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DIFFERENTIAL EXPRESSION OF SPECIFIC GENES REGULATING 'ROYAL GALA' (*Malus x domestica*) FRUIT DEVELOPMENT

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ABSTRACT

Apple (*Malus x domestica*) fruit size is facilitated by cell division and cell expansion, which in turn is regulated by plant hormones such as auxins, gibberellins, and cytokinins. In this research, we investigated the role of cell division and cell expansion in apple growth. Royal Gala cultivar was analysed over one season of harvest. Throughout the fruit development, gene expression and cell area measurement were done. The expression of cell division markers; *MdCDKB2:2* is correlated with *MdANT2* which both showing high expression pattern on early stages of time course and gradually decreasing towards the end of the time course. Cell expansion markers; *MdEXP2*, showed up-regulated expression as the cells expanded, while *MdARF106* is expressed in both cell division and cell expansion stages. Ripening related genes; *MdACO1* and *MdPG1*, were highly expressed during the ripening stage. From this research, it is found that the expressions of all the genes are specific, thus, can be ideal markers for Royal Gala fruit development in further studies.

Key words: Apple, fruit size, fruit development, cell division, cell expansion, ripening

INTRODUCTION

Fruits are reproductive organs unique to the Angiosperms that have evolved to promote seed dispersal. Fleshy fruits assist seed dispersal by attracting animals due to their colour, flavour, nutritional content and textures. While much work has gone into understanding fruit development and ripening in apples, much remains to be elucidated. One issue in studying cell development and ripening in apple fruit is the lengthy annual fruit development cycle. Fruit development starts with successful fertilisation and subsequently, the fruit sets. After fruit set, cells will differentiate into fruit cortex cells by undergoing cell expansion, following fruit maturation and ripening which all controlled by plant hormones (McAtee *et al.*, 2013). As shown in Figure 1, after flowering and pollination, apple fruit development takes place over 20-21 weeks which involves stages of fruit set, cell division, cell expansion, maturation and ripening which will lead to a crisp fruit with a waxy cuticle. Cell division starts during fruit set and continues until 3-4 weeks after pollination. Cell division will gradually cease to allow cell expansion to take place (McAtee *et al.*, 2013). This process will continue to occur until full ripening but it reaches its peak on 40-60 days after anthesis and become constant during ripening (Janssen *et al*, 2008; Kumar *et al.,* 2014). Fruits enter the onset of ripening at 90 Days After Full Bloom (DAFB) and continue until full ripening at 146 DAFB (Janssen *et al.*, 2008; Kumar *et al.,* 2014). Fruit ripening is a developmental process involving physiological and metabolic changes which result in changes in colour, texture, aroma and nutritional status. These changes help promote seed dispersal by making it attractive to birds and animals (Giovannoni, 2007; Rugkong *et al.*, 2011; Qin *et al.,* 2016).

The early phase in apple fruit development involves rapid cell division where the cell number is greatly amplified before exit from the cell division

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at approximately 24 DAFB (Malladi & Johnson, 2011; Li *et al.,* 2016). Final fruit size is determined by the progression of cell division and cell expansion during fruit growth and development, thus, understanding the cell cycle regulation and cell expansion will provide a good picture of fruit development in plants (Malladi & Hirst, 2010; Li *et al.,* 2016). Progression of the cells into each phase is controlled by specific checkpoints. The cells progression from each checkpoint is driven by a large family of serine/threonine protein kinases: the cyclin-dependent kinases (CDKs), an activating subunit, the cyclins and CDK inhibitors (Malladi & Johnson, 2011). A number of studies reported on *CDKB2:1* (Kitsios & Doonan, 2011; Endo *et al.*, 2012) but *CDKB2:2* is scarcely studied especially on apple fruit; probably due to some plants do not possess it. Apple *MdCDKB2* gene study has been contributed by Janssen *et al*. (2008) by identifying it from a pool of apple genes microarray data. The other research that thoroughly studied apple cell cycle genes is by Malladi and Johnson (2011) which explains about the expression of the genes on early apple development. Our interest the using *CDKB2:2* for marker on cell division is due to its distinct down-regulated expression towards the end of early development. Other related research is either scarce or unrecognised. Therefore, it is tantalising to investigate how *CDKB2:2* gene regulates the cell cycle in apple fruit.

AINTEGUMENTA (*ANT*) is a plant-specific gene and active in developing a flower and vegetative tissues (Klucher *et al.*, 1996). *ANT* consists of the floral transcription factor genes family of *APETALA 2* (*AP2*). The action of cell division during organogenesis in plants is essential to facilitate growth and consequently, influence the final size of the plant's organs (Mizukami & Fischer, 2000). In *Arabidopsisthaliana*, it is reported to control cell proliferation stage in integuments during ovule growth and important in floral growth (Mizukami and Fischer, 2000). It is supported by a decrease of cell division activity in loss of *ANT* function mutant and reducing organ size. Due to limited gene data resources of apple, it was speculated that apple homologs *ANT* genes also act similar function as in *A. thaliana* in promoting cell division and fruit growth (Dash & Malladi, 2012). In this research, we opted to use the apple gene, *MdANT2*, as another potential marker for cell division phase in apple fruit development and is also studied at different phase throughout the development.

Previous research observed up-regulation of *MdANT1* and *MdANT2* at early stages of fruit growth until the end of cell division stage which is due to the maintaining competency of undifferentiated cells for cell division, and then down-regulated towards ripening stage when the meristematic cells gradually loses its competency (Mizukami & Fischer, 2000; Dash & Malladi, 2012). This has, however, proven the hypothesised function of these genes in apple during fruit growth by directly involved in cell proliferation activity. *MdANT1* and *MdANT2* genes are both displayed higher and longer expression in larger fruit phenotype 'Golden Delicious Smoothee' (GS) (Dash & Malladi, 2012), thus, suggesting that this gene is a good marker in cell division studies in apple fruit development. Furthermore, these genes have a similar expression pattern in cell division stage, and they are also showed a positive correlation with A- and B-type CDKs in regulating cell production and cell cycle in apple (Dash & Malladi, 2012).

Apple Auxin Response Factor 106 (*MdARF106*) is an apple transcription factor which found co-localised with QTL-mapped fruit growth genes population, thus, implicating that the gene regulates apple fruit growth; consistent with the evidence of its expression during cell division and cell expansion stages in apple fruit growth (Devoghalaere *et al.*, 2012). Due to this finding, it is tempting to reveal more on the gene function in apple fruit growth.

Expansins are a family of proteins that catalyse cell wall expansion (Cosgrove, 2016). In *A. thaliana*, the expansin family consists of nearly 30 genes which divided into two subfamilies; α- and β-expansin, based on sequence divergence and biochemical activity (Cho and Kende, 1997; Cosgrove, 2000; 2016). Cell expansion is a compulsory process in which a plant cell needs to undergo in order to grow. In order for the process to occur, the plant cell wall needs to be synthesised more, which then leads to the plant growth (Percy *et al.*, 1998; Cosgrove 2016). In apple fruit size studies, high expression of *MdEXP3* during expansion phase of fruit development (at 35 and 49 DAFB) of big size apple *M. domestica* 'Sekaiichi' compared to small size apple *M. floribunda* (Harada *et al.*, 2005), supported the role of expansin in fruit growth, particularly during cell expansion. Low expression of *MdEXP3* was also observed on 21 DAFB fruit (Harada *et al.*, 2005), indicating its specific role in cell expansion stage during fruit development. However, there is a report on *MdEXP3* that has linked the gene with ripening, and that it is regulated by ethylene (Wakasa *et al.*, 2003) whereas *MdEXP2*, however, was reported to be specifically expressed during cell expansion stage of fruit development (Wakasa *et al.*, 2003). Another expansin, *MdEXPA10:1*, also showed cell expansion role in apple shaded-induced fruits where its low activity during the fruit growth has declined the fruit growth as a result of a low cell wall expansion activity (Dash *et al.*, 2013).

MATERIALS AND METHODS

The gene expression studies were carried out at two consecutive seasons and represented as two sets of data (repetitions) (named as Rep1 and Rep2) with ten time points on each repetition. Rep 1 was prepared in September until January 2012/2013 while Rep 2 in September until January 2013/2014. For each time points, three individual flower buds or fruits were obtained as biological replicates. Simultaneously, the cell area was measured on three individual fruits for each repetition and time points. Because the cells were counted within the designated grid area. Thus, the cell area reflects cell numbers. In other words, when the cell area is larger, it means there was a lesser cell number within the grid. The smaller the cell area, the higher number of cells fit in the grid.

Pollinations

The sampling was carried out in Plant and Food Research's orchard at Hawke's Bay, Napier, New Zealand. Pollen from Granny Smith was used to hand-pollinate the flowers of Royal Gala. To pollinate a flower, a small paintbrush was used to dip into the pollen. A blooming flower was brushed with the pollen. The pollination process was done before 11 a.m. The unpollinated blooming flower was distinguished by looking at the stigma of the flower. Unpollinated stigma was in white colour while pollinated stigma was brown in colour. Each of the pollinated flowers was tagged for recognition during sample collections. In total, around 108 flowers were pollinated to be harvested at nine time points, excluding the first time point (0 DAFB) which were un-bloomed flower buds.

Harvest

Hypanthium of unpollinated flowers was collected for each cultivar to be used as control (un-bloomed flowers) and noted as 0 DAFB. Pollinated flowers or fruits for each time points were collected and dissected. For the histology sample, flowers were fixed in fixative solutions before stored in 4°C. For gene expression, RNA sample preparation was done at the orchard. Hypanthiums of flowers were dissected to discard the ovaries using a scalpel before it was immediately frozen in liquid nitrogen. Excess of liquid nitrogen was discarded before the frozen samples were stored in -80°C freezer. Figure 1 shows hypanthium, flowers, and fruits harvested at ten time points.

Design of markers

The qPCR markers were designed using Primer3 software. The markers sequences were as shown in Table 1. Each of the primer pairs was optimised by using 10-fold serial dilutions in the same run according to generate standard curves.

Gene expression sample preparation

RNA extraction was done on frozen samples following protocol used on pine needles (Chang *et al.*, 1993; Schaffer *et al.*, 2007) and was cleaned

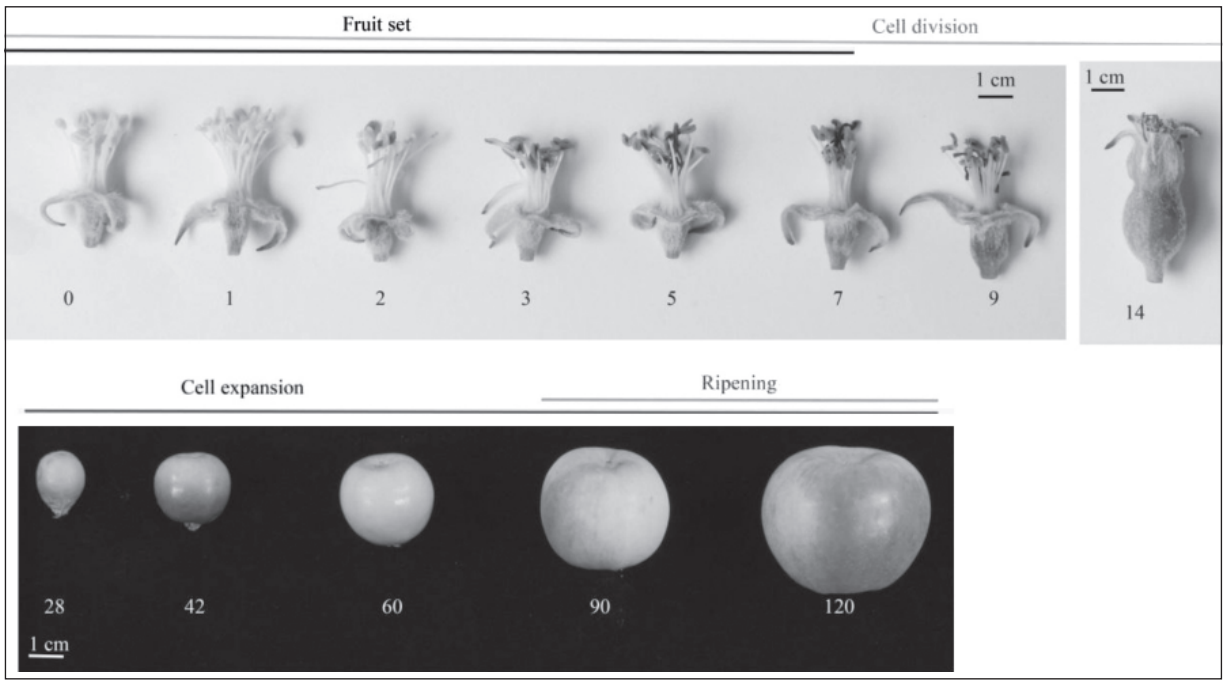

Fig. 1. 'Royal Gala' fruit development. Samples numbered with 0 until 14 were collected as DAFB and 28 until 120 as DAFB. Fruit set, cell division, cell expansion, and ripening durations are as shown by the lines.

Table 1. Primer sequences for qPCR analysis

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

using RNeasy Mini Kit (Qiagen) and Ambion Turbo DNase treatment Kit according to the manufacturer's protocol. First strand cDNA was synthesised using First strand superscript III cDNA supermix kit (Invitrogen). qPCR was then carried out and the data was normalised using housekeeping genes *MdGAPDH* (Table 1).

Histology slides preparation and cell area measurement

Fixed hypanthium and fruits were embedded in paraffin wax. Specimen slides were prepared using microtome with 10 µm thickness slices setting. The specimen was later stained with 1% Safranin- 0.5% Fast Green (Sass, 1968). Microscopy images were taken using Leica 500 Microscope using bright field. This experiment was carried out on three individual hypanthium/fruits for each time point and cultivar. By using the images, cell areas were measured using ImageJ software.

Statistical analysis

Two-way ANOVA (Analysis of Variance) or student's t-test analysis was conducted using the SPSS software.

RESULTS AND DISCUSSION

The relationship among fruit diameter and cell area in Royal Gala

Naturally, there is a distinct fruit size difference among apple cultivars (McAtee *et al.*, 2009; 2013). After fruit set, cells in the hypanthium will rapidly divide and then expand which results in lateral growth of the hypanthium. Towards the end of development, the hypanthium will grow into fruit flesh (mesocarp). Figure 2 shows the cell area measured within 42×10^3 um^2 of Royal Gala mesocarp tissues. These results indicate the influence of cell area in determining the final fruit size. In many fruits, final fruit size is determined by cell division on early development and following with cell expansion in final development stages. Studies on fruit size control in apples showed cell division and an enhanced cell expansion determined the fruits final size (Harada *et al.*, 2005). It is observed that the cell area increased towards the final stages of development. Correlated with an increment of cell area towards the final development process (Figure 2), these results showed a cell division occurs at early stages and gradually ceases which then taken place by cell expansion until the end of the development process. Figure 3 shows the obvious difference in cell area increment of the fruit

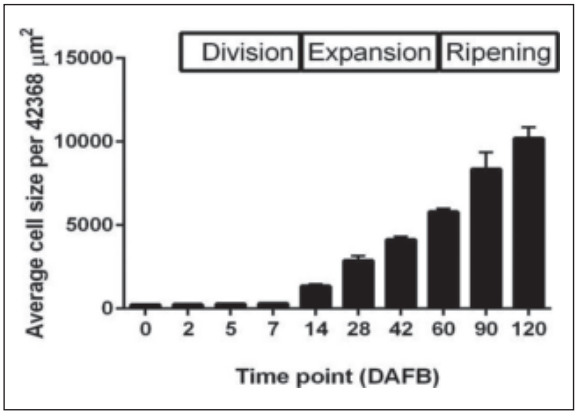

Fig. 2. Cell size of Royal Gala at 10 time points during one complete season of harvest. The measurements from 14 to 120 DAFB were statistically different using student's t-test, p-value ≤ 0.05.

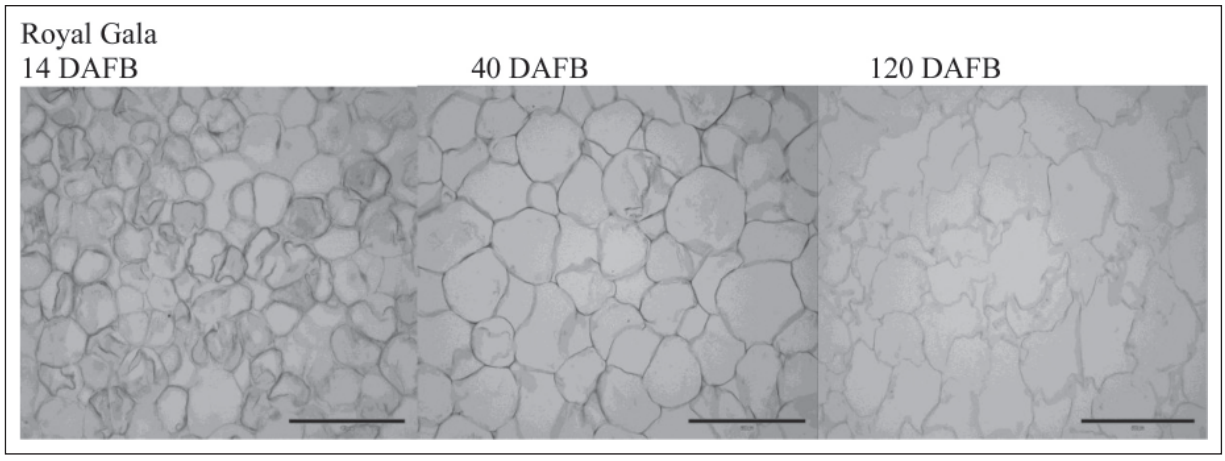

Fig. 3. Microscopy images of 'Royal Gala' mesocarp showing a distinct cell size increment throughout fruit development. Scale bars represent 500 μm. DAFB=Days after pollination, DAFB=Days after full bloom.

mesocarp during cell division (14 DAFB), cell expansion (40 DAFB), and ripening (120 DAFB).

Expressions of selected genes during fruit development *Malus domestica* 'Royal Gala'

As shown in Figure 4, during the first season, pollinated Royal Gala flowers and fruits derived were harvested to test the designed markers (gene of interest) to ensure its robustness. Our findings were consistent with previous reports by Dash and Malladi (2012), where the apple cell cycle gene *MdCDKB2:2* was highly expressed on early time points and correlated with *MdANT2*. *MdCDKB2:2* showed increment in expression up to about 5.5-fold at 7 DAFB from the control 0 DAFB in Rep1 and decrease afterwards. Likewise, in the Rep2, the gene reaches its peak at 5 and 7 DAFB with 2-fold increment from the control 0 DAFB, and then down-regulated towards the end of time course. The same pattern of expression was also showed with *MdANT2* on both Reps (repetitions), confirming the positive correlation between the two markers. Sequence of markers is stated in Table 1. Both genes have indicated their involvement in cell division during early fruit development and they are later down-regulated towards ripening stage, consistent with previous published reports by Dash and Malladi (2012). There are two types of *CDKB*s, *CDKB1* and *CDKB2*, which are plant-specific (Endo *et al.,* 2012). *CDKB1* is expressed during late S to M phase while *CDKB2* is expressed during G2 to M phase (Iwakawa *et al.,* 2006; Endo *et al.,* 2012). Apple, like Arabidopsis, possesses two types of *CDKB1*s; *CDKB1:1* and *CDKB1:2* and two sets of *CDKB2*s - *CDKB2:1* and *CDKB2:2* which are homologues of each other (Malladi & Johnson, 2011; Endo *et al.,* 2012). Some plants, such as rice, possess only the *CDKB2:1* gene (Endo *et al.,* 2012). In tobacco, impaired stomatal development was observed in a dominant-negative *CDKB1* mutant as a result of a block in early cell division stage, showing that *CDKB1* is required at the transition point of G2/M (Iwakawa *et al.,* 2006). Suppression of *CDKB1* has also been reported to induce endoreduplication in Arabidopsis (Boudolf *et al.,* 2004; Verkest *et al.,* 2005). Microarray data of *CDKB2:2* expression has shown a more distinct peak during early fruit development in apple fruit compared to *CDKB2:1* (Janssen *et al.,* 2008). Comparing apple gene data with Arabidopsis cell cycle data shows a high similarity of apple *CDKB2:2* gene (Accession number: CN943384) with the Arabidopsis cell cycle gene (Accession number: At1G20930.1), with the expected value of 1 × e-102. This suggests that both genes have a similar function in the cell cycle (Janssen *et al.,* 2008). A number of studies have examined *CDKB2:1* (Kitsios & Doonan, 2011; Endo *et al.,* 2012) but *CDKB2:2* is less understood, especially in apple fruit.

Meanwhile, *MdEXP2* was up-regulated at the later stages during development and reached its peak at 90 DAFB on both Reps with vast increment 20-fold from the control at Rep1 and 70-fold at Rep2. The expression was decreased as fruit enter ripening stage which is at 120 DAFB. In apple fruit size studies, high expression of *MdEXP3* occurs during the expansion phase of fruit development (at 35 and 49 DAFB) of large size apple, *M. domestica* 'Sekaiichi', compared to small size apple, *M. floribunda* (Harada *et al.,* 2005). This confirms the role of expansin in fruit growth, particularly during cell expansion. In fact, low expression of *MdEXP3* was also observed in 21 DAFB fruit (Harada *et al.,* 2005). Another expansin, *MdEXPA10:1*, also exhibited cell expansion in apple shade-induced fruits where lower activity occurs as fruit growth is reduced (Dash *et al.,* 2013). Another cell expansion gene, *MdARF106* is expressed highly on both cell

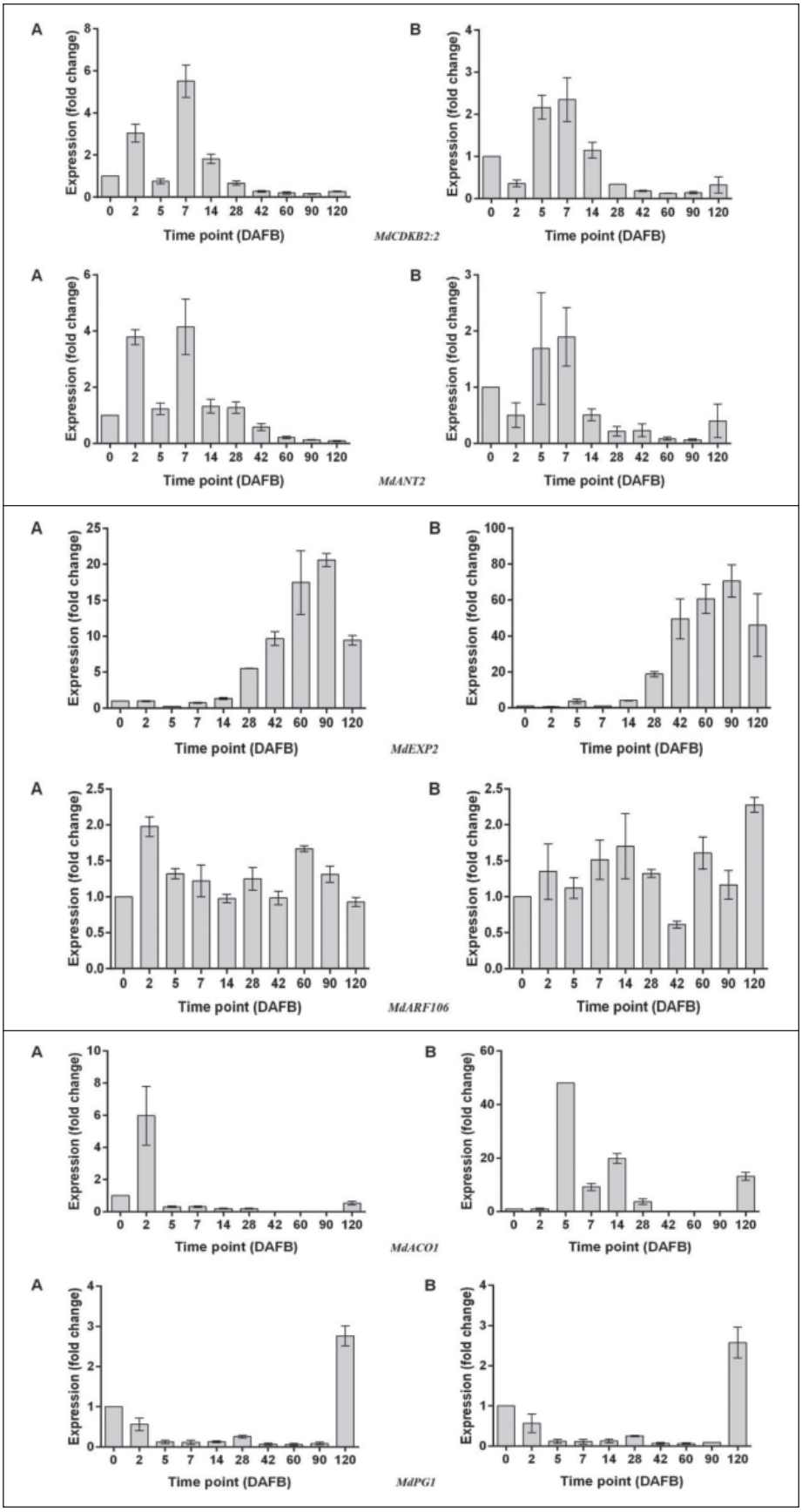

Fig. 4. Relative expression of selected genes in 'Royal Gala' during two consecutive seasons of harvest relative to *MdGAPDH*. (A) Expression pattern of repetition 1 (Rep1). (B) Expression pattern of repetition 2 (Rep2). Error bars = SEM; n=3 (technical replicates). The expressions between time points were statistically different using ANOVA (two way), p-value ≤ 0.05.

division and cell expansion stage of fruit growth which reported by Devoghalaere *et al*. (2012). Correlates with our observation in this research, the highest peak of the expression is at 2 DAFB (near to 1-fold increment) and 60 DAFB (0.6-fold increment) on Rep1 and at 14 DAFB (by 5.5-fold increment) and 60 DAFB (9-fold increment) on Rep2. This difference was due to the gene expression activity occurs in both cell division and cell expansion stages (Devoghalaere *et al.,* 2012).

At the ripening stage, *MdPG1*, as hypothesised, is expressed highly in ripening stage which is at 120 DAFB at both Reps with 2.5-fold increment at Rep1 and about 250-fold increment at Rep2. However, *MdACO1* is expressed higher in 2 and 5 DAFB than 120 DAFB which also indicate its role in fruit abscission as the activity is mainly involved auxin. *PG1* is a well-regarded gene for pectin hydrolysis and is encoded by multigene family (Atkinson & Gardner, 1993). Its function is associated with fruit softening, a process that is part of ripening (Tacken *et al.,* 2010). Resultantly, *PG1* action is supported or initiated through activation of the *ACO1* gene (Costa *et al.,* 2010). In apple fruits, the suppression of *MdACO1* also causes the *MdPG1* gene to be suppressed and results in a low softening rate (Tacken *et al.,* 2010). This is indicative of their cooperative role action in ripening, and therefore, *MdPG1* alongside *MdACO1* were employed as late maturity and ripening markers. Although *PG1* is associated in fruit ripening, this gene, when triggered by other hormones such as ethylene and ABA, is also reported to facilitate floral organ growth in dry fruits like Arabidopsis (McAtee *et al.,* 2013; Kumar *et al.,* 2014).

Several of early research has revealed that cell division influent fruit size in apples (Bain & Robertson, 1950; Denne, 1963). Cell number comparison between big-sized apples and small-sized apples at maturity revealed that both cultivars have similar cell size at maturity, whereby has led to the conclusion that high cell numbers produce the large size of fruit in apples (Smith, 1940). Until now, studies on fruit size control is carried out to increase the understandings about the process. High cell number production that has led to larger fruit growth was also found in other species such as rabbiteye blueberry [*Vaccinium ashei* (Johnson *et al.*, 2011)]; sweet cherry [*Prunus avium* (Olmstead *et al.,* 2007)]; and tomato [*Solanum lycopersicum* (Bertin *et al.,* 2003; Bohner & Bangerth, 1988)]. There are reports on apple fruits that concluded both cell division and cell expansion involved in huge fruit production, however, to what extent the cell expansion influence the final fruit size was not determined (Harada *et al.*, 2005; Malladi & Hirst, 2010).

Most of apple fruit size control studies were performed using cellular and/or physiological analysis (Bain & Robertson, 1950; Bertin *et al.*, 2003). Other research has been done on apple fruits using gene expression approaches, however, Northern blot. In this research, we performed real-time RT-PCR (qPCR) to investigate the differential expression of specific markers throughout fruit development. This approach is more reliable and adds more depth to the analysis. Specific markers of cell division, *MdCDKB2;2* and cell expansion, *MdEXP2* were designed by targeting to span introns sequences, making sure that any genomic DNA contamination does not interfere with qPCR. Another cell division marker, *MdANT2* was used as a confirmation to *MdCDKB2;2* expressions. This gene was chosen due to its positive correlation with *MdCDKB2;2* and its function in controlling cell division (Dash & Malladi, 2012). However, the role of this gene in apple fruits was not yet fully studied. Due to this reason, *MdANT* functions were hypothesised to be similar as *A. thaliana* (Dash & Malladi, 2012), which has been known to be involved in floral organ initiation and growth (Kriezek, 1999). In order to confirm this gene function, we have first tested it on Royal Gala cultivars. It showed up-regulated expression pattern during early development before decrease towards the ripening stage.

CONCLUSION

Molecular approaches have been used in many apple research to dissect the complex interaction and hormones interplay during fruit growth. From this research, it is found that the cell division gene is highly expressed during the early stage of development whereas cell expansion and ripening genes were highly expressed towards the end of development. It is also indicated that the genes especially *MdCDKB2:2*, *MdEXP2*, *MdANT2*, and *MdPG1* were specific and ideally used as markers in further research.

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