

Molecular Physiology of Fruit Growth in Apple

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ABSTRACT

Fruit growth and development are processes of primary biological importance and of considerable commercial significance. In apple, the fleshy fruit is derived largely from non-ovarian tissue. Regulation of fruit growth in apple is therefore likely distinct from that in other model fleshy fruit species. Fruit growth is an integration of multiple processes that are regulated through developmental factors, phytohormones, and availability of metabolic resources. These factors differentially influence growth during diverse stages of development, and across different tissues within the fruit. In recent years, substantial progress has been made in identifying some of the major molecular components and mechanisms involved in the regulation of apple fruit growth. This review presents a comprehensive analysis of our current knowledge of the molecular physiology of fruit growth in apple and identifies gaps where future research is needed to expand our knowledge of the regulation of this trait.

KEYWORDS: cell division; cell expansion; fruit development; fruit size; organ growth

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I. INTRODUCTION

Apple (*Malus × domestica*) is one of the most widely grown temperate fruit crops in the world. Fruit growth and development are not only of botanical significance but are also of vast economic significance in apple production. In this review, growth is defined as the increase in size of the organ, while development is defined as the progression of the organ through various phenological stages. The main emphasis of this review is on the processes and factors mediating fruit growth. However, often, growth of an organ and the processes that mediate it are intimately associated with its development. Hence, where applicable, these inter-relationships will also be discussed.

II. MORPHOLOGY AND ANATOMY OF THE APPLE FRUIT

The apple fruit is botanically a “pome.” Fruits of this class are characterized by the presence of fleshy exocarp and mesocarp tissues and a cartilaginous endocarp. The majority of the fruit tissue is comprised of accessory tissue (Pratt 1988). The central region of an apple fruit is typically constituted by five locules that are derived from five carpels from a syncarpous ovary. Each of these carpels may contain up to four ovules which upon fertilization can yield one to four seeds (Pratt 1988). The seeds are surrounded by the cartilaginous endocarp tissue at maturity. A ring of five sepal and five petal vascular traces occurs towards the periphery of the locules and is often referred to as being the core-line. Tissue outside of this core-line develops into the major fleshy and economically significant part of the apple fruit (Figure 1.1). At maturity, this tissue may constitute over 80% of the fruit volume (Tukey and Young 1942; Goffinet et al. 1995).

Ontogeny of the fleshy region of the fruit outside of the core-line and the precise localization of ovarian tissue inside of it have been debated extensively and reviewed previously (Pratt 1988). Briefly, two conflicting hypotheses have been proposed to explain the ontogeny of fruit tissues. According to MacDaniels (1940), the receptacular hypothesis indicates

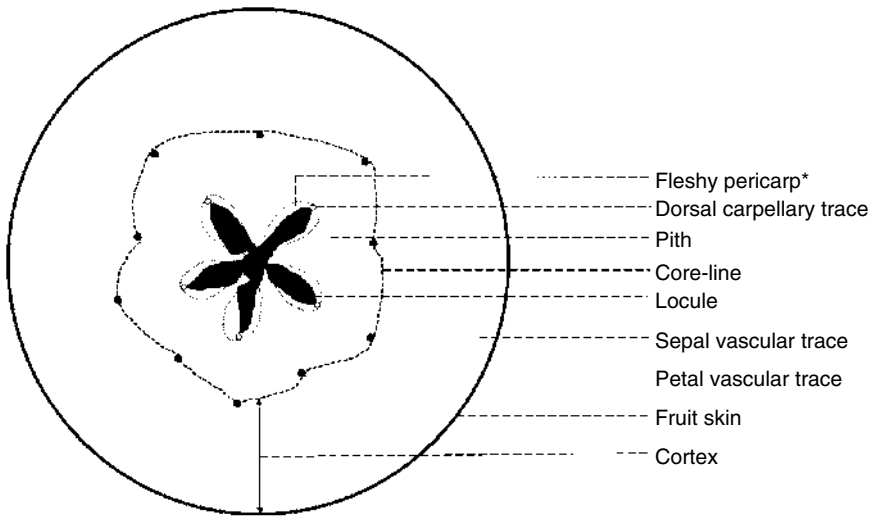


Figure 1.1 Transverse section of the apple fruit displaying the primary tissues.

*: The fleshy pericarp here is shown to indicate interpretation of the fruit morphology according to the receptacular hypothesis. According to this interpretation, tissue immediately surrounding the locule constitutes the pericarp. The appendicular hypothesis considers tissue inside of the core-line to be of ovarian origin as described in the text.

that the major fleshy region of the fruit is derived from axial tissues. The central region of the fruit extends from the pedicel into the fruit and the outer fleshy tissue represents an extension of the cortical region peripheral to the vascular tissue within the stem. Hence, this tissue is referred to as the cortex (Figure 1.1). As an extension of this terminology, tissue inside of the core-line (vascular tissue) is referred to as the pith. The location of the ovarian tissues is within the pith region and restricted to cell layers immediately surrounding the locules (Figure 1.1). Further, the true fruit is composed of five drupe-like structures characterized by a cartilaginous endocarp. As the cell layers constituted by the exocarp and mesocarp tissues are few, most of the pith is comprised of parenchymatous cells of non-ovarian origin. The alternative appendicular hypothesis presents a divergent view. In this context, the tissue peripheral to the core-line originates from the fusion of the basal tissues of appendages: multiple floral organs including the petals, sepals, and stamens (MacDaniels 1940). Hence, this tissue is often described as a floral tube or a hypanthium derived from fused basal regions of floral appendages. Tissue inside of the core-line is considered of ovarian origin, such that the innermost layer of this tissue is the endocarp while the rest is constituted by fleshy exocarp and mesocarp tissues. The core-line is regarded as the line of fusion between the floral tube

and the ovary. The relative merits of each of these theories have been evaluated with many recent authors preferring the appendicular hypothesis (Pratt 1988), largely owing to interpretations from comparative vascular anatomies across Rosaceae family fruits, as elegantly described by MacDaniels (1940), as well as data from cytochimeras summarized by Pratt (1988). Very few studies since the 1950s have addressed the origin of these tissues, despite its botanical significance.

An effective way to determine the origin of these tissues in the apple fruit is through the application of specific tissue-based markers for development. As the fruit and some of its constituent parts are derived from specific floral organs, identification of markers defining these floral organs in the fruit tissues, especially during early fruit development, can provide clues to the origin of these tissues. The ABC model describes development of floral organ identity (Bowman et al. 1991; Coen and Meyerowitz 1991) and has been extensively validated across many plant systems (Bowman et al. 2012; Irish 2017). According to this model, members of the A class of gene products determine sepal identity, and in interaction with those of the B class gene products, the identity of petals. Interaction of the B and C class gene products influences stamen development, while the C class gene products regulate gynoecium development (Irish 2017). Putative homologs of these classes of genes, many of which are MADS box transcription factors, have been identified in apple. The apple *APETALA2* (*AP2*) is a putative A class gene, the transcripts for which were shown to be abundant in sepal tissues (Kotoda et al. 2000) and in the cortex/floral tube region during early fruit development (Yao et al. 1999). This suggested that the fleshy region of the apple fruit was likely derived at least from sepal tissues. Further, facultatively parthenocarpic spontaneous mutants of apple, ‘Rae Inne’ and ‘Spencer Seedless’ were identified to be defective in one type of B class genes, *PISTILLATA* (*PI*; Yao et al. 2001). Transcript accumulation of the *PI* gene was abundant within the petals but could not be observed in the cortex/floral tube region of the developing apple fruit at four weeks after bloom (Yao et al. 2001, 2018). These data suggested that petal and stamen tissues did not likely contribute substantially to development of the cortex/floral tube region of the fruit. The A class gene defining sepal identity, *AP2*, is regulated post-transcriptionally by *microRNA 172* (*miR172*). In apple, *miR172* has been associated recently with regulation of fruit growth and final size (Yao et al. 2015). Higher levels of *miR172* were associated with a reduction in fruit growth while the opposite was true under lower levels of *miR172*. Overexpression of *miR172p* (one of several active *miR172*) in transgenic ‘Royal Gala’ plants resulted in a dramatic reduction in fruit size (Yao et al. 2015). The authors proposed

that the cortex/floral tube was largely derived from the base of the sepals and post-transcriptional alteration of the A class gene product, *AP2*, in these transgenic plants leads to altered growth of tissue derived from this floral organ. Together, these data strongly support the appendicular theory of apple fruit development and suggest that the basal regions of the floral organs, particularly the sepals, contribute greatly to development of the major fleshy tissue of the apple fruit. There are, however, a few limitations to the above approaches. Many of the genes described above have been identified and described in apple as floral organ identity genes that have clearly defined roles during flower development, but their roles in post-flowering fruit development are not as well characterized (Yao et al. 2016). While their transcript and protein accumulation in specific parts of the flower would be clearly indicative of organ identity, the significance of such accumulation at later stages and during fruit growth may need to be interpreted with caution. Further, in case of *miR172*, substantial growth and development of the fruit cortex/floral tube tissue was still noted as the hypanthium in transgenic lines, where it was overexpressed, was reduced by only about 25% during early fruit development (Yao et al. 2015). Presumably, basal regions of the petals and stamens may still contribute to the growth and development of this tissue. The current availability of additional floral tissue identity genes and newer approaches that aid in isolating specific tissues of the developing flower/fruit, such as laser capture micro-dissection, need to be applied to clearly identify the origin of this tissue.

III. FLOWER GROWTH BEFORE BLOOM

Apple flower buds are induced and initiated in the previous season and display substantial growth and development prior to dormancy (Buban and Faust 1982). Eight stages of progression in apical meristem morphology prior to winter dormancy have been described (Foster et al. 2003). Broadening of the apex of the meristem was identified as a key morphological feature signaling commitment to floral induction. This transition to floral induction peaked around 53 days after full bloom (DAFB) but continued until 127 DAFB. Similarly, broadening of the apex was also considered as a signal committing the meristem to inflorescence initiation (Pratt et al. 1959; Pratt 1988). Doming of the apex was rapid and occurred largely between 96 and 109 DAFB. Lateral floral meristem initiation and terminal floral meristem initiation followed this induction. By winter dormancy (~280 DAFB), floral meristems have clearly discernable initiation of sepals. At least in the terminal flower, further differentiation of floral organs and their development is also evident

before endo-dormancy (Pratt 1988). During the period of endo-dormancy and subsequent eco-dormancy, little growth and development are observed. Further flower development including microsporogenesis and macrosporogenesis is completed following bud break (Pratt 1988).

The period from bud break to anthesis/bloom is associated with substantial growth of the floral tube (Smith 1950; Malladi and Johnson 2011): a substantial increase in floral tube diameter from around three weeks before, until full bloom was reported (Smith 1950; Malladi and Johnson 2011). However, growth during this period was not uniform and involved rapid growth during the early part of this period followed by a cessation in growth before bloom (Malladi and Johnson 2011). A similar temporary reduction in growth around bloom was noted previously in several cultivars (Smith 1950), indicating that this may be an important feature of pre-bloom growth in apple. Reductions in growth prior to pollination and fertilization have also been reported previously in tomato (Vriezen et al. 2008; de Jong et al. 2009). Growth during the period from bud-break to bloom was largely associated with an increase in cell number, indicating that the majority of this growth was supported by an increase in the extent of cell production, although cell size also increased slightly during this period. The cessation in growth prior to bloom was associated with a quiescence in cell production (Malladi and Johnson 2011). Transcript accumulation of multiple positive regulators of cell production, such as the *CYCLIN DEPENDENT KINASE B1;2* (*CDKB1;2*), was reduced by greater than twofold during this period. Conversely, transcript accumulation of two *KIP RELATED PROTEINS* (*KRP4* and *KRP5*), which function as negative regulators of cell production by inhibiting progression of the cell cycle, were enhanced by over fourfold. Together, these data suggest coordinated transcriptional regulation to decrease cell production prior to bloom. Such reduction in growth may serve to restrict further nutrient investment in this organ until after successful pollination and fertilization. Alternatively, this reduction in growth may be reflective of extensive competition for limited resources during this period. Early growth, including flower growth after bud-break and before bloom, is largely supported by carbohydrate and nutrient resources remobilized from stored reserves (Hansen 1971; Titus and Kang 1982). Flower growth and development likely competes with vegetative growth for these resources, including that of spur leaves during the pre-bloom period. It may be hypothesized that such competition for resources leads to reduced allocation of these reserves for further growth of the flower before bloom. However, growth cessation during this period is temporary and resumes after successful pollination and fertilization, indicating that internal factors beyond the availability of resources may also temporarily limit growth during the pre-bloom

period. There is a general paucity of information regarding the role(s) of such internal factors prior to bloom. It is possible that changes in phytohormone content, transport, or signaling play important roles in regulating growth during this period. In unpollinated ovaries of garden pea, abscisic acid (ABA) concentration was found to be generally higher (Rodrigo and Garcia-Martinez 1998). Similarly, transcripts of genes associated with ABA and ethylene biosynthesis and signaling were generally upregulated in unpollinated tomato ovaries (Vriezen et al. 2008), suggesting these phytohormones, particularly ABA, may serve as negative regulators of cell production to limit growth during this period. Interestingly, KRPs are known to be positively regulated by ABA (Wang et al. 1998; Vergara et al. 2017). It may be speculated that similar regulation may occur in apple flowers before bloom to alter cell cycle progression and allow for quiescence in cell production. However, this needs to be experimentally verified. Measurement of phytohormone concentrations during the pre-bloom and bloom stages would be insightful in this context. Further, analysis of transcriptome changes during the pre-bloom period and in relation to cessation of growth is likely to provide further insights into its regulation.

IV. FRUIT SET

The term “fruit set” has different implications in apple botany and production. Botanically, fruit set refers to the transition from a flower to fruit upon successful pollination and fertilization. The alternative, and a common use of the term in apple literature, is in reference to the total amount of fruit retained on the tree after bloom (“initial set;” Lakso and Goffinet 2017) or after subsequent events of young fruit abscission (“final set”). In this review, the term is used to refer to the botanical interpretation of fruit formation associated with seed formation.

Pollination and fertilization lead to seed set. In other fruits such as tomato, seed set is thought to alter phytohormone synthesis and signaling, such as that of auxin, resulting in the resumption of ovary growth and the initiation of fruit development. Similarly, pollination, fertilization, and/or seed set may result in the generation of signals that trigger the resumption of growth within the fleshy regions of the apple fruit. Malladi and Johnson (2011) studied growth of the floral tube region and associated cell production and expansion in pollinated and unpollinated flowers. Growth of the floral tube region resumed between 3 to 10 DAFB in pollinated flowers but not in unpollinated flowers. This resumption in growth was associated with a rapid increase in cell production. Re-initiation of growth and cell production following pollination

was further associated with coordinated changes in accumulation of multiple transcripts associated with the regulation of cell production (Malladi and Johnson 2011). Many of the positive regulators of the cell cycle such as the *A* and *B*-type *CYCLINS* and several *CDKBs* displayed a clear increase in transcript abundance during the fruit set period (8–11 DAFB). Further, their abundance was dramatically reduced in unpollinated flowers consistent with a reduction in cell production and growth in these flowers. Negative regulators such as the CDK inhibitor, *KRP4*, displayed severely reduced transcript accumulation during the initial phases of fruit set and enhanced accumulation in unpollinated flowers. Together, these data indicate a coordinated transcriptional regulation of fruit set. Factors that coordinate such a response are not well understood in apple. In other fruits such as tomato, auxins and gibberellins (GAs) are known to regulate fruit set. Exogenous applications of auxin can induce parthenocarpic fruit growth in tomato (Serrani et al. 2007) and other fruits. Transcriptome analysis of pollinated tomato flowers during fruit set indicated alteration of multiple auxin signaling-related components (Vriezen et al. 2008). One set of these are the *AUXIN RESPONSE FACTOR* genes, *ARF7* and *ARF9*, which are transcription factors that coordinate auxin-dependent transcriptional responses (de Jong et al. 2009, 2015). *ARF7* transcript abundance is reduced during the fruit set period in pollinated tomato flowers. Downregulation of *ARF7* in transgenic tomato plants results in parthenocarpic fruit development (de Jong et al. 2009), indicating that it is involved in downregulating growth until pollination and fertilization occur in tomato flowers. *ARF9* overexpression in transgenic tomato lines decreased fruit size through negative regulation of cell production during early fruit development (de Jong et al. 2015). Its downregulation enhanced final fruit size in a complementary way, further implicating it in regulating early fruit growth. These data illustrate the important role of auxin in fruit development.

Exogenous applications of GA also induced parthenocarpic fruit development in tomato and multiple other fruits (Bukovac 1963; Serrani et al. 2007; Watanabe et al. 2008; Liu et al. 2018). The mode of GA action in inducing fruit set may differ from that of auxins with GAs promoting cell expansion while auxins promote cell division during early fruit development, at least in tomato (Serrani et al. 2007). In apple, unlike in tomato, auxin applications do not always induce parthenocarpic fruit development (Hayashi et al. 1968; Watanabe et al. 2008), while GA applications have been demonstrated to be effective in inducing parthenocarpy (Bukovac 1963; Bukovac and Nakagawa 1967). Among the various GAs, GA_4 and GA_7 were likely more effective in initiating parthenocarpic fruit set than GA_3 (Bukovac 1963; Bukovac and Nakagawa 1967). An interesting feature of GA applications

in inducing parthenocarpic fruit set in apple is an associated change in fruit shape. GA treated fruit often tend to display greater growth along the polar diameter and similar or lesser growth along the transverse diameter (Nakagawa et al. 1967). Increase in growth along the polar diameter is associated with a larger cortex width at the distal end of the fruit through an increase in cell number and size (Nakagawa et al. 1967). Such an inducible system for fruit set and parthenocarpic fruit growth offers an excellent system to investigate molecular components associated with quiescence in fruit growth at bloom, in fruit set, and in early fruit growth. However, this has not yet been explored in apple.

In the closely related fruit, pear, similar induction of parthenocarpic fruit growth in response to GAs has been reported and has been used to understand transcriptional processes involved in the regulation of fruit set (Liu et al. 2018). These data indicated an increase in transcript abundance of auxin transport-related genes in pollinated and parthenocarpic fruit and a corresponding decrease in abundance of transcripts associated with ABA biosynthesis. Further, the abundance of positive regulators of cell production and expansion related transcripts was up-regulated in pollinated and GA-treated fruit. Together, coordinated transcriptional re-programming of the developing flower/fruit appears to regulate the progression of fruit set. The data in pear are consistent with similar changes in the abundance of cell production-related gene products in apple during the fruit set period (Malladi and Johnson 2011).

A recent study has investigated changes in the transcriptome, in relation to early flower growth and development, associated with differential chilling accumulation in apple (Kumar et al. 2017). Genes associated with post-embryonic development were substantially enriched in transcript abundance during bud break and subsequent flower development leading to fruit set, suggesting coordinated regulation of this stage of fruit development. Further application of such transcriptomics and proteomics approaches to study fruit set using the inducible/parthenocarpic system is likely to provide important insights into the molecular factors that regulate this key transition in fruit growth and development.

V. FRUIT GROWTH

A. Components of Fruit Growth: Cell Production, Expansion, and Void Spaces

Organ growth is mediated by three main processes: cell production; cell expansion; and void space development. The relative contribution of these three processes to growth is highly variable depending

on the organ and the plant species under consideration. However, in many organs including apple fruit, final size is often correlated with cell number rather than cell size (Harada et al. 2005; Johnson et al. 2011). However, it is important to emphasize that both factors contribute greatly to the final size of the fruit and that cell production and expansion are closely inter-related processes (Harada et al. 2005; Malladi and Hirst 2010). While regulation of cell production and expansion have been studied in detail in many plant systems, including in apple, development of void space remains a poorly understood process in spite of its relatively significant contribution to the final size of the organ. Cell production in this review refers to the generation of new cells through the process of cell division. It is distinguishable from cell division itself owing to the non-synchronous nature of the population of cells in the organ. Cell division refers to the process of new cell generation at the level of an individual cell. In this context, an existing cell undergoes growth, duplication of the genome, and subsequently mitosis to generate a daughter cell. However, in a population of cells, as in organs such as the fruit or even within a specific tissue of the fruit, not all cells are involved in division. Further, even among those cells that are involved in the division process, not all are at the same stage of the cell division cycle. These factors together influence the rate at which new cells are produced from an existing population of cells. As measurements of cell number within a tissue over time typically do not account for the above factors, it is more appropriate to use the term cell production rather than cell division (Beemster and Baskin 1998; Baskin 2000). The use of these terms is more than just semantics. A change in cell production rate can be achieved due to an increase in the proportion of cells dividing in the population. This would involve acquisition of competency to divide by more cells. Alternatively, cell production rate could increase due to an increase in the cell division rate: the rate at which individual cells within the population complete their cell cycle. These two processes are likely regulated through different mechanisms and their relative contribution to changes in cell production rates may vary depending on the tissue type. One approach to determining the average cell division rate is to divide the relative cell production rate (RCPR) by the proportion of dividing cells, when the latter information is available. During the early period of fruit growth in apple, RCPR peaks around 0.26 cells per cell per day (Dash and Malladi 2012). During this period, flow cytometry analysis indicates that around 15% of the cells display nuclear DNA content of 4C, indicating that they are involved in cell division (Malladi and Hirst 2010). Further, a small proportion of cells displays nuclear DNA content between 2C and 4C, likely representing

nuclei in the process of DNA replication. Hence, around 25% of cells may be estimated to be involved in active cell division through flow cytometry analyses. Together, these data suggest a peak cell division rate of around 1.0 cell per cell per day, indicating a cell doubling time of approximately 24 h. This duration is broadly consistent with previous reports of cell division rates across other types of plant cells (Baskin et al. 1995; Beemster and Baskin 1998). However, it should be noted that flow cytometry provides only a snapshot and likely underestimates the proportion of dividing cells. Hence, the average cell division rate and the cell doubling time may, in fact, be lower and higher, respectively.

An important but often underappreciated aspect of cell production is the need for cell growth prior to cell division. The typical plant cell cycle involves four stages: G1: an initial gap phase associated with growth of the new cell; S: a synthesis phase for DNA replication and doubling of nuclear DNA content; G2: a second gap phase associated with more growth and correction of errors in DNA replication; and M: a mitotic phase where cell division occurs. Between the two M phases, the cell at least doubles its volume so that the two daughter cells formed at the end of a division cycle are at least of the same size as the original cell. Measurement of cell size during periods of active cell production generally indicates little change in size, further suggesting that cells are at least doubling in volume prior to their division. This implies that rapid cell growth occurs such that the volume of the cell doubles prior to its division. Under active cell production, as in the example of the apple fruit described above, this may occur within a 24 h period. Increase in cell volume during this short period is associated with synthesis of new organelles, membranes, cytoplasmic content, and cell wall. Hence, cell production requires a large input of carbon (C), nitrogen (N), and other resources to support this rapid growth of the cell (Verbancic et al. 2018). Further, cell growth and associated high metabolic activity during this period are likely to incur substantial energy demands. This aspect of growth and rapid doubling of cell volume associated with cell division is defined here as cell growth. The rate of cell growth associated with the cell production period may, in fact, be substantially higher than the rate of cell expansion observed at later stages of growth. This is an important consideration that explains the high demand for resources during growth phases mediated by cell production.

An increase in cell size during later stages of fruit growth following the cell production period is referred to in this review as post-mitotic cell expansion or simply as cell expansion. The relative cell expansion rate (RCER) is initially high immediately following the cell production period until around 40 DAFB (from Dash and Malladi 2012). Following

this period, RCER appears to substantially decline before increasing again slightly during the late stages of fruit development (Dash and Malladi 2012; Dash et al. 2013). These data suggest several phases of cell expansion rather than a uniform increase in cell size during fruit development. Post-mitotic cell expansion immediately following the cell production period may involve an increase in cellular content and organelles, as well as substantial alteration of the cell wall material. Following this period, cell expansion may be driven largely by an increase in vacuolar volume facilitated by uptake of water into the organelle. Such expansion may be metabolically less intensive than that during cell production or in the rapid early cell expansion period.

B. Fruit Growth and its Regulation

During fruit set and immediately thereafter, a rapid increase in the size of the organ is observed (Tukey and Young 1942; Smith 1950; Malladi and Johnson 2011). This pattern continues for several weeks, after which growth occurs at a more linear rate throughout the rest of fruit development. The increase in fruit size may continue until later stages of fruit development, sometimes into maturity and ripening, and may depend on environmental factors (Lakso and Goffinet 2017). Fruit growth in apple has been typically described as displaying a sigmoid growth pattern (Pratt 1988; Ryugo 1988). Increase in size is initially low around bloom, is rapid during fruit set and immediately thereafter, proceeds at a linear rate until later stages of fruit development, and tapers off at late stages. However, studies where growth was monitored on individual fruit suggested a double-sigmoid fruit growth pattern similar to that observed in some other Rosaceae members such as peach (Magein 1989). This has been questioned due to the relatively short lag period in apple which could potentially be attributed to adverse environmental conditions (Lakso et al. 1995). Schechter et al. (1993) suggested two linear phases of fruit growth in apple coincident with the cell production and expansion phases. However, while growth during later stages does often suggest a more linear pattern, initial growth deviates substantially from this. Hence, Lakso et al. (1995) proposed an expolinear model to describe apple fruit growth. Such a model appears to fit measured fruit growth, especially under non-limiting conditions such as when seed set is optimal, crop load on the tree is non-limiting, and when environmental conditions are favorable: a set of conditions that allow for inherent growth characteristics to manifest. In the expolinear model, the early period of fruit growth is considered exponential and associated primarily with the cell production period. The rest of fruit development

displays a linear growth and is largely associated with cell expansion. This suggests a nearly constant rate of cell expansion throughout most of fruit development and an associated constant demand for C and other resources during this period (Lakso et al. 1995). Using the model parameter, a nearly constant rate of cell expansion was estimated across different crop load reduction treatments (Lakso et al. 1995).

Early fruit growth in apple is largely associated with intensive cell production. A review of previous studies across multiple genotypes suggests that this duration typically lasts until 3–4 weeks after full bloom (WAFB; Blanpied and Wilde 1968; Malladi and Hirst 2010; Dash and Malladi 2012), but may continue until around 7 WAFB (Denne 1960). This duration may vary depending on several factors. Genotypic differences in the duration of the cell production period have been reported (Blanpied and Wilde 1968; Hirst and Malladi 2008). Duration of the cell production phase may be affected by environmental factors such as temperature. Warrington et al. (1999) conducted a series of experiments subjecting potted apple trees to various temperature regimes during different stages of early- to mid-fruit development. They indirectly assessed the duration of cell production and reported an inverse relationship between this factor and mean temperature. Additionally, the duration of cell production may potentially be affected by availability of resources (Dash and Malladi 2012; Dash et al. 2013). Together, these results suggest some plasticity in the duration of the cell production period in apple. Such plasticity may allow for fine-tuning fruit growth responses to external environmental factors and availability of resources.

C. Cell Production Related Genes and Regulation of Fruit Growth

Progression of cell production during early fruit growth involves several cycles of cell division. The plant cell cycle involves progression of the cell through multiple phases leading up to the division of the cell and generation of daughter cells. As discussed above, the plant cell cycle consists of four phases: 1. G1 phase – a gap phase immediately following the generation of a new cell, and associated with intensive cell growth; 2. S phase – a synthesis phase involving DNA replication and doubling of the nuclear genome; 3. G2 phase – another gap phase involving cell growth and DNA repair; and 4. M phase – a phase involving mitosis and generation of daughter cells (Inze and De Veylder 2006; Francis 2007). Key transitions in progression of the cell cycle are from G1 to the S phase and another from G2 to the M phase (Inze and De Veylder 2006; Francis 2007). Multiple gene products are associated with the regulation of the cell cycle. In the model plant *Arabidopsis thaliana*,

around 70–80 genes are reported to be closely associated with cell cycle regulation and are considered to constitute the core cell cycle machinery (Menges et al. 2005). Although there are multiple components to this machinery, the key units are the CYCLINS (CYC) and CYCLIN DEPENDENT KINASES (CDKs). CYCs accumulate and are degraded during specific phases of the cell cycle. They associate with CDKs to regulate downstream targets through phosphorylation. Multiple CYCs have been identified in plants, generally more than those identified in other eukaryotes (Inze and De Veylder 2006). Similarly, multiple types of CDKs have been identified in plants, some of which – such as the B-type CDKs (CDKBs) – are considered plant specific. Specific interactions of CYCs with CDKs control progression of the cell cycle through its phases and through the key transitions. For example, an association of D-type CYCs (CYCDs) with A-type CDKs (CDKAs) regulates G1–S phase transition while CYCA and CDKA interactions regulate progression through the S phase and transition to the M phase (Scofield et al. 2014). CYCB associations with CDKAs and CDKBs regulate progression through G2, transition from G2–M, and progression through the M phase during the cell cycle (Inze and De Veylder 2006). Knowledge of the downstream targets of the core cell cycle machinery is rather poorly developed in plants. Some estimates suggest that over a thousand gene products display cell cycle modulated changes in transcript accumulation (Breyne et al. 2002; Menges et al. 2002). It is likely that many of these are downstream targets of the core cell cycle machinery. For example, phosphorylation of two downstream targets (mitotic kinesin-like protein and a MAP kinase kinase kinase) by CDKA regulates transition to cytokinesis in plants (Sasabe et al. 2011). Several regulators of the cell cycle are included within the core cell cycle machinery. Plant CDKs are negatively regulated by KINASE INHIBITOR PROTEIN (KIP) RELATED PROTEINS (KRPs). KRPs can bind to and negatively regulate the activity of CDK/CYCLIN complexes (Verkest et al. 2005; Inze and De Veylder 2006; Van Leene et al. 2010). In *Arabidopsis*, KRP2 inhibited CDKA;1 activity and was itself regulated through phosphorylation by CDKB1;1 and subsequent proteasome mediated degradation (Verkest et al. 2005). Such regulation allows for fine-tuning of the cell cycle. The core cell cycle regulators can alter competency for cell division of individual cells. Further, the rate of progression through the cell cycle can also be affected by the activities of the cell cycle machinery. Together, these two processes can have a strong impact on the extent of cell production and can thereby influence growth rates of organs. However, multiple studies involving direct manipulation of the cell cycle machinery components have indicated that growth may not be

directly altered by their mis-expression in part due to “compensation” mechanisms (Hemerly et al. 1995; De Veylder et al. 2001; Dewitte et al. 2003; Verkest et al. 2005). Compensation refers to an active process where alteration in the extent of cell production is countered at least in part by a complementary change in the extent of cell expansion (Horiguchi and Tsukaya 2011). Nevertheless, as cell cycle regulators are key components controlling progression through the cell division process, it is likely that they are the key downstream effectors facilitating organ growth.

In apple, multiple members of the cell cycle machinery were identified and their transcript accumulation patterns during different stages of fruit growth and development were characterized (Malladi and Johnson 2011). Based on pre-bloom, fruit set, and fruit developmental patterns of transcript abundance, 14 genes were identified as being positively associated with cell production. Transcripts of these genes accumulated in response to fruit set and during early stages of fruit growth (3–4 WAFB). Several *CDKBs* and multiple *A*- and *B*-type *CYCLINS* were included within this group, suggesting that availability of these G2/M phase-related factors may be limiting for cell production during apple fruit growth. Similar results were reported in one of the earliest microarray-based transcriptome studies of fruit development in apple which evaluated eight stages of fruit growth and development (Janssen et al. 2008), and in another study describing impaired fruit growth responses to elevated temperature (Flaishman et al. 2015). Further, proteomics studies of fruit development also indicate similar changes in cell production regulator abundance (Li et al. 2016). Transcript abundance of several of these genes (*CYCAs*) was altered by severe shading during early fruit development, a treatment which reduces the extent of cell production (Dash et al. 2012). Conversely, many of the *CDKBs* and *CYCAs* displayed higher transcript abundance in response to a reduction in fruit load, a treatment that enhances growth through cell production (Dash et al. 2013). Duan et al. (2017) suggested a potential role for several *CYC* genes in evolution of fruit size during domestication as they were co-localized to regions associated with selective sweeps. Five genes were also identified as potential negative regulators of cell production by Malladi and Johnson (2011), two of which were *KRPs* (*KRP4* and *KRP5*). These *KRPs* displayed transcript accumulation patterns complementary to those of the positive regulators; generally declining during early fruit development and increasing in abundance during transition from cell production to expansion (Malladi and Johnson 2011). Further, both these *KRPs* displayed increased transcript abundance in unpollinated fruit and in response to severe shading,

instances associated with reduced cell production (Malladi and Johnson 2011; Dash et al. 2012). Together, these data suggest important roles for these genes in regulating cell production and growth during early stages of fruit development.

D. Organ Size Related Genes and Regulation of Fruit Growth

While the core cell cycle machinery facilitates cell production, additional factors may also be involved in regulating this process. Many organ growth-related genes have been identified in other plant systems such as *Arabidopsis* and tomato. Some of these genes affect cell production and their orthologs in apple are likely to perform similar functions. For example, the *FRUIT WEIGHT 2.2 (FW2.2)/ CELL NUMBER REGULATOR (CNR)* gene was initially identified in tomato as being associated with fruit weight (Frary et al. 2000). Differential temporal transcript accumulation of this gene during fruit development is thought to negatively regulate cell production and thereby influence fruit size across tomato genotypes (Nesbitt and Tanksley 2001; Cong et al. 2002). Multiple putative *CNR* homologs were identified in peach and cherry (Franceschi et al. 2013). The genomic position of two of these co-localized with that of two quantitative trait loci (QTLs) associated with fruit size in cherry (Franceschi et al. 2013). Hence, these genes may play similar roles in Rosaceae family members as well. No reports on the roles of these genes in regulating apple fruit growth are yet available.

Transcription factors that can modulate the transcript abundance of downstream targets associated with cell division are potential candidates associated with the regulation of fruit growth. One such transcription factor belonging to the plant specific APETALA2 (AP2) domain family is AINTEGUMENTA (ANT). In *Arabidopsis*, ANTs positively regulate cell production and thereby influence organ growth (Mizukami and Fisher 2000), although in a different *Arabidopsis* ecotype, they were reported to affect cell size (Krizek 1999). Loss of its function reduces overall organ size while its overexpression enhances shoot organ size in *Arabidopsis* (Krizek 1999; Mizukami and Fisher 2000). ANTs are thought to regulate the duration of cell production and its overexpression can affect cell cycle progression by enhancing *CYCD3* transcript abundance (Mizukami and Fisher 2000). However, such a direct relationship to *CYCD3* expression and the cell cycle has recently been questioned (Randall et al. 2015), and it may be likely that alternative mechanisms are involved. Putative homologs of *ANT*, *ANT1* and *ANT2*, and *ANT-like (AIL)* genes were identified and characterized in apple (Dash and Malladi 2012). The two genes, *ANT1* and *ANT2*,

share high sequence similarity and are likely duplicated forms of the apple *ANT* gene. Expression of *ANT1* and *ANT2* in apple is coincident with transcript accumulation of many positive regulators of cell production (Dash and Malladi 2012). Transcript abundance of both these genes increases during early fruit growth during the period of cell production and dramatically declines during exit from this phase (Dash and Malladi 2012). Reduction in fruit load enhances cell production in apple and this is associated with an increase in transcript abundance of at least *ANT1*. Further, comparison of two genotypes differing in final fruit size potential through differences in cell number, indicated substantial differences in transcript abundance profiles of these *ANT* genes. These data are consistent with a role for the apple *ANT* genes in regulating cell production during early fruit growth. Further analysis of their function and their downstream targets is necessary to clearly determine their specific roles in promoting cell production during early fruit growth.

Daccord et al. (2017) performed re-sequencing of the apple genome and genome-wide DNA methylation analysis in apple. In their study of fruit development, they utilized a double haploid ‘Golden Delicious’ apple genotype (GDDH13) and compared it to an isogenic line (GDDH18) derived from the same haploid. While these two genotypes were phenotypically largely similar, they differed primarily in their fruit size, with GDDH18 displaying smaller fruit owing to reduced cortex cell number. Multiple single nucleotide polymorphisms (SNPs), with a few resulting in potential amino acid changes, were identified through the comparison of these genotypes. Further, comparison of DNA methylation across these two genotypes over different developmental stages identified 22 differentially methylated genes including several transcription factors, and a gene associated with ethylene biosynthesis (Daccord et al. 2017). Several of these genes are homologous to genes with potential organ-growth regulating roles in other plants. Functional characterization of these genes may provide useful insights into regulation of cell production during early fruit growth in apple. This study also implicates DNA methylation as a potential fruit growth regulatory mechanism in apple (Daccord et al., 2017).

E. Floral Homeotic Genes and Regulation of Fruit Growth

Multiple floral organ identity genes have been defined in studies on apple and many of these genes appear to have functions beyond regulation of flower development, such as in fruit growth and ripening. In fact, several of these studies are examples of the clearest genetic case studies

for regulation of apple fruit growth. Potential changes in tissue and cell layer specifications during flower development can have profound effects on organ growth at later stages and may explain some of these effects on growth. The microRNA, *miR172*, was recently described as being associated with regulation of fruit size in apple (Yao et al. 2015). Overexpression of *miR172p* in apple resulted in multiple phenotypic changes in floral and fruit morphology, some of which were associated with the intensity of its overexpression. A transgenic apple line overexpressing *miR172p* by 15-fold displayed a dramatic reduction in fruit size, reducing 'Royal Gala' fruit size to that of a crabapple fruit. This was associated with a reduction in the size of the hypanthium at bloom and during early fruit development. During this period, cell size was similar in wild-type and transgenic lines indicating that a reduction in cell production capacity during early fruit growth may contribute partially to the reduced fruit size phenotype in these plants (Yao et al. 2015). An allele of *miR172* displaying a transposon insertion and potentially reduced expression was associated with larger fruit size across multiple *Malus* accessions and a segregating population. The wild type allele was named as the *CRAB APPLE FRUIT SIZE (CAFS)* locus (Yao et al. 2015). The *miR172* may regulate expression or translation of *AP2* genes which are floral homeotic genes regulating floral organ identity. This work in apple is somewhat contrary to a fruit growth promoting role proposed for *miR172* in *Arabidopsis* (Ripoll et al. 2015). However, this apparent discrepancy has been attributed to the fundamentally different origins of the fruit tissue in these two species with *Arabidopsis* forming a silique derived primarily from the ovary (Yao et al. 2015; 2016). In the proposed model, *AP2* may function to promote sepal identity and growth and its downregulated activity in genotypes with higher *miRNA172p* expression results in reduced fruit size as this cortex tissue is largely derived from the fused basal regions of floral appendages including sepals. In addition to *miRNA172p*, Duan et al. (2017) identified two other *miR172* genes that may also be associated with fruit size evolution in apple.

Another floral homeotic gene belonging to the class B of organ identity genes, *PI*, has also been associated with regulation of localized fruit growth in apple (Yao et al. 2018). Alteration of *PI* expression was previously reported to be associated with parthenocarpic apple fruit development (Yao et al. 2001). Over and ectopic expression of *PI* in 'Bolero' apple resulted in the conversion of sepals to petals and altered fruit morphology by altering fruit shape (Yao et al. 2018). Beginning from early stages of fruit growth (eight days after pollination), transgenic lines overexpressing the *PI* gene displayed characteristic longitudinal

grooves on the fruit and a temporary transverse groove. By maturity, the fruit displayed a reduced height to width ratio and a flattened fruit shape. While cell size was reduced within the groove regions, cell production may also have been potentially altered in this region resulting in the observed changes in fruit morphology. The authors hypothesized that *PI* may heterodimerize with another homeotic gene product *AP3* to suppress growth in the fruit and that the localized accumulation of *AP3* may contribute to such spatial growth regulation. However, the localized accumulation of *AP3* transcripts or the protein remains to be characterized. A potentially conserved role for *PI* in regulating fleshy fruit growth is supported by work in grape where a *fleshless berry (flb)* mutant was found to contain a transposon insertion within the promoter region of *PI* resulting in its ectopic expression during early fruit development and an associated loss of fleshy tissue growth due to reduced cell division and expansion (Fernandez et al. 2006, 2007, 2013).

A MADS box gene belonging to the class E floral homeotic gene family (*SEPALLATA1/2*; *SEP1/2*), a class of gene products that function along with other floral homeotic gene products to determine floral organ identity, has also been associated with regulation of fruit growth and development in apple (Ireland et al. 2013). Antisense suppression of one of these genes (*MADS8*) in apple also affected transcript accumulation of several closely related genes: *MADS9* and *MADS7*. Transgenic apple lines displaying reduced expression of these genes displayed floral, fruit growth, and ripening related phenotypes. Floral morphology was altered through the development of sepalloid petals and reduced post-bloom petal abscission. Histological analysis revealed that in these transgenic lines, the outer hypanthium tissue development was already affected at bloom. Further, fruit from these transgenic lines displayed reduced growth primarily due to a reduction in growth within the cortex tissue. Cells within the cortex were substantially reduced at maturity, indicating that cortex cell expansion was altered in these lines resulting in a dramatically reduced fruit size phenotype. Interestingly, reduced expression of a closely related *MADS9* genes appears to similarly alter fleshy fruit growth in strawberry, at least in some severe phenotype lines (Seymour et al. 2011), suggesting a more general role for these genes in the growth of fleshy fruits.

F. Cell Wall Modifying Genes and Regulation of Fruit Growth

Multiple cell wall modifying genes have been investigated in apple in relation to their potential roles in regulating fruit growth. Cell expansion is primarily driven by turgor within cells (Cosgrove 2018). Increase in

volume of cells, either in relation to cell growth or in relation to post-mitotic cell expansion, is associated with loosening of the cell wall and the associated relaxation of wall stress resulting in an irreversible increase in the surface area (Cosgrove 2016, 2018). Multiple enzymes such as expansins, endoglucanases, endotransglucosylases, and pectin-modifying enzymes such as pectinmethylesterases and polygalacturonases, have been associated with cell wall loosening in relation to growth and other responses (Cosgrove 2016). Only a few of these have been systematically evaluated in apple in relation to cell expansion during fruit growth. An initial study evaluated the transcript abundance of six *EXPA* (α -*EXPANSIN*) genes in apple and identified at least one for which the expression pattern coincided with the cell expansion period of fruit growth (Wakasa et al. 2003). Dash et al. (2013) also evaluated the transcript accumulation patterns of several *EXPA* genes during fruit development and in relation to reduction in fruit load. These *EXPAs* displayed varying transcript abundance during fruit development but at least two of them displayed an increase in abundance during mid-fruit development coincident with the post-mitotic cell expansion phase of fruit growth. One of these, *EXPA10;1*, displayed a reduction in transcript abundance in response to severe shading, a treatment associated with a reduction in cell expansion (Dash et al. 2012). A systematic genome-wide analysis of *EXPANSINs* in apple identified 41 such genes that could be classified into four sub-groups (Zhang et al. 2014). While the transcripts of many of these genes were identified within the fruit, a detailed analysis of abundance was performed primarily in relation to ripening (Zhang et al. 2014).

While many putative endoglucanase, endotransglucosylase, and pectin modification-associated genes have been identified in transcriptome studies and other studies in apple (Atkinson et al. 2012; S. Jing and others, unpubl.), analysis of their expression profiles and their responses to factors that alter growth is still lacking, although they have been studied in relation to fruit ripening (e.g., Atkinson et al. 2012). A proteomic study indicated higher expression of several endotransglucosylase and pectin modification-associated gene products during mid-fruit development coincident with the period of cell expansion (Li et al. 2016). Additionally, two beta-galactosidase genes were co-localized to a locus associated with fruit size evolution during domestication (Duan et al. 2017). The potential roles of these genes in regulating apple fruit growth is unclear. In strawberry, downregulation of the expression of these genes reduced fruit weight likely due to impaired pollen tube growth, fertilization, and reduced achene numbers, but not by altering fruit growth processes directly (Paniagua et al. 2016).

An additional set of cell wall modifying factors are the gene products of the *COBRA* (*COB*) and *COBRA-LIKE* gene families. *COB* was identified in *Arabidopsis* as a gene associated with oriented cell expansion (Schindelman et al. 2001). Further analysis suggested crystalline cellulose and microfibril orientation were significantly impacted in the *cob* mutant. These data indicated a role for *COB* in regulating the deposition of cellulose microfibrils during cell elongation (Roudier et al. 2005). A putative *COB* homolog, *COB1*, was identified in apple and its transcript accumulation during fruit development was characterized (Dash et al. 2012, 2013). *COB1* transcript abundance was relatively constant during the cell production period but rapidly increased by over fourfold following exit from this phase and remained high and stable during the rest of fruit development. Interestingly, the period of rapid increase in *COB1* transcript abundance in apple coincides with the period of highest RCER during fruit growth (Dash et al. 2013). Further, its transcript abundance was slightly but significantly reduced in response to severe shading which induced a reduction in cell production and expansion (Dash et al. 2012). Together, these data suggest a potential role for the *COB1* gene product in facilitating rapid cell expansion during fruit development. In tomato, a putative homolog of *COB* was identified and functionally characterized (Cao et al. 2012). The tomato *COB* displays higher transcript abundance during early fruit development coincident with the period of rapid cell expansion and declines towards ripening, a period where no additional cell expansion occurs in tomato. Fruit-specific downregulation of the tomato *COB* due to co-suppression leads to reduced crystalline cellulose content and fruit cracking. Further, pericarp cell walls appeared to be compromised in these lines, leading to the collapse of some cells. Its overexpression resulted in increased cellulose content, fruit firmness, and shelf-life (Cao et al. 2012). Together, these data suggest an important role for *COB* in regulating cell wall remodeling during cell expansion mediated fruit growth. In apple, much of the information on regulation of cell expansion remains to be functionally analyzed. A systematic functional analysis of some of the above described genes in relation to cell expansion during fruit growth is necessary to determine the key components associated with cell wall loosening in relation to fruit growth.

G. Metabolism and Regulation of Fruit Growth

Fruit metabolism is an integral component of fruit development and plays primary roles in determining the rate of growth and final fruit quality. Integration of molecular regulation of fruit metabolism with

regulation of growth in the organ is only recently being achieved in model fruit systems. In apple, further work is necessary to achieve a better understanding of the role of fruit metabolism in the molecular physiology of fruit growth. A brief discussion of apple fruit metabolism is presented below to allow for an appreciation of the inter-dependence of these processes.

The apple fruit possesses significant photosynthetic ability (Blanke and Lenz 1989), but it also functions as a heterotrophic organ. Blanke and Lenz (1989) proposed that apple fruit photosynthesis was intermediate between that of C3 and C4/CAM. Such photosynthetic activity allows for the fruit to partially meet (5–20%) its carbohydrate demands for growth, at least during early fruit development (Blanke and Lenz 1989; Lakso and Goffinet 2017). Hence, the apple fruit still functions as a major sink and is greatly dependent on resource import to meet its metabolic demands. Much of the carbohydrate and other nutritional requirements before bloom are supported by remobilized reserves from the previous season in roots and stems (Hansen 1971; Titus and Kang 1982). Around bloom or slightly after, there appears to be a transition such that current photosynthesis from newly emerged and photosynthetically established leaves begins to support the majority of growth of the fruit (Hansen 1967, 1971; Quinlan, 1969; Oliviera and Priestley 1988; Forshey and Elfving 1989). The cell production phase of growth creates intensive demands for carbohydrates and other nutrients. Cell growth requires additional cytoplasmic material, cell wall components, membranes, and organelles. Subsequently, there is also an increased energy demand to facilitate the high metabolic activity during this period. In fact, the rate of respiration during fruit development is highest during this growth period (Blanke and Lenz 1989; Beshir et al. 2017). Post-mitotic cell expansion involves multiple metabolic changes which allow for some cytoplasmic growth of the cell but also allow for a large increase in vacuolar volume through the intake of water, a process mediated by the accumulation of osmoticum within these organelles. Further, this period of growth also involves transitory accumulation of carbohydrate in the form of starch (Berüter 1985). This is subsequently metabolized during later stages to support accumulation of other sugars and to meet the respiratory demands of ripening. These aspects of carbohydrate and nutrient requirement clearly demonstrate the dependence of the fruit cells on resource import to sustain growth. Further, a reduction of fruit load often leads to enhanced fruit growth, especially if performed early in the season (Auchter 1920; Denne 1960; Westwood et al. 1967; Dennis 2000). This increase in fruit growth is mediated by an increase in cell production but can also involve an

increase in cell expansion (Denne 1960; Westwood et al. 1967; Goffinet et al. 1995; Dash et al. 2013). Such an increase in growth is one of the main reasons for application of extensive crop load management practices in apple (Wunsche and Ferguson 2005; Kon and Schupp 2019).

Carbon may enter apple fruit primarily in the form of sorbitol (Sor) or sucrose (Suc), with the extent of carbon in the form of Sor being several-fold higher than that in the form of Suc (Webb and Burley 1962; Berüter and Droz 1991). Phloem unloading in apple occurs apoplastically during early fruit development (Zhang et al. 2004), requiring the function of transporters to allow for its intake into fruit cells. Several putative sugar transporters have been identified and their transcript abundance characterized in apple, some of which may be involved in the entry of Sor and Suc into fruit cells (Gao et al. 2005; Li et al. 2012; Wei et al. 2014). Although Sor serves as one of the main forms of carbon entry into the fruit, it does not typically accumulate during fruit development (Berüter 1985; Yamaki and Ishikawa 1986; Zhang et al. 2010). Instead, Sor is rapidly converted to fructose (Fru) by sorbitol dehydrogenase (SDH) (Yamaki and Ishikawa 1986; Archbold 1999). Multiple genes coding for SDH have been identified in apple and the expression patterns of some of them are consistent with changes in Sor concentration, especially during early fruit development, indicating that SDH activity can affect sink strength of apple fruit (Nosarszewski et al. 2004).

Sucrose concentration increases during most of fruit development (Berüter 1985; Yamaki and Ishikawa, 1986), mediated by the entry of Suc into the fruit cells, and extensive interconversions during Suc metabolism. Sucrose metabolism in apple fruit may occur through the activity of multiple enzymes. Invertases convert Suc into Fru and glucose (Glc). Cell wall invertases may metabolize Suc following its apoplastic unloading, but such invertase content was found to be low in the fruit cortex (Li et al. 2016). In tomato, a cell wall invertase gene (*LIN5*) was identified as the underlying gene for a major QTL for fruit sugar content and yield, and its downregulation resulted in reduced fruit size (Fridman et al. 2004; Zanol et al. 2009), supporting a substantial role for these genes in regulating fruit growth. Neutral invertases (NINV) function in the cytoplasm and one of the genes coding for an NINV, *NINV5*, was postulated to be associated with Suc unloading in apple (Li et al. 2012, 2016). Vacuolar invertases (VINV) may also metabolize Suc leading to the production and potential accumulation of Fru and Glc in the vacuole. Acid invertase activity is generally higher during early fruit growth (Berüter 1985) and may therefore serve as an important mechanism for Suc metabolism during this period. Sucrose may also be metabolized

by sucrose synthase (Susy), yielding Fru and UDP-Glc. Potentially, such Susy activity can feed into cellulose synthesis to support the new cell wall growth requirements associated with rapid early fruit growth (Verbancic et al. 2018). In fact, Susy activity was generally higher during early fruit development and subsequently declined at later stages (Li et al. 2012). Further, Suc metabolism in the apple fruit also involves a Suc–Suc cycle contributing to the accumulation of Suc during fruit development through the activity of sucrose phosphate synthase (SPS; Li et al. 2012). Starch breakdown during late fruit development may also contribute to Suc accumulation (Berüter and Feusi 1997).

Fructose and Glc generated from the metabolism of carbon imported into the fruit can have multiple metabolic fates. Fru and Glc can enter glycolysis following their phosphorylation by fructokinase and hexokinase, respectively. Fructokinase protein content and activity are higher during early fruit growth (Li et al. 2012, 2016). Similarly, hexokinase activity and transcript abundance of hexokinase genes was greater during early fruit growth (Li et al. 2012; S. Jing and A. Malladi, unpubl.). Such flux into glycolysis and subsequent respiration is critical to meet energy and carbon skeleton demands of early fruit growth. Glycolytic flux was in fact reported to be high during early fruit development (Beshir et al. 2017) and to decline at later stages (Li et al. 2016). In tomato, overexpression of an *Arabidopsis* hexokinase gene reduced fruit growth underlining the important role of these genes in supporting organ growth (Menu et al. 2004). Hexose phosphates can also be converted to nucleotide-sugars which then serve as precursors for the synthesis of non-cellulosic cell wall material (Verbancic et al. 2018). Another metabolic fate for these sugars is their accumulation in the vacuoles during early fruit development (Berüter 1985; Zhang et al. 2010; S. Jing and A. Malladi, unpubl.). This may serve multiple purposes, including that of increasing osmoticum to allow for cell expansion at later stages. Fructose, in fact, continues to accumulate during mid and late stages of fruit development at levels higher than that of Glc such that it is typically the most abundant simple sugar at maturity (Berüter 1985; Zhang et al. 2010). These sugars may also contribute to transitory starch biosynthesis and accumulation that occurs during mid stages of fruit development, coincident with the period of cell expansion. This metabolic transition away from glycolysis during mid fruit development may reflect lower demands for carbon skeletons and energy to facilitate cell expansion-mediated growth.

Malic acid (malate) accumulates during fruit development in apple and is the most abundant organic acid in apple fruit (Ulrich 1970; Berüter 2004; Walker and Famiani 2018). Fixation of CO₂ (HCO₃⁻) by

phosphoenolpyruvate carboxylase (PEPC) with PEP serving as the other substrate to generate oxaloacetic acid (OAA) has been reported in apple (Blanke and Lenz 1989). Malate dehydrogenase (MDH) activity may subsequently convert OAA to malate (Blanke and Lenz 1989). In fact, such a pathway for malate synthesis appears to be common across multiple fruits and may serve as the major route for its synthesis (Etienne et al. 2013; Walker and Famiani 2018). Malate concentration increases gradually during early fruit development reaching a peak towards the end of the cell production period of fruit growth (Zhang et al. 2010; S. Jing and A. Malladi, unpubl.). Putative *PEPC* genes and *MDH* genes have been isolated from apple and partially characterized (Yao et al. 2009, 2011). Transcript abundance of some *PEPC* genes increases during early fruit growth and development, while that of some *MDH* genes is also high during this period, consistent with a role for PEPC and MDH in generating malate during early fruit development (Yao et al. 2009, 2011; S. Jing and A. Malladi, unpubl.). Malate generated through such metabolism or through the tricarboxylic acid (TCA) cycle may be transported into vacuoles for storage allowing for its accumulation. The capacity for storage may determine the ability of a given apple cultivar to accumulate malate (Berüter 2004). Further, a potential gene responsible for a low fruit acidity phenotype was found to be an aluminum activated malate transporter (Bai et al. 2012). The physiological significance for such accumulation of malate is not entirely clear. One hypothesis is that malate accumulation in the vacuole during early fruit growth may serve as an osmoticum that allows for cell expansion at later stages, as has been proposed for tomato (Guillet et al. 2002), indicating direct implications for cell expansion-mediated fruit growth. Alternatively, malate may serve as a storage form of carbon that can re-enter respiration (TCA cycle) at later stages such as during the respiratory climacteric. Overall, malate accumulation influences fruit growth and development, and contributes to final fruit quality.

An area that has had relatively little study is N metabolism during fruit growth. Much of the N required to support pre-bloom growth until full bloom is likely to be supported by remobilization of N reserves (Titus and Kang 1982; Malaguti et al. 2001; Guak et al. 2003). Remobilization of N continues to support early fruit growth, although current N acquisition also contributes to growth during this period (Titus and Kang 1982; Malaguti et al. 2001; Guak et al. 2003). The entry of N into the fruit is likely in the form of amino acids during this period, predominantly in the form of asparagine (Asn), aspartate (Asp), glutamine (Gln), and arginine (Tromp and Ovaa 1971; Malaguti et al. 2001). In fact, within the fruit tissue, Asn is the most abundant amino acid during

early fruit development (Zhang et al. 2010; Beshir et al. 2017). How these forms of N imported into the fruit are metabolized to support N demands during early fruit growth remains unclear. It is likely that Asn is metabolized by asparaginase to yield Asp and ammonium (NH_4^+ ; Gaufichon et al. 2016). Aspartate may have several metabolic fates including conversion to OAA, and as the N source for the biosynthesis of other amino acids. The NH_4^+ released may be re-assimilated through the glutamine synthetase–glutamine 2-oxo-glutarate aminotransferase (GS–GOGAT) pathway leading to the synthesis of glutamate. Owing to higher N demand during cell production, it may be speculated that such N metabolism is substantially higher during early fruit growth. Nitrogen availability does in fact limit fruit growth and increased N availability increases cell production and fruit size (Xia et al. 2009). Future studies should evaluate the contribution of N metabolism and its molecular regulation to fruit growth and development as this represents a major gap in knowledge.

H. Phytohormones and the Regulation of Fruit Growth

Phytohormones are key factors that coordinate cell production and expansion and may therefore have profound effects on fruit growth in apple. Multiple phytohormones are thought to regulate apple fruit growth. Auxins have been implicated in the regulation of various aspects of fruit growth and development including cell production, expansion, and ripening (Srivastava and Handa 2005). In apple, synthetic auxins such as naphthalene acetic acid (NAA) are regularly used for crop load management and can therefore influence fruit growth indirectly (Dennis 2000; Zhu et al. 2011). It is likely that endogenous auxins play significant roles in regulating fruit growth as well. Consequently, detailed genomic analysis of the potential role of auxins, their metabolism, transport, and signaling, has been reported in apple (Devoghalare et al. 2012). Free auxin (indole-3-acetic acid; IAA) concentration increased in the fruit cortex during mid-fruit growth coincident with the period of post-mitotic cell expansion. Interestingly, IAA concentration in the seeds was substantially higher and continued to increase during fruit development. Injection of IAA into the fruit at 30 DAFB enhanced growth, resulting in larger fruit. This was primarily achieved through an increase in cell size indicating a role for IAA in promoting cell wall loosening and facilitating cell expansion (Devoghalare et al. 2012). Transcript abundance of six classes of auxin-related genes – including those coding for putative receptors, signaling proteins, and transport proteins – were evaluated during fruit development and found to be

dynamically altered. One such class evaluated included auxin metabolism-related genes (*GH3*) involved in its conjugation. Transcript abundance of several of these genes declined during cell expansion, suggesting a decrease in IAA conjugation during this period allowing for a larger pool of free and active auxin (Devoghalare et al. 2012). A key aspect of that study was the identification of several fruit size-related QTLs using two mapping populations. One of the auxin signaling-related genes, *ARF106*, was co-localized to a QTL associated with fruit size. ARFs are transcription factors involved in mediating auxin responses through binding to auxin response elements within promoters of auxin-inducible genes. Modulation of gene expression by ARFs is repressed by their interaction with AUX/IAA proteins. Once auxin binds to the TIR1–AUX/IAA co-receptor complex, AUX/IAA is degraded and its repression of ARF activity is released. Subsequently, the ARFs dimerize and, by binding to promoter regions of auxin-inducible genes, regulate downstream transcription of those genes (Leyser 2018). *ARF106* transcript abundance was higher during cell production and expansion phases, consistent with a potential role in promoting growth through both processes (Devoghalare et al. 2012; Dash et al. 2013). These data implicate endogenous auxins in regulating fruit growth, but further studies are clearly needed to determine mechanisms mediating this response. For example, identification of ARF targets may provide mechanistic explanations for the roles of auxins in regulating apple fruit growth.

Cytokinins are known to promote cell production in a range of plant systems by potentially altering cell cycle progression (Schaller et al. 2014). *CYCD3* transcript abundance has in fact been demonstrated to be affected by cytokinin availability (Riou-Khamlichi et al. 2000). Hence, cytokinins may also regulate cell production during early fruit growth (Srivastava and Handa 2005). The plant growth regulators (PGRs), 6-benzyl adenine (6-BA) and N-(2-chloro-4-pyridyl); N'-phenylurea (CPPU) have been studied in reference to crop load management and enhancement of fruit size in apple (Greene 2001; Stern et al. 2003, 2006). Applications of 6-BA can result in a reduction in fruit load which can indirectly allow for enhanced fruit growth of remaining fruit (Greene et al. 2016). However, a more direct effect of 6-BA on cell division within apple fruit has also been suggested (Wismer and Procter 1995). Stern et al. (2003, 2006) reported an increase in fruit size by early applications of 6-BA and CPPU at lower rates than those used for crop load reduction. The molecular components facilitating such an increase in cell production in relation to these PGR applications remain to be identified. Analysis of changes in cell production, and

changes in the transcript abundance of genes associated with regulating cell production, would be an initial step in this direction. Owing to its importance in regulating cell production, it is essential to characterize spatial and temporal changes in cytokinin metabolism and changes in associated transcript abundance during the cell production phase of fruit growth. A related study in tomato indicated high cytokinin metabolism during the cell production phase of fruit growth and high cytokinin biosynthesis and signaling-related transcript accumulation during this period (Matsuo et al. 2012).

Endogenous abscisic acid (ABA) concentration in the fruit cortex appears to decline during fruit development, although this was studied during only a short period spanning four days (Giulia et al. 2013). Transcriptomics analysis of shaded, 6-BA treated, and NAA treated fruit in relation to fruit abscission revealed substantial alteration in ABA biosynthesis and signaling suggesting a role for this phytohormone in mediating this response (Botton et al. 2011; Zhu et al. 2011). In fact, exogenous ABA applications are able increase fruit drop (Giulia et al. 2013). However, whether endogenous ABA contributes to fruit abscission by altering growth characteristics directly remains to be evaluated.

Ethylene is a gaseous phytohormone with well-defined roles in fruit ripening and mature fruit drop in apple (Li et al. 2015). Ethylene concentration and emission are often affected by PGRs and other chemical agents functioning as chemical thinners in apple (Zhu et al. 2011), but the role of ethylene in regulating apple fruit growth remains unexplored. Endogenous ethylene emission decreases during the first few weeks of fruit development following petal fall (Walsh and Solomos 1987). Large variability in ethylene emission characteristics during this period were noted, but this was not necessarily correlated with abscission potential. Whether this is related to the growth potential of the fruit remains to be established. This is a potentially important aspect considering that emerging evidence in model plant systems suggests that ethylene signaling can regulate organ growth by altering cell production (via the cell cycle) and expansion (via cell wall loosening agents) in a context-specific manner (Dubois et al. 2018). For example, while ethylene inhibits young leaf growth in *Arabidopsis*, it can promote cell expansion in grape berries (Chervin et al. 2008; Dubois et al. 2018).

Jasmonates are another class of phytohormones with potential roles in regulating apple fruit growth and development. The concentrations of jasmonates, jasmonic acid (JA), and methyl-jasmonate (MeJA) were high during early fruit development coincident with the period of cell production, declined at later stages, and increased during ripening

(Kondo et al. 2000). A clear role for jasmonates in regulating fruit ripening in apple by alteration of ethylene biosynthesis has recently been demonstrated (Li et al. 2017). However, the role of jasmonates during early fruit growth and development remains unclear. Jasmonates are generally associated with negative regulation of cell cycle progression (Pauwels et al. 2008; Noir et al. 2013). The spatial and temporal context of elevated concentrations of jasmonates during early fruit growth needs to be better characterized to understand their roles in regulating fruit growth. Further, the molecular mechanisms associated with jasmonate metabolism and signaling need to be explored to better understand their roles in fruit growth and development.

I. A Note on the Measurement of Growth

Future research on understanding molecular regulation of fruit growth should aim to integrate spatial and temporal characteristics of growth with molecular mechanisms of their regulation. Hence, it is important that fruit growth and its contributing components are measured accurately and with high spatiotemporal resolution. Diverse methods of growth measurement have been used in apple and other fruits. One of the most effective methods to monitor growth is through measurement of increase in dry weight over time. However, this method is not widely used owing to the destructive nature of sampling. Growth is most frequently measured by monitoring changes in fruit diameter over time, a relatively simple and non-destructive method that can allow for repeated measurements of individual fruit. However, fruit diameter is a one-dimensional measure of a three-dimensional organ and is therefore limited in the information it provides, especially if growth is monitored over the entire fruit developmental period as this can involve substantial changes in fruit shape. An approach often used by multiple authors is to establish a relationship between fruit diameter and weight for the genotype under consideration (e.g., Warrington et al. 1999). Estimations of fruit weight from these data can be used to monitor growth. An alternative is the estimation of fruit volume using fruit diameter data. However, this can also be complicated by changes in fruit shape over development and the fact that the typical apple fruit often deviates from a simple sphere. In this context, measurement of fruit diameter and length can provide better estimates of volume, which can then be related to fruit weight. Advances in the application of imaging tools are necessary to non-destructively measure fruit growth parameters. One such approach is the use of three-dimensional imaging methods such as light detection and ranging (LiDAR) to obtain dense-point clouds

from which fruit morphology metrics can be extracted. A recent study demonstrated such an application of multiview stereovision to obtain fruit height, width, volume, and other metrics from mature strawberry fruit (He et al. 2017). Similar tools are also being employed in other types of fruit such as peach (D. Chavez, pers. commun.). Some limitations of current technology are low spatial resolution and issues with interference from alternative light sources making their application more challenging on younger fruit and under field conditions. However, further improvements in these imaging technologies and their decreasing costs should allow for their application to non-destructively monitor individual fruit growth under field conditions. Such developments are essential to improve high throughput fruit growth phenotypic characterization, an essential metric in studies of molecular regulation of fruit growth.

An inherent issue with all of the above methods of growth measurement is that they limit monitoring to growth across the entire organ. However, the apple fruit is not homogenous and is constituted by multiple tissue types. It is likely that growth across all of these tissues is not uniform (Tukey and Young 1942; Herremans et al. 2015; S. Jing and A. Malladi, unpubl.). Better characterization of fruit growth, therefore, requires spatial resolution in measurement and integration with overall organ growth. Emerging imaging technologies need to be employed to make progress in this area. Such tools have recently been employed in some initial efforts to obtain information on void space and vascular tissue development within the fruit. Three-dimensional imaging analysis through X-ray micro-tomography was used to determine and compare void spaces in apple and pear fruits and was effective in providing micro-meter resolution of some internal features of the fruit (Mendoza et al. 2007; Verboven et al. 2008). These studies indicated a higher void fraction within the cortex of mature apple fruit (~23%) than in pear fruit (~5%; Verboven et al. 2008). The estimate for apple is consistent with previous studies that used light microscopy-based estimates of void space (Goffinet et al. 1995). These tools were further employed to determine temporal and spatial growth and development of void fractions within the fruit (Herremans et al. 2015). Two key aspects of growth in void space emerged from these analyses. Firstly, there was a progressive increase in porosity within the fruit cortex from around 5 to around 22 WAFB (from about 10% to around 25% of the fruit volume). Secondly, spatial differences in the development of these voids were identified. The pith of the fruit displayed clearly lower porosity at maturity (<15%) and this was associated with a lack of change in porosity during later stages of fruit growth, unlike that observed in the

cortex. Also, the pore spaces were found to be more fragmented and smaller in the pith than in the cortex, from relatively early stages of fruit growth. This technology was also used to determine the development of vasculature within the fruit, especially at later stages of growth. Total vascular length increased during fruit development, although initially at a rate lower than that of fruit growth (Herremans et al. 2015). Further, much of the increase in vascular length during later stages was associated with increased branching, especially near the peripheral regions of the fruit. Tools such as X-ray micro-tomography have some limitations in terms of their application to younger fruit but are still very promising in providing deep insights into internal fruit growth and developmental characteristics. Such tools need to be integrated with molecular and physiological measurements to develop a better understanding of fruit growth (Cieslak et al. 2016). An example of such an application is the integration of fruit porosity and pore space connectivity measurements with the development of post-harvest physiological disorders (Herremans et al. 2013, 2014). A further advantage of such methods could be the ability to automate measurements of cell number and size which would greatly aid in generating key information on fruit growth. Substantial limitations of this approach currently are high cost, low throughput, and limited data processing tools, many of which could potentially become smaller barriers for their application in the future.

VI. CONCLUSIONS

Considerable progress has been made in recent years in identifying the molecular mechanisms and components regulating fruit growth in apple. Genes associated with floral organ development, phytohormone biosynthesis or signaling, and regulation of cell production and expansion have been some of the key components identified to date. A summary of some of that current understanding is presented in Figure 1.2. Future efforts should be aimed at gaining a deeper understanding of molecular mechanisms and at integration of knowledge to develop a comprehensive model(s) for regulation of fruit growth. Wider approaches – such as transcriptome, proteome, and methylome analyses – are being increasingly applied to study fruit growth and development (Janssen et al. 2008; Li et al. 2016; Daccord et al. 2017). Insights provided by such studies will be critical in the identification of molecular components involved in the regulation of fruit growth. An aspect of great importance here is spatial resolution. The fruit is not homogenous and is comprised of multiple tissue types, as described

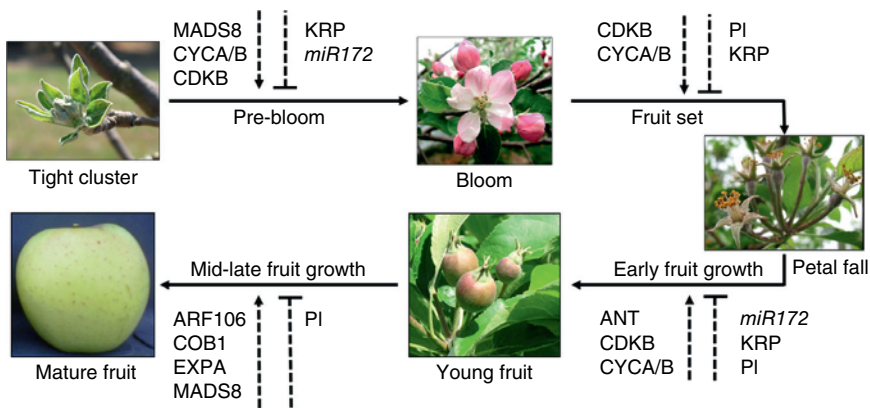


Figure 1.2 A summary of the currently known potential regulators of flower and fruit growth in apple. The various stages during which these regulators are functional are indicated. Arrows indicate positive regulation while the dashed line with a perpendicular line at the end indicates negative regulation.

earlier. Detailed analyses of growth with high spatiotemporal resolution is required to further our understanding of its regulation. Effective coupling of growth analysis, using high resolution and high throughput options, with the “omics” approaches indicated above will be required to make meaningful advances in our understanding of fruit growth regulation. In this context, comparison of multiple genotypes with diverse spatiotemporal growth patterns presents a useful approach. An additional key component is the integration of fruit metabolism with fruit growth. Metabolomics approaches are beginning to be applied to study fruit growth in apple (Li et al. 2012; Beshir et al. 2017). These need to be performed at higher spatiotemporal resolution and should be integrated with growth analyses to better understand features of metabolic regulation of fruit growth.

Several QTLs associated with fruit size have been identified in apple (Devoghalare et al. 2012; Potts et al. 2014). Identification of genes underlying these QTLs can lead to great insights into fruit growth regulation. Additionally, genetic approaches such as those involving large scale genome re-sequencing followed by association analyses (e.g., Duan et al. 2017) will also be critical in identifying loci and genes associated with fruit growth regulation. An approach that needs to be explored further is the use of mutants with altered fruit growth phenotypes. Molecular, genetic, and physiological characterization of such mutants can be an effective approach to identifying key molecular players and processes regulating fruit growth (e.g., Malladi and Hirst 2010).

A potential approach here may be the development of mutant (induced and natural) collection resources for apple, as has been done previously for other species such as *Arabidopsis* and tomato. This presents an opportunity for collaborative efforts of molecular geneticists, breeders, and fruit physiologists. Together, these efforts can help fill current gaps in knowledge of fruit growth regulation in apple and provide tools to optimize this critical trait.

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