reproductive organs unique to the Angiosperm phylum that have evolved to promote seed dispersal. Fleshy fruits assist seed dispersal by attracting animals due to their colour, flavour, nutritional content, and texture. Despite being a major contributor to the human diet, little is known about how the flesh tissue develops, differentiates, and ripens. Fruit development starts with successful fertilisation. This event releases the hormones necessary to consecutively induce the stages of fruit set, cell division, cell expansion, and ripening [10,11]. The entire process takes 20 to 21 weeks, which culminates with the production of a crisp fruit with a waxy cuticle.

The cell division stage begins during fruit set, a stage characterised by the rapid amplification of cell numbers [11,12]. Division ceases three to four weeks after pollination to allow expansion to take place [10,11]. Although cell expansion continues until full ripening, the process reaches its peak 40 to 60 days after anthesis [8,11]. The onset of ripening occurs at 90 DAFB, after which the fruit would continue to mature until fully ripened by 146 DAFB [8,11]. The ripening stage is particularly crucial as it entails the changes necessary to increase the fruit's attractiveness to agents of seed dispersal such as birds and animals [11,13,14].

Final fruit size is determined by cell division and expansion. Thus, an understanding of cell cycle regulation would provide a good picture of fruit development in plants [5,15]. Cell cycle progression is a controlled process involving specific checkpoints monitored by a large family of serine/threonine protein kinases, such as the Cyclin-Dependent Kinases (CDKs), an activating subunit, the cyclins, and CDK inhibitors [12,15]. Although *CDKB2;1* is relatively well studied [16,17], *CDKB2;2* is not, likely due to its absence in some plant species [11]. In apples, the *MdCDKB2* gene has been identified by Janssen et al. [8] from a microarray data pool, findings which were then refined and elaborated upon by Malladi and Johnson [12]. Our interest in utilising *CDKB2;2* as a marker of cell division lies in its distinct downregulation towards the end of the early developmental stages, an observation that may have alluded to the participation of *CDKB2;2* in the cell cycle regulation of the apple fruit.

AINTEGUMENTA (*ANT*) is a plant-specific gene of the *APETALA 2* (*AP2*) family of floral transcription factors and is active in developing flower and vegetative tissues [2,18]. In *Arabidopsis*, *ANT* was demonstrated to control cell proliferation in integuments during ovule growth [2,19]. The loss of *ANT* function decreased cell division activity, resulting in reduced floral organ size. However, apple-specific homologues of *ANT* are yet to be shown to function similarly to their *Arabidopsis* counterparts [1,2]. Consequently, we opted to analyse the apple *ANT* gene, *MdANT2*, as another potential marker of the cell division phase in apple fruit development.

Several other genes have been identified for their potential involvement in apple fruit growth. Previous research observed the upregulation of *MdANT1* and *MdANT2* during the early stages of fruit growth. Their high expression levels were maintained to the end of the cell division stage to propel the division of competent undifferentiated cells. However, meristematic cells eventually lose their competence with both *ANT* variants becoming downregulated as the fruit enters the ripening stage [1,2,19]. These observations were nonetheless critical in demonstrating that the cyclical expression of *MdANT1* and *MdANT2* coincided with the developmental stage of cell division during fruit growth. Both genes are known to be more lengthily and highly expressed in larger fruits within the cultivar Golden Delicious Smoothie (GS) [1,2]. Moreover, *MdANT1* and *MdANT2* have exhibited a positive correlation to A- and B-type CDKs during the regulation of cell production and cell cycle in apples [1,2].

Meanwhile, the Apple Auxin Response Factor 106 (*MdARF106*) is a transcription factor discovered due to its QTL-mapped co-localisation with a population of fruit growth genes. Analysis of its expression patterns during the cell division and expansion stages of development then supported the hypothesis that *MdARF106* too might regulate fruit growth [20,21].

Expansins are a family of proteins that catalyse cell wall expansion [22,23]. In *Arabidopsis*, the expansin family consists of nearly 30 genes divided into two subfamilies, designated as either α or β, based on sequence divergence and biochemical activity [23–25]. Cell expansion entails the synthesis of new plant cell walls around nascent cells, a process that inevitably enlarges cellular size, thus leading to plant bulking and growth [23,26]. In apple fruit size studies, higher expression levels of *MdEXP3* were observed in the big-sized cultivar *M. domestica* "Sekaiichi" in comparison to that of the small-sized *M. floribunda* during their expansion phases (35 and 49 DAFB), thus supporting the role of expansins during this stage of fruit growth [27]. Their effects are also cell-expansion-specific, as *MdEXP3* was only negligibly detectable during the preceding cell division stage (21 DAFB) [27]. Another study then suggested that *MdEXP3* may be linked with ripening or and ethylene regulation [28]. Nonetheless, expansins undoubtedly play a vital role in cell expansion as the low activity of another gene family member, *MdEXPA10;1*, caused a decline in fruit growth due to low cell wall expansion activity [29].

2. Materials and Methods

Entire experiments were repeated over two consecutive seasons. Gene expressions were analysed with samples from two biological replicates (Rep1 and Rep2). Cell area and therefore size were determined by measuring three individuals at each time point.

2.1. Pollination

Pollen obtained from Granny Smith was used to hand-pollinate the flowers of Twenty Ounce, Royal Gala, and Crabapples. The Granny Smith variety is one of the most common and compatible pollinisers for many apple varieties including these three cultivars studied. All pollination was performed early in the morning (before 11 a.m.), which is when the most flowers are first open, in order to minimize competition with other pollinators such as bees. An unpollinated blooming flower was distinguished by its white stigma, as opposed to the brown stigma of pollinated blooms. To pollinate a flower, a small paint brush was first dipped into the pollen, then gently brushed against the stigma of a blooming flower. Each flower was tagged for recognition during sample collection. A total of 108 flowers were pollinated during Season 1 and 300 during Season 2. We opted to pollinate more flowers during Season 2 in order to obtain a higher concentration of RNA.

2.2. Harvest

The hypanthium of unpollinated flowers was collected from each cultivar as controls (un-bloomed flowers). Pollinated flowers or fruits were collected and dissected according to the pre-determined time points. Samples were collected for three types of experiments, i.e., physiology, histology, and gene expression. For physiology studies, the equatorial diameter of a fruit was determined using callipers. Measurements were taken from three individual fruits, after which they were quickly imaged. For histology experiments, flowers were fixed in a fixative solution before storage in 4 °C. For gene expression analyses, fresh samples were processed on-site at the orchard to retain RNA integrity during the process of their transport back to the laboratory. Briefly, the hypanthiums of flowers were dissected with a scalpel to discard their ovaries, then flash frozen in liquid nitrogen. Samples were immediately stored in a −80 °C freezer upon arrival.

2.3. RNA Isolation and qPCR Analyses

The RNA was extracted from frozen samples. Due to the low concentration of RNA in the mesocarp region of fruits, we opted to follow a protocol that was previously described

for pine needles [30,31]. This protocol works in isolating a higher concentration of RNA from even small-sized tissues. Clean-up was achieved using the RNeasy Mini Kit (Qiagen) and Ambion Turbo DNase treatment Kit, both performed as per the manufacturer's instructions. First-strand cDNA was synthesised using the First Strand Superscript III cDNA Supermix Kit (Invitrogen). Two biological replicates were prepared for each time point and cultivar. The qPCR runs were executed on the Applied Biosystems Real-Time PCR platform alongside the housekeeping gene *MdGAPDH* for normalisation purposes. Three replicates of each reaction were prepared for each of the 8 markers used. qPCR was performed on both biological replicates (Rep1 and Rep2) for each season's samples. The data were analysed using the Livak method ($\Delta\Delta C_T$) [32] or using the ΔC_t method and normalised against *MdGAPDH*.

To ensure RNA-specific targeting during qPCR, primers for a marker of cell division, *MdCDKB2;2*, and one of cell expansion, *MdEXP3*, were designed to span the exons of their genomic sequences. The specific qPCR marker of the *MdCDKB2:2* gene was designed by using the GeneBank accession number (CN943384) that was published in a report [33]; the cDNA sequence was searched on the NCBI website. The sequence was then copied and blasted on the Genome Database of Rosaceae (GDR) website. The results indicated that there were three chromosomes where *CDKB2:2* could be located—Chromosome 6, Chromosome 6 (Haploid 1), and Chromosome 9. The accession number/code of the three sequences were analysed against the GDR database. The gene model sequence code for Ch6 was MDP0000418062. The gDNA, which was in reverse sequence, was saved in Notepad (Microsoft Corporation, Redmond, WA, USA). The cDNA sequence was also saved in a separate Notepad file. At this point, there were three gDNAs and cDNAs obtained. Using Geneious Pro software (http://www.geneious.com, accessed on 15 January 2015 [34]), each of the gDNA files was imported and reverse complemented. All cDNA files were also imported into the software.

Within Geneious, all three cDNAs were aligned, and the consensus sequence was saved in a Notepad file. All three gDNAs (reversed) were also aligned and saved in another Notepad file. The aligned consensus sequence of both the cDNA and gDNA were then further aligned, and exons and introns could be predicted. The forward primer was designed by taking the 10 mer from the edge of exon1 and the 10 mer from the edge of exon2 in the start region (using Primer 3 online freeware; www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/, accessed on 15 January 2015). The reverse primer was designed by taking the 20 mer from another exon so that the product would be in the range of 80–100 bp. The primer sequence was then analysed using online software, IDT Oligo Analyser (http://sg.idtdna.com/calc/analyzer, accessed on 15 January 2015). Several parameters were considered, such as the G/C percentage (at least 50%), the possibility of a primer dimer, hairpin, the melting temperature between the pair (65 °C and above; the T_m difference between forward and reverse must not be as high), and the product size (between 80–100 bp only). The designed primers were next opened using IDT to be synthesised. Primer efficiency was evaluated using qPCR.

The *MdEXP3* accession number is MDP0000670959. Primers were designed following the same protocol of the MdCDKB2:2 primer design work. Primer efficiency was evaluated using qPCR. The primer sequence for *MdANT2* was re-synthesised from a published report [1]. An *MdARF106* primer was designed using the same method described for *MdCDKB2:2*. The *MdARF106* accession number is MDP0000232116 [20]. A pair of primers was created. The *MdACO1* primer was re-synthesised from a published work [35,36]. The *MdPG1* primer was obtained from an unpublished work of the Plant Molecular Biology Lab at the University of Auckland, New Zealand. The primer of *MdActin* was re-synthesised from a published work [35]. *MdGAPDH* was re-synthesised from a published work [29]. Table 1 shows the primer sequences generated. Each of the primers' efficiency was evaluated using qPCR.

Table 1. Primer sequences for qPCR analysis.

Primer	Sequence 5′-3′
MdCDKB2:2	TGCACAGGGATCTTAAGC
MdCDKB2:2	ATACTTCTTGAGTGGCAC
MdANT2	CCAAGGTGATCGAACCTAACATTGCAG
MdANT2	TCCTCCAATGCCATTGAGAATGAGAGA
MdARF106	GAGGGGAAGCCGTTTGAGGT
MdARF106	GCCGTCCAAAACACCTTCAAT
MdEXP3	GATGCAGGAGAAGAGGAGGC
MdEXP3	ATTGCACATCTCCAGCACCA
MdACO1	CAGTCGGATGGGACCAGAA
MdACO1	GCTTGGAATTTCAGGCCAGA
MdPG1	TGAACACTTTGCAGCACGAT
MdPG1	GGCGGTTCAAGTGAAAAATG
MdActin	ACCATCTGCAACTCATCCGAACCT
MdActin	ACAATGCTAGGGAACACGGCTCTT
MdGAPDH	TGAGGGCAAGCTGAAGGGTATCTT
MdGAPDH	TCAAGTCAACCACACGGGTACTGT

2.4. Histology Slide Preparation and Cell Area Measurement

Hypanthium segments and fruits were fixed and embedded in paraffin wax. Specimen slides were prepared on a microtome (Leica Biosystems, Deer Park, IL, USA) with a thickness setting of 10 µm per slice. Specimens were stained with 1% Safranin- 0.5% Fast Green [37]. Brightfield microscopy images were obtained using the Leica 500 Microscope, then exported to the ImageJ software for cell area measurements. These steps were repeated with two more individual hypanthium/fruits for each time point and cultivar, for a total of three replicates.

2.5. Phylogenetic Analysis

A phylogenetic tree was generated in order to investigate the relatedness of the apple CDK family with two well-known model plants, which are *Arabidopsis* for dry fruit and tomato for fleshy fruit. A number of CDK genes from *Arabidopsis*, apple, and tomato were taken from reports [12,38–41]. Sequences were analysed using the Basic Local Alignment Search Tool (BLAST). All sequences then underwent BLASTn and tBLASTn to identify the homology. Nucleotide sequences were translated into protein sequences before protein homology between samples was validated using BLASTp. Sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) before a neighbour-joining phylogenetic tree was generated using the Geneious Pro™ software (Auckland 1010, New Zealand, www.geneious.com, accessed on 15 January 2015) with 500 bootstraps [35].

2.6. Statistical Analysis

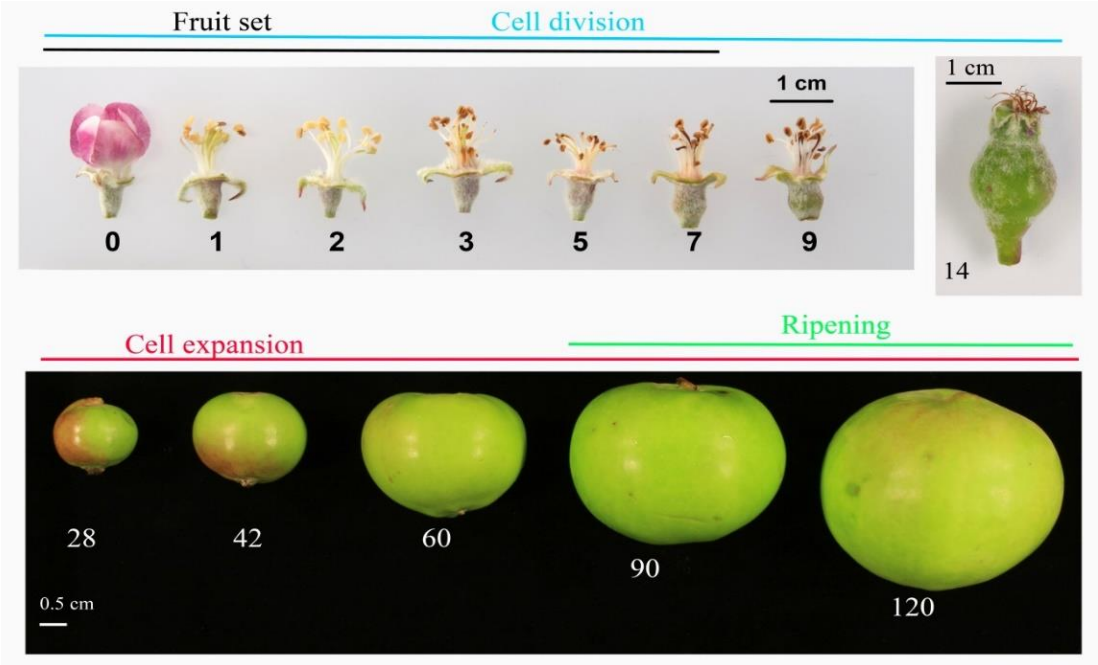
All data in this research were analysed by ANOVA using SPSS version 22 (IBM Corporation, Armonk, NY, USA) or the *t*-test method using Windows Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). Data were judged with a p-value < 0.05 to accept the significant difference. Post-ANOVA, when a significant difference was obtained, Tukey's HSD test was carried out for the separation of means at significance <0.05.

3. Results

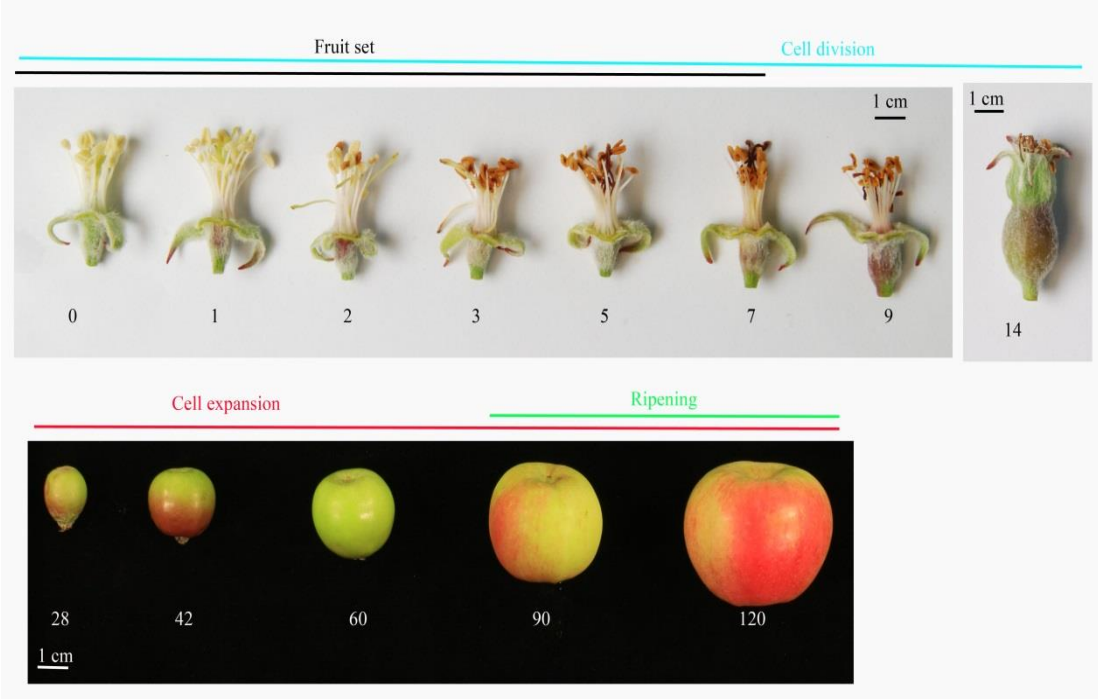
3.1. Cell Coverage Area at before Full Bloom and at Maturity

In this study, fruit development was characterised by its width and cell coverage area measurements (Figure 1A–C). Physiological testing revealed similar hypanthium widths among the three cultivars before full bloom, or at 0 DAFB (Figure 2). As such, the eventual differences in fruit width at maturity (120 DAFB) were instead attributable to differences in total cell number. The Twenty Ounce cultivar experienced a faster growth pace from

early to late development, consequently producing the largest fruit compared to the other cultivars (Figure 2). The most rapid period of fruit width gain for Twenty Ounce was the cell expansion stage (42 DAFB to 120 DAFB). This observation indicated that along with cell division, cell expansion was also accountable for the big size of Twenty Ounce. Histology and gene expression studies were subsequently conducted to further support these hypotheses.

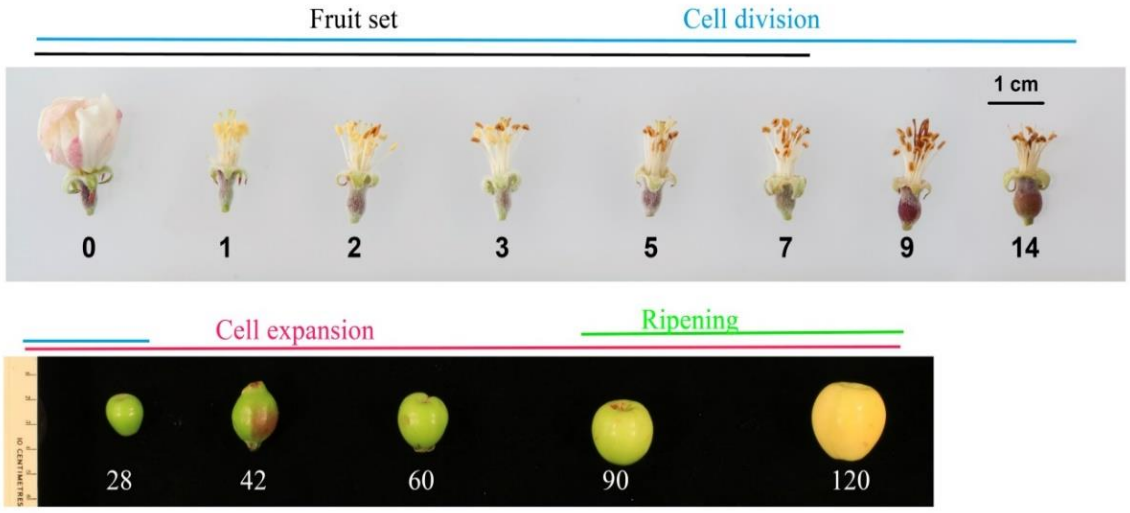


(**A**)



(**B**)

Figure 1. *Cont.*



(**C**)

Figure 1. (**A**) The morphology of the "Twenty Ounce" cultivar at various timepoints (DAFB) throughout the developmental stages of fruit set, cell division, cell expansion, and ripening. (**B**) The morphology of the "Royal Gala" cultivar at various timepoints (DAFB) of the fruit development process such as fruit set, cell division, cell expansion, and ripening. (**C**) Crabapple fruit morphology at various timepoints (DAFB) throughout the development stages of fruit set, cell division, cell expansion, and ripening.

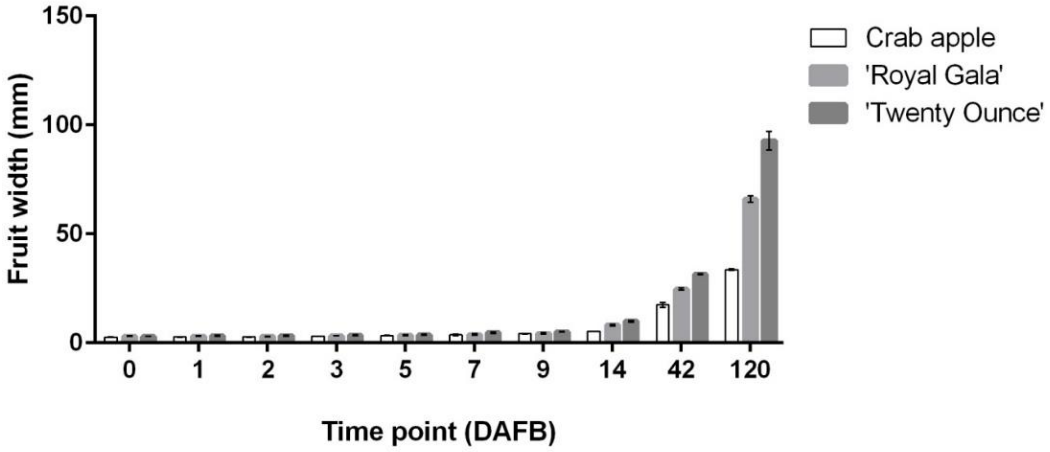


Figure 2. Average fruit diameter of the three cultivars from three individual replicates (Season 2). The differences in measurements between cultivars at 14, 42, and 120 DAFB were statistically different (Tukey's HSD). ANOVA (two-factor), p-value ≤ 0.05.

During the first experimental season, flowers and fruits were collected at 10 time points from 0 to 120 DAFB. Due to the need for more data from the early developmental stages, the number of collection time points was increased to 12 throughout the second experimental season. The cell coverage area of either the floral hypanthium or fruits was measured. Cell area measurements corroborated these findings as they were similar for all three cultivars along the same time points. Such outcomes were consistently obtained across the two seasons and further showed that cells in all cultivars expand at the same pace throughout fruit development (Figure 3). Microscopy images of the hypanthium showed no significant differences in the cell sizes of the cultivars at either 0 or 120 DAFB (Figure 4).

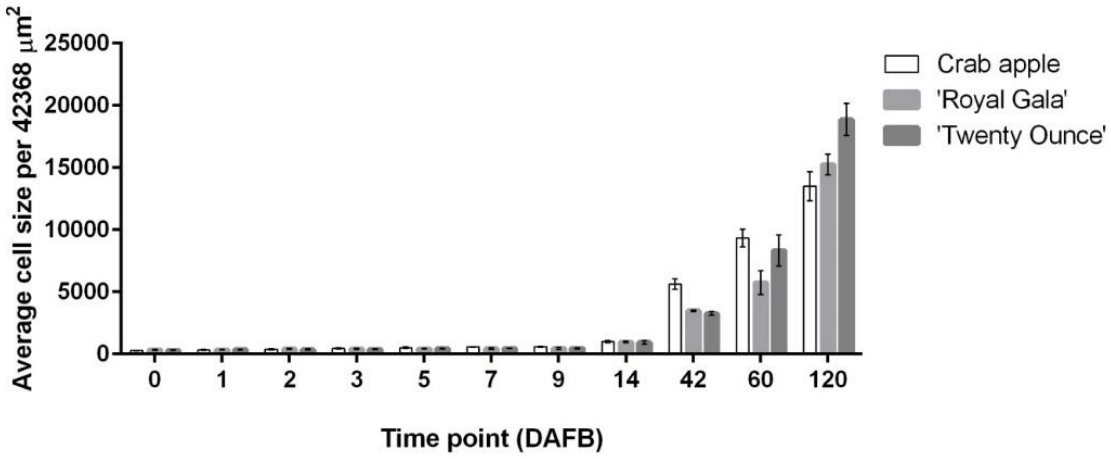


Figure 3. Average cell size of three cultivars from three individual replicates (Season 2). Error bars represent standard deviations. The difference in measurements between cultivars at 42 until 120 DAFB was statistically different (Tukey's HSD), ANOVA (two-factor), $p \leq 0.05$.

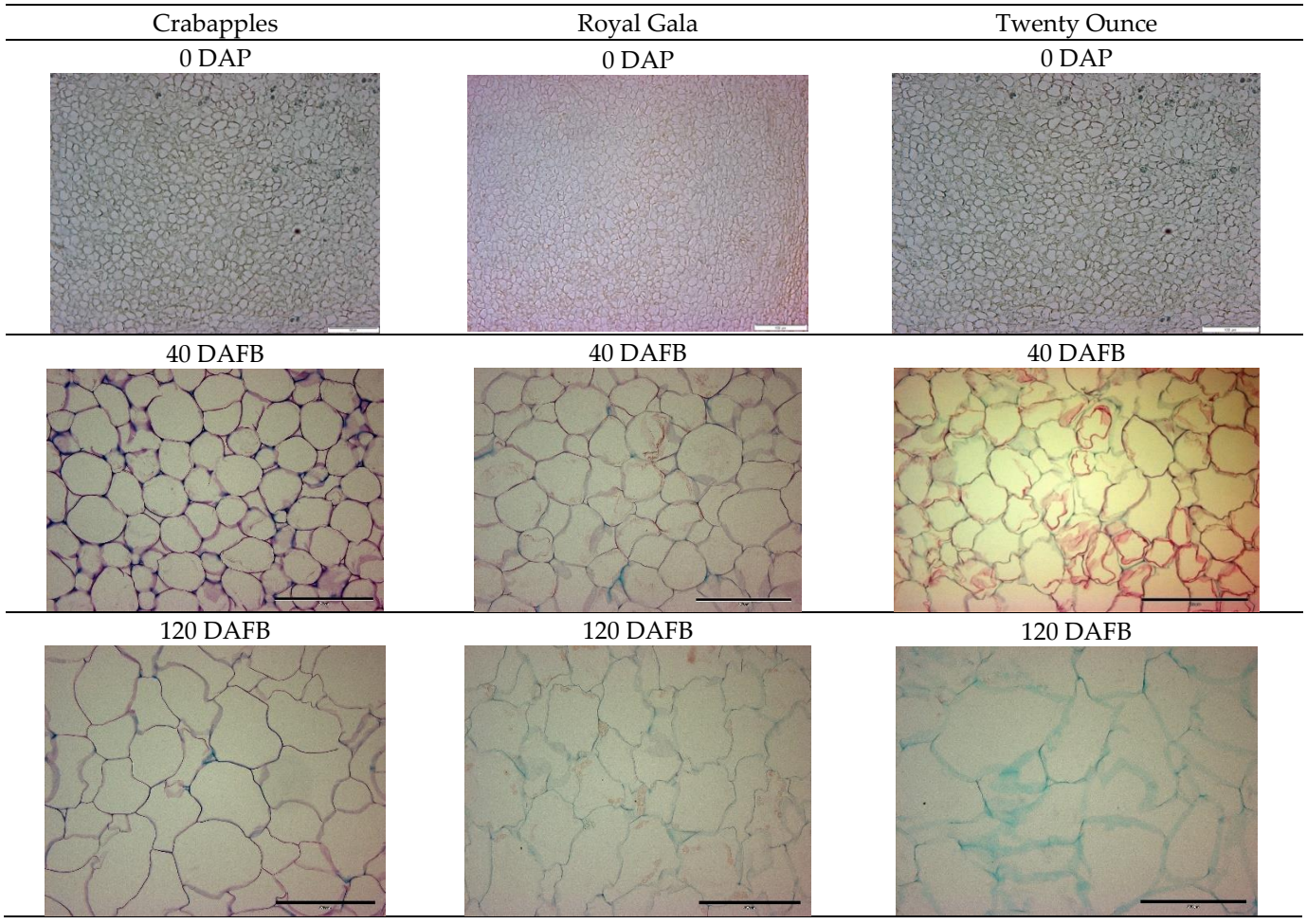


Figure 4. Microscopy images of three cultivars showing a distinct cell size increment amongst the cultivars. Scale bars represent 500 µm.

Crabapple fruits nonetheless grew in size at a slower pace than those of either Royal Gala or Twenty Ounce (Figures 1A–C and 2), the latter of which was the fastest-growing cultivar. This was demonstrated by the exponential size gain of Twenty Ounce after 42 DAFB, consequently producing the largest fruits amongst the three apple variants.

3.2. Relationship between Fruit Diameter, Cell Area, and Cell Number in Three Apple Cultivars

Data collected from Season 2 showed the positive correlation between fruit diameter and cell area among the three cultivars ($R^2 = 0.9245$, $p < 0.05$ (Figure 5)), an indication of the deterministic influence of cell area on final fruit size. Naturally, cell coverage areas would depend on cell division during early development, as well as cell expansion in the final developmental stages. Both cellular processes have indeed been reported to determine final fruit size [27]. Figure 3 depicts the number of cells counted within a set image area of 42×10^3 μm^2. The graph shows an inverse relationship between cell coverage area and cell numbers towards late development, whereby cell numbers were decreased despite an expansion of the cell area (Figure 5). Collectively, these results indicated that whilst cell division occurs rapidly in the early stages, it is gradually overtaken by cell expansion as fruit development progresses into the late stages.

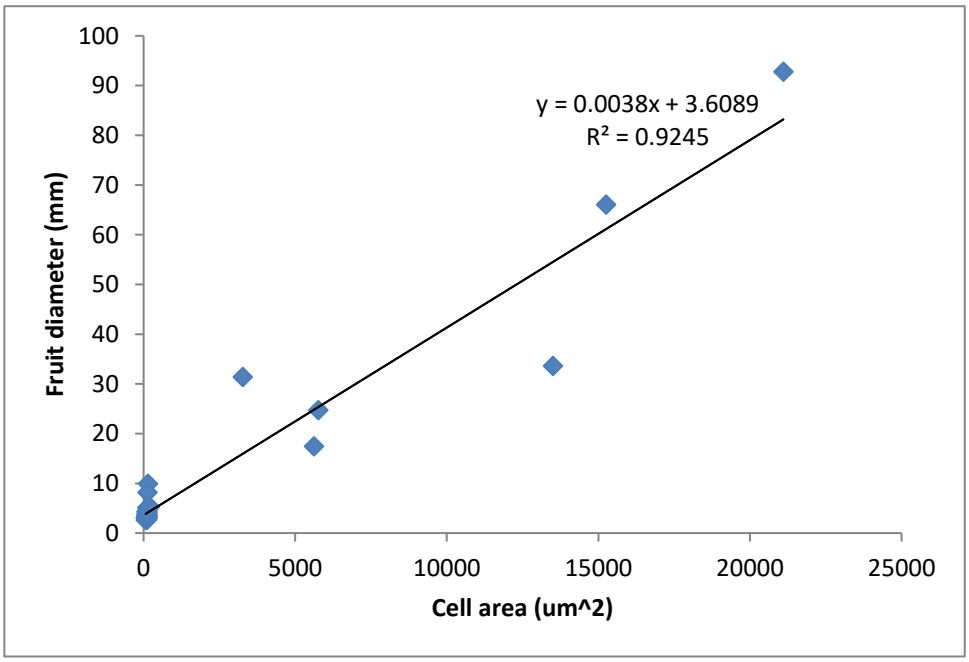


Figure 5. A graph depicting the relationship between fruit diameter and cell area for the three cultivars across 10 time points during harvest (Season 2). The best fit line indicates the linear correlation of the two variables ($p < 0.05$, y = 0.0038x + 3.6089).

3.3. Comparison of Expression Patterns among Three Cultivars

Preliminary testing on samples harvested during Season 1 (data not shown) found that *MdCDKB2;2* was upregulated during early development, but was downregulated afterwards. In preparation for Season 2, flowers from the Twenty Ounce, Royal Gala, and Crabapple cultivars were again hand-pollinated and harvested. The time course was changed slightly to include narrower intervals within the earlier stages of fruit development. Gene expression analyses were conducted with RNA samples derived from two biological replicates for all three cultivars, referred to as Rep1 and Rep2. As anticipated, a similar expression pattern was observed in Season 2 replicates where *MdCDKB2;2* was highest in Twenty Ounce during early development (Figure 6). The cultivar displayed the most significant surges in *MdCDKB2;2* expression at 0, 3, and 5 DAFB.

The expression pattern of *MdANT2*, a second cell division gene, was then found to be similar to that of *MdCDKB2;2*. Though expressed highly in the earlier time points, Figure 7 demonstrates the distinct downturn of *MdANT2* in the later stages. Similar to *MdCDKB2;2*, *MdANT2* expression was also the greatest in the big-sized Twenty Ounce, followed by the middle-sized Royal Gala and the small Crabapple.

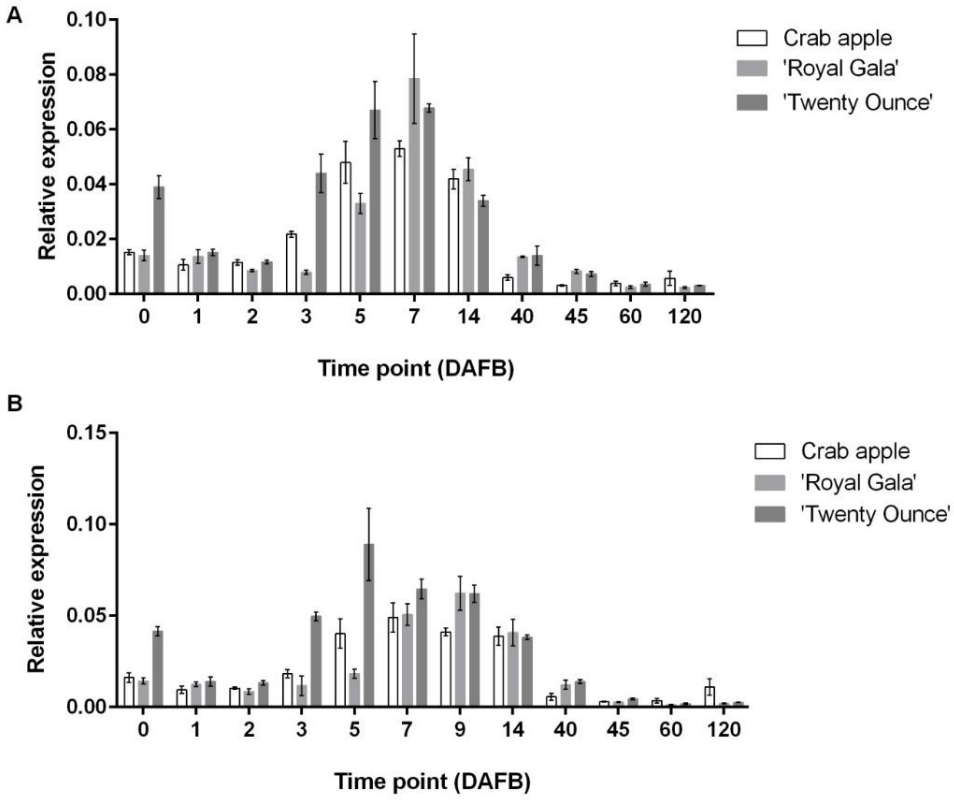


Figure 6. Differential expression of *MdCDKB2:2* in three cultivars during Season 2, relative to the control gene, *MdGAPDH*. (**A**) Relative expression in biological Replicate 1 (Rep1). (**B**) Relative expression in biological Replicate 2 (Rep2). Error bars = standard deviation; n = 3 (technical replicates). Expression levels between time points and cultivars were statistically different, ANOVA (two-factor), p-value ≤ 0.05.

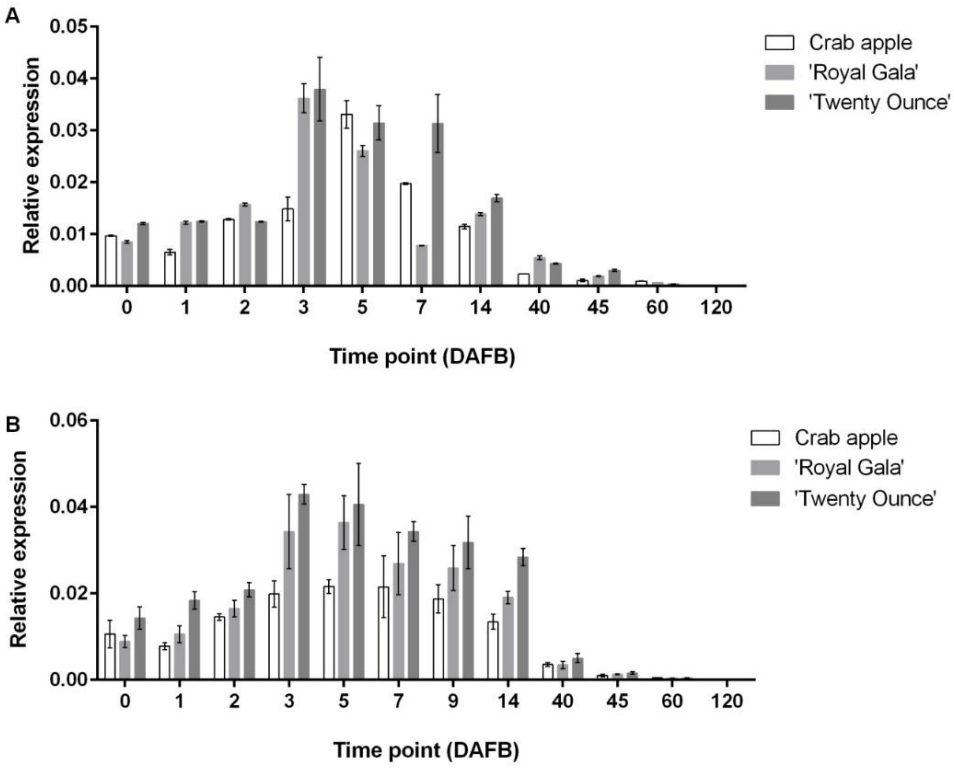


Figure 7. Differential expression of *MdANT2* in three cultivars during Season 2 relative to *MdGAPDH*. (**A**) Relative expression in biological Replicate 1 (Rep1). (**B**) Relative expression in biological Replicate 2 (Rep2).

Error bars = standard deviation; n = 3 (technical replicates). The expressions between markers were statistically different, ANOVA (two-factor), p-value ≤ 0.05.

Season 1 testing of *MdEXP3* expression in Royal Gala flowers and fruits revealed its high levels during the cell expansion stages. In Season 2 (Figure 8), *MdEXP3* was most prominently found in Crabapple throughout the earlier stages. By contrast, Twenty Ounce expressed the highest levels of the cell expansion gene only as the fruit approached maturity (120 DAFB). These observations importantly signified that although each cultivar may do so in a temporally dissimilar manner, the cells of its fruit would nevertheless undergo the expansion process; this explains why all cultivars produced similar cell sizes before full bloom (0 DAFB) and at full ripening (120 DAFB).

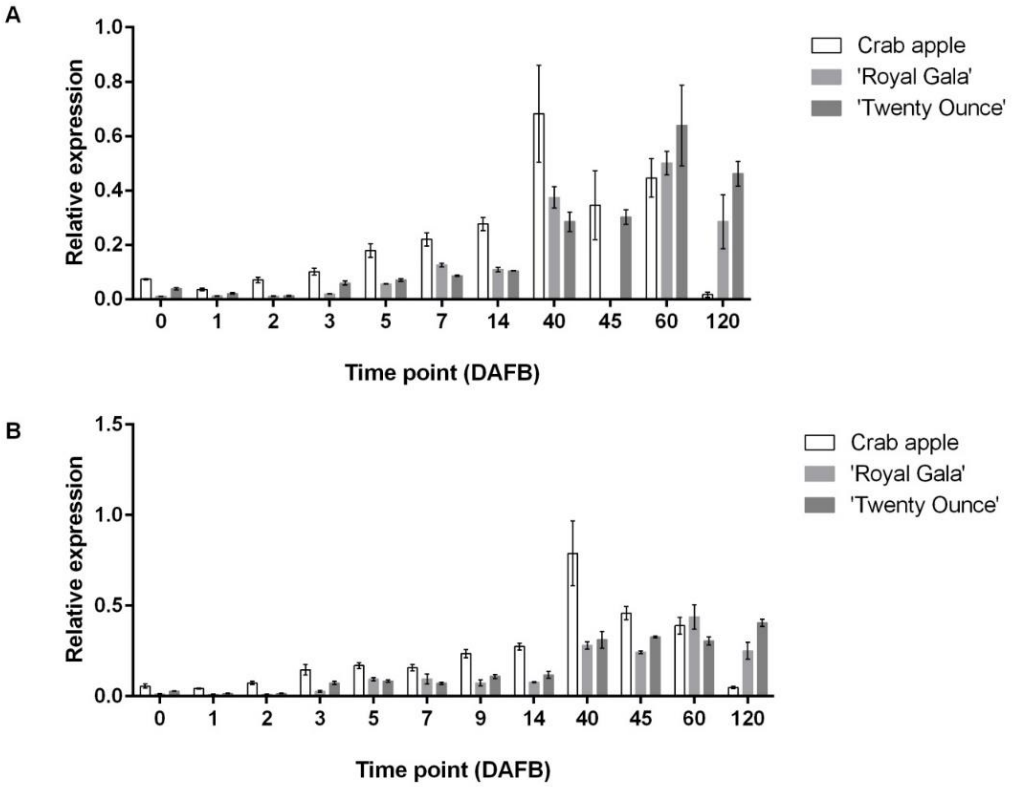


Figure 8. Differential expression of *MdEXP3* in three cultivars during Season 2, relative to the control gene, *MdGAPDH*. (**A**) Relative expression in biological Replicate 1 (Rep1). (**B**) Relative expression in biological Replicate 2 (Rep2). Error bars = standard deviation; n = 3 (technical replicates). Expression between time points and cultivars were statistically different, ANOVA (two-factor), p-value ≤ 0.05.

More anomalous outcomes were derived from gene expression analyses of two genes, namely *MdARF106* and *MdACO1.* The transcription factor *MdARF106* was highly expressed throughout the time course and without significant differential expression among cultivars, an implication of its role as a facilitator rather than a driver of both the cell division and expansion stages (Figure 9). The ripening marker *MdACO1* was then shown to be strongly produced in Royal Gala over the ripening period. Unexpectedly, *MdACO1* was almost absent from the other two cultivars (Figure 10).

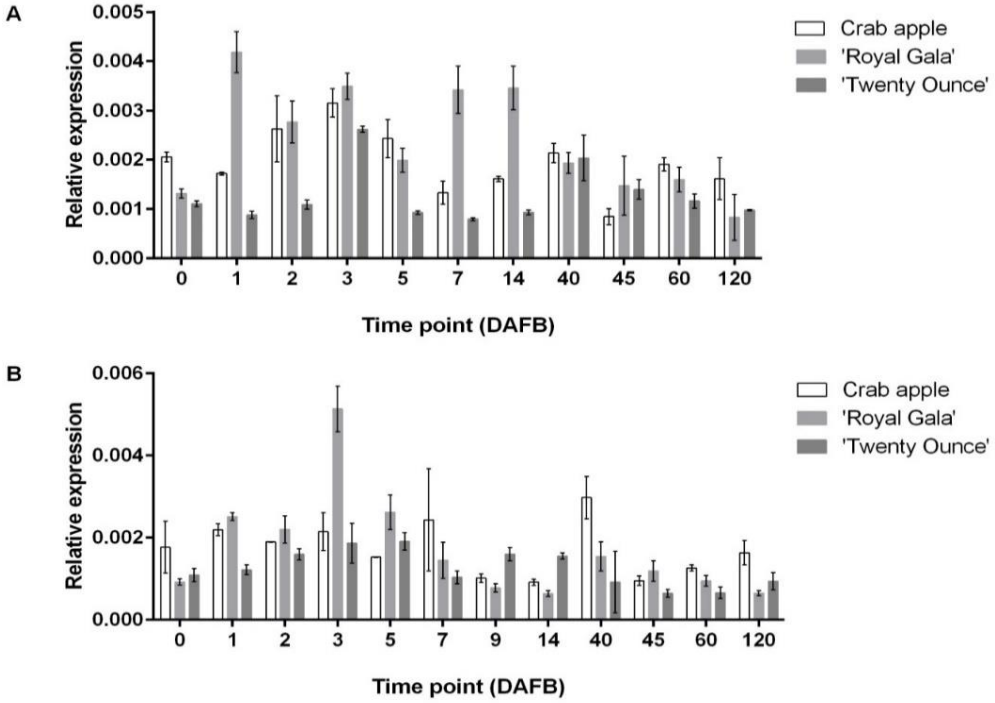


Figure 9. Differential expression of *MdARF106* in three cultivars during Season 2, relative to the control gene, *MdGAPDH*. (**A**) Relative expression in biological Replicate 1 (Rep1). (**B**) Relative expression in biological Replicate 2 (Rep2). Error bars = standard deviation; n = 3 (technical replicates). The expression between time points and cultivars were statistically different, ANOVA (two-factor), p-value ≤ 0.05.

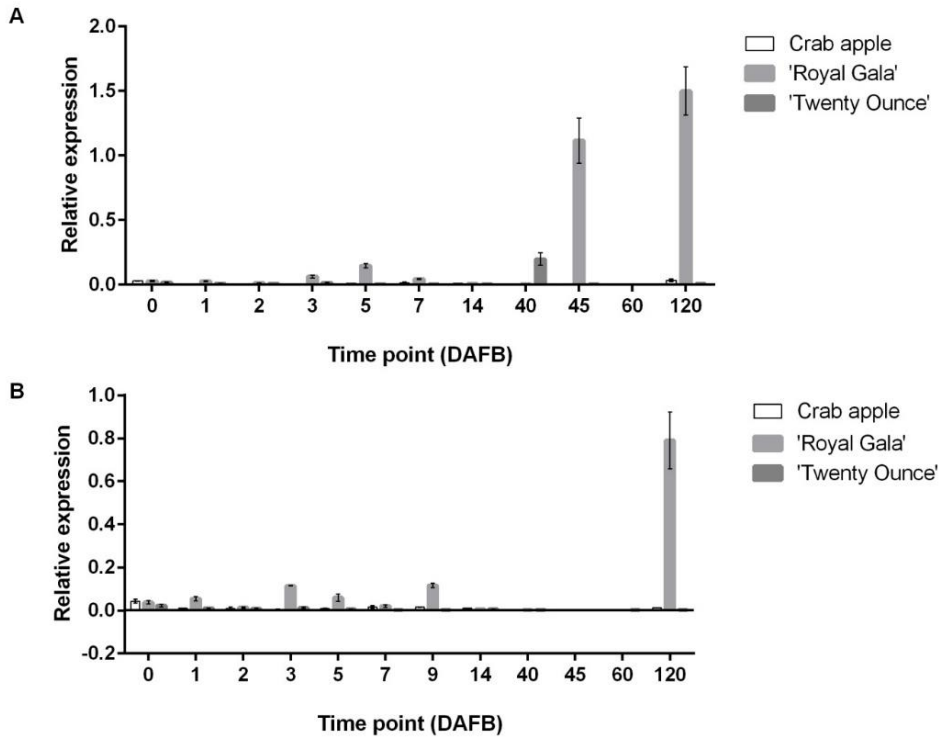


Figure 10. Differential expression of *MdACO1* in three cultivars during Season 2, relative to the control gene, *MdGAPDH*. (**A**) Relative expression in biological Replicate 1 (Rep1). (**B**) Relative expression in biological Replicate 2 (Rep2). Error bars represent standard deviation (n = 3).

3.4. Phylogenetic Analysis of CDK Genes

A phylogenetic tree of apple, *Arabidopsis*, and tomato CDK genes was generated to study the relationship between members of this gene family across different species (Figure 11). CDK genes were divided into seven classes (A to F), based on their roles in the different stages of cell cycle [16,22]. Here, *CDKB2;2* was selected as a marker for cell division in apple fruit and cell culture. *CDKA* and *CDKB* are core cell cycle genes [15,42–44]. Two types of *CDKB*s, i.e., *CDKB1* and *CDKB2*, are plant-specific. *CDKB1* is expressed from the late S to M phases of the cell cycle, whereas *CDKB2* is expressed specifically from the G2 to M phases [15,44]. Like *Arabidopsis*, the apple plant possesses two types of *CDKB1* (*CDKB1;1* and *CDKB1;2*) and two types of *CDKB2* (*CDKB2;1* and *CDKB2;2*), all of which are homologous to each other [12,15,44]. Microarray expression data from previous studies determined that *CDKB2;2* forms a more distinct peak during early apple fruit development compared to *CDKB2;1* [8,22]. Despite the scarcity of data available about apple fruit genomic sequences, alignment of the cell cycle genes from apple and the well-established *Arabidopsis* successfully established the high similarity of the apple *CDKB2;2* gene (accession number: CN943384) to the *Arabidopsis CDKB2* (accession number: At1G20930.1). With an expected value of $1 \times e^{-102}$, the degree of likeliness between the two was deemed great enough to indicate a shared function in cell cycle regulation [8]. The accession number of each gene is shown in Table 2.

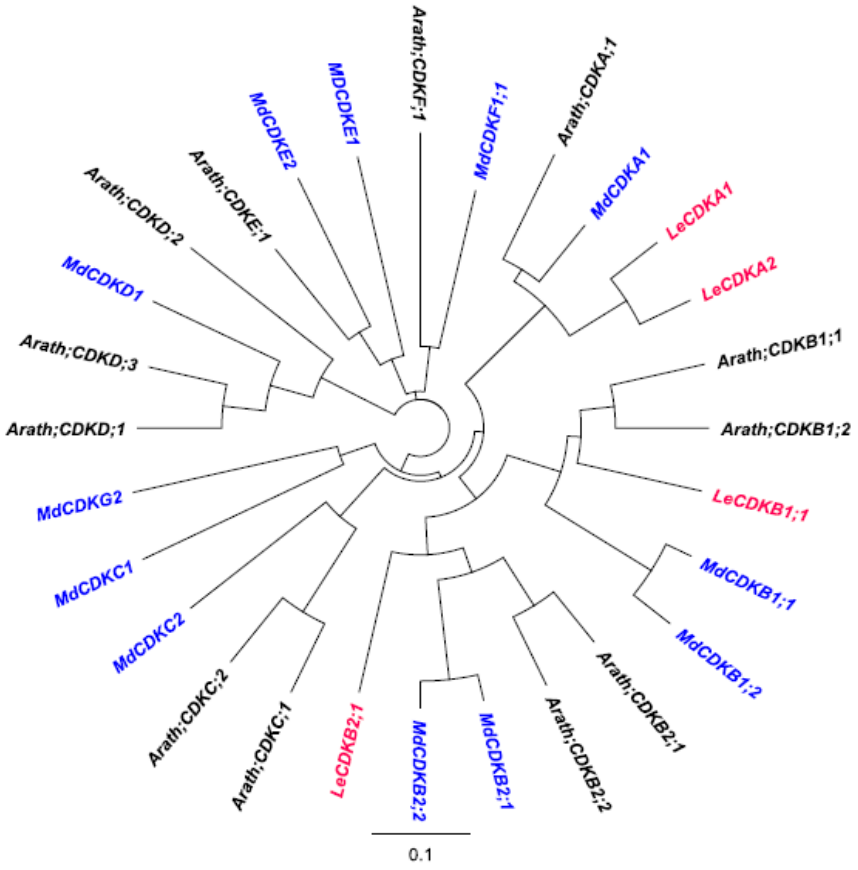


Figure 11. Phylogenetic tree of the Cyclin Dependent Kinases (CDKs). The CDKs from apple (blue), *Arabidopsis* (black,) and tomato (red) were aligned on the MUSCLE software. Parameters for phylogenetic tree building were set to neighbour joining and 500 bootstraps. Md: *Malus x domestica*, Arath: *Arabidopsis*, Sl: *Solanum lycopersicum*.

Table 2. Gene name and accession number of CDKs from three different plant species.

Gene Name	Accession Number
Arath; CDKA;1	AT3G48750.1
Arath;CDKB1;1	AT3G54180.1
Arath;CDKB1;2	AT2G38620.2
Arath;CDKB2;1	AT1G76540.1
Arath;CDKB2;2	AT1G20930.1
Arath;CDKC;1	AT5G10270.1
Arath;CDKC;2	AT5G64960.1
Arath;CDKD;1	AT1G73690.1
Arath;CDKD;2	AT1G66750.1
Arath;CDKD;3	AT1G18040.1
Arath;CDKE;1	AT5G63610.1
Arath;CDKF;1	AT4G28980.2
MdCDKA1	MDP0000185491
MdCDKB1;1	MDP0000223519
MdCDKB1;2	MDP0000240040
MdCDKB2;1	MDP0000418062
MdCDKB2;2	MDP0000722904
MdCDKC1	MDP0000243737
MdCDKC2	MDP0000253325
MdCDKD1	MDP0000128357
MdCDKE1	MDP0000206441
MdCDKE2	MDP0000235270
MdCDKF1;1	MDP0000303768
MdCDKG2	MDP0000263387
SlCDKA1	Solyc08g066330.1.1
SlCDKA2	Solyc12g095860.1.1
SlCDKB1;1	Solyc10g074720.1.1
SlCDKB2;1	Solyc04g082840.2.1

4. Discussion

Given that there are over 7000 varieties of apples known to mankind, it is only natural that distinct fruit size differences would be found among apple cultivars [10,11]. Once fertilised and having entered the fruit set stage, cells in the hypanthium will rapidly divide and expand, thus resulting in the organ's lateral growth; specifically, the hypanthium would have developed into fruit flesh (mesocarp) by the end of the development process. Interestingly, we found that the cell size of all three cultivars was very similar before full bloom (0 DAFB), as well as at ripening (120 DAFB), despite their vast final fruit size differences. The same findings were reported before in other apple cultivars [15,45]. As such, these observations affirm that, ultimately, fruit size is dependent not on cell size, but instead cell numbers, the growth of which is determined by cell division and expansion genes. By contrast, the smaller fruits of a blueberry cultivar were shown to produce smaller cells at full bloom compared to the bigger fruits of the same plant. However, it was later concluded that the cell size differences at full bloom did not actually influence final fruit size in the cultivar [15,46].

Physiologically, Twenty Ounce grows at a faster rate than the other cultivars. At ripening, its fruit width was three-times larger than that of Crabapple. However, our histology studies demonstrated that the cells of the three cultivars expanded at a similar pace throughout development, an implication of the weak impact of cell expansion in determining fruit size. Even though expansion itself is a major cause of cell growth, its contribution to the final fruit size among apple variants was unclear [15,46]. In this study, the low expression of *MdEXP3* in big-sized Twenty Ounce proved that cell expansion is not deterministic for fruit size differences among the three cultivars. Rather, the high expression of *MdCDKB2;2* observed in Twenty Ounce asserts that cell division more prominently influenced the final fruit size of the cultivar. Nonetheless, such a straightforward relationship appears unlikely. As high levels of *MdCDKB2;2* are speculated to encourage fruit growth, its upregulation at

an earlier period would benefit fruit size gain more effectively than later surges. However, should this be the only contributing factor to fruit size, then Crabapple fruits would grow larger than Royal Gala fruits, as the *MdCDKB2;2* spike in the former precedes that of the latter by two days. There must thus be intrinsic genetic factors that override cell division genes in the determination of final fruit size.

Early research has revealed that cell division influences fruit size in apples [4,47,48]. Cell number comparisons between big-sized and small-sized apples indicated that the cells of either cultivar would be similarly sized at maturity, insinuating that cell population numbers as a whole are more impactful on fruit size in apples [35,49]. A high cell number has indeed been shown to lead to larger fruit sizes in other species such as the rabbit-eye blueberry (*Vaccinium ashei* [46]), sweet cherry (*Prunus avium* [32]), and tomato (*Solanum lycopersicum* [33,50]). In apple fruits, reports preceding the present study concluded that both cell division and expansion are involved in large-fruit production, although to what extent expansion could influence final fruit size was not determined [27,51].

Most of the pre-existing apple fruit size control research involved cellular and/or physiological assessments [4,50], and few have incorporated gene expression analysis. In this study, we performed real-time quantitative PCR (RT-qPCR) to investigate the differential expression of specific markers throughout fruit development. This approach is more reliable and adds more depth to the overall analysis. Another cell division marker, *MdANT2*, was chosen due to its positive correlation with *MdCDKB2;2* and might thus be used to reaffirm their expression patterns. Though it is a known regulator of cell division control in other species [1,22], the role of *MdANT* in apple fruits is not yet studied. *MdANT* was hypothesised to function similarly to its homologue in *Arabidopsis* [1,2], a gene involved in floral organ initiation and growth [2,44]. To investigate the truth to this assumption, we first quantified *MdANT* expression levels in the Royal Gala cultivar harvested over Season 1. *MdANT* was shown to be upregulated during early development before declining towards the ripening stage. With the Season 2 harvest, we observed the positive correlation of *MdANT* and *MdCDKB2;2*, in line with findings from a previous report [1]. We also determined that cell division more actively occurs in the young fruits of Twenty Ounce than those of either the Royal Gala or Crabapple variants. The high expression of *MdANT2* in Twenty Ounce before full bloom and the cultivar's rapid cell production rate together implicate the gene in cell division and population growth.

The transcription factor *MdARF106* has been previously identified as a highly present gene throughout early development [1,20]. Our analysis of *MdARF106* duly showed that the gene was expressed during both cell division and cell expansion, with no significant level differences among cultivars. As the utilisation of stage-specific markers was crucial to our experimental design, *MdARF106* was disregarded in favour of the expansin *MdEXP3* [28,52]. Indeed, *MdEXP3* displayed an expression pattern that was temporally in tune with cell expansion; curiously, however, the gene was more modestly expressed in Twenty Ounce than in both Royal Gala and Crabapple. Contrarily, preceding studies have shown *MdEXP3* to exhibit high-expression profiles in big-sized apple variants, leading to the conclusion that cell expansion genes could influence fruit production in those cultivars [28,33]. Taken together, these observations therefore suggest that the effects of cell expansion on final fruit size may be more prominent in some apple cultivars over others.

5. Conclusions

Molecular approaches have been utilised to great effect in apple research to dissect the complex interaction and interplay between hormones during fruit growth. Data from three apple cultivars were collected over a two-year period (two seasons of harvest), where it was found that cell numbers determined the final fruit size. Additionally, the expression of certain genes during early fruit development directly influenced fruit size. The biggest cultivar within this study, Twenty Ounce, evidently demonstrated such a connection; in direct contrast, the middle-sized Royal Gala and small-sized Crabapple exhibited the highest levels of cell division genes during early fruit development.

Regarding fruit size control, cell division appears key to fruit size differences among the apple cultivars studied. Although the *MdCDKB2:2* gene has been characterised, it would be of interest to further research the function of this gene during apple fruit development. One way to assess its function would be through the introduction of the gene on an auxin-specific promoter into developing fruits. Since *MdCDKB2:2* was correlated with auxin action in promoting cell cycle promotion, the promoter should increase the cells' sensitivity to auxin. The application of antisense or knock-out could provide a tool to study *MdCDKB2:2* function in apple fruit. From this study, it was found that "Twenty Ounce" had a higher expression of *MdCDKB2:2* compared to the other cultivars, suggesting it plays a major role in determining the large size of the fruit. Through introduction of this gene by antisense, its function in influencing "Twenty Ounce" final fruit size could be further examined, particularly in how it may affect cell number production.

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