FRUIT GROWTH IN APPLE: ANALYSIS OF THE MOLECULAR MECHANISMS AND EVALUATION OF THE ROLE OF THE *AINTEGUMENTA* (*ANT*) GENES

by

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(Under the Direction of Anish Malladi)

ABSTRACT

Fruit size in apple (*Malus* x *domestica*) is of great economic significance. A thorough comprehension of mechanisms that regulate fruit growth and development is essential to optimize fruit size. In this study, the factors affecting shade-induced and thinning-induced alteration in fruit growth were determined. The results demonstrate that shade-induced reduction in fruit growth and thinning-induced increase in fruit growth is facilitated by coordinated changes in the expression of carbohydrate metabolism-related genes, transcription factors associated with fruit growth, and key genes associated with cell production and expansion. The changes in the expression of these genes may regulate fruit growth by altering the key processes of cell production and expansion. *AINTEGUMENTA (ANT)*, an AP2 domain transcription factor, controls organ size in Arabidopsis by regulating the duration of cell production and is a candidate for fruit growth regulation in apple. Two genes homologous to the Arabidopsis *ANT*, *MdANT1* and *MdANT2*, were isolated from apple. The expression of these genes was analyzed during fruit development, in response to factors affecting fruit size, and across genotypes. The results demonstrate that the expression of these *ANTs* is closely associated with cell production during fruit development. Additionally, wild-type Arabidopsis plants were transformed with

Act7::*MdANT1/2* and *Act2*::*GFP:MdANT1/2* constructs. The transgenic plants obtained can be used for functional characterization of *MdANT1* and *MdANT2*, to determine their roles in regulating organ size in plants.

INDEX WORDS: Cell division, cell expansion, fruit development, fruit size, organ growth

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DEDICATION

I dedicate this thesis to my family for believing in me and for their tremendous support and encouragement.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Regulation of fruit growth and size in apple

Apple (*Malus* × *domestica* Borkh.), the most widely cultivated temperate tree fruit, belongs to the family Rosaceae. Apple fruits are easy to ship, resistant to disease, and popular for their flavor which can vary depending on the variety. Fruit size is an important quality-related trait and is a key marketing parameter in many fruit including apple. Consumers as well as wholesalers prefer large-sized apple fruit [1]. In apple, fruit size is not only of economic significance but also an important factor determining its keeping quality during storage [2].

Apple is classified as a pome, a false fruit consisting of two distinct parts: an expanded ovary corresponding to the "core" which is homologous to the tomato fruit; and the cortex or edible portion of the fruit [3]. The fleshy part or the cortex is derived either from the receptacle or the floral tube [4]. Fruit development in apple has been divided into three phases [5]. The first phase is characterized by pollination and fertilization followed by the phase of fruit growth, leading finally to the fruit maturation phase. Apple fruit growth follows a sigmoid curve where fruit growth begins after bloom proceeding slowly followed by a gradual increase in growth and finally a gradual decline in growth until harvest. Fruit growth in apple is mainly mediated by cell division and cell expansion [6-11]. Tetley (1930, 1931) and McArthur and Wetmore (1939, 1941) studied the cytology and development of different apple varieties and established that cell

production ceases within a few weeks after fruit set. Fruit growth during the rest of fruit development is mostly facilitated by cell expansion.

The rate of cell proliferation and expansion, which is coordinated by cellular mechanisms, is critically important in determining final fruit size. Bain and Robertson (1951) reported that variation in size of fruits at maturity within a given variety is mostly due to variation in cell size. However, Denne (1960) and Harada *et al.,* (2005) compared five apple cultivars and concluded that fruit size is regulated by a combination of greater cell production capacity and an enhanced degree of cell enlargement. Therefore, despite the fact that cell expansion may account for the greatest increase in fruit volume, cell production is an essential factor affecting fruit growth as it determines final cell number within the fruit. Final fruit size is, in part, dependent on a defined number of cell divisions that occur during early development.

Since, larger fruit have a higher per fruit economic value than smaller fruit; growers spend significant efforts to optimize this trait using horticultural practices such as pruning and thinning. Final fruit size in apple is determined by several factors like the environment, cultural practices and the intrinsic genetics. Much research has been performed in apple to understand the mechanisms that control fruit size, and many of these studies have described the effects of environmental and cultural factors on fruit size [13-15]. However, in spite of its commercial importance, little is known about the genetic and molecular mechanisms regulating fruit size in apple. A thorough comprehension of these mechanisms that regulate fruit growth and development is essential to optimize fruit size in apple.

Effects of Environmental Factors on Fruit Growth

The environment within which a fruit grows, tempers the genetic potential and determines the final fruit size attained. Therefore, the environmental conditions during the period

of apple fruit development play a very important role in determining the fruit size. Many studies have reported positive correlation between temperatures immediately following bloom in the field and fruit size at harvest [16-18]. Ford (1979) exposed small trees to two contrasting temperature systems for 3 weeks post-bloom and demonstrated a strong impact of temperature on mean fruit diameter. Warrington et al., (1999), performed a controlled environment study of the impacts of temperature on five different apple cultivars and found that post-bloom temperature markedly affected fruit expansion, final fruit weight and fruit maturation. This study showed that mean fruit weight from warm post-bloom treatments was up to four times greater at harvest maturity than that from cool temperature treatments.

In addition to temperature, light also influences growth and development of many fruits including apple. Several studies have shown that shading during early apple fruit development has a detrimental effect on fruit growth and induces fruit abscission [20,21]. During early fruit development, active sinks such as the growing shoots and fruit compete for limited carbohydrate and nutrient resources [22]. Low light levels during this period can reduce fruit growth and increase shoot extension [21,22]. Shading of isolated branches during early fruit development resulted in reduced fruit growth, but allowed for continued shoot growth [23].

Effect of Cultural Practices and Crop Load on Fruit Growth

In addition to environmental factors fruit size can also be influenced by various horticultural practices such as training, pruning and fruit thinning. High-density plantings and training the tree to the required architecture can increase light interception. However, Hampson et al., (2002) have shown that different training systems have no impact on the final fruit size. Similar to canopy training, trees are pruned to ensure adequate light penetration into the tree canopy which may also affect final fruit size.

In apple, a significant amount of research has been focused on understanding the impact of crop load on final fruit size since there is a substantial incentive for growers to optimize fruit size. Crop load is defined as the number of fruit produced per tree or branch unit. Most apple trees tend to bear more fruit than they can support to maturity. While such over-cropping may help ensure reproductive success, it can lead to branch damage, low quality fruit and drastic reductions in cropping in the following year. Consequently, crop load is a key cultural component affecting final fruit quality. Hence, information on crop manipulation and effects of harvest time and fruit maturity are of particular importance to growers in enhancing the proportion of the crop achieving desired qualities. Reducing crop load or thinning is very important for commercial apple production. It has been shown that crop reduction at the appropriate time can lead to optimization of fruit size. In apple and other fruit trees, thinning at the appropriate time allows the remaining fruits to attain their maximum potential size [25-27]. Goffinet et al., (1995) compared 'Empire' apple fruit size in manually thinned and un-thinned trees and found that thinning allowed cell division to progress under less competition after bloom, resulting in an increase in final fruit size. Growers use several techniques, like manual, mechanical and chemical thinning, to reduce crop load. However, application of chemical thinning agents for crop load reduction does not always result in an increase in fruit size. For example, in a study by Marini et al., (2004), ethephon applications did not increase fruit size in apple although there was a significant reduction in crop load. Other chemical thinners such as Benzyl Adenine (BA) have been shown to increase fruit size by promoting cell division [28]. These chemical agents are most effective if applied within a short period after bloom. Hence, chemical thinning is the most commonly used technique since application of certain chemical agents during early fruit development can help in attaining optimal fruit size. Shading trees

during early stages of fruit development has been shown to increase fruit size by inducing fruit abscission and thereby reducing crop load [20,21,29]. However, fruit abscission due to shading has been shown to be preceded by a drastic reduction in the fruit growth rate [30]. Little progress has been made towards understanding the mechanisms causing a reduction in fruit growth due to shading, thus making it difficult to apply this technique commercially.

Effect of Carbohydrate Availability and Carbohydrate Metabolism on Fruit Growth

Apple and other Rosaceae tree fruits synthesize sorbitol and sucrose in source leaves, and both sorbitol and sucrose are translocated to fruit. Sorbitol accounts for about 60–70% of the photosynthates translocated to fruit [34]. Apple fruit growth and development may be mediated by source-sink relationships between photosynthetic source leaves and vegetative and reproductive sinks [31]. Fruit set and development is dependent on both carbohydrate availability as well as carbohydrate metabolism [32-35]. Fruit development in the first few weeks after full bloom is essentially supported by carbohydrate supply from spur leaves, whereas actively growing extension shoots utilize endogenously synthesized carbohydrates for their own development [36,31]. The supply of carbon to individual fruitlets may be limiting during this stage due to competition from other fruitlets and other sinks such as the rapidly growing shoots [37]. High crop density during early fruit growth results in fruit demand for assimilates that exceeds carbohydrate availability leading to fewer fruit cells and reduced final fruit size at harvest and/or increased fruit abscission [37,21]. When assimilate is limited, fruit competition results in fruit drop in apple [38]. The mechanism of dominance among fruits may be regulated by sink strength of developing fruit which in turn may depends upon cell number and cell size [39]. Reducing crop load in tomato has been shown to increase carbohydrate availability which resulted in higher fruit growth rate due to higher cell number [40]. Additionally, increase in

carbohydrate availability in tomato also resulted in an increase in the expression of genes positively associated with cell production. Increase in cell number may lead to higher sink activity in developing fruit. Early fruit development in apple, a period of higher cell production, has been shown to be associated with low level of sugar accumulation and higher activity of enzymes associated with carbohydrate metabolism [41]. However, the molecular mechanism regulating fruit growth in response to change in carbohydrate availability due to thinning or shading is not well understood in apple.

Molecular Mechanisms Regulating Fruit Growth

Apparent differences in fruit size among different apple genotypes clearly demonstrate that fruit size is greatly determined by its genetic potential. Liebhard et al., (2003) reported that at least eight loci were associated with variability in fruit size. Recently, multiple quantitative trait loci (QTLs) were observed in two mapping populations phenotyped for fruit size [43]. One QTL mapped to a region containing the *Auxin Response Factor 106* (*ARF106*) which is expressed during the cell division and expansion phases. As final fruit size is affected by changes in cell production or expansion, genes regulating these processes may be potential candidates for controlling final fruit size. Analysis of the expression of 59 cell cycle genes during multiple stages of fruit growth and development in apple resulted in the identification of 14 and five genes, positively and negatively associated respectively, with cell production and fruit growth in apple [44]. Besides these reports, very few studies have been performed to understand the molecular mechanisms affecting fruit size regulation in apple.

Recently, several studies have characterized molecular events associated with growth through their influence on cell division or cell expansion, primarily during the development of leaves, roots, and shoot apical meristems. A majority of such studies on developmental and

molecular mechanisms regulating fruit size have been performed in tomato. A major quantitative trait locus *FW2.2* accounts for as much as 30% of the difference in fruit size between wild and cultivated tomatoes by modulating cell production during early tomato fruit growth [45,46]. *FW2.2* has been shown to interact with a putative cell cycle regulator [46]. In addition, *FASCIATED*, a YABBY-like transcription factor also affects fruit size by altering carpel number during flower and fruit development [47]. Some of the predicted genes for *FW3.2*, a major locus associated with fruit mass in tomato, were shown to be homologous to genes associated with cell production, such as *KLUH*, and cell expansion, such as *COBRA-LIKE* genes (*COBL2* and *COBL4*) [48]. *KLUH*, a cytochrome P450 gene (*CYP78A5*), has been shown to control organ size in Arabidopsis through promoting cell production [49,50]. *COBRA-LIKE* genes encode glycosylphosphatidylinositol (GPI)-anchored proteins that are thought to be involved in directional cell expansion in Arabidopsis [51,52]. In tomato, fruit development and final cell size has been shown to be associated with an increase in the DNA content of cells through endoreduplication [53-55]. *WEE* and the anaphase promoting complex/cyclosome (APC/C) activator, *CCS52* (*Cell Cycle Switch 52*), have been shown to alter tomato fruit growth through their effects on endoreduplication and cell expansion [56-58]. *KIP RELATED PROTEINS* (*KRPs*) that are key facilitators of exit from mitotic cell production [59], have been also shown to be involved in promoting entry into endoreduplication during tomato fruit development [60].

Regulation of organ size in plants

Like fruit growth, organ growth can be divided into an initial phase of cell production followed by the phase where growth is mainly mediated by cell expansion. Several studies have revealed the role of cell number in the determination of organ size in plants [61-63]. Various genetic

factors regulate the proliferation capacity of an organ or determine the timing at which cells exit from proliferative growth into cell differentiation and expansion. Many cell cycle factors regulate the final organ size through their regulation of cell proliferation [63,64]. Overexpression of the mitotic cyclin gene *CYC1A* enhances cell proliferation in the roots and leads to the development of a greatly enlarged root system [65].

Some of the genes regulating cell proliferation are transcriptional regulators that either positively or negatively regulate organ growth. Members of the transcription factor family, *GROWTH REGULATING FACTORs* (*GRFs*), regulate cell production and thereby control organ size [66,67]. In addition, these transcriptional regulators are differentially regulated by environmental factors and photosynthesis [68,69]. *ARGOS* is an auxin-induced transcription factor that regulates cell proliferation and lateral organ growth [70]. Genetic interaction studies with genes involved in regulating lateral organ size in Arabidopsis indicate a link between *ARGOS* and another transcription factor *AINTEGUMENTA (ANT)* [70]. *ANT*, an AP2 (APETALA2) domain transcription factor has been shown to be a key regulator of organ growth and final size in *Arabidopsis* [71,62]. Besides ANT, various other factors, such as C2H2 zinc-finger proteins, JAGGED (JAG) and its homologue NUBBIN (NUB), promote organ growth by regulating cell proliferation [72].

***Aintegumenta* (*ANT*)**

ANT and *AINTEGUMENTA LIKE* (*AIL*) genes encode transcription factors of the AP2/ethylene response element binding protein (EREBP) family that make up the largest transcription factor family in *Arabidopsis* [73]. *Arabidopsis* has 146 genes belonging to the AP2/ERF family whereas apple genome reportedly consists of 58 transcription factors belonging

to this family [74]. These proteins contain either one (ERF, DREB, RAV subfamily and others) or two (AP2 subfamily) copies of a ~70 amino acid domain termed as the AP2 repeat because of its initial description in the floral homeotic protein, APETALA2 (AP2) [75]. AP2/EREBP family members are involved in flower development, hormone signal transduction and cellular differentiation, and various other aspects of plant growth and development including responses to biotic and abiotic stresses. *ANT* and *AIL* genes belong to the AP2 subfamily which consist of 15 members in *Arabidopsis* [76]. All members of AP2 subfamily share homology within both the AP2 domains and the connecting linker region. The *AIL* group in *Arabidopsis* consists of seven genes that are mostly similar to the *ANT* gene. The *AIL* group members *AtBBM/AIL2*, *PLETHORA1* (*PLT1)/AIL3* and *PLETHORA2* (*PLT2*)*/AIL4*, are required for specification and maintenance of stem cells within the root meristem [77]. Ectopic expression of two other *AIL* members, i.e., *AIL5* and *PLETHORA3* (*PLT3*)/*AIL6* results in larger floral organ phenotype [76,78,79].

ANT promotes growth within floral meristems and developing organ primordia. It is required for integument initiation in ovules and plays important roles in gynoecium and petal development. Loss-of-function mutations of *ANT* in *Arabidopsis* exhibit reduced number and size of floral organs, and decreased leaf *size* [80,81,62]. Ectopic expression of *ANT* under the control of the constitutive CaMV:*35S* promoter increases the size of shoot organs, such as leaves and stems, as well as of floral organs and siliques [71,62]. *ANT* regulates mature organ size by enhancing cell proliferation and thereby increasing final cell number [62]. Further examination of cell number and cell size in developing petals and leaves in the above plants revealed that *ANT* controls the duration of organ growth and cell production during organ development [62]. One of the target genes for *ANT* is thought to be *CYCD3;1*, a cell cycle gene, which may be

involved in transducing growth signals leading to fruit growth by cell division [62,82]. In addition, *Arabidopsis ANT* expression in transgenic tobacco plants resulted in increased organ size [62]. Furthermore, ectopic expression of *BnANT*, an *ANT* ortholog from *B. napus*, resulted in organ enlargement in *Arabidopsis* [62]. The data support the existence of a conserved *ANT* function in organ-size control in different plant species.

Significance and hypothesis

Fruit growth and development, and the molecular mechanisms involved in its regulation have been typically studied in model fruit such as tomato. Little progress has been made in identifying genes regulating fruit size in other fruit crops. Although fruit development in apple shares certain common features with that in tomato, there are considerable differences in their fruit morphology and growth mechanisms. The unique fruit morphology of apple makes it an interesting system to investigate processes involved in fruit growth and development. In tomato, the fruit is primarily derived from the ovary wall [83]. This contrasts with the apple fruit, which as mentioned earlier, is a pome, a false fleshy fruit where the majority of the fleshy tissue is derived from either the receptacle or the floral-tube tissue surrounding the ovary [3]. Post-mitotic cell expansion in fruit such as tomato is associated with an increase in the DNA content of cells through endoreduplication [53]. However, in apple post-mitotic cell expansion usually occurs in the absence of an increase in the ploidy levels of the cells [84]. Hence, it is likely that additional mechanisms and/or genes are involved in controlling fruit size in apple. Therefore, studies in fruit crops like apple may greatly enhance our understanding of the mechanism of fruit development. Until recently, the lack of appropriate genomic tools has hampered advanced molecular studies in apple, but extensive apple EST resources are currently available in public databases (NCBI and GDR). Additionally, the apple genome has recently been sequenced [85].

The above resources make molecular investigations of regulatory genes associated with apple fruit growth feasible.

The key to understanding fruit growth is to identify the genetic program regulating the process of fruit development and their response to various environmental and cultural factors. Therefore, the knowledge of molecular mechanisms affecting fruit growth in response to factors such as shading and thinning is an essential component to understand fruit size regulation in apple. It is hypothesized that shading and thinning alter carbohydrate availability and subsequently alter fruit growth by mediating changes in cell production and/or cell expansion.

Since *ANT* and/or the *AIL* genes are involved in vegetative and floral organ size regulation it may be a potential candidate involved in fruit size regulation. Hence, it is hypothesized that apple *ANT* and/or the *AIL* genes homolog(s) contribute to the regulation of fruit growth in apple by regulating cell production during fruit development. Detailed characterization of their function and their target genes may allow insights into not only fruit but also overall organ size regulation in plants.

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CHAPTER 2

SEVERE SHADING REDUCES EARLY FRUIT GROWTH IN APPLE BY DECREASING CELL PRODUCTION AND EXPANSION

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Abstract

Shading during early fruit development reduces fruit growth and initiates fruit abscission in apple (*Malus* × *domestica*). The mechanisms mediating the decline in fruit growth in response to shading are not well understood. In this study, the effects of shading during early fruit development on cell production and expansion were investigated. Additionally, the effects of shading on the expression of genes associated with carbohydrate metabolism, fruit growth, and cell production and expansion were investigated to develop a better understanding of the molecular mechanisms, and to identify genes, that mediate the reduction in fruit growth. Shading of isolated branches or entire trees around 15-18 days after full bloom resulted in a sharp decline in fruit growth by 3 days after treatment. Reduction in fruit growth was consistently mediated by a decline in cell production within 3 days after treatment. Reduced fruit growth was also associated with lower cell size by 3-7 days after shading in two different years. These data indicate that the reduction in fruit growth as a result of shading is mediated by a reduction in cell production and expansion. The expression of two *Sorbitol dehydrogenase* (*SDH*) genes, *MdSDH1* and *MdSDH2,* was higher in the shaded fruit by up to 10-fold, suggesting an increase in SDH activity to meet the immediate respiratory demands of the developing fruit. The *Auxin response factor* (*ARF*), *MdARF106*, displayed ≈three-fold higher expression in the shaded fruit, suggesting its involvement in regulating mechanisms that mediate the reduction in fruit growth. Two A2-type *Cyclins*, *MdCYCA2;2* and *MdCYCA2;3,* which are positively associated with cell production, displayed lower expression in the shaded fruit by up to 4.6-fold. Conversely, *MdKRP4* and *MdKRP5,* cell cycle genes negatively associated with cell production, displayed 3.9- and 5.3-fold higher expression in the shaded fruit, respectively. Additionally, two genes associated with cell expansion, *MdCOB1* (*Cobra1*) and *MdEXPA10;1* (*Expansin*), displayed

lower expression in the shaded fruit. Together, these data indicate that shading results in coordinated changes in the expression of carbohydrate metabolism-related genes, key transcription factors related to fruit growth, and genes associated with cell production and expansion. These changes may subsequently decrease the progression of the primary processes that mediate fruit growth.

Introduction

Shading during early apple (*Malus* × *domestica*) fruit development decreases fruit growth and induces fruit abscission, and has been used to understand processes that affect thinning [1-4]. During early fruit development, active sinks such as the growing shoots and fruit compete for limited carbohydrate and nutrient resources [5]. Shading during this period rapidly reduces canopy photosynthesis resulting in the decreased availability of assimilates, and further enhances the competition among these sinks [3-8]. In such a context, the available carbohydrate and nutrient resources are channeled primarily in favor of shoot growth while fruit growth is reduced. For example, shading of isolated branches during early fruit development results in reduced fruit growth, but allows for continued shoot growth [9]. The reduction in fruit growth and a subsequent decrease in sink strength may lead to the induction of fruit abscission mechanisms [10-12]. A decrease in fruit relative growth rate (RGR) was apparent within 2 d after shading [6,8]. Shade-induced fruit abscission occurred around 5 to 10 d after shading and peaked around 15 d [12-14]. Hence, reduction in fruit growth is an earlier response to shading. Although progress has been made in understanding the mechanisms that mediate shade-induced fruit abscission [11,12], mechanisms regulating the shade-induced reduction in fruit growth are not well understood.

Reduction in fruit growth due to shading may be mediated by a decrease in the extent of cell production and/or cell expansion, the primary mechanisms that mediate fruit growth. The contribution of these processes to shade-induced reduction in fruit growth has not yet been determined. Shading during early fruit development, a period of intensive cell production, is particularly effective in reducing fruit growth and inducing abscission [3,7]. Further, cell production has a high requirement for resources and this phase of fruit development displays the

highest amount of respiration on a per unit fruit weight basis [15]. Hence, cell production may be particularly sensitive to lower assimilate availability due to shading. However, considerable cell expansion also occurs during early fruit growth [16]. Hence, it is also likely that shading may reduce fruit growth by altering the extent of early cell expansion.

The molecular mechanisms leading to a reduction in fruit growth due to shading are not well understood. Shade-induced reduction in fruit growth may be mediated by genes regulating carbohydrate metabolism, transcription factors associated with fruit growth, and final effectors of cell production and expansion. Changes in the expression of genes associated with sorbitol metabolism, such as *Sorbitol dehydrogenase* (*SDH*), in response to shading may enable the developing fruit to respond to the decreased availability of assimilates. For example, *SDH* expression in the fruit decreased in response to shading [12]. Transcription factors such as the *Auxin response factor*, *MdARF106*, a gene putatively associated with the regulation of fruit growth [17], may coordinate changes in gene expression to facilitate a reduction in fruit growth in response to shading. Core cell cycle genes such as the B-type *Cyclin dependent kinases* (*CDKs*), and A- and B-type *Cyclins* (*CYCs*) are positively associated, while others such as the *Kip related proteins*, *MdKRP4* and *MdKRP5*, are negatively associated with cell production during different stages of apple fruit growth [16]. *Expansins* (*EXPAs*) and *Cobra* (*COB*) genes are associated with cell growth and orientation of cell expansion [18-20]. The above genes may function as the downstream effectors of cell production and expansion and may aid in coordinating changes in these processes in response to shading. Analyses of the changes in the expression of the above genes due to shading may allow for a better understanding of the molecular mechanisms that facilitate the reduction in fruit growth.

It was hypothesized that a decrease in cell production and expansion contribute to the reduction in fruit growth in response to shading, and that genes associated with these processes mediate the shade-induced reduction in fruit growth. To address these hypotheses, the effects of shading on fruit size, cell number and cell area were analyzed at different stages of early fruit growth, and changes in the expression of key genes associated with the regulation of fruit growth and particularly, cell production and expansion, were investigated.

Materials and methods

Plant material

Mature trees of 'Golden Delicious Smoothee' on Malling 7a (M.7a) rootstocks were used in this study. Trees were grown and maintained at the Mountain Research and Education Center, University of Georgia, Blairsville, GA. The trees were maintained according to commercial apple production practices. Chemical or hand thinning were not performed in either of the two years of this study.

Shading treatment

In 2009, eight uniform trees were selected and assigned randomly either to the Shaded treatment or used as control trees (n = 4). One major scaffold branch per tree, on the west side, was selected for these treatments. At 15 d after full bloom (DAFB; ≈11 mm fruit diameter) the selected branch within the Shaded treatment was covered with black polypropylene, 80% shade material using a wire support framework built around the branch. Previous studies have reported significant fruit growth reduction in response to similar levels of shading [9,11]. The branches were shaded throughout the duration of the experiment (15-25 DAFB). The base of the branch, close to the tree trunk was girdled to isolate the branch from the rest of the tree [9]. Girdling has

been found to have little direct impact on fruit growth for at least 10 d [9]. The branches on the control trees were also girdled but were left uncovered. Temperature within the canopy of the shaded and control branches was recorded using sensors placed inside radiation shields. The average daily temperature during the period of the experiment was 18.9 ± 0.6 °C and 18.6 ± 0.7 °C within the branches in the Shaded and the Control treatments, respectively. The light levels within the canopy of the Shaded and Control treatments were measured during early afternoon at 0, 3, 7 and 10 d after treatment, using a 1-m line quantum sensor (LI-191, LI-COR, Lincoln, NE). During the experimental period, the light levels within the canopy of the control branches were around 585.1 μmol· m^{-2}· s^{-1}, while the canopy within the shaded branches received around 59 μmol· m^{-2}· s^{-1}, indicating ≈90% shading. Ten fruit on each experimental unit (branch) were tagged and fruit diameter was recorded over the duration of the experiment. Fruit RGR (mm· mm^{-1}· d^{-1}) was calculated from the fruit diameter data using the formula, $(\ln[D_2]-\ln[D_1])/T_2-T_1$, where D_2 and D_1 are fruit diameter at time points, T_2 and T_1. Five fruit from each replicate were sampled at 0, 3, 7 and 10 d after shading. All of the samples were collected during the afternoon period (around 1400 HR). The samples were fixed in CRAF III fixative (3% chromic acid, 20% acetic acid, and 10% formalin) for histology or immediately frozen in liquid N_2 for gene expression analyses.

In 2010, four trees each were assigned randomly to either the Shaded or the Control treatments (n = 4). Entire trees within the Shaded treatment were individually covered with black polypropylene, 80% shade material using a metal framework support constructed around the trees at 18 DAFB (≈11 mm fruit diameter). The trees were shaded throughout the duration of the experiment (18-28 DAFB). Temperature sensors housed within a radiation shield and light sensors (Apogee SQ100 quantum sensor, Apogee Instruments, Logan, UT) were placed at 1 m

above the ground level close to the tree canopy. The average daily temperature during the duration of the experiment was 18.1 ± 1.3 °C and 17.6 ± 1.3 °C in the Shaded and the Control treatments, respectively. The average daily light integrals (DLI) over the duration of the experiment (18 to 28 DAFB) were 25.9 ± 3.3 mol· m^{-2}· d^{-1} (maximum and minimum of 38 and 13 mol· m^{-2}· d^{-1}, respectively) and 3.8 ± 1.7 mol· m^{-2}· d^{-1} within the canopies of the control and shaded trees, respectively. Severe weather at 6 d after the initiation of the experiment resulted in partial opening of the shade material over three of the replicates, but was fixed on the same day. Analysis of the DLI data did not indicate an increase in light levels within the Shaded treatment during this period. The ambient light levels were low from 6 to 10 d after treatment. Twenty fruit per tree were tagged at 0 d after treatment and were used to determine fruit diameter. Fruit RGR (mm· mm^{-1}· d^{-1}) was calculated as described above. Fruit were sampled from the trees at 0, 1, 2, 3, 6 and 10 d after treatment and fixed in CRAF III fixative for histology. All of the samples were collected during the afternoon period (around 1400 HR). One branch (average of 42 fruit per branch) on each tree was tagged at 1 d after treatment and the number of fruit on it was monitored during the duration of the experiment (18-28 DAFB).

Cell number and cell area measurement

The number of cell layers in the cortex and the cortex cell area were measured as described previously in Malladi and Johnson (2011). Briefly, samples fixed in CRAF III were sectioned using a vibratome (Micro-cut H1200, Bio-Rad, Hercules, CA). The sections were stained in toluidine blue and images were captured using a microscope (BX51, DP70, Olympus, Center Valley, PA). The number of cell layers between the petal vascular trace and the epidermis were counted manually to obtain the cell number data. The number of cells was determined at three

locations within the fruit cortex, between the petal vascular trace and the epidermis. The average cell area from the three locations was used to determine the cortex cell area.

RNA extraction and cDNA synthesis

RNA was extracted from fruit collected at 0 and 3 d after the initiation of the treatment in 2009. The extraction was performed as reported previously in Malladi and Hirst (2010). One microgram of total RNA was used for cDNA synthesis using oligo dT primers and ImProm II reverse transcriptase (Promega, Madison, WI) after treatment with DNase (Promega). Synthesis of cDNA was performed in a total volume of 20 µL which was subsequently diluted six-fold and stored at -20 °C until further analysis.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

All quantitative RT-PCR analyses were performed using a Light Cycler 480 (Roche Applied Sciences, Indianapolis, IN). One microliter of the diluted cDNA was used in a final reaction volume of 12 µL. The Light Cycler 480 SYBR Green I Master mix (Roche Applied Sciences) was used in all gene expression analyses. The reaction conditions involved the following cycles: 95 °C for 10 min and 40 cycles of 95 °C for 30 s, 60 °C for 1 min. Melt curve analyses performed at the end of the PCR cycles indicated a distinct single peak for all of the amplicons analyzed. Controls without a template and without the reverse transcriptase were used. Rarely, some negative controls displayed low amplification but this occurred only during the late stages of PCR cycling. The genes analyzed and the gene-specific primer sequences used in this study are indicated in Table 2.1. The primer efficiency was determined using LinRegPCR (Ruijter et al., 2009) using converted fluorescence data from the Light Cycler 480, and ranged from 1.58 to 1.91. All gene expression was normalized to the expression of three reference genes, *MdACTIN* (*Actin*), *MdGAPDH* (*Glyceraldehyde 3-phosphate dehydrogenase*) and *MdCACS* (*Clathrin*

adaptor complexes medium subunit family protein). Gene expression was calculated using the Cq (cycle number where the fluorescence threshold was crossed) values with correction for amplification efficiency (Pfaffl, 2001). The relative quantities ($1/E^{Cq}$, where E is the amplification efficiency) were normalized using the geometric mean of the relative quantities of the reference genes. The expression of a given gene relative to its expression in the control fruit at 0 d after treatment is reported here. The standard error of the expression was calculated as described in Rieu and Powers (2009).

Statistical analyses

All statistical analyses and graph preparation were performed with JMP software (version 9; SAS Institute, Cary NC) and Sigmaplot 11 (Systat Software, San Jose, CA). The main effects of Shading and Time after treatment, and their interaction effects were tested using repeated measures. Wherever the interactions were significant between the main factors, the simple effects were further analyzed using test of effect slices. For the gene expression data, the normalized relative quantities were transformed ($\log_2$) prior to statistical analyses. For the gene expression analysis, the genes of interest were ones that displayed significant interaction effects between the main factors, as in these cases the effect of Shading depended on the Time after treatment.

Results

Shading reduces fruit growth by decreasing cell production and expansion

In 2009, fruit within the Control treatment increased in size by 8.3 mm between 15 and 25 DAFB, but little change in fruit diameter (2.2 mm) was observed in the shaded fruit during this period (Fig. 2.1). Fruit diameter was significantly smaller in the shaded fruit from 3 d after treatment. Similar to data from the 2009 study, shaded fruit in the 2010 study displayed only a

minor increase in size (2.4 mm), while the control fruit displayed an increase in fruit diameter by 8.7 mm between 18 and 28 DAFB (Fig. 2.1). A significantly lower diameter was evident in shaded fruit from 3 d after treatment. Therefore, in both years of the study shading resulted in a reduction in fruit growth within 3 d after treatment. In 2009, the fruit RGR ($mm \cdot mm^{-1} \cdot d^{-1}$) was significantly different at 3 d after treatment (control fruit: 0.05 ± 0.01 $mm \cdot mm^{-1} \cdot d^{-1}$ and shaded fruit: 0.02 ± 0.01 $mm \cdot mm^{-1} \cdot d^{-1}$; $P = 0.018$). In 2010, fruit RGR was significantly different between control and shaded fruit at 2 d after treatment (control fruit: 0.06 ± 0.01 $mm \cdot mm^{-1} \cdot d^{-1}$ and shaded fruit: 0.02 ± 0.01 $mm \cdot mm^{-1} \cdot d^{-1}$; $P = 0.005$). During the duration of the experiment (18-28 DAFB), shading did not have a significant effect on the extent of fruit drop. At 10 d after treatment, around 54% of the fruit in the Shaded treatment abscised while 38% of the fruit abscised in the Control treatment ($P = 0.06$, Student's *t*-test).

In 2009, the number of cell layers within the cortex increased by 1.47-fold over the duration of the experiment in the control fruit, but only by 1.32-fold in the shaded fruit (Fig. 2.1). The number of cell layers within the fruit cortex of shaded fruit was significantly lower than that in the control fruit from 3 d after treatment. By the end of the experiment, cell number in the shaded fruit was only 82% of that in the control fruit. A similar pattern of change in cell number was observed in the 2010 study and the number of cell layers was significantly lower in the shaded fruit from 3 d after treatment (Fig. 2.1). Over the duration of the experiment, the cortex cell area increased by 1.72-fold and 1.48-fold in the control fruit, in 2009 and 2010, respectively. In the shaded fruit, cortex cell area increased only by 1.17-fold and 1.13-fold in 2009 and 2010, respectively. In 2009, significant differences in cell area between the control and shaded fruit were evident at 7 and 10 d after treatment (Fig. 2.1). In 2010, lower fruit cortex cell area in the shaded fruit was evident from 3 d after treatment. Together, these data clearly indicate that the

shade-induced decrease in fruit growth was associated with a reduction in cell production and expansion in the fruit cortex.

Altered expression of carbohydrate metabolism- and fruit growth-related genes due to shading

The expression of two *Sorbitol dehydrogenase* genes, *MdSDH1* and *MdSDH2*, was higher in the shaded fruit at 3 d after shading by around 10-fold and two-fold, respectively (Fig. 2.2). The expression of two transcription factors (*ARFs*) putatively associated with fruit growth was investigated. The interaction effect between the factors, Shading and Time after treatment, was not significant for *MdARF6* (Fig. 2.3). *MdARF106* displayed higher expression in the shaded fruit at 3 d after treatment by 2.9-fold (Fig. 2.3).

Altered expression of cell production- and expansion-related genes due to shading

The expression of ten genes positively associated with cell production and two genes negatively associated with cell production was investigated (Fig. 2.4). Many of the positive regulators of cell production including four B-type *Cyclin dependent kinase* (*MdCDKB*) genes, *MdCYCA2;1* and two B1-type *Cyclins* (*MdCYCB1;1* and *MdCYCB1;2*) were affected by the main factors, Shading and Time after treatment. Significant interaction effects of these factors on the expression of the above genes were not observed. At 0 d after treatment, the expression of the above genes in the shaded fruit was never lower than 1.9-fold of that in the control fruit and were not significantly different (Pairwise comparisons using Tukey's HSD) except for *MdCYCB1;1*. The expression of the B2-type cyclin, *MdCYCB2;2* was affected only by the factor, Time after treatment. The expression of two A2-type cyclins, *MdCYCA2;2* and *MdCYCA2;3*, and two *KRP* genes, *MdKRP4* and *MdKRP5*, displayed significant interaction effects between Shading and Time after treatment. The expression of *MdCYCA2;2* and *MdCYCA2;3* was lower in the shaded fruit by 4.6-fold and 3.6-fold, respectively, at 3 d after treatment. The expression of *MdKRP4*

and *MdKRP5* was higher in the shaded fruit by 3.9-fold and 5.3-fold, respectively, at 3 d after treatment, consistent with their proposed roles as negative regulators of cell production during apple fruit growth.

The expression of two genes putatively associated with directional cell expansion, *MdCOB1* and *MdCOBL4* (*Cobra1* and *Cobra-Like4*), and several α–type *Expansin* (*MdEXPA*) genes putatively involved in the loosening of cell walls, was investigated (Fig. 2.5). *MdCOB1* expression in the shaded fruit was slightly higher (1.2-fold) at 0 d after treatment and was 1.6-fold lower at 3 d after treatment, than that in the control fruit. *MdCOBL4* expression was 1.9-fold higher in the shaded fruit at 3 d after treatment. The expression of *MdEXPA10;1* was lower in the shaded fruit by 4.6-fold, at 3 d after treatment. The expression of the other *MdEXPA* genes analyzed here was not significantly affected by shading.

Discussion

Several studies have indicated a reduction in fruit growth prior to fruit abscission in response to shading during early fruit development [6,11,12,8]. In both years of the current study, fruit growth was significantly lower in shaded fruit from 3 d after treatment indicating that a reduction in fruit growth is an early response to shading. The fruit RGR declined by 2 to 3 d after shading. These data are consistent with previous studies where shading reduced fruit RGR by around 50% at 2 d and fruit growth by 58% at 3 d after treatment [6,11]. A reduction in fruit growth may decrease sink strength and subsequently lead to the activation of abscission mechanisms. Although, the effect of shading on fruit drop was not significant within the duration of the experiment, it is likely that fruit abscission under shading continued at later stages. The high

levels of shading used in this study have previously been shown to induce extensive abscission [7,12].

The shading treatments were imposed during the phase of fruit development involving intensive cell production. In both years of this study, shading resulted in a rapid decline in cell production within 3 d after treatment, coincident with the decrease in fruit growth. These data indicate that the reduction in fruit growth was partly mediated by a decline in cell production. While the majority of cell expansion typically occurs during the later stages of fruit development, a considerable increase in cell area occurs during early fruit development and may contribute to early fruit growth. In fact, cell area in the control fruit increased by around 1.5- to 1.7-fold over the duration of the experiment. These data are consistent with previous studies in which considerable cell expansion was noted during early fruit growth [21, 16]. In the 2009 study, cell expansion was affected by shading at 7 d after treatment, and in the 2010 study, it declined by 3 d after shading. Therefore, the data indicate that a decline in cell expansion contributes to the reduction in fruit growth. Together, these data indicate that shade-induced reduction in fruit growth is mediated by a decline in cell production and expansion. Progression of these processes mediating growth is dependent on the availability of carbohydrates. A reduction in photosynthate availability and subsequent changes in carbon metabolism due to shading may rapidly decrease the rates of cell production and expansion, thereby reducing fruit growth.

Sorbitol is the main translocated carbohydrate in apple and is converted to fructose through the activity of SDH [25]. Interestingly, the expression of *MdSDH1* and *MdSDH2,* genes known to be expressed in the fruit cortex [26,27], was higher in the shaded fruit, suggesting higher SDH activity. A decrease in light levels, and subsequently photosynthesis, potentially

decreases the extent of sorbitol translocated into the developing fruit. It may be hypothesized that the fruit responds through a rapid (and potentially transient) increase in SDH activity, which allows for a higher rate of conversion of the available sorbitol into fructose, thereby allowing the fruit to meet its immediate and high respiratory demand [5]. Hence, the effects of shading on the carbohydrate status within the developing fruit, and its metabolism, warrant further investigation. The expression of another *SDH*, *MdSDH5*, reportedly decreased initially in the fruit cortex in response to shading [12]. This may reflect differences in the expression among different *SDH* genes.

The shade-induced reduction in fruit growth may be mediated by changes in the expression of key transcription factors. The transcription factors, *MdARF6* and *MdARF106*, have been investigated in relation to their potential roles in the regulation of fruit size [17]. *MdARF106* was co-localized to a region on chromosome 15 containing a quantitative trait locus (QTL) associated with fruit size. Also, *MdARF106* was expressed during the cell production and expansion phases of fruit growth consistent with a proposed role in mediating the effects of auxin on fruit growth [17]. In the current study, *MdARF106* expression was higher by 2.9-fold in the shaded fruit. The increase in *MdARF106* expression may, in-turn, regulate mechanisms that mediate the shade-induced reduction in fruit growth.

The shade-induced decline in cell production was associated with coordinated changes in the expression of core cell cycle genes which are key facilitators of cell production [16]. The expression of *MdCYCA2;2* and *MdCYCA2;3*, genes positively associated with cell production [16], was lower in the shaded fruit. A2-type cyclins mediate the progression of the G2/M phase of the cell cycle. Members of this class of cyclins associate with CDKBs to prevent exit from mitotic cell production [28]. Lower expression of these genes in response to shading may

therefore facilitate the exit from cell production within the developing fruit cortex. KRPs are key facilitators of exit from mitotic cell production and are also involved in promoting entry into endoreduplication in other plants [29,30]. In apple, *MdKRP4* and *MdKRP5* display an increase in expression in un-pollinated fruit, and during later stages of fruit development, consistent with their proposed roles in mediating the exit from cell production [16]. *MdKRP4* and *MdKRP5* displayed a sharp increase in expression by around four- to five-fold in response to the decrease in light levels, indicating that they may mediate the shade-induced exit from cell production. Together, these data indicate that shading during early fruit development results in the coordinated alteration of core cell cycle gene expression, which may subsequently mediate the reduction in cell production and fruit growth.

The expression of several genes associated with cell growth was reduced by shading and preceded the decline in cell expansion. *COBRA* encodes a glycosyl-phosphatidyl inositol (GPI)-anchored protein which may regulate cell growth by affecting cellulose biosynthesis and by determining the orientation of cellulose microfibrils within the cell wall [19,20]. *MdCOB1* expression was lower in the shaded fruit at 3 d after treatment. Lower *MdCOB1* expression in response to shading may impair cell wall extensibility and contribute to the subsequent decline in cell expansion. Interestingly, expression of the *COBRA-LIKE* gene, *MdCOBL4*, was higher in the shaded fruit. Members of the *COBL4* sub-group of *COB* genes are thought to function in secondary cell wall synthesis and may primarily contribute towards maintaining the mechanical strength of tissues [31-33]. As cell production and expansion decline, mechanisms involved in secondary cell wall synthesis may be activated and the higher *MdCOBL4* expression may potentially be part of such a mechanism in the shaded fruit. The *Expansin* family consists of multiple genes which encode extracellular proteins that facilitate the loosening of cell walls,

thereby allowing for cell expansion [18,34]. The expression of the *α-Expansin*, *MdEXPA10;1,* was lower by over four-fold in shaded fruit. Such a reduction in *MdEXPA10;1* expression may reduce cell wall extensibility and contribute to the shade-induced decline in cell expansion and fruit growth.

The high level of shading used in the current study is known to induce extensive fruit abscission [7,12]. Hence, it is possible that some of the fruit sampled for growth and gene-expression analyses were derived from a population of fruit, some of which were destined to abscise. It will be interesting to investigate in future studies whether the shade-induced changes in fruit growth-related parameters and gene expression reported here are applicable to fruit that display a reduction in growth but are not programmed for abscission.

Data from this study are consistent with our hypotheses and clearly demonstrate that shade-induced reduction in fruit growth is facilitated by a decrease in cell production and expansion. Further, the data indicate that the decrease in the extent of cell production and expansion due to shading may be mediated by coordinated changes in the expression of carbohydrate metabolism-related genes, transcription factors associated with fruit growth, and key genes associated with cell production and expansion.

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Table 2.1. *List of the apple genes and the sequence of primers used in quantitative RT-PCR analyses.*

Gene	Accession number[z]	Primer sequence (5′ to 3′)[y]
MdCACS	MDP0000291148	TCTTCCAAAGGTAGTGTTCTGCGC
		GGACGGGCTTTAAGTTGCGAC
MdACTIN	EB127077	ACCATCTGCAACTCATCCGAACCT
		ACAATGCTAGGGAACACGGCTCTT
MdGAPDH	EB146750	TGAGGGCAAGCTGAAGGGTATCTT
		TCAAGTCAACCACACGGGTACTGT
MdCOB1	MDP0000288732	GCAATCATGGATCCAGGACCCAGA
		GGGTCCATCTCCTTTTGTCCGAC
MdCOBL4	MDP0000895592	CCCTGGCTGGACTCTCGG
		ACGCCACCTTTGCAGCAATTAGAA
MdEXPA8;1	MDP0000138500	CTTCAACCTACATCTGCATGGTGTG
		TCAAAGCTGCAGTGTTGGTTCCATAT
MdEXPA8;2	MDP0000431696	GGGTCTTGCTATGAGATGAAATGTGG
		CACCATTGTCGTTGGCCTGC
MdEXPA10;1	MDP0000681724	GGGTGCGGATCTTGCTACG
		GGAGGGCGTTGTTTGGTGGA
MdSDH1	MDP0000932467	GAGTCTTGGCGCAGATGCAGT
		ACAGTCGAAGGTTACATCCACTCCATT
MdSDH2	MDP0000874667	CATTGCCAGCAGTGCAAAGGC
		GGCAATTTAAAGCACAGATCCGCG
MdCDKB1;1	CV085424	CGATTGATCTGCGTCGAGCATGTT
		CGGATTCGGCCCCTTCCG

MdCDKB1;2	EB138473	GATTGCTCTGCGTCGAACACGTC
		CCCAGGATTCGGCCCCTTTCT
MdCDKB2;1	CV129014	GGTAACAGAGATGCGCTCTGTAGTAGT
		GAGATTGTTGAGTTGTTGAATCCTATGGA
MdCDKB2;2	CV086331	AGAGAAGCGCTCTGTACTACTGAAGTT
		AAAGCTACTTGCAAATTGTAAACACCAC
MdCYCA2;1	CO416185	CAATTGAACACCACCGGTTGTCC
		ACTCGAAGCACCTGAATGGAGG
MdCYCA2;2	CO722204	CAATTGAACACGACTGGTTGCCT
		CCTCAAACTCAAAGTACCCGAATGCAAA
MdCYCA2;3	CO415585	GCAAGAATTACAGTTGAACACTAGTGGTT
		CCGGAAAGTGTACATGTCACAGTCTCT
MdCYCB1;1	CN579062	AGACACTCAAGCTTCACACTGGTTTC
		AGCAGTGCAACAGCTCCGTG
MdCYCB1;2	CV084069	GTTCTGGTAACCCTTCATTCGGCA
		AGAAGAGCAACCGCGCTACG
MdCYCB2;2	CV628904	GTGAAGGAGGTTGGACCGAATC
		CAGTATACGAGCTCAGTTTCTTAGCTTCC
MdKRP4	CV084380	GCTTGCAGAATTCGGCGATGGAAC
		CTCCTCCTCCGCCTCGGA
MdKRP5	CN912198	CCGTCGTCGTATGACGTGGC
		GCCGTCGTTGGAAGTCCGT
MdARF6	MDP0000256621	CTTCTCTCTCACCTCCAACTCATCC

		AACCGAGTCCTGAGGAGCGA
MdARF106	MDP0000232116	GAGGGGAAGCCGTTTGAGGT
		GCCGTCCAAAACACCTTCAAT

[z]The Genbank accession numbers or the accession numbers from the apple genome database are indicated [35].

[y]The forward (top) and reverse (bottom) primers are indicated for each of the genes analyzed.

Figure 2.1. *Effects of shading on apple fruit growth, cell number and cell size.*

Shading was performed on branches in 2009 and entire trees in 2010 using 80% shade material. Fruit diameter (mm), number of cell layers in the fruit cortex, and cortex cell area (μm^2) were determined in fruit sampled from the Shaded and Control treatments in 2009 and 2010. Error bars indicate the SE of the mean (n = 4). Significant interaction effects between the factors, Shading and Time after treatment, were observed for all the fruit growth-related parameters in both years of the study ($P < 0.05$). Simple effects were analyzed using the test of effect slices. Asterisk indicates significant difference between the shaded and control fruit within the indicated time after treatment, as determined using the test of effect slices. All the differences indicated by asterisks were significant at $\alpha = 0.01$ except for fruit diameter at 3 d after treatment in 2010 (P = 0.012).

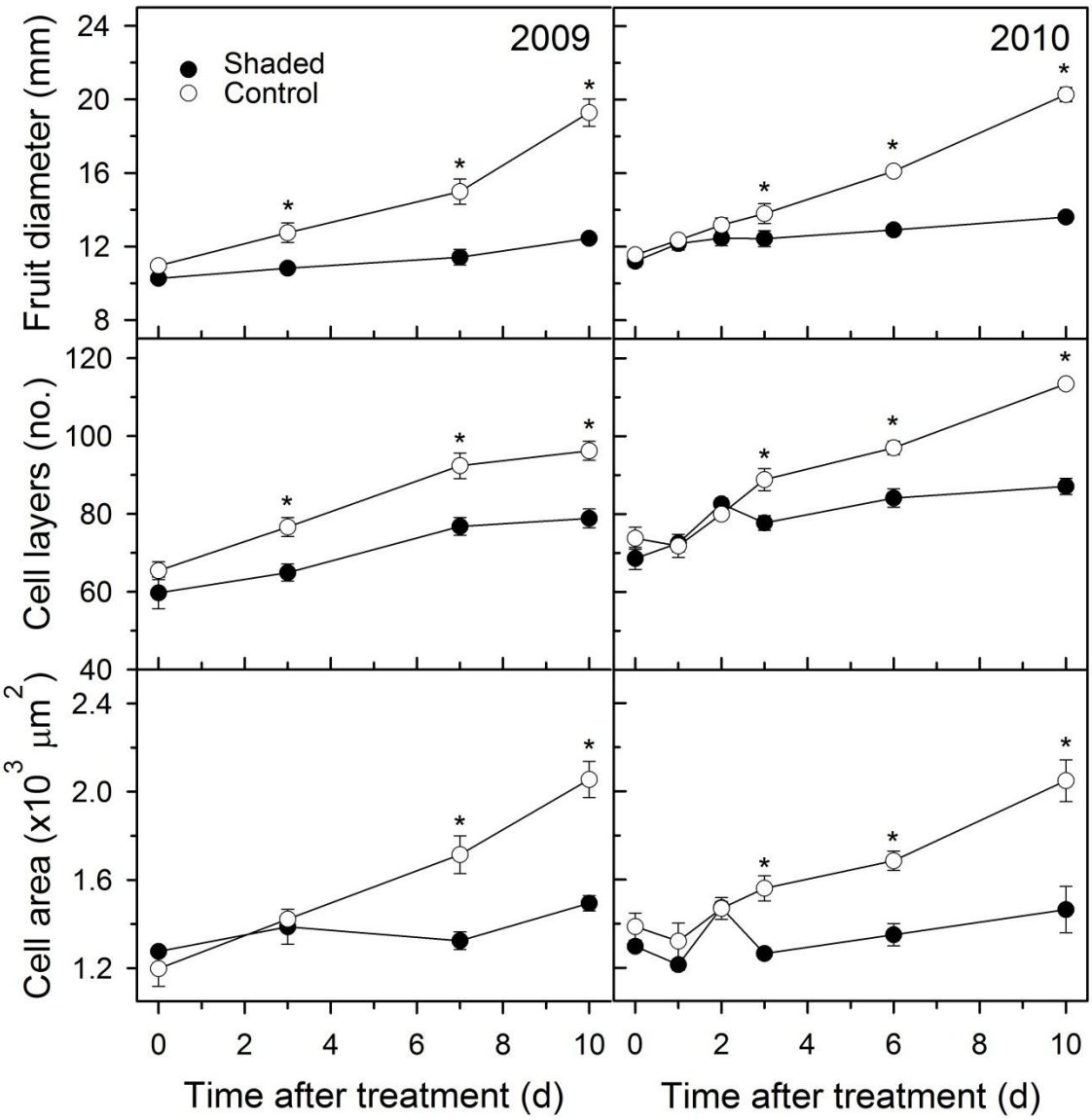

2009
2010
Shaded
Control
Fruit diameter (mm)
Cell layers (no.)
Cell area (x10^3 μm^2)
Time after treatment (d)
Time after treatment (d)

Figure 2.2. *Effect of shading on the expression of two Sorbitol dehydrogenase (SDH) genes in apple fruit.*

Shading was performed on branches in 2009 using 80% shade material. Open box represents control fruit and closed box represents shaded fruit. Expression was determined using quantitative reverse transcriptase-polymerase chain reaction. The expression of a gene in relation to its expression at 0 d after treatment in the control fruit is presented. Error bars indicate the SE of the mean ($n = 4$). Both the genes displayed a significant interaction between the factors, Shading and Time after treatment ($P < 0.05$). Simple effects were analyzed using test of effect slices. Asterisk indicates significant difference between the shaded and control fruit at the indicated time after treatment as determined using the test of effect slices ($P < 0.01$).

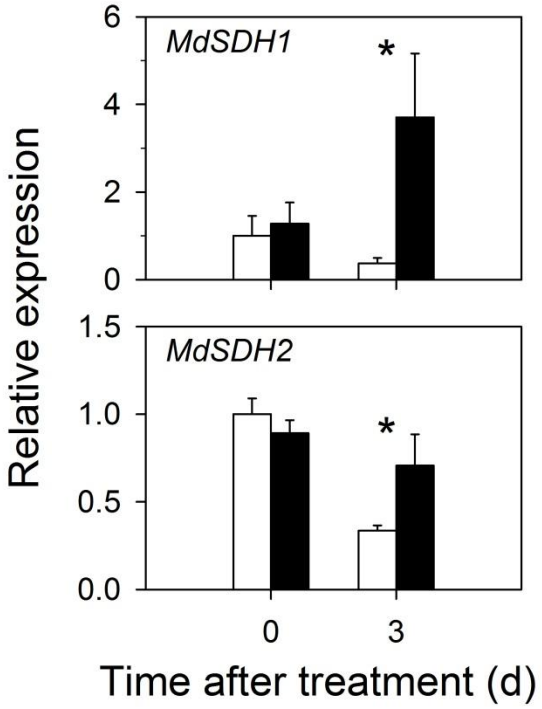

MdSDH1
MdSDH2
*
*
Relative expression
Time after treatment (d)
0
2
4
6
0.0
0.5
1.0
1.5
0
3

Figure 2.3. *Effect of shading on the expression of two transcription factors putatively associated with fruit growth in apple.*

Individual branches were shaded in 2009 using 80% shade material. Open box indicates control fruit and closed box indicates shaded fruit. Expression was determined using quantitative reverse transcriptase-polymerase chain reaction. The expression of a gene in relation to its expression in the control fruit at 0 d after treatment is presented. Error bars indicate the SE of the mean ($n = 4$). Only *MdARF106* expression displayed a significant interaction effect between the factors, Shading and Time after treatment ($P < 0.05$). Simple effects were analyzed using test of effect slices to determine differences between shaded and control fruit at each time after treatment for *MdARF106*. Asterisk indicates significant difference between the shaded and control fruit within the indicated time after treatment as determined by the above test for *MdARF106* ($P < 0.01$).

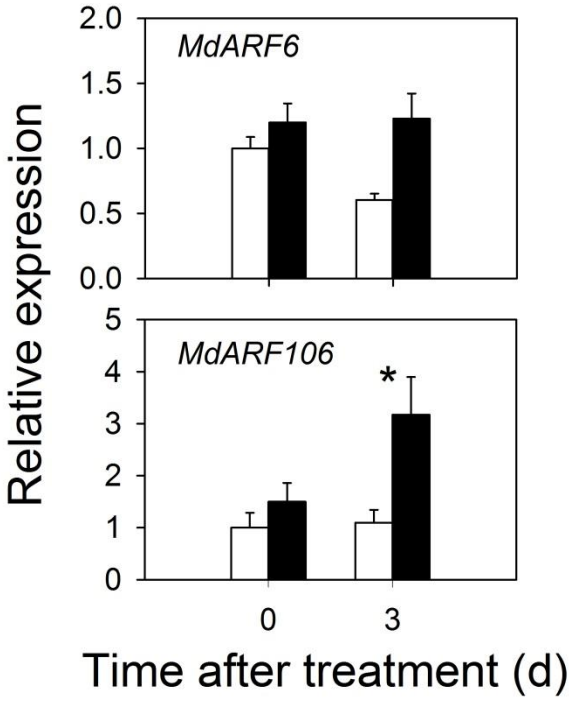

MdARF6
MdARF106
Relative expression
Time after treatment (d)
2.0
1.5
1.0
0.5
0.0
5
4
3
2
1
0
0
3
*

Figure 2.4. *Effect of shading on the expression of core cell cycle genes associated with cell production in apple fruit.*

Shading was performed using 80% shade material on individual branches in 2009. Open box represents control fruit and closed box represents shaded fruit. Expression analysis was performed using quantitative reverse transcriptase-polymerase chain reaction. Expression of a gene relative to its expression in the control fruit at 0 d after treatment is presented. Error bars indicate the SE of the mean (n = 4). The interaction effects between Shading and Time after treatment were significant ($P < 0.05$) for *MdCYCA2;2*, *MdCYCA2;3*, *MdKRP4* and *MdKRP5* only. Asterisk indicates significant difference between shaded and control fruit at the indicated time after treatment for the above genes as determined by the test of effect slices ($P < 0.01$). All the other cell cycle genes (except for *MdCYCB2;2*) displayed significant main effects of Shading and Time after treatment, but non-significant interaction between these factors. For *MdCYCB2;2*, only the factor, Time after treatment, was significant.

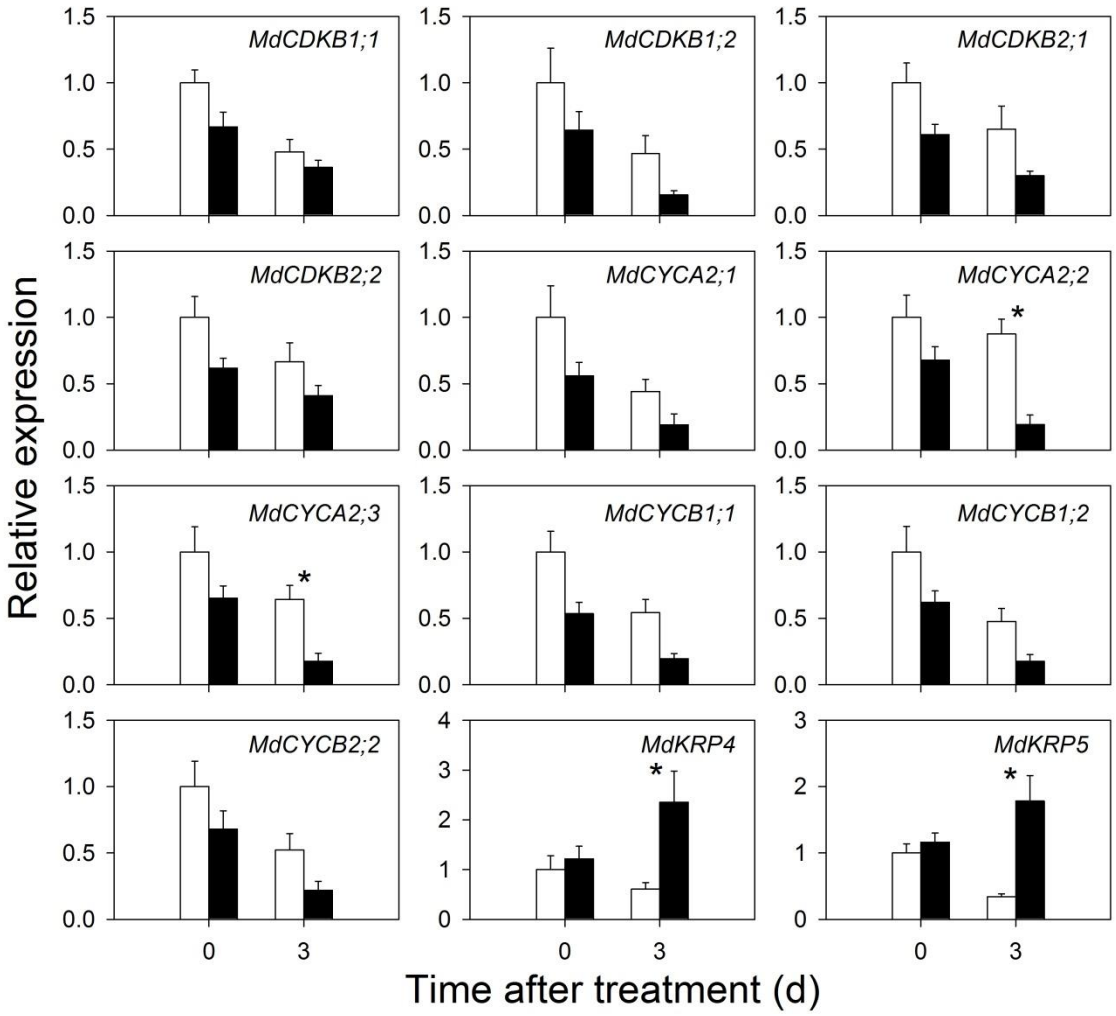
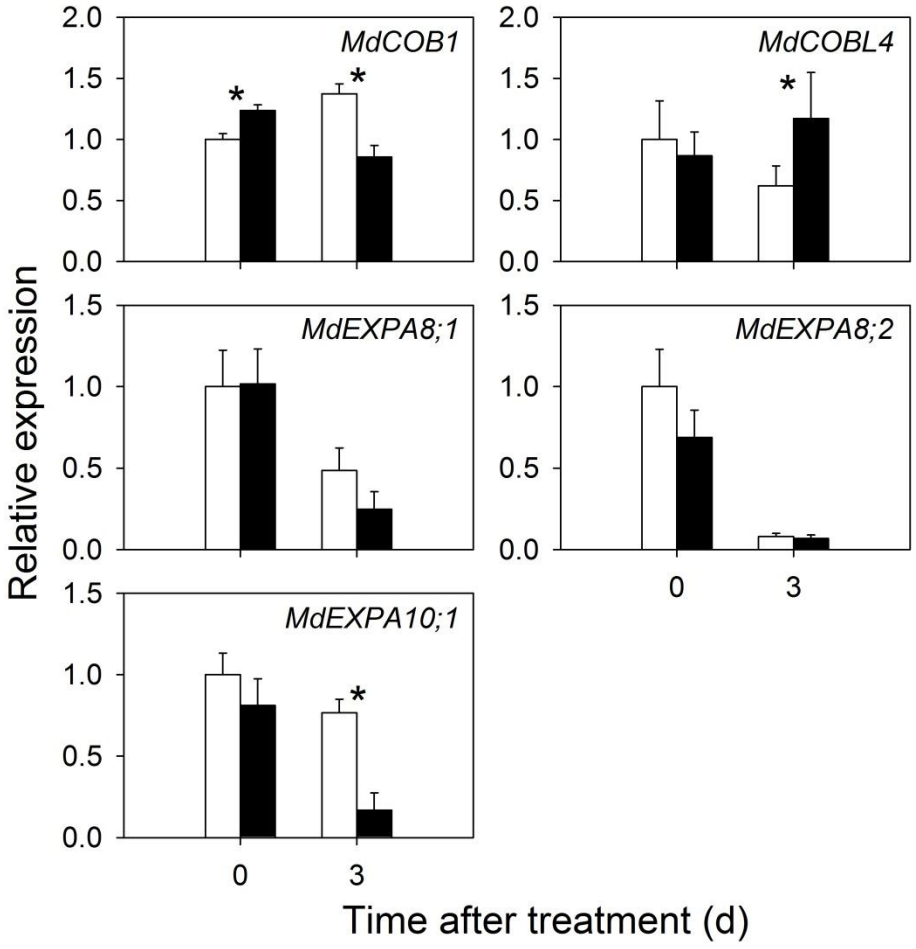

MdCDKB1;1
MdCDKB1;2
MdCDKB2;1
MdCDKB2;2
MdCYCA2;1
MdCYCA2;2
MdCYCA2;3
MdCYCB1;1
MdCYCB1;2
MdCYCB2;2
MdKRP4
MdKRP5
Relative expression
Time after treatment (d)
0
3

Figure 2.5. *Effect of shading on the expression of genes associated with cell expansion in apple fruit.*

Shading was performed using 80% shade material on individual branches in 2009. Open box indicates control fruit and closed box indicates shaded fruit. Expression was measured using quantitative reverse transcriptase-polymerase chain reaction. Expression of a gene relative to its expression at 0 d after treatment in the control fruit is presented. Error bars indicate the SE of the mean (n = 4). The interaction effects between Shading and Time after treatment were significant for *MdCOB1*, *MdCOBL4*, and *MdEXPA10;1* only ($P < 0.05$). Asterisk indicates significant difference between shaded and control fruit at the indicated time after treatment for these three genes as determined by the test of effect slices. Expression of *MdCOB1* at 0 d after treatment, and *MdCOBL4* at 3 d after treatment were significantly different between the shaded and control fruit at $\alpha = 0.05$, while *MdCOB1* at 3 d after treatment and *MdEXPA10;1* at 3 d after treatment were significantly different between shaded and control fruit at $\alpha = 0.01$. *MdEXPA8;1* and *MdEXPA8;2* were not affected by shading.


MdCOB1
MdCOBL4
MdEXPA8;1
MdEXPA8;2
MdEXPA10;1
Relative expression
Time after treatment (d)
0
3
0.0
0.5
1.0
1.5
2.0
*

CHAPTER 3

THE *AINTEGUMENTA* GENES, *MdANT1* AND *MdANT2*, ARE ASSOCIATED WITH THE REGULATION OF CELL PRODUCTION DURING FRUIT GROWTH IN APPLE (*Malus* × *domestica* Borkh.)

Dash, M. and Malladi, A. 2012. *BMC Plant Biology* 12:98. Reprinted here with permission of publisher

Abstract

Background

Fruit growth in apple (*Malus* × *domestica* Borkh.) is mediated by cell production and expansion. Genes involved in regulating these processes and thereby fruit growth, are not well characterized. We hypothesized that the apple homolog(s) of *AINTEGUMENTA* (*ANT*), an APETALA2–repeat containing transcription factor, regulates cell production during fruit growth in apple.

Results

Two *ANT* genes, *MdANT1* and *MdANT2*, were isolated from apple and their expression was studied during multiple stages of fruit development. *MdANT1* and *MdANT2* expression was high during early fruit growth coincident with the period of cell production, rapidly declined during exit from cell production, and remained low during the rest of fruit development. The effects of increase in carbohydrate availability during fruit growth were characterized. Increase in carbohydrate availability enhanced fruit growth largely through an increase in cell production. Expression of *MdANT1* and *MdANT2* increased sharply by up to around 5-fold in response to an increase in carbohydrate availability. Expression of the *ANT* genes was compared across two apple genotypes, 'Gala' and 'Golden Delicious Smoothee' (GS), which differ in the extent of fruit growth, largely due to differences in cell production. In comparison to 'Gala', the larger fruit-size genotype, GS, displayed higher levels and a longer duration of *MdANT1* and *MdANT2* expression. Expression of the *ANTs* and cell cycle genes in the fruit core and cortex tissues isolated using laser capture microdissection was studied. During early fruit growth, expression of *MdANT2* was higher within the cortex, the tissue that constitutes the majority of the fruit.

Additionally, *MdANT1* and *MdANT2* expression was positively correlated with that of A- and B-type *CYCLINS*, B-type *CYCLIN-DEPENDENT-KINASES* (*CDKBs*) and *MdDEL1*.

Conclusions

Multiple lines of evidence from this study suggest that *MdANT1* and *MdANT2* regulate cell production during fruit growth in apple. ANTs may coordinate the expression of cell proliferation genes and thereby affect the competence of cells for cell production during fruit growth. Together, data from this study implicate *MdANT1* and *MdANT2* in the regulation of fruit growth in apple.

Introduction

Apple (*Malus* × *domestica* Borkh.) fruit growth is mediated by cell production and expansion. After bud-break, rapid growth within the ovary and floral-tube tissues is facilitated by intensive cell production. This phase is followed by a period of temporary cessation of growth around bloom associated with quiescence in cell production, a phenomenon which likely prevents fruit growth in the absence of pollination and fertilization [1]. Cell production is re-initiated in response to signals generated during pollination and/or fertilization resulting in fruit set. Early fruit development is associated with intensive cell production-mediated growth which occurs until several weeks after fruit set [1-3]. Final cell number attained by the end of this period contributes greatly to the sink strength and thereby the growth potential of the fruit. Subsequent fruit growth is associated with post-mitotic cell expansion, a process which continues until maturity and contributes to the majority of fruit growth and increase in fruit size [1,3]. Enhanced fruit growth and increase in fruit size are mediated by changes in cell production or expansion. For example, increase in fruit growth under higher carbohydrate availability during early fruit development is primarily associated with an increase in cell production [4]. Also, variation in fruit growth potential and fruit size across genotypes is associated with differences in cell number and size [3,5]. Although it is apparent that cell production and expansion are important determinants of fruit growth, our understanding of the molecular mechanisms and genes that regulate these processes remains limited.

Cell production during fruit growth is potentially regulated by genes controlling the progression of the cell cycle [1,6,7]. Previous research indicated coordinated changes in the expression of core cell cycle genes during different phases of fruit growth in apple [1]. Expression of 14 such genes including B-type *CYCLIN DEPENDENT KINASES* (*CDKs*), A- and

B-type cyclins, a WEE kinase (*MdWEE1*), and an atypical E2F transcription factor (*MdDEL1*) was positively associated with cell production during fruit growth and development. These genes displayed high expression before bloom and during early fruit development, stages primarily associated with rapid growth mediated by cell production. Subsequently, these genes displayed a sharp reduction in expression coincident with exit from cell production during fruit development. Additionally, five cell cycle genes including the *KIP RELATED PROTEINS* (*KRPs*), *MdKRP4* and *MdKRP5*, were negatively associated with cell production during different phases of fruit growth and development. It is likely that upstream regulatory genes may, either directly or indirectly, coordinate changes in the expression of these cell cycle genes as well as other genes associated with cell proliferation, thereby regulating cell production during fruit growth. Such upstream regulators of cell production during fruit growth have not yet been definitively identified in apple. Recently, an *AUXIN RESPONSE FACTOR* (*ARF106*) expressed during cell division and expansion phases of apple fruit development was co-localized to a major fruit size QTL, suggesting its involvement in regulating fruit growth [8]. In other fleshy fruit such as tomato (*Solanum lycopersicum*), *FW2.2*, a fruit size regulator, inhibits cell production potentially through its association with a cell cycle gene, and thereby regulates fruit growth [9,10]. Also, *SUN*, a gene involved in the regulation of tomato fruit shape may affect the patterns and orientations of cell proliferation during early fruit growth [11,12]. Beyond the above examples, little information is available regarding upstream regulators of cell production during growth of fleshy fruit. Identification and characterization of such genes is essential to develop a better understanding of fleshy fruit growth.

Genes controlling organ growth are potential candidates for the regulation of growth of fleshy fruit. Many genes that regulate organ growth have been identified in Arabidopsis

(*Arabidopsis thaliana*) and other plants [13-15]. One such gene, *AINTEGUMENTA* (*ANT*) is a key regulator of organ growth in Arabidopsis. *ANT* is involved in the regulation of ovule development, floral organ growth and development, and organ size in Arabidopsis [16-21]. Arabidopsis *ant* mutants display pleiotropic effects including a reduction in the size of floral organs and leaves [16,17,19]. Over-expression of *ANT* in Arabidopsis results in an increase in the duration of cell proliferation and enhances organ size in leaves, floral organs and siliques [19]. Additionally, ANT mediates the effects of other genes involved in regulating organ growth. In Arabidopsis, *ARGOS* (*AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE*) promotes cell production and growth, and positively regulates final organ size in an auxin-dependent manner [22]. Over-expression of *ARGOS* in Arabidopsis increases *ANT* expression, and the effects of *ARGOS* on organ growth are attenuated in the *ant* mutant, suggesting that *ANT* mediates *ARGOS*-dependent effects of auxin on organ growth. *ANT* expression is also affected by *AUXIN RESPONSE FACTOR2* (*ARF2*), a negative regulator of cell production and organ size in Arabidopsis [23].

ANT is a member of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of transcription factors and is grouped within the AP2 sub-family. Members of the AP2 sub-family are defined by the presence of two AP2 domains separated by a conserved linker region which together constitute the DNA binding domain [24,25]. Genes within the AP2 sub-family, including several *AINTEGUMENTA-LIKE* (*AIL*) genes, are involved in the regulation of a multitude of plant growth and developmental processes. For example, *APETALA2* (*AP2*) is involved in determining floral organ identity, regulating flower development, maintaining the stem cell niche in the shoot apical meristem, and regulating seed size [26-29]. *AP2* negatively regulates replum growth and valve margin formation during Arabidopsis fruit development [30].

PLETHORA (*PLT*) genes are *AILs* which function as master regulators of root growth and development in Arabidopsis partly through their effects on promoting cell proliferation [31,32]. *AtBBM* (*BABYBOOM/AIL2*) promotes cell production, and regulates embryo development and root growth [32,33]. Additionally, *AIL5* and *AIL6/PLT3* are positive regulators of cell production and organ growth in Arabidopsis as their over-expression leads to enhanced floral organ growth [21,34,35].

Whether *ANT* and/or the *AIL* genes are involved in regulating the growth of fleshy fruit has not been investigated previously. It was hypothesized that the apple *ANT* homolog(s) regulate cell production during fruit development and therefore contribute to regulation of fruit growth. Here, the isolation and characterization of two *ANT* genes from apple is reported. Evidence supporting the role of these genes in regulating cell production during different stages of fruit growth, across genotypes differing in fruit growth potential, and in response to carbohydrate availability is presented. Data from this study implicate *ANTs* in the regulation of fleshy fruit growth.

Materials and methods

Plant material

Mature 'Gala' and 'Golden Delicious Smoothee' (GS) trees growing on M.7 and M.7a rootstocks respectively, at the Georgia Mountain Research and Experiment Station in Blairsville, GA, USA were used in this study. Fruit growth and development was studied using four randomly selected 'Gala' trees at the above location in 2009. Each of these trees was treated as an independent replicate (n=4). Trees were manually thinned to one lateral fruit per cluster at 10 DAFB. Fruit diameter was measured from bloom until maturity on 20 fruit per replicate. At each

stage, fruit were randomly sampled from different parts of the canopy between 12 pm and 2 pm, independently from each replicate. At each stage, four fruit from each replicate were fixed in CRAF III fixative for cytology. At each stage, fruit tissue from at least four fruit was pooled within each replicate and frozen in liquid N_2 for gene expression analyses. To determine the affect of carbohydrate availability on fruit growth, four randomly selected GS trees were subjected to the thinning treatment while four other trees were left un-thinned in 2009. Each tree was treated as an independent replicate (n=4). Thinning involved the manual removal of all fruit within a cluster except for one lateral fruit at 11 DAFB. Fruit diameter was measured on 20 fruit per replicate from bloom until maturity. Fruit were sampled at different stages of development for cytology and gene expression analyses as described above. All trees used in the above studies were maintained according to commercial apple production practices except for the application of chemical thinning agents.

In 2010, three 'Gala' trees, each of which was treated as an independent replicate (n=3), were used to determine the localization of expression of several genes using laser capture microdissection (LCM). For this study, lateral flowers/fruit were randomly sampled from different parts of the tree canopy at -7, 0, 10 and 15 DAFB. At least four individual flowers/fruit from each replicate were used at each stage in this experiment. All sampling was performed between 12 pm and 2 pm. The ovary and floral-tube tissues, or the fruit was dissected and fixed in freshly prepared Farmer's fixative containing 75% (v/v) ethanol and 25% (v/v) acetic acid, and stored at 4°C until further analysis. Manual thinning or application of chemical thinning agents was not performed in this study.

Measurement of cell number and cell area

Cell number and cell area were determined as described previously [1]. Briefly, sectioning of flower/fruit was performed using a vibratome (Micro-cut H1200, Bio-Rad, Hercules, CA, USA). Cell number was determined by counting the number of cell layers between the petal vascular trace and the epidermis in sections stained with toluidine blue. The relative cell production rate (RCPR) was determined from the cell number data as: $[Ln(C_2)–Ln(C_1)]/T_2–T_1]$, where C_1 and C_2 denote the cell number at time points T_1 and T_2, respectively. To measure the cell area, the number of cells within a defined area was determined at three locations between the epidermis and the petal vascular trace. Cell area was calculated using this value and the average cell area from the three locations was used as the cortex cell area of the fruit sample.

Comparison of various parameters such as fruit growth, cell number, cell area and gene expression was performed across the genotypes, 'Gala' and GS. Data from the fruit development study in 'Gala' and the thinning study in GS (only thinned fruit) described above were used for this comparison (2009). As the genotypes differed significantly in terms of the time of full bloom (by around 1 week), cumulative growing degree days (GDD) from the time of the respective full bloom dates were used to allow for this comparison. GDD was determined using temperature data obtained from the Georgia weather network (www.georgiaweather.net). A base temperature of 10°C was used for GDD determination. If the average daily temperature was below 10°C, GDD accumulation was set to zero [46].

Isolation of the apple ANT genes

Publicly available apple expressed sequence tag (EST) database (National Center for Biotechnology Information-NCBI) was mined to identify genes with homology to the

Arabidopsis *ANT* (*AtANT*; [GenBank:At4G37750]). Eight potential genes with similarity to the *AtANT* and other *AIL* genes were identified. The EST displaying highest homology to *AtANT* was designated as *MdANT*. Preliminary gene expression analyses were performed to determine the pattern of expression of these genes during apple fruit development. Expression analyses was performed using fruit collected from mature 'Gala' trees in 2008 (n=4; previously described in [1]). *MdANT* displayed higher expression during the cell production phase of fruit development and was selected for further analysis.

To isolate the full-length cDNA of the *MdANT* gene, 5′ and 3′ RACE (Rapid Amplification of cDNA Ends) were attempted. Total RNA was extracted from 'Gala' fruit at 10 DAFB as the gene displayed high expression at this stage in the preliminary analysis. First strand cDNA synthesis and amplification were performed using the SMART RACE cDNA Amplification kit (Clontech Laboratories Inc., CA, USA) following the manufacturer's instructions. The 5′ and 3′ gene-specific primers for this analysis were designed using the EST sequences of *MdANT*. The 3′ RACE analysis of *MdANT* yielded two products which were subsequently cloned into the pGEM-T Easy vector (Promega Corporation, WI, USA) and sequenced. The 3′ RACE products displayed high homology (>90% identity) with each other, and were designated as *MdANT1* and *MdANT2*. Several attempts were made to isolate the 5′ sequences of *MdANT1* and *MdANT2*. Techniques such as 5′ RACE and degenerate PCR using primers designed from the highly conserved regions of multiple *ANT* genes {*Arabidopsis thaliana* (*AtANT*; [GenBank:ABR21533]), *Vitis vinifera* (*VvANT*; [GenBank:AM444297]), *Brassica napus* (*BnANT*; [GenBank:ABA42146]), *Populus trichocarpa* (*PtANT*; [GenBank:AC210555]), *Nicotiana tabaccum* (*NtANT*; [GenBank:AAR22388]), *Artemisia annua* (AaANT; [GenBank:ACY74336])} were used. However, these attempts were largely

unsuccessful. Following the release of the peach (*Prunus persica*) genome, primers were designed using the peach *ANT* (*PpANT*; [Genome database for Rosaceae:ppa023077m]). The 5′ sequence of *MdANT2* was amplified, cloned and sequenced using this approach. Following the release of the apple genome [47], *MdANT1* and *MdANT2* were identified from the apple genome database (http://genomics.research.iasma.it) using the sequence information derived from the above approaches. Primers were designed for full-length amplification of *MdANT1* and *MdANT2*. The PCR amplified products were cloned into pGEM-T Easy vector (Promega Corporation, WI, USA) and sequenced. Accession numbers for *MdANT1* and *MdANT2* are MDP0000175309 and MDP0000190889, respectively [Apple genome database (http://genomics.research.iasma.it)]. Sequence of the above genes obtained in this study differed from the predicted sequence available in the apple genome database primarily with respect to the presence of a 'VYL' motif within the DNA binding domain. Primer sequences used in the above approaches for cloning the apple *ANT* genes are provided in Table 3.1.

Phylogenetic analysis

Plant ANT sequences were retrieved from the NCBI database, Genome Database for Rosaceae and The Arabidopsis Information Resource (TAIR). Multiple alignments of apple and other plant ANT transcription factors were performed using MUSCLE (Multiple Sequence Comparison by Log-Expectation; http://www.ebi.ac.uk/Tools/msa/muscle/). Phylogenetic tree construction was performed using the neighbor joining distance method of the MEGA5 (Molecular Evolutionary Genetics Analysis) software [36].

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

RNA extraction from flower and fruit was performed using the method described previously [3], except that the extraction buffer contained 150 mM Tris-HCl instead of Tris-Borate. The cDNA synthesis was performed as described previously [1] using 1 μg of total RNA after removal of genomic DNA with a DNase treatment (Promega Corporation, WI, USA). Reverse transcription was performed using ImProm II reverse transcriptase (Promega Corporation, WI, USA) and oligo dT (15) primers. The cDNA was diluted 5-fold for all gene expression analyses. Gene-specific primers for qRT-PCR analyses of *MdANT1* and *MdANT2* were designed from regions sharing low homology and are shown in Table 3.2. Primer efficiency was determined for the primer pairs and ranged from 1.85 to 1.97. The 2X SYBR GREEN master mix (Applied Biosystems, Carlsbad, CA, USA) was used for all analyses. All the qRT-PCR analyses were performed using the Stratagene Mx3005P real-time PCR system as described previously [1]. Briefly, the reaction conditions were as follows: 95°C for 10 min; 40 cycles of 95°C (30 s) and 60°C (1 min). Melt-curve analyses were performed after the PCR. A single distinct peak was observed for all the genes studied indicating the specific amplification of a single product. No-template controls were included in each run of the qRT-PCR. Relative expression was calculated using a modified Pfaffl method [37] and as described in [50]. Relative quantity (RQ) for each sample was calculated using the formula, $1/E^{Cq}$, where Cq is the quantification cycle (threshold cycle). The RQ was normalized using two reference genes, *MdACTIN* and *MdGAPDH* (accession numbers [Genbank:EB127077] and [Genbank:EB146750], respectively; described previously in [1]). The geometric mean of expression of the two reference genes (normalization factor) was used for normalization. The normalized RQ (NRQ) values were $\log_2$ transformed and used for statistical analyses. The standard error of the means was calculated as described in [38]. Expression of a gene relative to its expression at full bloom (0 DAFB) is presented for the fruit

development study in ‘Gala’. For the thinning study in GS, expression of a gene relative to its expression at full bloom (0 DAFB) in thinned fruit is presented. For the study involving comparison of gene expression between ‘Gala’ and GS, expression of a gene relative to its expression at 0 DAFB in ‘Gala’ is presented. In all the above studies, four independent biological replicates were used for the qRT-PCR analyses.

Laser capture microdissection (LCM)

Flower (or fruit) sampled at -7, 0, 10 and 15 DAFB and fixed in Farmer’s fixative were rehydrated in a graded series of ethanol (2 h each in 75%, 50%, 30% and 0% ethanol prepared with DEPC-treated water) at 4^{o}C. The samples were embedded in 6% agarose (prepared in DEPC-treated water) and sectioned using a vibratome. All surfaces of the vibratome were cleaned with RNaseZAP solution (Ambion, Inc., TX, USA) and rinsed with DEPC-treated water before use. The sections were placed on a glass slide and LCM was performed using the PALM MicroBeam system (Carl Zeiss Microscopy, LLC, NY, USA). LCM was performed with the laser beam set to a power of 60 to 90 mW. Microdissected cells were collected in the lid of a 0.6 mL reaction tube containing the RNA extraction buffer (150 mM Tris-HCl, 50 mM EDTA, 2% SDS, and 1% β-mercaptoethanol). The microdissected cells from flowers/fruit within a replicate were pooled for RNA extraction. Captured tissues were transferred to a tube containing the extraction buffer followed by the addition of PVPP. To this mix, 0.1 volumes of 5 M potassium acetate and 0.25 volumes of ethanol were added and the mixture was extracted with chloroform:iso-amyl alcohol (24:1 v/v), followed by extraction with phenol:chloroform:iso-amyl alcohol (25:24:1 v/v) and chloroform:iso-amyl alcohol (24:1 v/v). The aqueous supernatant was precipitated with isopropanol (1:1 v/v) at room temperature for 15 min, followed by precipitation in 3 M lithium chloride (4^{o}C) for 2 h. RNA was subsequently washed with 70% ethanol, air

dried, and dissolved in DEPC-treated water. Total RNA (0.5 µg) was used for cDNA synthesis. cDNA synthesis and and qRT-PCR analyses were performed as described above. Only *MdACTIN* was used as the reference gene as *MdGAPDH* did not display stable expression across the samples in this study. Calculation of gene expression was performed as described above. The cell cycle genes, *MdCYCA2; 3* [Genbank:CO415585], *MdCYCB1;1* [Genbank:CN579062], *MdCDKB1;2* [Genbank:EB138473], *MdCDKB2;1* [Genbank:CV129014], *MdDEL1* [Genbank:CV631574], *MdKRP4* [Genbank:CV084380] and *MdKRP5* [Genbank:CN912198] were used in this study and have been described previously [1]. *MdMADS5* (Apple Genome Database:MDP0000013331) and *MdMADS10* [Genbank:AJ000762] were used to confirm the isolation of core and cortex tissues by LCM. Primer sequences for the two *MdMADS* genes are provided in Table 3.2. Expression of a gene relative to its expression at 0 DAFB in the cortex tissue is presented here. Three independent biological replicates were used for the qRT-PCR analysis.

Statistical analysis

Statistical analyses were performed using SAS 9.0 (SAS Institute Inc., NC, USA) and SigmaPlot 11 (Systat Software Inc., San Jose, CA). Fruit diameter, cell layers, cell area and qRT-PCR data were compared between thinned and un-thinned treatments using two-way ANOVA. The paired *t* test was used for statistical comparison of gene expression between the core and cortex tissues isolated by LCM. Pearson product moment correlation analysis was used to analyze the association between the expression of *MdANT1*, *MdANT2* and the cell cycle genes. NRQ values (log_2 transformed) were used for the above analyses.

Results

Isolation of the apple ANT genes

Eight expressed sequence tags (ESTs) with homology to the Arabidopsis *ANT* were identified from publicly available apple EST databases. The EST displaying the highest similarity with the *AtANT* was designated as *MdANT* and selected for the isolation of the full-length gene. The 3′ RACE analysis of *MdANT* revealed the presence of two *ANT* genes which were designated as *MdANT1* and *MdANT2*. Full-length sequences of these genes were determined as described in the 'Materials and Methods' section. *MdANT1* and *MdANT2* shared 93% homology at the nucleotide level (coding region) and 90% identity at the amino acid level. Nucleotide sequence identity also extended into the 5′ (~1 kb) and 3′ (~0.5 kb) regions of the open reading frame. Both genes encode putative protein products with 651 amino acids. Phylogenetic analysis of different plant ANT transcription factors, including MdANT1 and MdANT2, using their predicted protein sequences is shown in Fig. 3.1. MdANT1 and MdANT2 displayed higher sequence similarity with AtANT than with the other AILs from Arabidopsis (Fig. 3.1). MdANT1 and MdANT2 shared >50% amino acid identity with the Arabidopsis ANT and >75% identity with the peach (*Prunus persica*) ANT. MdANT1 and MdANT2 displayed high sequence similarity with other plant ANTs within a stretch of around 170 amino acids containing the AP2-domain repeats and the linker region (Fig. 3.2). MdANT1 and MdANT2 displayed greater than 88% identity with AtANT in this region. MdANT1 and MdANT2 contained a basic motif (TKKR) similar to the nuclear localization signal in AtANT (KKKR; [39]). Nineteen amino acids within the two AP2-domain repeats and the linker region essential for the DNA binding activity of AtANT were identified in Arabidopsis [25]. All of these residues were conserved within the two apple ANTs. Seven potential apple *AILs* were identified from the EST databases

and subsequently five were confirmed following comparisons with the apple genome database (Fig. 3.1). All these genes contained the well conserved AP2-domain repeats and the linker region.

Expression of MdANT1 and MdANT2 is associated with cell production during fruit growth

Fruit diameter in 'Gala' increased by over 2-fold between 7 and 25 DAFB and continued to increase linearly during the rest of fruit development (Fig. 3.3A; Table 3.3). Analysis of cell production within the fruit cortex indicated little change in cell number between 0 DAFB and 7 DAFB (Fig. 3.3B). A rapid increase in cell number (3.6-fold) was observed between 7 DAFB and 15 DAFB. This was also reflected in the high relative cell production rates (RCPR) observed especially around 10 and 15 DAFB (Fig. 3.3C). While the cell number continued to increase between 15 and 32 DAFB (Fig. 3.3B), this occurred at a slower rate than that between 7 and 15 DAFB. The RCPR declined rapidly during this period, and reached basal levels by around 32 DAFB. Cell number did not change greatly after this period. The cortex cell area displayed little change during early fruit growth but increased from around 25 DAFB, coincident with the period of decline in cell production (Fig. 3.3D). Most of the increase in cell area occurred during the later stages of fruit development and was associated with the majority of increase in fruit size (Fig. 3.3D).

MdANT1 and *MdANT2* displayed largely similar patterns of expression during fruit development (Fig. 3.4; Table 3.3). Expression of *MdANT1* and *MdANT2* was generally high from bloom until around 15 DAFB (peak in expression around 7 DAFB), coincident with the period of rapid cell production. A sharp decline in expression was noted between 15 DAFB and 25 DAFB by ~8-fold and ~3-fold in *MdANT1* and *MdANT2*, respectively, and was coincident

with the initial decline in cell production. The expression of these genes declined further between 32 and 39 DAFB, coincident with exit from cell production, and remained low throughout the rest of fruit development. The above data indicate that the expression of *MdANT1*and *MdANT2* was closely associated with cell production during early fruit growth.

In addition to *MdANT1* and *MdANT2*, the expression of five *AILs* was studied during fruit development in 'Gala'. *MdAIL1*, *MdAIL2*, and *MdAIL3* displayed highest expression primarily before full bloom (Fig. 3.5). Their expression declined rapidly during early fruit development and remained low throughout the rest of fruit development (Fig. 3.5). *MdAIL4* and *MdAIL5* also displayed a similar pattern except that the expression of these genes transiently increased by ~6 and ~12-fold, respectively, between 14 to 18 DAFB and was followed by low levels of expression throughout the rest of fruit development (Fig. 3.5).

Expression of MdANT1 and MdANT2 is enhanced in response to increase in carbohydrate availability

In 'Golden Delicious Smoothee' (GS), manual thinning at 11 DAFB led to enhanced fruit growth and an increase in fruit size (Fig. 3.6A; Table 3.4). A 16% increase in fruit diameter was observed in thinned fruit by around 25 DAFB (P=0.004), indicating that thinning resulted in a rapid increase in early fruit growth. At maturity, thinned fruit had higher fruit diameter (~16%; P<0.001) and fruit weight (35%; P<0.001) than un-thinned fruit. Enhanced fruit growth during early fruit development in thinned fruit was primarily associated with an increase in cell production in the fruit cortex. Cell production was similar between thinned and un-thinned fruit until around 18 DAFB. The extent of cell production in the fruit cortex was higher in thinned fruit between 18 and 25 DAFB, than that in un-thinned fruit. Cell number in un-thinned fruit was

lower than that in thinned fruit by ~30% (P<0.001) at 25 DAFB, and remained lower during the rest of fruit development (Fig. 3.6B). The RCPR was 3-fold higher in thinned fruit at 25 DAFB (Fig. 3.6C). Cell area within the fruit cortex was significantly higher in thinned fruit in comparison to that in un-thinned fruit at 128 DAFB (~12%; P=0.019) and 150 DAFB (~11%; P<0.001; Fig. 3.6D). These data indicate that increase in carbohydrate availability due to thinning enhanced fruit growth primarily by increasing cell production during early fruit growth and cell expansion at later stages.

Expression of *MdANT1* and *MdANT2* was not significantly different between thinned and un-thinned fruit until after 18 DAFB (Fig. 3.7; Table 3.4). In comparison to un-thinned fruit, expression of *MdANT1* was almost 2-fold higher (P=0.005), while that of *MdANT2* was around 5-fold higher in thinned fruit at 25 DAFB (P<0.001). Expression of *MdANT1* in thinned fruit was also significantly higher at 32 DAFB (P<0.001), while that of *MdANT2* was significantly higher at 32 DAFB (~2-fold; P=0.009) and 50 DAFB (~2-fold; P=0.009). Interestingly, thinning resulted in a transient up-regulation in the expression of *MdANT2*. At 25 DAFB, expression of *MdANT2* was >3-fold and >2-fold higher than that at 11 and 18 DAFB respectively, in thinned fruit. Together, the above data indicate that enhanced expression of *MdANT1* and *MdANT2* due to thinning was associated with an increase in cell production.

MdANT1 and MdANT2 are differentially expressed across different fruit size genotypes during early fruit growth

Fruit growth and development were compared across ‘Gala’, a medium fruit size genotype, and GS, a large fruit size genotype. ‘Gala’ flowers were in full bloom ~7 days prior to that of GS. Hence, growing degree days after bloom (GDD) were used to allow for comparison of fruit

growth, cell production and gene expression parameters between ‘Gala’ and GS. Both genotypes displayed a similar pattern of fruit growth, except that GS had a longer growing period of 1544 GDD in comparison to 1187 GDD in ‘Gala’ (Fig. 3.8A; Table 3.5). The initial phase of fruit growth in ‘Gala’ involved a rapid increase in fruit diameter which continued until around 237 GDD after which fruit diameter increased linearly until fruit maturity. In GS, the initial phase of rapid fruit growth continued for a longer period (around 404 GDD) after which fruit diameter increased linearly until maturity. Final fruit diameter in GS was 23% higher than that in ‘Gala’. Both genotypes displayed a similar number of cell layers within the floral-tube at full bloom (0 GDD; Fig. 3.8B inset). ‘Gala’ and GS displayed differences in the pattern of progression in cell production within the fruit cortex. In ‘Gala’, cell number within the fruit cortex increased rapidly until around 62 GDD, continued to increase at a lower rate between 62 and 198 GDD, and remained largely unchanged thereafter. In GS, increase in cell number within the fruit cortex was observed from around 48 GDD until around 184 GDD after which it remained largely unchanged (Fig. 3.8B inset). Cell number at maturity in GS was almost 54% higher than that in ‘Gala’. The RCPR maxima in ‘Gala’ was around 0.14 cell $cell^{-1}$ GDD^{-1},while that in GS was around 0.024 cell $cell^{-1}$ GDD^{-1} (Fig. 3.8C). However, the peak in RCPR in ‘Gala’ was attained around 19 GDD while that in GS was attained at around 73 GDD (Fig. 3.8C). Potentially higher RCPR levels were maintained in GS than that in ‘Gala’ from around 73 GDD until the end of the cell production period. Final area of the fruit cortex cells in GS was around 2-fold higher in GS than that in ‘Gala’ (Fig. 3.8D).

MdANT1 expression in ‘Gala’ increased after bloom but was subsequently similar to that in GS until around 62 GDD (Fig. 3.9 inset; Table 3.5). After 62 GDD, transcript abundance of *MdANT1*declined rapidly in ‘Gala’ but continued to remain high in GS until around 184 GDD.

Around this period, *MdANT1* expression was substantially higher in GS (3- to 10-fold) in comparison to that in 'Gala'. Expression of *MdANT2* appeared to be slightly higher in GS than that in 'Gala' until around 48 GDD (Fig. 3.9 inset; Table 3.5). Between 62 and 198 GDD, the expression of *MdANT2* declined in 'Gala' by around 3-fold. During a similar period (73-184 GDD) the expression of *MdANT2* in GS increased by > 2-fold. Around 184 GDD, *MdANT2* expression was ~6-fold higher in GS in comparison to that in 'Gala'. At later stages of fruit development, a period of post mitotic cell expansion-mediated growth, the *ANTs* displayed very low levels of expression in both the genotypes.

MdANT1 and MdANT2 are differentially expressed between the core and the cortex

Apple is an accessory fruit (pome) where the floral-tube tissue surrounding the ovary develops into the fleshy and edible region of the fruit (cortex) while the ovary develops into the core [40]. Laser capture microdissection (LCM) was used to isolate these tissues and the localization of *MdANT1* and *MdANT2* expression within these tissues during early fruit development was studied. Expression of two MADS box genes, *MdMADS5* and *MdMADS10*, was also analyzed. Previous research indicated that *MdMADS5* was predominantly expressed in the cortex and the skin while *MdMADS10* was primarily expressed in the core tissue [41]. In the present study, *MdMADS5* expression was clearly higher in the cortex tissue than in the core by 2.5- to 11-fold during different stages of flower development and early fruit growth (-7 to 15 DAFB; Fig. 3.10). Also, the expression of *MdMADS10* was consistently higher in the core tissue than in the cortex by 5- to 9-fold between -7 and 15 DAFB (Fig. 3.10). These data are consistent with the previous report [39], and demonstrate that the cortex and core tissues were effectively isolated using LCM.

MdANT1 and *MdANT2* were expressed in the core as well as the cortex tissues during different stages of flower and early fruit development. Expression of these genes in both tissues was high before bloom and declined by up to 3-fold between -7 DAFB and full bloom (Fig. 3.11). *MdANT1* expression was almost 2-fold higher in the ovary tissue than the floral-tube at -7 DAFB. Between bloom and 10 DAFB, *MdANT1* and *MdANT2* expression increased by 3-fold and 4-fold respectively in the cortex tissue, while it remained largely unaltered in the core. At 15 DAFB, expression of *MdANT2* within the cortex continued to be higher than that in the core, but the expression of *MdANT1* in the core reached levels similar to that in the cortex.

MdANT1 and MdANT2 expression is correlated with that of cell cycle genes

Expression of several cell cycle genes was investigated in the core and cortex tissues isolated using LCM (Fig. 3.11). Genes positively associated with cell production such as *MdCYCA2;3*, *MdCYCB1;1*, *MdCDKB1;2*, *MdCDKB2;1* and *MdDEL1* [1] displayed expression patterns similar to that of *MdANT1* and *MdANT2* during early fruit growth and development. At 10 DAFB, expression of these genes was up to 3-fold higher in the cortex in comparison to that in the core. *MdANT1* and *MdANT2* expression was significantly correlated with that of *MdCYCA2;3*, *MdCYCB1;1*, *MdCDKB1;2*, *MdCDKB2;1* and *MdDEL1* [R = 0.86, 0.49, 0.75, 0.58, and 0.68 (with *MdANT1*); 0.83, 0.62, 0.45, 0.73 and 0.69 (with *MdANT2*), respectively]. *MdKRP4*, a gene negatively associated with cell production, displayed a gradual increase in expression in the core tissue during early fruit growth while it declined steadily within the cortex. At 15 DAFB, *MdKRP4* displayed ~10-fold higher expression in the core in comparison to that in the cortex. *MdKRP4* expression was not significantly correlated with either of the apple *ANT* genes. *MdKRP5*, another gene negatively associated with cell production, displayed a minor increase in

expression until 10 DAFB in the core and the cortex. At 15 DAFB, *MdKRP5* expression increased greatly (~10-fold) within the core but only slightly in the cortex, resulting in > 6-fold difference in expression between these tissues. *MdANT1* expression was weakly correlated with that of *MdKRP5* (R = 0.51; *P*=0.01).

Discussion

Several lines of evidence from this study suggest that *MdANT1* and *MdANT2* function as transcription factors in apple. A motif of basic residues (KKKR) is essential for the nuclear localization of ANT, as replacement of two lysine residues within this motif resulted in a loss of nuclear localization in Arabidopsis [39]. In MdANT1 and MdANT2, a major part of this element is conserved (TKKR), strongly suggesting that these ANTs are targeted to the nucleus, consistent with their proposed roles as transcription factors. The Arabidopsis ANT binds to the DNA at a consensus site of 16 bases through two AP2 domains and a conserved linker region [24]. MdANT1 and MdANT2 shared greater than 88% sequence identity with the Arabidopsis ANT within these regions. All of the 19 residues identified as essential for the DNA binding activity of the Arabidopsis ANT [25] are conserved in the apple ANTs suggesting that they may bind to similar DNA elements, further supporting their role as transcription factors. Domains within the amino-terminal region are also essential for the transcriptional activation properties of the Arabidopsis ANT [39]. Although the apple ANTs display limited conservation of residues with that of the Arabidopsis ANT in this region, it should be noted that other plant ANTs also display significant sequence divergence within this region, indicating that distinct, species-specific features may be required for the transcriptional activation properties of the ANTs.

MdANT1 and *MdANT2* are expressed in regions associated with fruit growth and development [a) ovary and floral-tube tissues before bloom; b) core and cortex tissues during early fruit growth]. *MdANT1* and *MdANT2* display high expression before bloom in the ovary as well as the floral-tube regions, strongly suggesting their association with cell production-mediated growth of the ovary and floral-tube tissues before bloom. Expression of *MdANT1* and *MdANT2* declines within these tissues during the period of temporary cessation of growth and quiescence in cell production (around full bloom). Subsequently, the expression of the *ANT*s increases sharply within the cortex tissue while little change in their expression is observed within the core tissue between bloom and 10 DAFB, coincident with the resumption of growth and re-initiation of cell production in the cortex during early fruit development. The sharp increase in expression at 10 DAFB within the cortex is likely triggered by pollination and/or fertilization and may mediate fruit set. *MdANT1* and *MdANT2* expression is high during the cell production-mediated phase of early fruit growth and subsequently declines greatly during exit from this phase. This pattern of expression is conserved under conditions of different carbohydrate availability and across genotypes differing in their fruit growth potential. Together, the data presented here indicate that the expression of *MdANT1* and *MdANT2* is consistently and closely associated with cell production during fruit growth in apple. Therefore, it is proposed that *ANTs* are important components of a developmental program that controls the extent of cell production and thereby regulates fruit growth in apple.

Cell production and fruit growth are limited by carbohydrate availability in many plant species [4,42-45]. Consistent with previous studies, increase in carbohydrate availability through manual thinning during early fruit development in GS enhanced fruit growth and final fruit size. This was primarily achieved through sustained cell production in the fruit cortex during early

fruit growth and a higher relative cell production rate, especially towards the later stages of the cell production phase. These data indicate that carbohydrate limitation due to increased competition among sinks decreases the extent of cell production in the fruit cortex. Under conditions of higher carbohydrate availability, the expression of *MdANT1* and *MdANT2* was several-fold higher than that under carbohydrate limitation. Additionally, *MdANT2* was up-regulated (>3-fold at 25 DAFB compared to 11 DAFB) in response to an increase in carbohydrate availability in GS. These data suggest that an increase in carbohydrate availability enhances the expression of the *ANT* genes, especially *MdANT2*, thereby increasing the competence of the fruit cortex cells for cell production. Hence, it may be proposed that the *ANTs*, particularly *MdANT2*, mediate the effects of carbohydrate availability on cell production and fruit growth. Increase in competence for cell production may be achieved either through an increase in the proportion of fruit cortex cells undergoing proliferation or through an increase in the capacity of individual cortex cells for division. Increase in carbohydrate availability also led to a minor increase in cell area during later stages of fruit growth in GS, inconsistent with previously reported results in the apple cultivar, 'Empire' [4], but consistent with results in tomato fruit [42,45]. It is likely that an increase in sink strength as a result of higher fruit cortex cell number in thinned fruit may subsequently aid in increasing the extent of cell expansion.

Comparison of apple genotypes differing in their growth potential further supports the proposed roles of the *ANTs* in regulating cell production. Although, it is possible that some of the differences observed between the two genotypes are due to environmental effects, the overall patterns of fruit growth and gene expression reported here were consistent with that observed in other studies during different years (data not shown). The initial cell number and the duration of the cell production phase were similar in 'Gala' and GS (around 198 and 184 GDD after bloom,

respectively), indicating that the higher final cell number within the fruit cortex of GS in comparison to that in 'Gala' was due to differences in the pattern of progression in cell production during early fruit development. GS fruit cortex cells displayed a more gradual increase in cell number after bloom in comparison to those of 'Gala' which displayed a short-lived early burst in cell production. In fact, the RCPR in GS reached the maxima around 54 GDD after that in 'Gala'. Subsequently, the rate of cell production in GS was higher than that in 'Gala', especially between 73 and 184 GDD after bloom. Expression of *MdANT1* and *MdANT2* in the two genotypes matched their respective patterns of cell production. Expression of these genes in GS was sustained at higher levels for a longer duration while in 'Gala', the expression of these genes displayed an initial rapid burst followed by a rapid decline. The expression of both these genes was higher in GS than in 'Gala' during the final stages of the cell production phase (around 129 and 184 DAFB). Sustained competence for cell production as a result of this pattern of expression of the *ANTs* may allow for enhanced cell production and a higher final cell number in GS. Final cell number is often an important determinant of variation in fruit size across genotypes [5,9,46]. Differences in the pattern of expression of the *ANT* genes during early fruit growth may affect the final cell number and thereby final fruit size across genotypes. Similarly, differences in the pattern of expression of *FW2.2* are thought to determine fruit size differences across tomato genotypes [47]. Hence, it is likely that *MdANT1* and *MdANT2* also function as regulators of fruit size in apple.

Expression of the apple *ANT* genes was correlated with that of several positive regulators of the cell cycle, including B-type CDKs, A- and B-type cyclins, and *MdDEL1* during different stages of flower and early fruit development. During the period of exit from cell production (around 15-25 DAFB in 'Gala'), the expression of several cell cycle genes positively associated

with cell production declined, while that of genes negatively associated with cell production increased [1]. These changes in the expression of the cell cycle genes coincide with the decline in the expression of *MdANT1* and *MdANT2* observed in this study. In fact, the expression patterns of the *ANT* genes during fruit growth display high similarity with those of the core cell cycle genes involved primarily in the regulation of the G2-M phases of the cell cycle. Co-expression of these genes suggests coordinated regulation and their involvement in a common biological process [48]. Considering that the *ANT* genes may function as transcription factors, it is possible that *MdANT1* and *MdANT2* regulate the expression of the core cell cycle genes and thereby coordinate cell production during fruit growth. In Arabidopsis, increased cell production as a result of the over-expression of *ANT* was associated with the prolonged expression of D3-type cyclins [19]. Identification of the genes targeted for direct regulation by the ANTs is essential to test this hypothesis.

The general similarities in the expression patterns of *MdANT1* and *MdANT2* suggest overlapping roles for these genes in regulating flower and fruit development. In Arabidopsis, expression of four *PLT* genes (members of the AP2 sub-family) in overlapping as well as specific regions of the root allows for PLT concentration-dependent regulation of root growth and development [32]. Similarly in apple, the combined activity of MdANT1 and MdANT2 may have an additive effect on cell production and fruit growth. However, certain key differences between *MdANT1* and *MdANT2* were also noted. The expression of these genes in the core tissue differed slightly during early fruit development. MdANT1 and MdANT2 also differed within the AP2-repeats and linker region in three residues (A354-S352; T365-A363; S388-F386, MdANT1-MdANT2, respectively). If the DNA binding characteristics are affected by the above residues, MdANT1 and MdANT2 may regulate different pools of downstream target genes. Together, the

above data suggest that *MdANT1* and *MdANT2* may also have distinct roles in regulating fruit growth and development. Functional characterization of *MdANT1* and *MdANT2* and the identification of their downstream targets *in vivo* are essential to determine their specific roles in regulating fruit growth.

All of the *AIL* genes studied here contained the characteristic AP2-repeats and the conserved linker region suggesting that they function as transcriptional regulators. These genes displayed elevated expression during flower development and a sharp decline in expression during early fruit development, suggesting that they may be primarily involved in regulating flower growth and development in apple. In Arabidopsis, many of the *AIL* genes are involved in regulating floral organ growth and development [21,34,35]. MdAIL4 and MdAIL5 share significant amino acid identity with AtAIL5 and AtAIL6 respectively, genes which have been previously reported to regulate organ growth [21,34,35]. Further characterization of the tissue-specific patterns of expression and the functional characterization of the *AIL* genes is essential to determine their specific roles in apple.

Data presented here strongly suggest that *MdANT1* and *MdANT2* regulate cell production and fruit growth in apple by coordinating the expression of genes involved in cell proliferation. *MdANT1* and *MdANT2* are a significant addition to the limited list of candidate upstream regulatory genes involved in the control of growth of fleshy fruit. Functional characterization of these genes and the identification of their downstream targets may greatly aid in unraveling the mechanisms involved in the regulation of fruit growth in apple and other fleshy fruit.

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Table 3.1. *List of primer sequences used for sequencing MdANT1 and MdANT2.*

Primer Name and Orientation	**Type of Primer**	**Primer Sequence 5′-3′**
NANT Reverse	Gene specific primer for PCR amplification of *MdANT15′* end	CCAATGCCGTTGAGAAGGAAGGG
NANT' Reverse	Gene specific primer for PCR amplification of *MdANT2* end	TCCTCCAATGCCATTGAGAATGAGAGA
DP1 Forward	Degenerate primer for 5′ sequencing	ATGCCRCTNARRTCNGAYGG
DP2 Forward	Degenerate primer for 5′ sequencing	CCNAARCTNGARGAYTTYTT
GSANT1 Reverse	Gene specific primer for PCR amplification of *MdANT1*	GGGTTATGCTCAATGGCCAGG
GSANT2 Reverse	Gene specific primer for PCR amplification of *MdANT2*	TTATACTCAATGGCTGGCGCTG
PANTF1 Forward	Primer designed from Peach *ANT*	GTTCTCACTCTCACCCCACATGAA
PANTF2 Forward	Primer designed from Peach *ANT*	GTTGCTTCTGACCCTCATCAGCAT
PANTF3 Forward	Primer designed from Peach *ANT*	GGTCAGGCTTCTTCAGCTGCTG
PANTF4 Forward	Primer designed from Peach *ANT*	ATGAATGATCACAATAATAACAACAATGGA
PANTF5 Forward	Primer designed from Peach *ANT*	AACTGGTTGGGGTTCTCACTCTC
PANTR1 Reverse	Primer designed from Peach *ANT*	GTCACGCCTCGGTACATTGAAGC
PANTR2 Reverse	Primer designed from Peach *ANT*	CCAAGATAAAGATCCTTGTTCCCAGC

PANTR3 Reverse	Primer designed from Peach *ANT*	TCAAAGTTGGTGACCGCATTTGCG
S1Primer Forward	Forward primer for full length sequencing of *MdANT1*	CTGTCTTTAGAGAGAGAAACACAGTG
S2Primer Forward	Forward primer for full length sequencing of *MdANT2*	TGTGAGTGCATAGAAGGAAGTGTAT
S2PrimerR1 Reverse	Reverse primer for full length sequencing of *MdANT2*	CTCCACTAATTACTTAACCCTCACCTC
S2PrimerR2 Reverse	Reverse primer for full length sequencing of *MdANT2*	CATGCAAAAATCTTTGAAGGCATTTCAG
S1PrimerR1 Reverse	Reverse primer for full length sequencing of *MdANT1*	AGAATTTCCTCCACTAATTACTTACCCTAA
S1PrimerR2 Reverse	Reverse primer for full length sequencing of *MdANT1*	AATTTCTTCCCATTTTTCCTTGTTCAAT

Table 3.2. List of primers used for analysis of *ANTs*, *AILs*, and *MdMADS*' gene expression with qRT-PCR.

Gene	**Primer Orientation**	**Primer Sequence (5′-3′)**
MdAIL1	Forward	CCTCAGAACCCATGCGATGATCTTG
	Reverse	GCCATGTTGTTCTGGTCCATGGAA
MdAIL2	Forward	CATAACACGGTATGATGTGGACCGA
	Reverse	TCCCCATTGCCGATTTGCGAA
MdAIL3	Forward	CTTCGGCCAACGCACATCCATTTA
	Reverse	AGATCGTAGGCTCTTGCTGCCTTT
MdAIL4	Forward	GCGGCCATAAAGTTTAGGGGCATT
	Reverse	TCTGCTCTTCAGCTTCGAGTGAGAG
MdAIL5	Forward	CTCCCTTCTTGTCTGCACCACTTC
	Reverse	TCTTCGGGCTGAAATAAAGCGAAACTTG
MdANT1	Forward	CACCAAGGTGATCGAACCTAACATCCTG
	Reverse	CCAATGCCGTTGAGAAGGAAGGG
MdANT2	Forward	CCAAGGTGATCGAACCTAACATTGCAG
	Reverse	TCCTCCAATGCCATTGAGAATGAGAGA
MdMADS5	Forward	ATCCATCTCTGAGCTTCAGAGAAAGAG
	Reverse	GCTGTGGAAGCAGGTCAAGGC
MdMADS10	Forward	CACTTAATGGGAGATGCCTTGAGCACT
	Reverse	GCCTCTCGACTTCTGATACCTTAGTTCG

Table 3.3. *Growth and gene expression during fruit development in 'Gala'.*

The table displays data corresponding to Fig. 3.3 for Fruit diameter (Fig. 3.3A), Cell layers (Fig. 3.3), Relative cell production rate (RCPR; Fig. 3.3C) and Cell area (Fig. 3.3D). The table also displays expression data for *MdANT1* and *MdANT2* from Fig. 3.4. Fruit diameter was not measured at 10 days after full bloom (DAFB). RCPR data were rounded off to the third decimal point. Gene expression was normalized using *MdGAPDH* and *MdACTIN*. Expression of a gene relative to its expression at 0 DAFB is presented. The mean and standard error of four biological replicates are displayed.

DAFB	Fruit diameter (mm)	Cell layers	RCPR (cell cell^{-1} day^{-1})	Cell area (× 1000 µm^2)	*MdANT1* (Relative expression)	*MdANT2* (Relative expression)
0	2.7 ± 0.03	13.7 ± 0.51		0.22 ± 0.009	1 ± 0.07	1 ± 0.03
7	3.5 ± 0.05	15.3 ± 0.53	0.02 ± 0.003	0.23 ± 0.004	1.39 ± 0.17	1.36 ± 0.26
10	-	27.97 ± 0.80	0. 20 ± 0.003	0.27 ± 0.01	0.93 ± 0.17	0.92 ± 0.05
15	5.7 ± 0.16	55.1 ± 0.69	0.14 ± 0.006	0.39 ± 0.006	1.12 ± 0.12	0.97 ± 0.17
25	15.5 ± 0.32	68.8 ± 1.02	0.02 ± 0.005	0.73 ± 0.006	0.15 ± 0.05	0.38 ± 0.07
32	21.3 ± 0.22	76.8 ± 2.14	0.02 ± 0.002	1.60 ± 0.06	0.47 ± 0.03	0.38 ± 0.03
39	26.3 ± 0.22	78.1 ± 1.66	0.002 ± 0.001	3.00 ± 0.07	0.10 ± 0.03	0.20 ± 0.06
57	33.6 ± 0.34	80 ± 0.52	0.001 ± 0.001	5.67 ± 0.11	0.11 ± 0.02	0.21 ± 0.02
86	54.9 ± 0.94	79.8 ± 0.22	0.000 ± 0.000	17.31 ± 0.52	0.03 ± 0.01	0.09 ± 0.02
123	67.3 ± 1.74	80.3 ± 0.66	0.000 ± 0.000	20.67 ± 0.56	0.01 ± 0.004	0.004 ± 0.001

Table 3.4: *Growth and gene expression in thinned [A] and un-thinned fruit [B] of 'Golden Delicious Smoothee'.*

The table shows data corresponding to Fig. 3.6 for Fruit diameter (Fig. 3.5A), Cell layers (Fig. 3.6B), Relative cell production rate (RCPR; Fig. 3.6C) and Cell area (Fig. 3.6D). The table also shows expression data for *MdANT1* and *MdANT2* from Fig. 3.7. Fruit diameter was not measured at 11 days after full bloom (DAFB). RCPR data were rounded off to the third decimal point. Expression of a gene is presented relative to its expression at 0 DAFB in thinned fruit. Gene expression was normalized using *MdGAPDH* and *MdACTIN*. The mean and standard error of four biological replicates are presented.

DAFB	Fruit diameter (mm)	Cell layers	RCPR (cell cell^{-1} day^{-1})	Cell area (× 1000 µm^2)	*MdANT1* (Relative expression)	*MdANT2* (Relative expression)
0	3.2 ± 0.03	12.7 ± 0.73		0.30 ± 0.01	1.00 ± 0.21	1.00 ± 0.13
8	5.5 ± 0.03	23.7 ± 0.60	0.09 ± 0.006	0.38 ± 0.002	1.17 ± 0.22	1.67 ± 0.39
11	-	42.7 ± 0.60	0.20 ± 0.013	0.48 ± 0.05	0.91 ± 0.16	0.85 ± 0.10
18	11.9 ± 0.27	76.3 ± 0.89	0.09 ± 0.003	0.58 ± 0.01	0.71 ± 0.18	1.22 ± 0.14
25	20.2 ± 0.39	120.8 ± 1.07	0.06 ± 0.003	1.47 ± 0.04	1.04 ± 0.2	2.92 ± 0.62
32	26.5 ± 0.29	122.4 ± 1.05	0.002 ± .001	3.16 ± 0.11	0.29 ± 0.08	0.69 ± 0.08
50	41.0 ± 0.4	124.1 ± 0.34	0.001 ± 0.001	5.16 ± 0.17	0.12 ± 0.02	0.30 ± 0.05
79	59.9 ± 0.19	122.5 ± 0.28	0.000 ± 0.000	18.13 ± 0.74	0.03 ± 0.007	0.06 ± 0.02
128	78.7 ± 0.55	125.0 ± 1.73	0.000 ± 0.000	24.89 ± 0.46	0.21 ± 0.05	0.06 ± 0.02
150	83.0 ± 0.25	123.5 ± 0.34	0.000 ± 0.000	45.08 ± 2.33	0.01 ± 0.002	0.02 ± 0.003

Table 3.5. *Fruit growth and gene expression in 'Gala' [A] and 'Golden Delicious Smoothee' [B]*.

The table displays data corresponding to Fig. 3.8 for Fruit diameter (Fig. 3.8A), Cell layers (Fig. 3.8B), Relative cell production rate (RCPR; Fig. 3.8C) and Cell area (Fig. 3.8D). Fruit diameter was not measured at 19 growing degree days (GDD) after full bloom in 'Gala' and at 73 GDD after full bloom in 'Golden Delicious Smoothee'. RCPR data was rounded off to the third decimal point. The table also shows expression data for *MdANT1* and *MdANT2* from Fig. 3.9. Expression of a gene is presented relative to its expression at 0 GDD in 'Gala'. Gene expression was normalized using *MdGAPDH* and *MdACTIN*. Data for 'Golden Delicious Smoothee' are from thinned fruit only. The mean and standard error of four biological replicates are presented here.

GDD	Fruit diameter (mm)	Cell layers	RCPR (cell cell^{-1} degree day^{-1})	Cell area (× 1000 µm^2)	*MdANT1* (Relative expression)	*MdANT2* (Relative expression)
0	2.7 ± 0.03	13.7 ± 0.51		0.22 ± 0.009	1 ± 0.07	1 ± 0.03
15.0	3.5 ± 0.05	15.3 ± 0.53	0.008 ± 0.003	0.23 ± 0.004	1.39 ± 0.17	1.36 ± 0.26
19.3	-	27.97 ± 0.80	0.14 ± 0.01	0.27 ± 0.01	0.93 ± 0.17	0.92 ± 0.05
62.6	5.7 ± 0.16	55.1 ± 0.69	0.016 ± 0.000	0.39 ± 0.006	1.12 ± 0.12	0.97 ± 0.17
143.6	15.5 ± 0.32	68.8 ± 1.02	0.003 ± 0.000	0.73 ± 0.006	0.15 ± 0.05	0.38 ± 0.07
198.1	21.3 ± 0.22	76.8 ± 2.14	0.002 ± 0.000	1.60 ± 0.06	0.47 ± 0.03	0.38 ± 0.03
237.7	26.3 ± 0.22	78.1 ± 1.66	0.000 ± 0.001	3.00 ± 0.07	0.10 ± 0.03	0.20 ± 0.06
417.8	33.6 ± 0.34	80 ± 0.52	0.000 ± 0.000	5.67 ± 0.11	0.11 ± 0.02	0.21 ± 0.02
759.6	54.9 ± 0.94	79.8 ± 0.22	0.000 ± 0.000	17.31 ± 0.52	0.03 ± 0.01	0.09 ± 0.02
1187.2	67.3 ± 1.74	80.3 ±	0.000 ±	20.67 ± 0.56	0.01 ± 0.004	0.004 ±

		0.66	0.000			0.001

Figure 3.1. *Comparison of the predicted amino acid sequences of plant ANTs.*

(A) Phylogenetic analysis of two apple ANTs, and Arabidopsis ANT and AILs was performed using the neighbor joining distance method of MUSCLE. Sequences for Arabidopsis ANT and AILs were retrieved from the NCBI database. The accession numbers for Arabidopsis AILs are: AtAIL1 (AT1G72570); AtAIL2 (AT5G17430); AtAIL3 (AT3G20840); AtAIL4 (AT1G51190); AtAIL5 (AT5G57390); AtAIL6 (AT5G10510); AtAIL7 (AT5G65510) (B) Phylogenetic analysis of two ANTs and five AILs from apple. The apple AIL sequences were retrieved from the apple genome database. The accession numbers for the apple AILs are: AIL1 (MDP0000178745); AIL2 (MDP0000801540); AIL3 (MDP0000121984); AIL4 (MDP0000277643); AIL5 (MDP0000211931). (C) Phylogenetic analysis of ANTs from apple and other plants. Sequences for the ANTs used here were retrieved from the NCBI database and Genome Database for Rosaceae. *Arabidopsis thaliana* (AtANT; ABR21533), *Brassica napus* (BnANT; ABA42146), *Artemisia annua* (AaANT; ACY74336), *Triticum aestivum* (TaANT; AB458518.1), *Oryza sativa* (OsANT; AK106306.1), *Sorghum bicolor* (SbANT; XM_002468181.1), *Hordeum vulgare* (HvANT; AK375318.1), *Malus* × *domestica* (MdANT1), *Malus* × *domestica* (MdANT2), *Prunus persica* (PpANT; ppa023077m), *Fragaria* × *ananassa* (FaANT; scf0512968), *Nicotiana tabacum* (NtANT; AAR22388), *Vitis vinifera* (VvANT; AM444297), *Populus trichocarpa* (PtANT; AC210555).

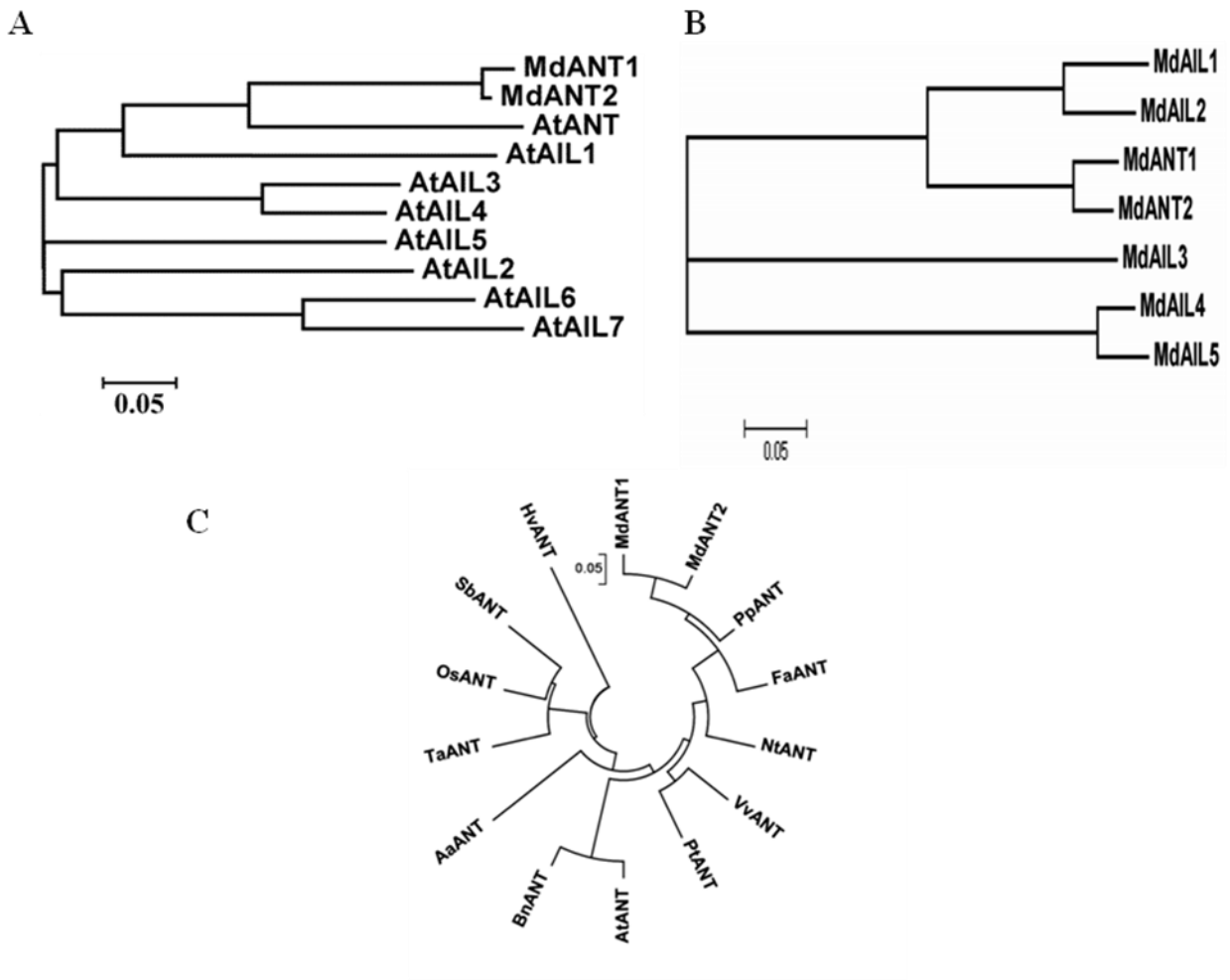
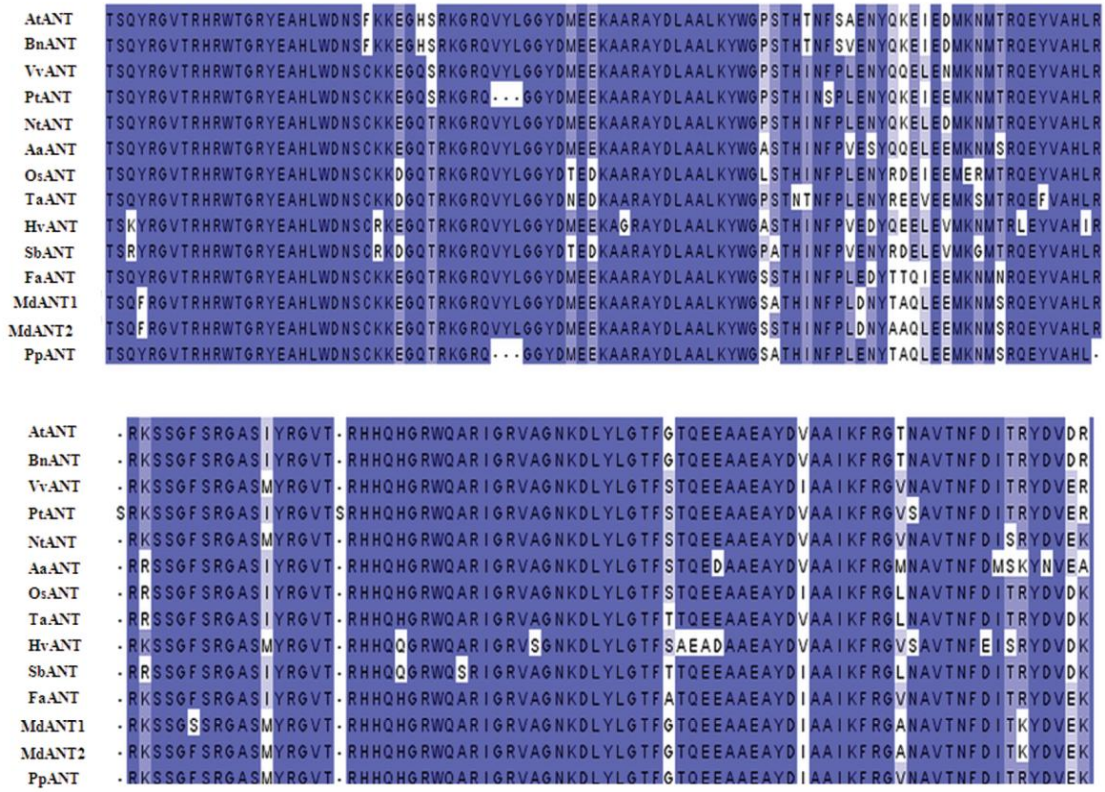

A
MdANT1
MdANT2
AtANT
AtAIL1
AtAIL3
AtAIL4
AtAIL5
AtAIL2
AtAIL6
AtAIL7
0.05
B
MdAIL1
MdAIL2
MdANT1
MdANT2
MdAIL3
MdAIL4
MdAIL5
0.05
C
HvANT
MdANT1
MdANT2
PpANT
FaANT
NtANT
VvANT
PtANT
AtANT
BnANT
AaANT
TaANT
OsANT
SbANT
0.05

Figure 3.2. *Comparison of predicted amino acid sequences within the DNA binding domain of ANT from higher plants.*

The predicted amino acid sequence of the two AP2 domains and linker region are shown for 14 ANTs from 13 plants. Sequences were aligned using MUSCLE software. The scientific name along with the protein name and the region corresponding to the AP2 domains and the linker regions, is indicated within the parenthesis. *Arabidopsis thaliana* (AtANT; 280-451), *Brassica napus* (BnANT; 284-454), *Artemisia annua* (AaANT; 157-327), *Triticum aestivum* (TaANT; 283-453), *Oryza sativa* (OsANT; 289-459), *Hordeum vulgare* (HvANT; 293-463), *Sorghum bicolor* (SbANT; 216-386), *Malus* × *domestica* (MdANT1; 291-462), *Malus* × *domestica* (MdANT2; 289-460), *Prunus persica* (PpANT; 305-471), *Fragaria vesca* (FvANT; 260-430), *Nicotiana tabacum* (NtANT; 309-479), *Vitis vinifera* (VvANT; 256-426), *Populus trichocarpa* (PtANT; 265-434).


AtANT TSQYRGVTRHRWTGRYEAHLWDNSFKKEGHSRKGRQVYLGGYDMEEKAARAYDLAALKYWGPSTHTNFSAENYQKEIEDMKNMTRQEYVAHLR
BnANT TSQYRGVTRHRWTGRYEAHLWDNSFKKEGHSRKGRQVYLGGYDMEEKAARAYDLAALKYWGPSTHTNFSVENYQKEIEDMKNMTRQEYVAHLR
VvANT TSQYRGVTRHRWTGRYEAHLWDNSCKKEGQSRKGRQVYLGGYDMEEKAARAYDLAALKYWGPSTHINFPLENYQQELENMKNMTRQEYVAHLR
PtANT TSQYRGVTRHRWTGRYEAHLWDNSCKKEGQSRKGRQ...GGYDMEEKAARAYDLAALKYWGPSTHINSPLENYQKEIEEMKNMTRQEYVAHLR
NtANT TSQYRGVTRHRWTGRYEAHLWDNSCKKEGQTRKGRQVYLGGYDMEEKAARAYDLAALKYWGPSTHINFPLENYQKELEDMKNMTRQEYVAHLR
AaANT TSQYRGVTRHRWTGRYEAHLWDNSCKKEGQTRKGRQVYLGGYDMEEKAARAYDLAALKYWGASTHINFPVESYQQELEEMKNMSRQEYVAHLR
OsANT TSQYRGVTRHRWTGRYEAHLWDNSCKHDGQTRKGRQVYLGGYDTEDKAARAYDLAALKYWGLSTHINFPLENYRDEIEEMERMTRQEYVAHLR
TaANT TSQYRGVTRHRWTGRYEAHLWDNSCKKDGQTRKGRQVYLGGYDNEDKAARAYDLAALKYWGPSTNTNFPLENYREEVEEMKSMTRQEFVAHLR
HvANT TSKYRGVTRHRWTGRYEAHLWDNSCRKEGQTRKGRQVYLGGYDMEEKAGRAYDLAALKYWGASTHINFPVEDYQEELEVMKNMTRLEYVAHIR
SbANT TSRYRGVTRHRWTGRYEAHLWDNSCRKDGQTRKGRQVYLGGYDTEDKAARAYDLAALKYWGPATHINFPVENYRDELEVMKGMTRQEYVAHLR
FaANT TSQYRGVTRHRWTGRYEAHLWDNSCKKEGQTRKGRQVYLGGYDMEEKAARAYDLAALKYWGSSTHINFPLEDYTTQIEEMKNMNRQEYVAHLR
MdANT1 TSQFRGVTRHRWTGRYEAHLWDNSCKKEGQTRKGRQVYLGGYDMEEKAARAYDLAALKYWGSATHINFPLDNYTAQLEEMKNMSRQEYVAHLR
MdANT2 TSQFRGVTRHRWTGRYEAHLWDNSCKKEGQTRKGRQVYLGGYDMEEKAARAYDLAALKYWGSSTHINFPLDNYAAQLEEMKNMSRQEYVAHLR
PpANT TSQYRGVTRHRWTGRYEAHLWDNSCKKEGQTRKGRQ...GGYDMEEKAARAYDLAALKYWGSATHINFPLENYTAQLEEMKNMSRQEYVAHL.

AtANT .RKSSGFSRGASIYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDVAAIKFRGTNAVTNFDITRYDVDR
BnANT .RKSSGFSRGASIYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDVAAIKFRGTNAVTNFDITRYDVDR
VvANT .RKSSGFSRGASMYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGVNAVTNFDITRYDVER
PtANT SRKSSGFSRGASIYRGVTSRHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDVAAIKFRGVSAVTNFDITRYDVER
NtANT .RKSSGFSRGASMYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDVAAIKFRGVNAVTNFDISRYDVEK
AaANT .RRSSGFSRGASIYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFSTQEDAAEAYDVAAIKFRGMNAVTNFDMSKYNVEA
OsANT .RRSSGFSRGASIYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFSTQEEAAEAYDIAAIKFRGLNAVTNFDITRYDVDK
TaANT .RRSSGFSRGASIYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFTTQEEAAEAYDVAAIKFRGLNAVTNFDITRYDVDK
HvANT .RKSSGFSRGASMYRGVT.RHHQQGRWQARIGRVSGNKDLYLGTFSAEADAAEAYDVAAIKFRGVSAVTNFEISRYDVDK
SbANT .RRSSGFSRGASIYRGVT.RHHQQGRWQSRIGRVAGNKDLYLGTFTTQEEAAEAYDIAAIKFRGLNAVTNFDITRYDVDK
FaANT .RKSSGFSRGASIYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFATQEEAAEAYDIAAIKFRGVNAVTNFDITRYDVEK
MdANT1 .RKSSGSSRGASMYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDIAAIKFRGANAVTNFDITKYDVEK
MdANT2 .RKSSGFSRGASMYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDIAAIKFRGANAVTNFDITKYDVEK
PpANT .RKSSGFSRGASMYRGVT.RHHQHGRWQARIGRVAGNKDLYLGTFGTQEEAAEAYDIAAIKFRGVNAVTNFDITRYDVEK

Figure 3.3. *Fruit and cell growth-related parameters during fruit development in 'Gala'.*
(A) Fruit diameter, (B) cell number (layers), (C) relative cell production rate (RCPR) and (D) cell area were measured from full bloom until maturity. Error bars represent standard error of the mean and are smaller than the symbol when not visible. Four biological replicates were used in this study (n=4).

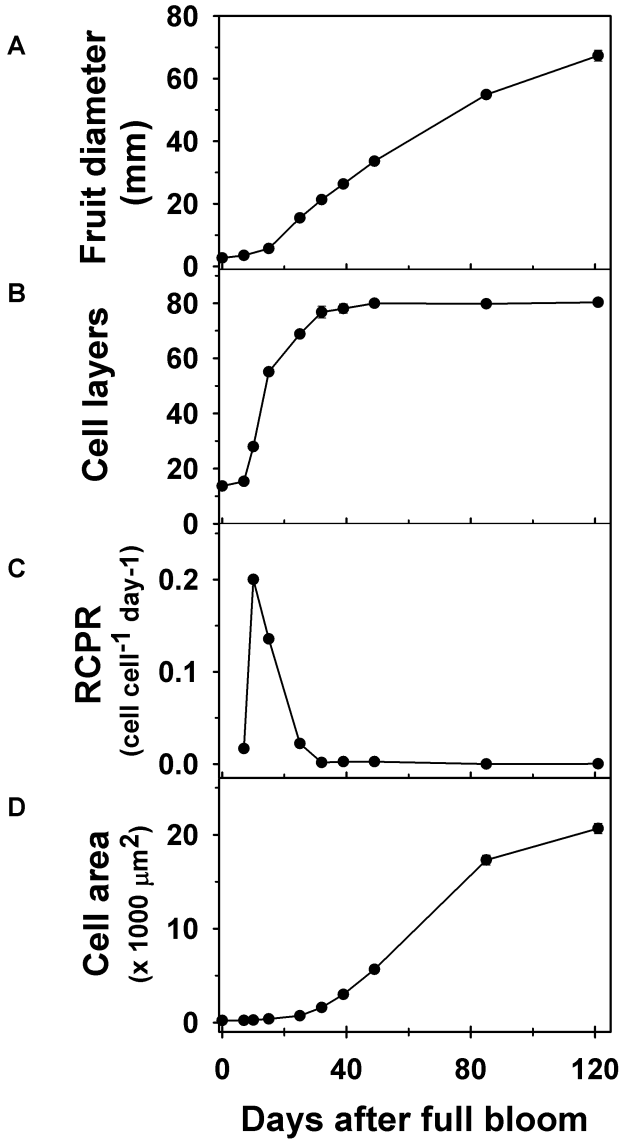

A
Fruit diameter (mm)
80
60
40
20
0
B
Cell layers
80
60
40
20
0
C
RCPR
(cell cell-1 day-1)
0.2
0.1
0.0
D
Cell area
(x 1000 μm2)
20
10
0
0
40
80
120
Days after full bloom

Figure 3.4. *Expression of MdANT1 and MdANT2 during fruit development in 'Gala'.*

Fold-change in the expression of a gene relative to its expression at full bloom (0 DAFB) is presented here. *MdACTIN* and *MdGAPDH* were used as the reference genes. Error bar represents the standard error of the mean of four biological replicates (*n*=4).

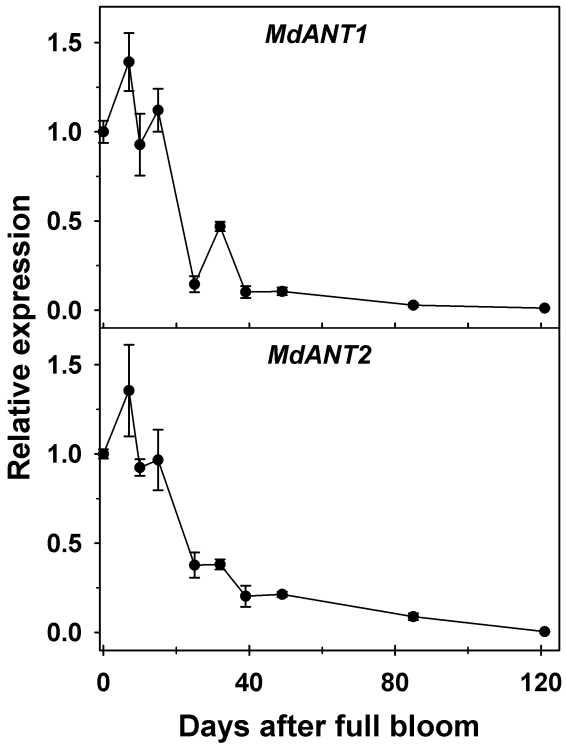

MdANT1
MdANT2
Relative expression
1.5
1.0
0.5
0.0
0
40
80
120
Days after full bloom

Figure 3.5. *Expression of the AIL genes during fruit development in 'Gala'.*

The normalization factor was determined as the geometric mean of expression of *MdGAPDH* and *MdACTIN*. Fold change in expression is presented relative to expression during full bloom. Error bar represents the standard error of the mean of four biological replicates (*n*=4).

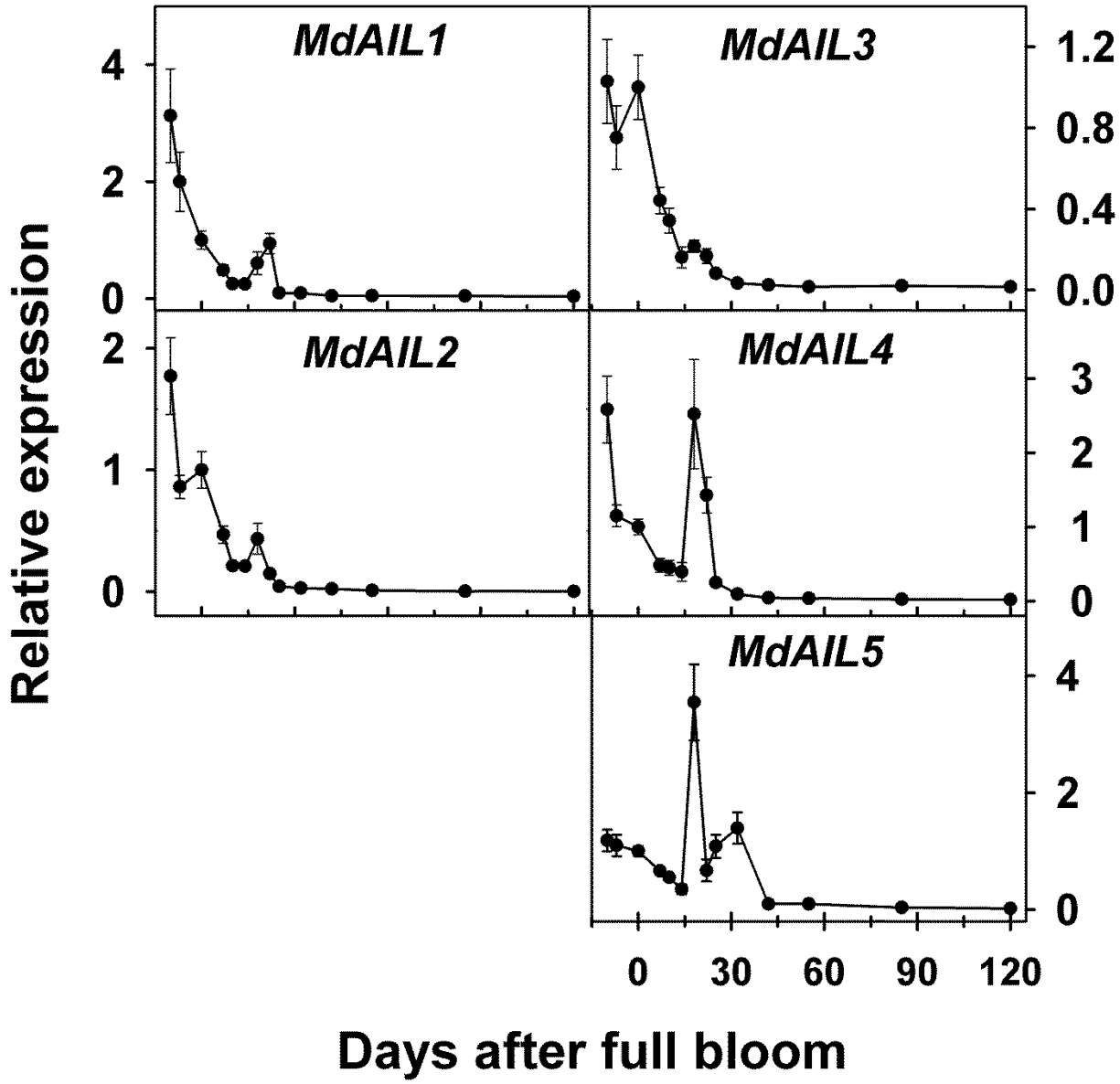

MdAIL1
MdAIL3
MdAIL2
MdAIL4
MdAIL5
Relative expression
Days after full bloom
0
30
60
90
120

Figure 3.6. *Fruit growth and cell growth-related parameters during fruit development in thinned and un-thinned fruit of 'Golden Delicious Smoothee' (GS).*

(A) Fruit diameter, (B) cell number (layers), (C) relative cell production rate (RCPR) and (D) cell area were measured from bloom to maturity. Closed circles represent un-thinned fruit and open circles represent thinned fruit. Dotted line represents the day of manual thinning (11 DAFB). Error bars represent the standard error of the mean of four biological replicates (n=4).

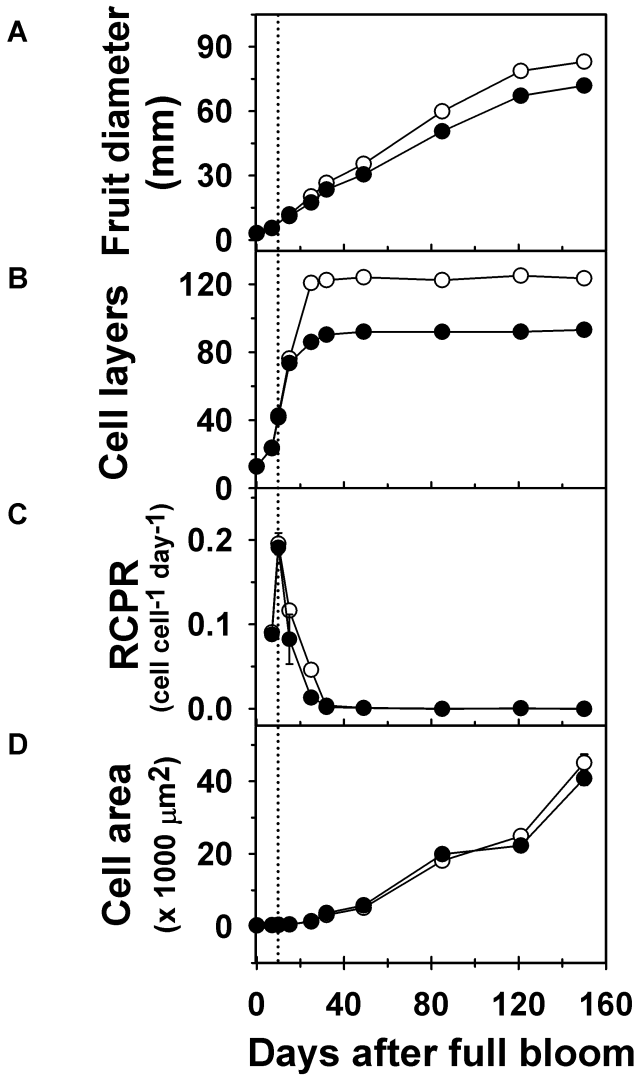

A
Fruit diameter (mm)
90
60
30
0
B
Cell layers
120
80
40
0
C
RCPR
(cell cell-1 day-1)
0.2
0.1
0.0
D
Cell area
(x 1000 μm2)
40
20
0
0
40
80
120
160
Days after full bloom

Figure 3.7. *Expression of MdANT1 and MdANT2 in thinned and un-thinned 'Golden Delicious Smoothee' (GS) fruit.*

Gene expression was analyzed from bloom to maturity. Dotted line represents the day of thinning (11 DAFB). Fold-change in the expression of a gene relative to its expression in thinned fruit at full bloom (0 DAFB) is presented here. *MdACTIN* and *MdGAPDH* were used as the reference genes in this study. Error bar represents the standard error of mean of four biological replicates (n=4). Closed circles represent un-thinned fruit and open circles represent thinned fruit.

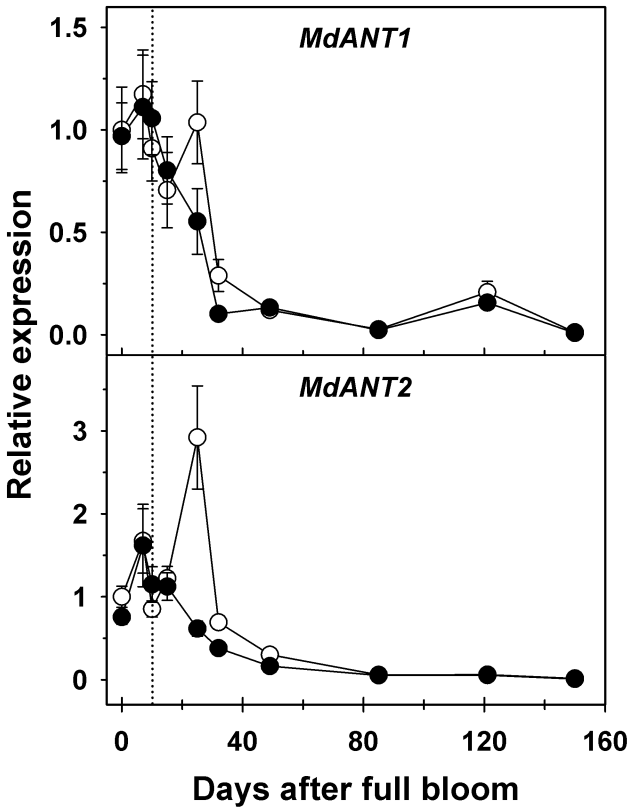

MdANT1
MdANT2
Relative expression
Days after full bloom
1.5
1.0
0.5
0.0
3
2
1
0
0
40
80
120
160

Figure 3.8. *Comparison of fruit growth and cell growth-related parameters during fruit development in 'Gala' and 'Golden Delicious Smoothie' (GS).*

(A) Fruit diameter, (B) cell number (layers), (C) relative cell production rate, and (D) cell area were measured from bloom until maturity. Closed circles represent 'Gala' and open circles represent GS. Error bar represents the standard error of the mean of four biological replicates (*n*=4). Data presented here are from Figures 2 and 4 (thinned fruit only). Cumulative growing degree days after full bloom (GDD) were used to allow for comparison across the two genotypes. Insets display changes in fruit diameter, cell number and RCPR during early fruit development.

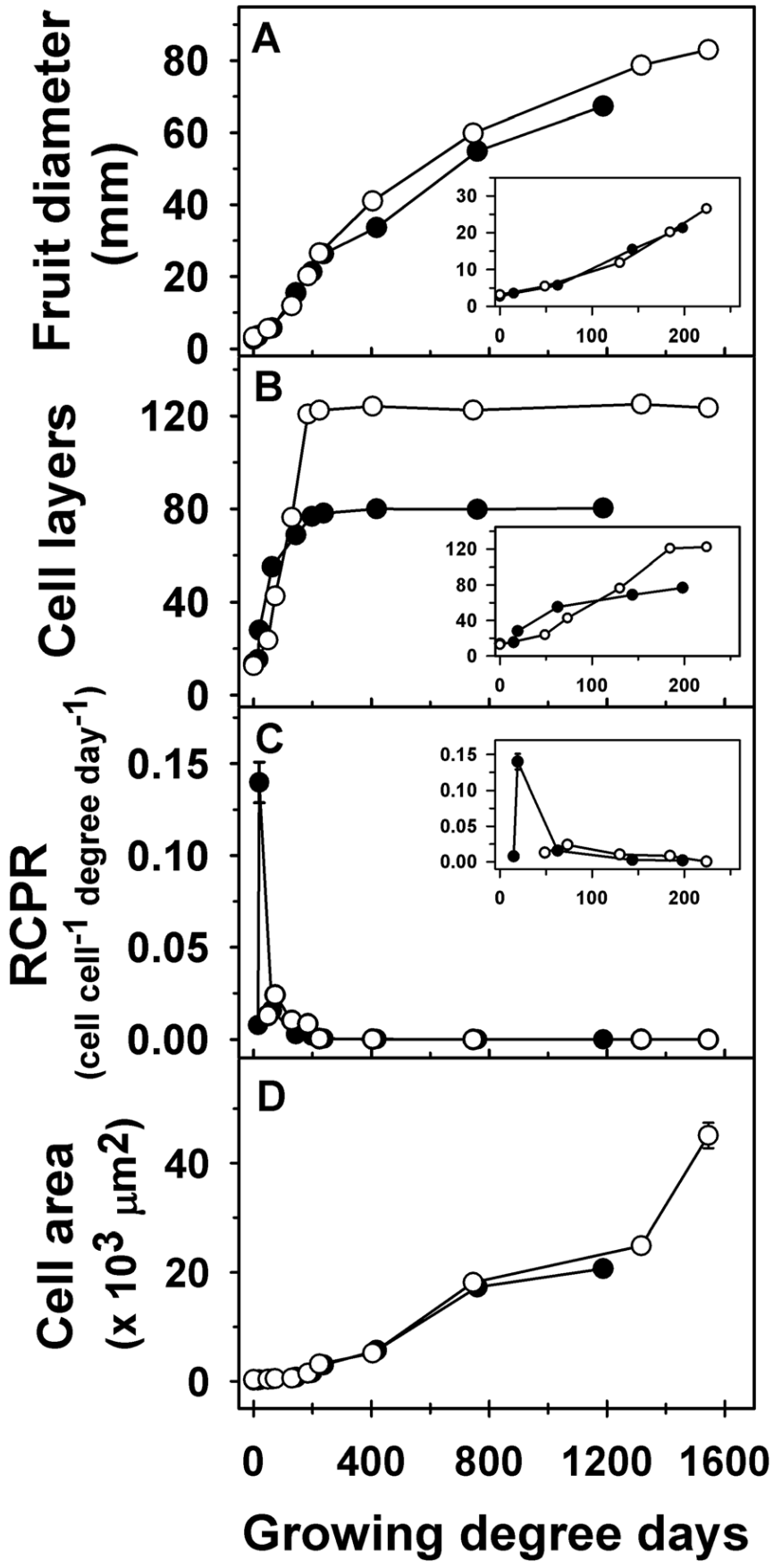

A
B
C
D
Fruit diameter (mm)
Cell layers
RCPR (cell cell-1 degree day-1)
Cell area (x 10^3 μm2)
Growing degree days
0
400
800
1200
1600

Figure 3.9. *Comparison of expression of MdANT1 and MdANT2 during fruit development in 'Gala' and 'Golden Delicious Smoothie' (GS).*

Closed circles represent 'Gala' and open circles represent GS. Data presented here are from Figures 3 and 5 (thinned fruit only). Cumulative growing degree days after full bloom (GDD) were used to allow for comparison of the two genotypes. Fold-change in the expression of a gene relative to its expression at full bloom (0 DAFB) in 'Gala' is presented. Error bar represents the standard error of mean of four biological replicates (n=4). The reference genes *MdACTIN* and *MdGAPDH* were used in this study. Insets display the expression of the genes during early fruit development.

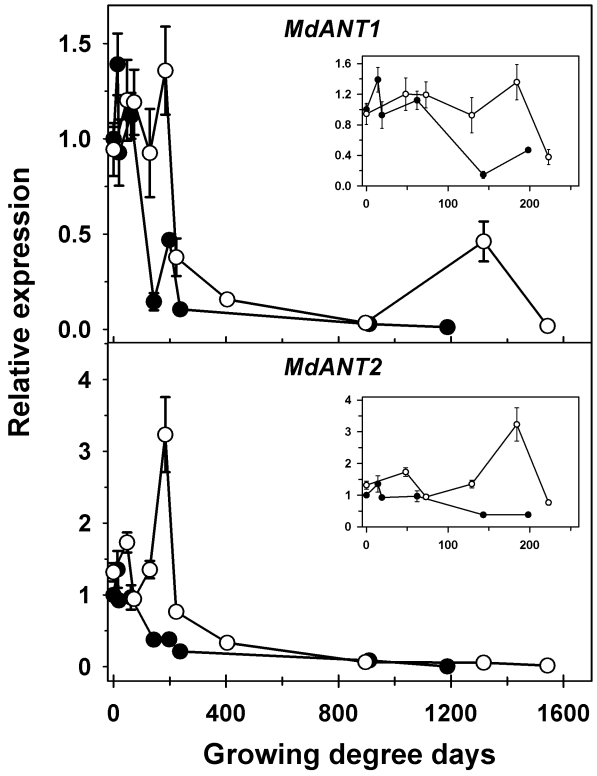

MdANT1
MdANT2
Relative expression
Growing degree days
0
400
800
1200
1600
0.0
0.5
1.0
1.5
0
1
2
3
4
0.0
0.4
0.8
1.2
1.6
0
100
200
0
1
2
3
4

Figure 3.10. *Expression of MdMADS5 and MdMADS10 in the core and the cortex tissues of 'Gala' apple during flower and early fruit development.*

Closed circles represent cortex tissues and open circles represent core tissues. Core and cortex tissues were separated using laser capture microdissection. Gene expression was determined using quantitative RT-PCR and was normalized using *MdACTIN*. Expression of the gene is presented as the fold-change in relation to its expression at 0 DAFB. Error bar represents standard error of the mean of three biological replicates.

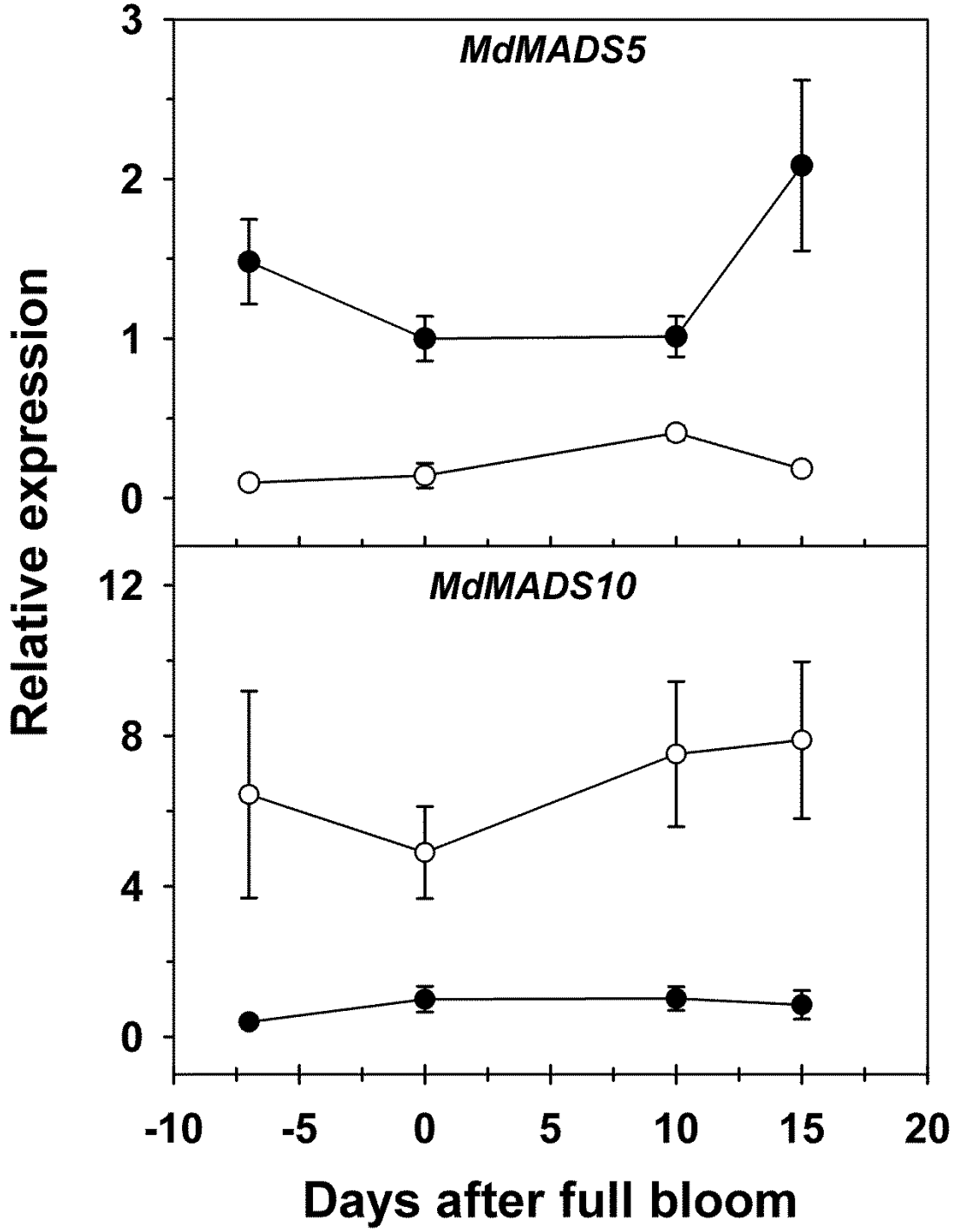
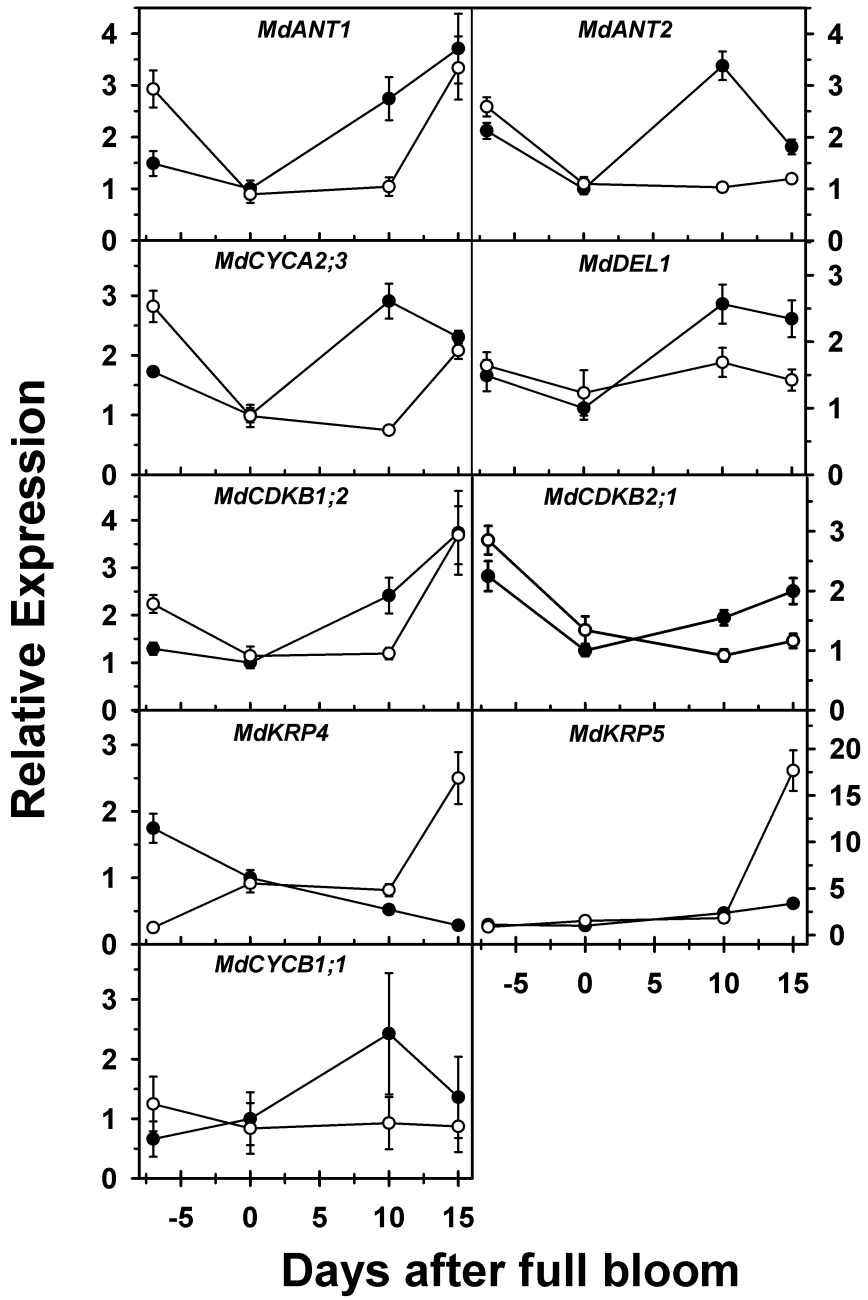

MdMADS5
MdMADS10
Relative expression
Days after full bloom
3
2
1
0
12
8
4
0
-10
-5
0
5
10
15
20

Figure 3.11. *Expression of MdANT1, MdANT2 and cell cycle genes in the core and cortex of 'Gala' during flower and early fruit development.*

Open circles represent the core tissue (ovary at -7 DAFB) while closed circles represent the cortex tissues (floral-tube at -7 DAFB). These tissues were isolated using laser capture microdissection. Gene expression was determined using qRT-PCR and was normalized using *MdACTIN*. Expression of a gene relative to its expression at full bloom (0 DAFB) is presented here. Error bar represents the standard error of the mean of three biological replicates (n=3).


MdANT1
MdANT2
MdCYCA2;3
MdDEL1
MdCDKB1;2
MdCDKB2;1
MdKRP4
MdKRP5
MdCYCB1;1
Relative Expression
Days after full bloom

CHAPTER 4

CROP LOAD REDUCTION ENHANCES FRUIT GROWTH BY ALTERING CARBOHYDRATE METABOLISM AND INCREASING CELL PRODUCTION

Dash, M., Johnson, L.K. and Malladi, A. To be submitted to *Physiologia Plantarum*.

Abstract

Thinning is one of the most important management practices used to optimize final fruit size in apple. However, the molecular mechanisms regulating fruit size due to thinning are not well understood. In this study, the effects of thinning on cell production and expansion and on the expression of key genes associated with these processes were investigated. Manual thinning around 11 days after full bloom (DAFB) resulted in an increase in fruit growth. This increase in fruit growth was mediated by an increase in cell production and resulted in ~36% higher cell number in thinned fruits around maturity. The cell size was similar for thinned and control fruit during most of fruit development but was 11% higher in thinned fruit at maturity. To better understand the molecular mechanisms enhancing thinning induced fruit growth in apple, the expression of genes associated with fruit growth were also investigated. The *AUXIN RESPONSE FACTOR*, *MdARF106*, and one of the *AINTEGUMENTA* genes, *MdANT1*, genes putatively associated with the regulation of fruit growth displayed ~2-fold and ~7-fold higher expression in thinned fruit, respectively. Several cell cycle genes positively associated with cell production, e.g., A2-type and B2-type cyclins (*MdCYCA2;1*, *MdCYCA2;3* and *MdCYCB2;2*) and few cyclin-dependent kinases (*MdCDKB1;1*, *MdCDKB1;2* and *MdCDKB2;2*), displayed higher expression in thinned fruit. The *COBRA-LIKE4* gene, *MdCOBL4*, associated with cell expansion, displayed up to 4-fold higher expression in thinned fruit. The *EXPANSIN* genes, *MdEXPA8;1*, *MdEXPA8;2* and *MdEXPA10;1*, also associated with cell expansion, showed up to ~4-fold higher expression at maturity. Thinning also resulted in alteration in the expression of genes associated with carbohydrate metabolism. The expression of the *SORBITOL DEHYDROGENASE* gene, *MdSDH1*, was higher in the control fruit during most of fruit development. However, the *SUCROSE SYNTHASE* gene, *MdSUSY3*, displayed a higher expression level in response to

thinning suggesting an increased sink activity in response to higher cell production rate. Together, data from this study indicate that thinning induces changes in carbohydrate availability and metabolism which might thereby mediate changes in the expression of genes associated with cell production and expansion subsequently enhancing fruit growth in apple.

Introduction

Crop load reduction by thinning of fruit is one of the most important management practices performed by fruit growers to produce high quality apples. Over cropping can result in small fruit size and poor quality, breakage of limbs, biennial bearing and exhaustion of tree reserves. Thinning improves fruit size, color, and quality at harvest, and increases return bloom in the following year, thereby reducing biennial bearing [1]. In apple and in other fruit trees, thinning at the appropriate time allows the remaining fruit to attain their maximum potential size [2-6]. Increase in fruit size in response to thinning may be caused by an increase in cell production and/or cell expansion, the primary mechanisms that mediate fruit growth. Goffinet *et al.,* (1995) compared 'Empire' apple fruit size in manually thinned and un-thinned trees and found that thinning allowed cell division to progress under less competition after bloom, resulting in an increase in final fruit size. Application of chemical thinning agents such as Benzyl Adenine (BA) increases fruit size in 'Empire' by increasing the number of cells in the fruit cortex, whereas the increase in fruit size due to naphthalene acetic acid derivative (NAA) application is largely a consequence of increase in cell size [8]. Manual thinning in 'Golden Delicious Smoothie' increases fruit size due to increased cell production during early fruit development and enhanced cell expansion at later stages [9].

Thinning may increase the carbohydrate availability to the developing fruit and have an immediate effect on cell production and expansion, thereby enhancing fruit growth. Soluble sugars, including sucrose, glucose and fructose, are known to act as signal molecules to regulate the expression of many key genes involved in plant metabolic processes and defense responses, consequently regulating plant growth and development. Fruit number and fruit growth are determined by carbohydrate source-sink relationships between photosynthetic source leaves and

reproductive sinks [10]. It has been suggested that chemical agents used for thinning, such as NAA and BA, reduce the energy available to the developing fruit by interfering with photosynthesis [11,12]. In tomato it has been demonstrated that fruit load reduction leads to increased photoassimilate availability [13]. Additionally, fruit load reduction in tomato also resulted in an enhanced fruit size due to higher cell production and increased expression of genes positively associated with cell production. Therefore it can be suggested that, regulation of fruit growth due to thinning may be mediated by genes regulating carbohydrate metabolism, and genes associated with the regulation of cell production and expansion. However, the molecular mechanisms leading to increase in fruit size and carbohydrate availability due to thinning have not been well studied in apple.

Several genes, associated with the control of cell production and expansion, and potentially involved in the regulation of fruit growth in apple have been identified [14,9,15]. Pre-bloom and early stages of fruit development in apple, which are associated with growth mediated by cell production, have been shown to be positively associated with 14 core cell cycle genes including B-type CDKs and A- and B-type cyclins [14]. Additionally, five cell cycle genes including the *KIP RELATED PROTEINS* (*KRPs*), *MdKRP4* and *MdKRP5*, were negatively associated with cell production [14]. *EXPANSINS, COBRA* (*COB*) and *COBRA-LIKE* genes, have been shown to mediate plant cell growth and orientation of cell expansion in Arabidopsis [16-20]. Some of these genes may have similar functions in apple fruit development as changes in their expression are associated with a reduction in fruit growth in response to severe shading [15].

Besides the above genes, certain upstream regulatory genes may also be involved in coordinating changes in response to thinning. The expression of a member of the AP2/ERF

(APETALA2/ETHYLENE RESPONSE FACTOR)-domain family of transcription factors, *AINTEGUMENTA* (*ANT*), is associated with regulation of apple fruit growth by mediating cell production [9]. Additionally, an *AUXIN RESPONSE FACTOR*, *MdARF106*, has been shown to be putatively associated with the regulation of fruit growth and has been co-localized to a quantitative trait locus (QTL) regulating fruit size [21]. Several genes associated with carbohydrate metabolism, such as *SORBITOL DEHYDROGENASE* (*SDH*), *NEUTRAL INVERTASE* (*NINV*) and *SUCROSE SYNTHASE* (*SUSY*), may also be involved in regulating apple fruit development [22]. It is likely that thinning induced increase in fruit growth is mediated by the coordinated changes in the expression of the above genes.

Increasing knowledge of fruit growth response to thinning will aid in better understanding the mechanisms involved in regulating fruit growth in apple. Therefore, the main objectives of the current study were to determine the molecular mechanisms mediating thinning induced increase in fruit growth by investigating its effects on cell production and expansion, and the expression of key genes associated with these processes. Additionally, to better understand the effect of thinning on carbohydrate metabolism, the levels of key metabolites and the expression of genes associated with carbohydrate metabolism were also investigated.

Materials and methods

Plant material

Mature trees of 'Golden Delicious' Smoothie (GS) growing on M.7a rootstocks were used in this study. Trees were grown and maintained at the Mountain Research and Education Center, University of Georgia, Blairsville, GA. In 2010, four randomly selected GS trees were subjected to the thinning treatment while four other trees were left un-thinned (Control). Thinning involved

the manual removal of all fruit within a cluster except for one lateral fruit at 11 days after full bloom (DAFB). Fruit diameter was measured on 20 fruit per tree from bloom until maturity. Fruit were randomly sampled at different stages of development and were either fixed in CRAF III fixative (chromic acid, formaldehyde and acetic acid; for cytology) or frozen in liquid N_2 and stored at -80 °C (for gene expression analyses). All trees used in the above studies were maintained according to commercial apple production practices. Chemical thinning agent applications were not performed in this study.

Cell number and cell area measurement

The number of cell layers within the cortex and the cell area of cortex cells were measured as described previously in Malladi and Johnson (2011). Four fruit from each replicate were used for this analysis. Briefly, cell number was determined by counting the number of cell layers between the petal vascular trace and the epidermis. The number of cells within a pre-defined area was determined at three regions within the fruit cortex and the average cell area was calculated using these data.

RNA extraction and cDNA synthesis

RNA extraction from flower and fruit was performed using the method described in Dash and Malladi (2012). One μg of total RNA was used for cDNA synthesis, after removal of genomic DNA with a DNase treatment, using the method described previously in Malladi and Johnson (2011). The synthesis of cDNA was performed in a total volume of 20 μL, was diluted by 6-fold, and stored at -20 °C until further analysis.

Quantitative RT-PCR

Quantitative RT-PCR analyses were performed using the Stratagene Mx3005P. One μL of the diluted cDNA was used in a final reaction volume of 12 μL. The PCR conditions involved the

following cycles: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min. Melt curve analyses were performed at the end of the above cycles and indicated a distinct single peak for all the amplicons analyzed. Controls without any template and without the reverse-transcriptase were used. Primer efficiency was determined using LinRegPCR [23]. All gene expression was normalized to the expression of three reference genes, *MdACTIN*, *MdGAPDH* and *MdCACS2*. Gene expression was calculated with the Cq values using a modified Pfaffl method [24], involving correction for amplification efficiency. The relative quantities ($1/E^{Cq}$, where E is the efficiency for a given gene and Cq is the cycle number where the threshold fluorescence was crossed) were normalized using the geometric mean of the relative quantities of the reference genes. Expression of a given gene is in relation to its expression in the control fruit at 0 DAFB. The standard error of the expression was calculated as described in Rieu and Powers (2009).

Measurement of sugars

Soluble sugars were extracted and were analyzed as their trimethylsilyl (oxime; TMS) derivatives as described in Tisza et al., (1993). Briefly, 0.25 g of fruit tissue was extracted in 1.5 mL 80% methanol containing phenyl β-D-glucoside, as an internal standard. After centrifugation for 10 min at room temperature, 1 mL of the extract was used for sugar analysis. For derivatization, 100 µL of the extract was heated at 40 ^{o}C and the solvent was evaporated to dryness under a stream of dry nitrogen. Sugars were first converted to their oxime derivative by adding 25 µL hydroxylamine (25 mg mL^{-1} of pyridine) and heated to 75 ^{o}C for 30 min. They were then converted to their TMS derivatives by addition of 70 µL of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% Trimethylcholorosilane (TMCS). After cooling, the oxime-TMS derivatives of the sugars were analyzed using gas chromatography

(Hewlett-Packard). A standard solution was prepared by dissolving known quantities of sucrose, fructose, glucose, sorbitol, and phenyl β-D-glucoside in 80% methanol. The standard solution was derivatized using the method described above.

Statistical analyses

All statistical analyses were performed using SAS (version 9; SAS Institute, Cary, NC) and Sigmaplot 11 (Systat Software, San Jose, CA). Two-way ANOVA with repeated measures was performed in SAS. The two factors analyzed were: thinning and time after treatment. Wherever the interactions were significant, the simple effects were analyzed by using test of slices.

Results

Thinning increases fruit size by increasing cell production and cell expansion

Fruit growth was enhanced in response to crop load reduction by thinning (Fig. 4.1). Thinning led to a 19% increase in fruit diameter by around 24 DAFB ($P<0.05$), indicating a rapid response. In comparison to the control, thinned fruit had around 15% higher fruit diameter ($P<0.005$) at maturity. Enhanced fruit growth during early fruit development in thinned fruit was primarily associated with an increase in cell production in the fruit cortex. Both the thinned and control fruit displayed similar cell production until around 18 DAFB. Thinned fruit displayed 5.6% higher cell number ($P<0.005$) than control at 21 DAFB. While cell production in the fruit cortex continued in the thinned fruit until 28 DAFB, it began to slow down in the control fruit after 18 DAFB (Fig. 4.1). In comparison to the control fruit, cell number in the thinned fruit was higher by 33% ($P<0.005$) at 28 DAFB. Increase in cell number in thinned fruit was associated with 2-fold and 3.5-fold higher relative cell production rate (RCPR) than the control at 24 and 28 DAFB, respectively (Fig. 4.1). The cell number in thinned fruit remained higher during the rest

of fruit development and was around 40% higher than that in the control at maturity. Cell expansion was similar between thinned fruit and control fruit during early stages of fruit development (Fig. 4.1). Significant differences in cell area between the control and thinned fruit were evident at 40 DAFB and at maturity (Fig. 4.1). Cell area within the fruit cortex was slightly greater in the control fruit in comparison to that in the thinned fruit at 40 DAFB (~10%; $P<0.05$). However, at maturity thinned fruit displayed around 11% higher cell area ($P<0.005$) than that of control fruit. These data indicate that thinning enhances fruit growth primarily by increasing cell production during early fruit growth and possibly cell expansion at later stages of fruit development.

Expression of genes associated with fruit growth is altered in response to thinning

MdANT1 expression was similar in thinned and control fruit until around 18 DAFB (Fig. 4.2). In comparison to the control fruit, *MdANT1* expression was almost 3-fold higher ($P<0.005$) at 21 DAFB and around 5.7-fold higher at 24 DAFB, the period during which differences in cell production were apparent. In this study, the expression of *MdANT2* was not significantly affected by thinning. The *AUXIN RESPONSE FACTOR*, *MdARF6* and *MdARF106*, displayed ~2-fold higher expression in thinned fruit at 28 DAFB. *MdANT1* and *MdARF106* also displayed significantly higher expression in thinned fruit around 133 DAFB, a period when their general expression levels were considerably lower than that during early fruit development.

Expression of genes related to cell production and cell expansion is altered in response to thinning

The expression of many of the positive regulators of cell production displayed an increase in expression in thinned fruit during early stages of fruit growth, coinciding with the period of

increased cell production (Fig. 4.3). Three of the four B-type *CDKB* genes (*MdCDKB1; 1*, *MdCDKB1;2* and *MdCDKB2;2*) displayed around 1.6-fold to 5-fold ($P<0.005$) higher expression in thinned fruit at 28 DAFB (Fig. 4.3). The expression of two A2-type cyclins, *MdCYCA2;1*, and *MdCYCA2;3*, was also enhanced in response to thinning by up to 4-fold during the early stages of fruit growth. The expression of a B2-type cyclin, *MdCYCB2;2*, was around 2-fold and 3-fold higher in thinned fruit at 28 and 54 DAFB ($P<0.05$), respectively, in thinned fruit, while that of a B1-type cyclin was higher at 28 DAFB by 1.7-fold. The expression of the negative regulators of cell production, *MdKRP4* and *MdKRP5*, was reduced by 1.5-fold at 40 DAFB in response to thinning (Fig 4.3). *MdKRP5* displayed higher expression (~2-fold; $P<0.005$) in thinned fruit at 18 DAFB and 28 DAFB. Most of the above genes displayed a significantly higher expression in thinned fruit around 133 DAFB.

In thinned fruit, *MdCOB1* expression was ~2-fold lower than that in control fruit at 24, 28 and 133 DAFB (Fig 4.4). However, the expression of *COBRA-LIKE4*, *MdCOBL4*, was 3-fold to 4-fold higher in thinned fruit both during early fruit development (18, 24 and 28 DAFB) and later phase of fruit growth (133 DAFB). The expression of the *EXPANSIN* genes, *MdEXPA8;1* and *MdEXPA8;2* and *MdEXPA10;1*, was ~3-fold higher ($P<0.005$) in thinned fruit at maturity, coincident with an increase cell expansion within the thinned fruit (Fig 4.4). The expression of *MdEXPA10;1* was also higher in thinned fruit by 2- to 4-fold ($P<0.005$) during early fruit growth (11, 18, 21 and 28 DAFB). Together, the above data indicate that the expression of key regulators of cell production and cell expansion is greatly altered in response to thinning to facilitate an increase in cell production and cell expansion and subsequently fruit growth in response to thinning.

Effect of thinning on sugar metabolism and expression of genes related to carbohydrate metabolism

The expression of *MdSDH1* was 3-fold higher in thinned fruit at 18 DAFB ($P<0.005$). However, the expression of *MdSDH1* was significantly lower in the thinned fruit than that in the control fruit at later stages of fruit development (Fig. 4.5). In comparison to thinned fruit, *MdSDH2* displayed around 1.5-fold higher gene expression in control fruit at 40 DAFB (~1.5-fold, $P<0.05$) and also at maturity (2.5-fold, $P<0.005$). *MdSUSY3* expression was higher in thinned fruit during early fruit development at 18 DAFB (~2-fold; $P<0.05$) and 28 DAFB (~2.7-fold; $P<0.005$); and *MdSUSY5* expression was 5.5-fold higher expression ($P<0.005$) in thinned fruit during mid-fruit development (54 DAFB). However, the levels of the soluble sugars such as sorbitol, sucrose, glucose and fructose were not significantly different between thinned and control fruit at 28 DAFB (Table 4.1).

Discussion

Thinning in apple has been shown to increase cell production and/or cell expansion thereby enhancing fruit growth [27,7,28,9]. In the current study, crop load reduction through manual thinning led to an increase in cell production during early fruit development and a minor increase in cell size around maturity, consistent with a previous study in the same genotype [9]. Increase in cell number within the cortex of thinned fruit was primarily achieved between 21 and 28 DAFB. Thinned fruit displayed a higher RCPR during this period (at 24 and 28 DAFB). Additionally, in comparison to the control fruit, thinned fruit displayed a longer period of cell production. The data from this study as well as our previous study [9] show that thinning leads to an increase in cell production during early fruit growth thereby enhancing final fruit size. While

cell expansion was not affected by thinning during most of fruit development, thinned fruit displayed slightly larger cell size towards the period of fruit maturation. These data are consistent with a previous study where a similar increase in cell size was observed during later stages of fruit development in thinned fruit. Similarly, in other fruit, reduction in crops load has been shown to affect cell production as well as cell expansion. In tomato, proximal fruits displayed an increase in cell size whereas distal fruits had higher cell number in response to thinning [3].

MdANT1 may regulate fruit growth by regulating cell production during fruit development as it displayed higher expression during early fruit growth in thinned fruit. *MdANT1* expression was 3- to 6-fold higher in thinned fruit during the period when differences in cell production became apparent. These data strongly suggest that the increase in *MdANT1* expression may facilitate changes in the expression of downstream target genes associated with cell production thereby leading to an increase in the cell number. These data are consistent with a previous study where *MdANT1* expression was found to be higher in thinned fruit [9]. In the above study, *MdANT2* expression was also found to be higher in thinned fruit, but no significant differences in *MdANT2* expression were observed in the current study. MdANT1 and MdANT2 share around 91% sequence identity and may therefore regulate similar targets. It may be likely that the increase in the expression of one of these genes is sufficient to elicit cell production–related responses during fruit development. *MdARF6* and *MdARF106* have been investigated in relation to their roles in the regulation of fruit-size [21]. *MdARF106* expression was reported to be associated with cell production and expansion during fruit development. In the current study, the expression of *MdARF106* was higher in thinned fruit by ~2-fold at 28 DAFB suggesting that *MdARF106*

may be involved in regulating mechanisms that increase fruit growth in response to thinning, potentially by affecting cell production.

Thinning induced increase in cell production was associated with coordinated changes in the expression of cell cycle genes which have been shown to be core facilitators of cell production [14]. The expression of several such genes, *MdCDKB*s, two A2-type cyclins, and two B-type cyclins, positively associated with cell production was 1.5-fold to 5-fold higher in thinned fruit during early phase of fruit growth coincident with the period of increased cell production. A-type cyclins are thought to be associated with S and/or G2/M phase progression, and B-type cyclins regulate progression of cells through the G2/M phase [29]. The coordinated increase in the expression of these genes during early fruit growth may therefore facilitate the increase in cell production in response to a reduction in the crop load. Additionally, at least one of the genes negatively associated with cell production, *MdKRP4*, displayed a 1.5-fold reduction in expression in thinned fruit during early fruit development. These genes have been shown to be the mediators of exit from mitotic cell production [30,31]. These data indicate that a decrease in crop load due to thinning initiates mechanisms that result in the coordinated alteration of core cell cycle gene expression thereby facilitating an increase in cell production and fruit growth.

Increase in fruit growth as a result of thinning was also facilitated by increased cell expansion during late fruit development. The expression of several genes related to cell expansion such as, *COBRA-LIKE4* (*MdCOBL4*) and *EXPANSINs* (*MdEXP8;1*, *MdEXP8;2* and *MdEXP10;1*), was 3- to 4-fold higher in thinned fruit around maturity. *COBRA-LIKE* (*COBL*) genes have been associated with cellulose deposition during secondary wall biosynthesis [17,19, 32]. The *EXPANSIN* (*EXP*) family encodes extracellular proteins that are thought to be involved in the loosening of cell walls thereby facilitating cell expansion [33]. In this study, the change in

expression of these genes was coincident with an increase cell expansion within the thinned fruit. The expression of *MdCOBL4* and *MdEXP10;1* was also 2-to 4-fold higher during early fruit growth (18 to 28 DAFB) when no significant difference in cell size was observed between the thinned and the control fruit. However, a significant increase in cell production was observed in response to thinning during this period (18 and 21 DAFB). Cell division is preceded by a phase of cell growth during which amount of cytoplasm is increased through macromolecular synthesis [34]. An increase in the expression of genes (*MdCOBL4* and *MdEXPA10;1*) potentially associated with cell wall modifications in response to thinning may also be important facilitator of such cell growth during early fruit growth.

Together, these data indicate that increase in fruit size in response to thinning is mediated by an increase in cell production and expansion. Progression of these processes affecting growth is dependent on the availability of carbohydrates. It was hypothesized that an increase in carbohydrate availability due to a reduction in crop load and subsequent alteration in carbon metabolism may rapidly increase the rates of cell production thereby enhancing fruit growth. In tomato, thinning has been shown to result in increased carbohydrate availability and higher expression level of genes associated with cell cycle mediating higher cell production and increased fruit growth [13]. However, in this study we did not observe any change in sugar concentration between the thinned and control treatment at 28 DAFB (Table 4.1). However, thinning led to alteration in the expression of the genes related to sugar metabolism. Sorbitol dehydrogenase is the primary enzyme involved in the conversion of sorbitol to fructose [35]. Since, sorbitol is the main translocated carbohydrate in apple, sorbitol dehydrogenase may play an important role in defining sink activity [36]. Additionally, other enzymes involved in sucrose metabolism such as sucrose synthase (converts sucrose to fructose and UDP-glucose) and neutral

invertase (converts sucrose to fructose and glucose) may also regulate the carbohydrate availability. Expression of *MdSDH1* and *MdSDH2* was lower in the thinned fruit during most of fruit development. However, the *MdSUSY3* displayed a higher expression level in response to thinning at 18 DAFB and 28 DAFB. Expression of genes associated with sorbitol metabolism has been shown to be higher during the period of cell production with low sugar accumulation and allowing the fruit to meet its high respiratory demand [22]. Thinned fruit displayed a much higher cell production rate during early period fruit growth at 18 DAFB to 28 DAFB which may lead to increased sink activity and a potentially transient increase in expression level of *MdSUSY3* associated with sugar metabolism. However, the higher crop load in control fruit might have led to subsequent decrease in level of sorbitol which may have resulted in greater expression of the *SDH* genes in the control treatment. These data indicate that thinning mediates changes in expression of genes associated with sorbitol metabolism during early fruit growth. Further investigation of sugar level in thinned and control apple fruit tissue, especially during early fruit growth (such as at 18 DAFB) may establish if sugar accumulation is altered in response to thinning.

Data from this study clearly demonstrate that thinning enhances fruit growth by facilitating cell production and cell expansion. Fruit growth due to higher cell production and cell expansion appears to be mediated by coordinated changes in the expression of key genes associated with fruit growth, cell production and expansion. Thinning also alters the expression of genes associated with carbohydrate metabolism indicating their effect on sink activity and carbohydrate availability.

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Table 4.1. *Effect of thinning on sugar accumulation in apple fruit tissue.*

Soluble sugars were extracted and were analyzed as their TMS-oxime derivatives for ‘Thinned’ and ‘Control’ fruit at 28 days after full bloom (DAFB). Sugar composition is expressed as amount of sugar compound (µg) present per gram (fresh weight) of the fruit tissue (w/w). Values shown are means of three biological replicates pooled from two technical replicates ± S.E. No significant differences were observed between thinned fruit and control fruit samples.

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Compound	Thinned (µg/g) Mean ± S.E.	Control (µg/g) Mean ± S.E.
Sorbitol	2576.7 ± 177	3029.7 ± 179.9
Fructose	6528.5 ± 438.9	7156.7 ± 268.5
Glucose	4971.4 ± 320.2	5483.6 ± 161.9
Sucrose	2977.4 ± 324.5	3109.1 ± 69.3

Figure 4.1. *Effect of thinning on fruit growth and cell-growth related parameters.*

Fruit diameter (mm), number of cell layers in the fruit cortex, cortex cell area (μm^2) and relative cell production rate (RCPR) were determined in 'Thinned' and 'Control' fruit. Closed box indicates 'Control' fruit and open box indicates 'Thinned' fruit. Error bars indicate the S.E of the mean (n = 4). Large asterisk indicates significant difference between 'Thinned' and 'Control' fruit at $P < 0.005$; small asterisk indicates significant difference between 'Thinned' and 'Control' fruit at $P < 0.05$.

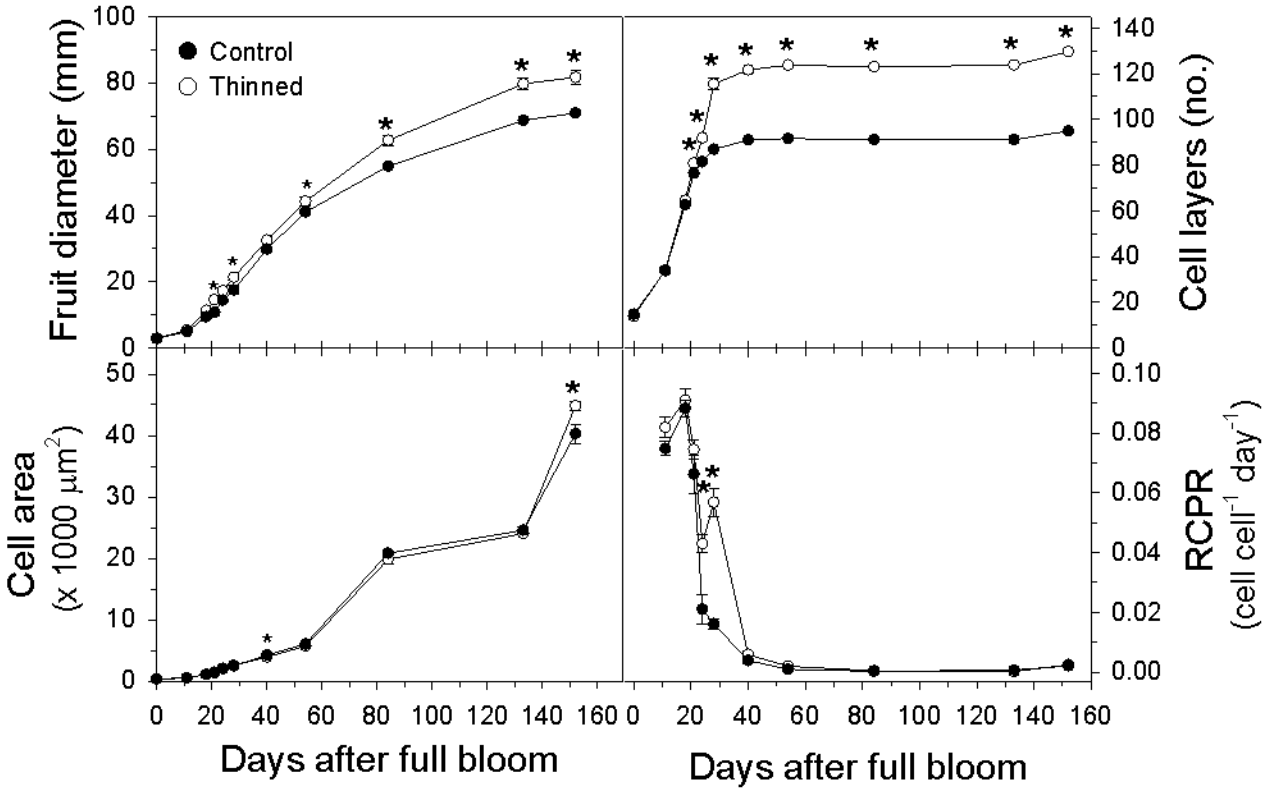

Control
Thinned
Fruit diameter (mm)
Cell layers (no.)
Cell area
(x 1000 μm²)
RCPR
(cell cell⁻¹ day⁻¹)
Days after full bloom
Days after full bloom

Figure 4.2. *Effect of thinning on the expression of transcription factors associated with fruit growth.*

Closed box indicates ‘Control’ fruit and open box indicates ‘Thinned’ fruit. The expression of a gene in relation to its expression in ‘Control’ fruit at 0 days after full bloom (DAFB) is presented. Error bars indicate the S.E of the mean (n = 4). Large asterisk indicates significant difference between ‘Thinned’ and ‘Control’ fruit at $P < 0.005$; small asterisk indicates significant difference between ‘Thinned’ and ‘Control’ fruit at $P < 0.05$.

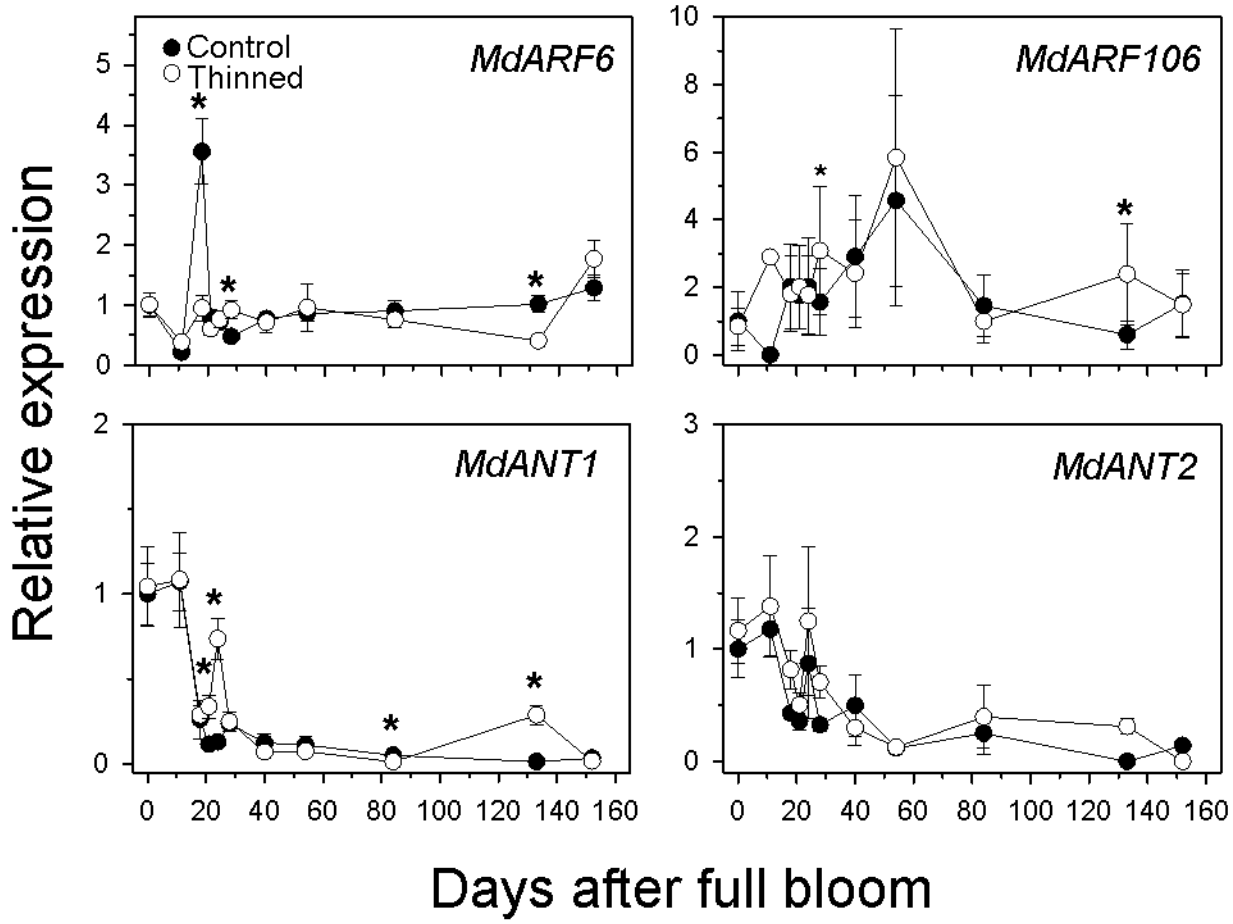

Control
Thinned
MdARF6
MdARF106
MdANT1
MdANT2
Relative expression
Days after full bloom

Figure 4.3. *Effect of thinning on the expression of core cell cycle genes associated with cell production.*

Closed box represents ‘Control’ fruit and open box represents ‘Thinned’ fruit. Expression of a gene relative to its expression in ‘Control’ fruit at 0 days after full bloom (DAFB) is presented. Error bars indicate the S.E of the mean (n = 4). Large asterisk indicates significant difference between ‘Thinned’ and ‘Control’ fruit at $P < 0.005$; small asterisk indicates significant difference between ‘Thinned’ and ‘Control’ fruit at $P < 0.05$.

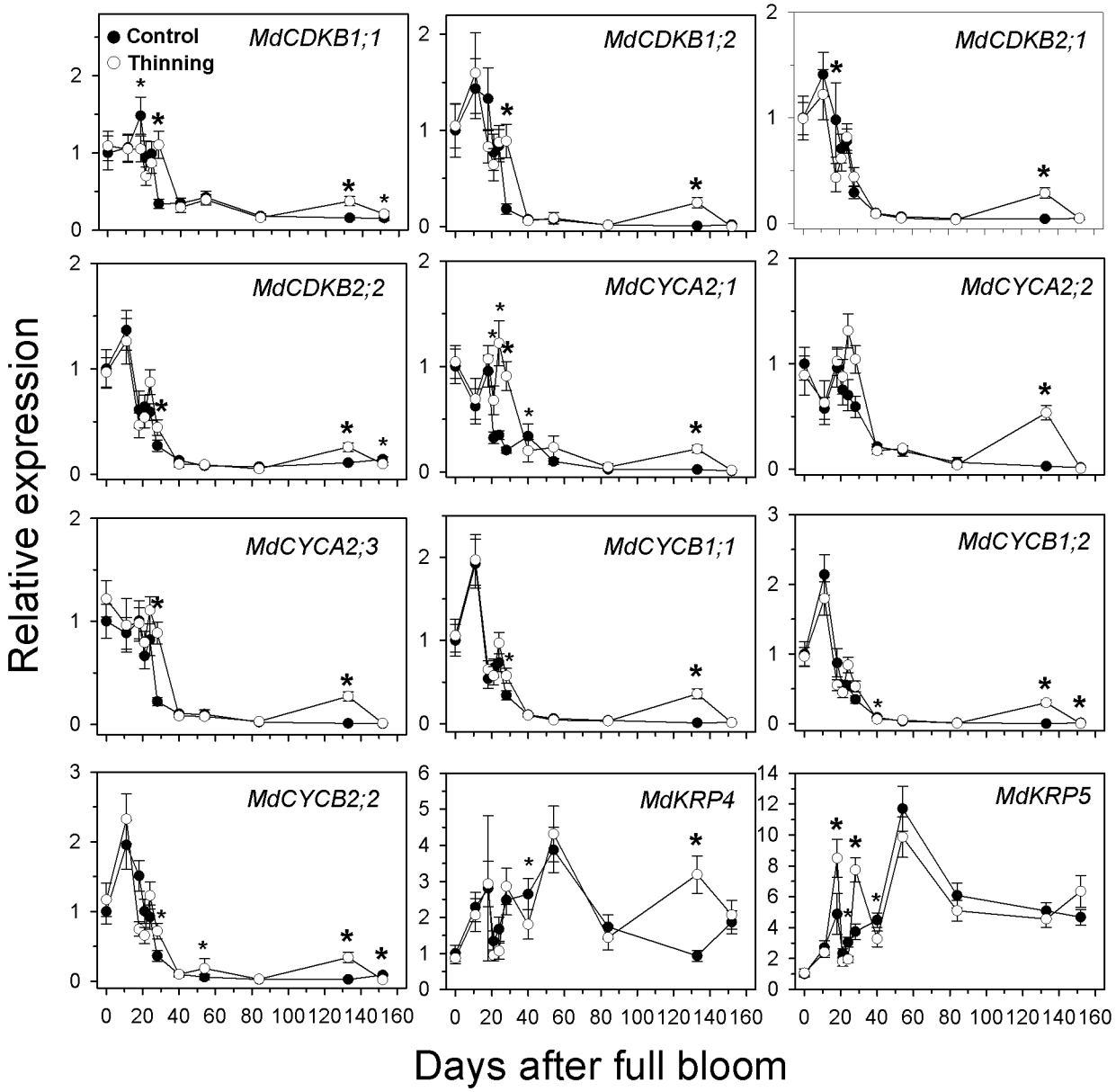

Control
Thinning
MdCDKB1;1
MdCDKB1;2
MdCDKB2;1
MdCDKB2;2
MdCYCA2;1
MdCYCA2;2
MdCYCA2;3
MdCYCB1;1
MdCYCB1;2
MdCYCB2;2
MdKRP4
MdKRP5
Relative expression
Days after full bloom

Figure 4.4. *Effect of thinning on the expression of genes associated with cell expansion.* Closed box indicates 'Control' fruit and closed box indicates 'Thinned' fruit. Expression of a gene relative to its expression at 0 days after full bloom (DAFB) in 'Control' fruit is presented. Error bars indicate the S.E of the mean (n = 4). Large asterisk indicates significant difference between 'Thinned' and 'Control' fruit at $P < 0.005$; small asterisk indicates significant difference between 'Thinned' and 'Control' fruit at $P < 0.05$.

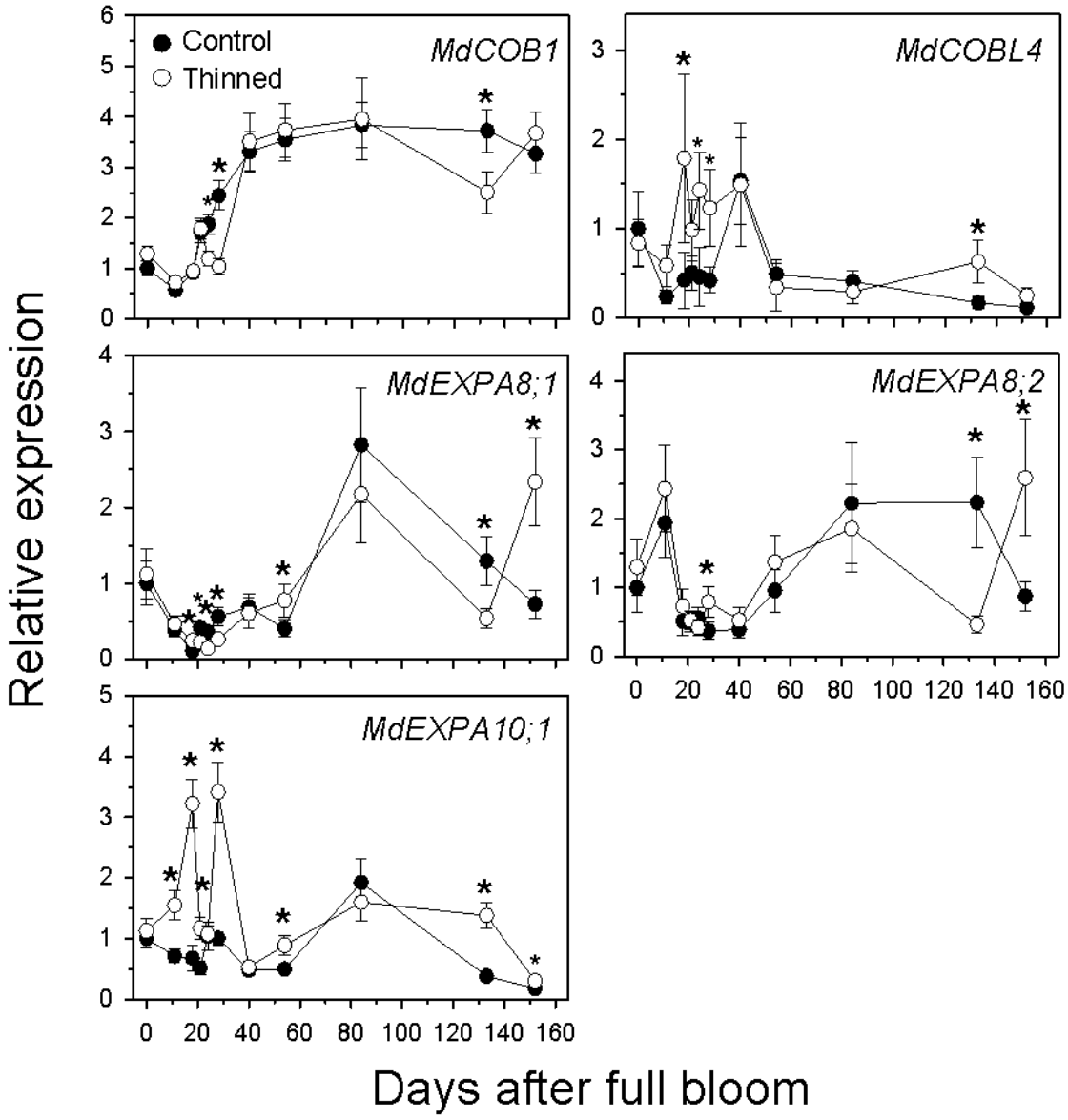
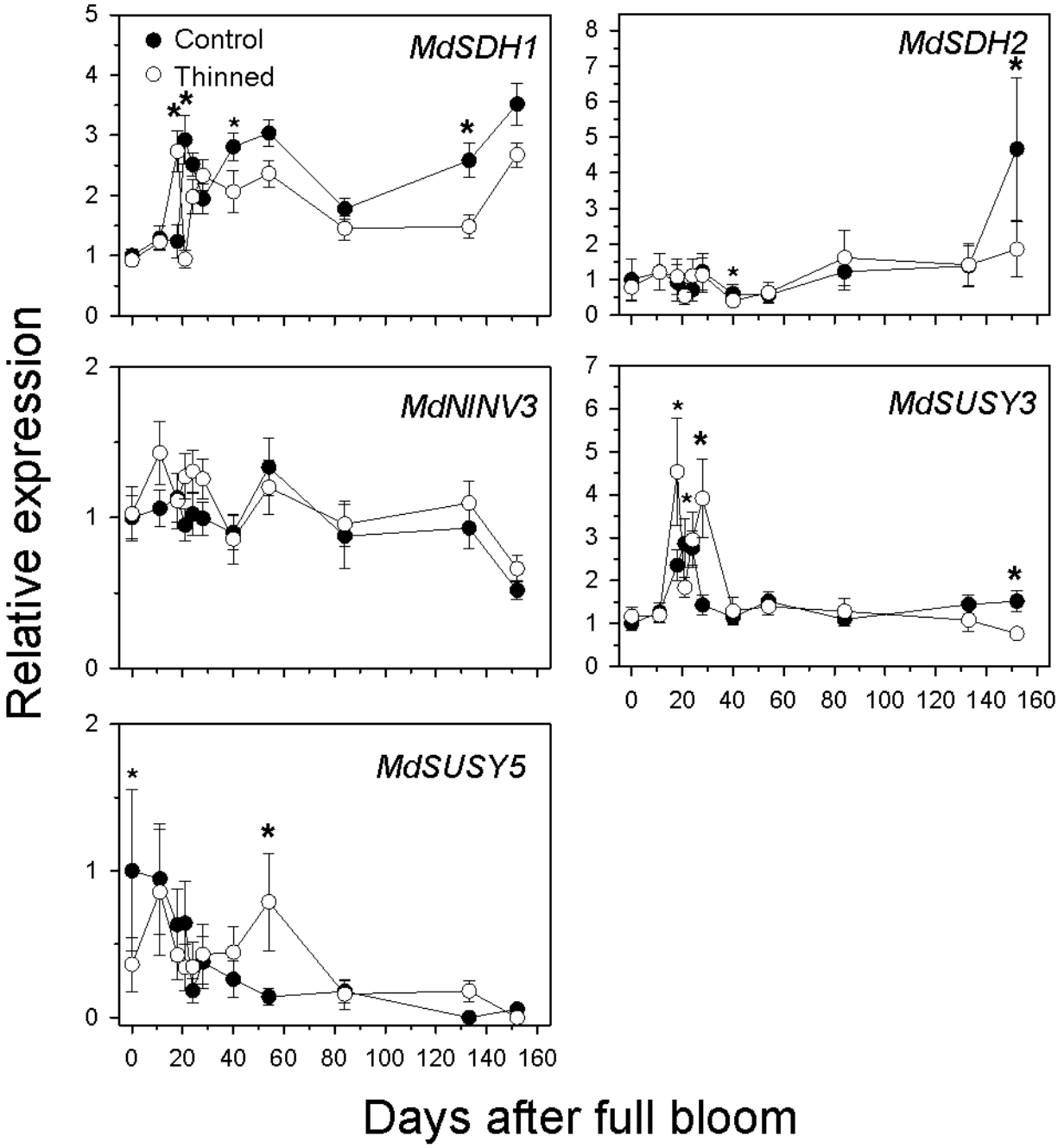

Control
Thinned
MdCOB1
MdCOBL4
MdEXPA8;1
MdEXPA8;2
MdEXPA10;1
Relative expression
Days after full bloom

Figure 4.5. *Effect of thinning on the expression of genes associated with carbohydrate metabolism.*

Closed box indicates ‘Control’ fruit and closed box indicates ‘Thinned’ fruit. Expression of a gene relative to its expression at 0 days after full bloom (DAFB) in ‘Control’ fruit is presented. Error bars indicate the S.E of the mean (n = 4). Large asterisk indicates significant difference between ‘Thinned’ and ‘Control’ fruit at $P < 0.005$; small asterisk indicates significant difference between ‘Thinned’ and ‘Control’ fruit at $P < 0.05$.


Control
Thinned
MdSDH1
MdSDH2
MdNINV3
MdSUSY3
MdSUSY5
Relative expression
Days after full bloom

CHAPTER 5

FUNCTIONAL CHARACTERIZATION OF *MdANT1* AND *MdANT2*

Dash, M., McKinney, E.C., Meagher, R.B. and Malladi, A. To be submitted to *Journal of Experimental Botany*.

Abstract

MdANT1 and *MdANT2* regulate cell production and fruit growth in apple. To determine the role of *MdANT* genes in organ size regulation, wild type Arabidopsis plants (*Columbia* ecotype) were transformed with the *MdANTs* under a constitutive promoter *Actin7* (*Act7*) promoter. Four transgenic plants for *MdANT1* and six transgenic plants for *MdANT2* were successfully obtained. *MdANT1* and *MdANT2* cDNA have been cloned in-frame with the GFP protein under the control of the constitutive promoter *Actin2* and the construct was transformed into Arabidopsis plants. Root of transgenic seedlings will be observed using confocal to determine MdANT-GFP localization. MdANT2 protein was cloned in-frame to maltose binding protein (MBP) and the fused protein was purified using amylose resin. Multiple bands were observed for the purified protein on an SDS-PAGE and on western blot suggesting protein degradation.

Introduction

Fruit size is an important quality-related trait and is a key marketing parameter in many fruit including apple. Enhancing fruit size can be economically beneficial to fruit growers [1]. Since, larger fruit have a higher per pound value than smaller fruit; growers spend significant efforts to optimize this trait using horticultural practices such as pruning and thinning. Additionally, breeding programs often include fruit size as a key trait targeted for selection. However, genes involved in regulating fruit growth in apple and other fleshy fruit are not well characterized.

Fruit growth in apple consists of an early period of cell division followed by subsequent cell expansion during later stages [2]. The rate of cell growth and cell proliferation is critically important in determining final fruit size [3-5]. *AINTEGUMENTA* (*ANT*) is involved in the control of organ growth during *Arabidopsis* flower development and has been shown to be a key organ size regulator [6-9]. Recently, two *ANT* homologs, *MdANT1* and *MdANT2*, have been identified in apple [10]. Further, expression analysis of the *ANT* genes from apple has demonstrated that *MdANT1* and *MdANT2* are associated with the regulation of cell production during fruit growth. In Arabidopsis, ANT has been shown to function as a transcription factor [6,7]. Predicted MdANT1 and MdANT2 sequences shared greater than 88% sequence identity with the Arabidopsis ANT within their DNA binding regions. *MdANT1* and *MdANT2* expression also showed significant correlation with genes positively associated with cell production such as the A2-type cyclin (*MdCYCA2;3*) and *MdCDKB1;*2, a B2-type cyclin-dependent kinases [10]. The above data strongly suggest that *MdANT1* and *MdANT2* may function as transcription factors in apple and regulate cell production and fruit growth in apple, potentially by coordinating the expression of genes involved in cell proliferation.

The main objective of this study was to determine the function of *MdANT1* and *MdANT2* genes in plant organ development. To achieve this objective, the apple *ANT* genes were expressed in Arabidopsis. Additional objectives of this study were to determine the sub-cellular localization of apple *ANT* genes and to identify genes that were potentially regulated by the *ANTs*.

Materials and methods

Generation of transgenic Arabidopsis lines expressing the apple ANTs

MdANT1 and *MdANT2* cDNA sequences were amplified using PCR [Forward primers 5'GCAGCCGAAGACATCATGAAGTCC (*MdANT1*) and 5'GCAGCCTCATGAAGTCCATGAAT (*MdANT2*); Reverse primer 5'GCAGCCGGATCCCTAGGTCTCATT]. The primers consisted of BbsI/BamHI (*MdANT1*) and BspHI/BamHI (*MdANT2*) restriction sites to allow cloning of the cDNA fragments. The cDNA fragments were digested with the respective restriction enzymes and were subcloned in sense orientation into polylinker region of a pBluescript vector downstream of the *Actin7* promoter (from Dr. Richard Meagher, UGA, GA, USA; [11]). The pBluescript vector constructs were digested with KpnI/SacI and the expression cassettes were cloned into the polylinker region of pCAMBIA binary vector (from Dr. Richard Meagher, UGA, GA, USA). The pCAMBIA vectors containing the appropriate expression cassette were subsequently used to transform the *Agrobacterium tumefaciens* strain C58. Arabidopsis plants (ecotype *Columbia*) were grown in growth chambers under 16 h fluorescent light, 8 h dark were used for transformation. Prior to infiltration, preformed siliques were removed and the plants were infiltrated with the *A.*

tumefaciens strain C58 carrying either the MdANT1 or MdANT2 expression cassettes. . Transformation was performed using the ‘‘floral dip’’ method described in [12]. Transgenic (T1) plants were selected on Murashige and Skoog (MS) medium containing 50 μg mL^{-1} hygromycin before being transferred to soil. PCR was performed on DNA extracted from Arabidopsis leaves to identify the plants containing the *MdANT1* and *MdANT2* genes. Forward primers used for PCR were 5’ TTCAATGTACCGAGGCGTGACAA (*MdANT1*) and 5’ATGGGAGATGGCAAGCTAGGATTG (*MdANT2*). Reverse primers used for PCR were 5’AATTTCTTCCCATTTTTCCTTGTTCAAT (*MdANT1*) and 5’CTCCACTAATTACTTAACCCTCACCTC (*MdANT2*).

Sub-cellular localization of MdANT1 and MdANT2

MdANT1 and *MdANT2* cDNA were PCR amplified using the primers mentioned above. The cDNA fragments were sub-cloned individually in into the pBluescript vector downstream of the *Actin2* promoter and *GFP* sequence (*Act2promoter-GFP-Actin2terminator* in pBluescript vector; from Dr. Richard Meagher, UGA, GA, USA). The vector was subsequently digested with KpnI/SacI and sub-cloned into the binary vector, pCAMBIA as described above. Wild type Arabidopsis plants (*Columbia* ecotype) were transformed using the ‘floral dip’ method described above. The pCAMBIA vectors containing the appropriate expression cassette were subsequently transformed into *Agrobacterium tumefaciens* strain C58. Additionally, Arabidopsis plants (*Columbia* ecotype) were transformed with *Agrobacterium tumefaciens* strain C58 carrying *Act2prom/GFP/Act2term* construct. The transgenic seedlings obtained from these plants were used as control.

MdANT2 protein expression and purification

MdANT2 cDNA was amplified using PCR with the forward primer, 5'GGCTGTGAATTCATGAAGTCCATGAAT (containing an EcoRI site) and the reverse primer, 5'TTCGATTCTAGACTAGGTCTCATTCCAG (containing an XbaI site). The amplified fragments were cloned into a pMAL vector (New England Biolabs, UK) in frame with the maltose-binding protein (MBP). The vector was transformed into the *E.coli* BL-21 (DE3) strain (Novagen, Darmstadt, Germany). Expression of MdANT2-MBP fusion protein was performed using 500 mL of Luria Broth (LB) cultures incubated at 37 °C until an OD_{600} of 0.4–0.6. Protein expression was induced by the addition of isopropyl β–D-1-thiogalactopyranoside (IPTG, final concentration of 600 mM and 1 M). Expression was also performed under different conditions: 24 h at 25°C, 24h at 37 °C, and 48 h at 37 °C. Cells were harvested by centrifugation, re-suspended in 1X phosphate buffered saline (PBS, 8 gL^{-1} NaCl, 0.2 gL^{-1} KCl, 1.78 gL^{-1} $Na_2HPO_4.2H_2O$, 0.27 gL^{-1} KH_2PO_4 and pH = 7.4) and lysed by passing through a French press at 1000 pounds per square inch. The soluble protein fraction was recovered by centrifugation. Expressed protein was purified from the soluble protein fraction using amylose resin (New England Biolabs, UK). The purified protein was eluted in 1X PBS containing 10 mM maltose. Recombinant protein expression was visualized by Coomassie Brilliant Blue R-250 staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. MdANT2-MBP fusion protein was further detected using an anti-MBP antibody (New England Biolabs, UK) through western blot analysis.

Results

Functional analysis of MdANT1 and MdANT2

With the aim of studying the function of *MdANT1* and *MdANT2* genes, the coding region of the *MdANT1* and *MdANT2* was cloned under the control of the *Actin7* promoter and the resulting construct containing the expression cassette was used to transform Arabidopsis plants. After hygromycin selection, 12 out of 21 independent T1 plants for *MdANT1* and 9 out of 25 T1 plants for *MdANT2* reached the mature stage. PCR was performed on DNA extracted from Arabidopsis leaves to determine plants containing *MdANT1* and *MdANT2* genes in their genome. PCR data indicated the presence of *MdANT1* in four transgenic plants while *MdANT2* was found in six transgenic plants (Fig. 5.1). Seeds from these plants have been obtained and transgenic plants will be further selected and characterized to determine the effect of the *MdANT* genes on organ size.

To determine the sub-cellular localization of the apple ANT proteins, *MdANT1* and *MdANT2* cDNA were cloned in frame with the GFP protein under the control of the constitutive promoter, *Actin2* (*Act2*). The transgenic seeds were germinated in MS medium containing 50 μg mL^{-1} hygromycin. The plates were oriented to grow vertically to increase the root length. Roots of the germinated seedlings were observed using the light microscope. However, high background fluorescence made it difficult to localize the expression the GFP-tagged protein. Further characterization using confocal microscope will help in determining the sub-cellular localization of the MdANT1 and MdANT2 proteins.

Expression of the MdANT2 protein

MdANT2 gene encodes a putative protein with 651 amino acids and an approximate molecular mass of 71.4 kDa, while the molecular mass of the MBP is 42.5 kDa. Therefore, the expected molecular mass of the fusion protein is around 114 kDa. The fusion protein expression was induced by growing the cultures for 24 h and 48 h at 37 ^{o}C. Expression of the recombinant protein was responsive to the temperature used for induction. At 25 ^{o}C, negligible amount of recombinant protein was expressed (Fig. 5.2). Increasing the IPTG concentration from 600 mM to 1 M, for cultures grown at 25 ^{o}C, also had no effect on the recombinant protein expression. Protein expression was higher at 37 ^{o}C. However, temperature had no effect on the solubility of the recombinant protein. Under all conditions, the fusion protein accumulated within the pellet fraction as compared to the soluble fraction (Fig. 5.3). The expression of the recombinant fusion protein was higher when induced for 48 h in comparison to 24 h (Fig. 5.3).

Purification of soluble MBP- MdANT2 protein

The expressed MBP-MdANT2 protein was purified from the soluble fraction using amylose resin. Purified protein was eluted in three fractions. Multiple protein bands were observed in the first and second elution (Fig. 5.4). The molecular weight for the protein bands varied from about 200 kDa to about 40 kDa. The 40 kDa band displayed the highest concentration on an SDS-PAGE. The purified protein was further analyzed using anti-MBP antibody using western blot analysis to determine if the eluted products were associated with MBP. The Western analysis also indicated the presence of multiple MBP-fused protein bands (Fig. 5.5). The protein band with the highest concentration was also of about 40 kDa molecular weight.

Discussion

In this study wild-type Arabidopsis plants were successfully transformed with *MdANT1* and *MdANT2* expressed under the control of the *Actin7* promoter. *Actin7* promoter has been shown to respond to several distinct developmental programs and therefore, promotes higher expression in rapidly developing vegetative as well as reproductive tissues [11]. *ANT* promotes growth within floral meristems and is required for integument initiation in ovules and plays important roles in gynoecium and petal development [6,7]. In Arabidopsis, *ANT* is highly expressed in the cells at the growing domain of the developing organs [6]. Mizukami and Fischer (2000) reported that *ANT* controls the duration of organ growth and cell production during organ development. Therefore, expression of *MdANT1* and *MdANT2* under the control of the *Actin7* promoter may help in determining the effect of ectopic expression of *MdANT1* and *MdANT2* on organ development in Arabidopsis. PCR analysis indicated the presence of *MdANT1* in four transgenic plants and that of *MdANT2* in six transgenic plants. Expression analysis of *MdANT1* and *MdANT2* in selected transgenic plants will help in screening plants not only positive for the genes but also displaying higher expression of *MdANT1* and *MdANT2*. Further phenotypic characterization in the transgenic plants expressing higher levels of *MdANT1* and *MdANT2* may aid in better understanding the function of these genes.

Arabidopsis plants were also transformed with GFP-tagged *MdANT1* and *MdANT2* under the control of the constitutive promoter *Actin2* (*Act2*). MdANT1 and MdANT2 have been shown to share >50% homology with the Arabidopsis ANT [10]. Transient expression analysis indicated that the GFP-AtANT was localized to the nucleus [13]. *ANT* belongs to the AP2-domain family encoding protein that contains two copies of a ~70-amino acid domain termed the

AP2 repeat. The two AP2-domain repeats and the linker region essential for the DNA binding activity of AtANT [14], are conserved in MdANT1 and MdANT2 [10]. Additionally, MdANT1 and MdANT2 also contain a basic motif (TKKR) similar to the nuclear localization signal in Arabidopsis ANT [13,10]. Further analysis of GFP-tagged transgenic root seedlings will help in determining if these proteins are localized to the nucleus, thereby supporting their function as transcription factors.

To identify the target genes for MdANTs, purification of the MdANT2 protein was attempted. *MdANT2* was chosen as a candidate for this study as initial studies suggested that its expression was closely associated with cell production [10]. Purified MdANT2 (tagged with MBP) may be further used to identify genes regulated by MdANT2 using DNA immunoprecipitation (DIP; [15]). A variety of factors, such as the expression vector, growth conditions, and culture temperature, can strongly influence the production and solubility of the recombinant protein. The aggregation reaction, leading to formation of inclusion bodies, is in general favored at higher temperatures due to the strong temperature dependence of hydrophobic interactions that determine the aggregation reaction [16]. Several studies have demonstrated increased solubility of recombinant proteins at lower cultivation temperatures [17,18]. However, expression of the recombinant protein (MdANT2-MBP) was not observed at 25 °C but only at 37 °C. Bacterial growth is generally decreased at lower temperature which might have resulted in decreased fusion protein production at 25° C. Increasing the incubation time after induction from 24 h to 48 h had no significant effect on the solubility of the recombinant but the expression was higher at 48 h. MdANT2 protein was also cloned in frame with an N-terminal polyhistidine-tags (His.tag, pET28a vector, EMD Bioscience). However, the His-tagged MdANT2 recombinant protein accumulated only in the inclusion bodies under all conditions (data not shown). MBP has

been shown to promote solubility and influences proper folding of its soluble partner [19]. We observed higher solubility of MBP-MdANT2 fusion protein in comparison to the polyhistidine-tag. However, majority of the expressed protein was accumulated in the inclusion bodies. Inclusion bodies are a set of structurally complex aggregates that occur due to the deposition of mis-folded or partially folded polypeptides. Inclusion bodies may result either from accumulation of high concentrations of folding intermediates, from inefficient processing by molecular chaperones, or through the exposition of hydrophobic patches and the consequent intermolecular interactions [20]. Most of the expression vectors consist of strong promoter system that might lead to partial or complete segregation of the recombinant protein with the inclusion bodies [17]. The pMAL vector consist of the hybrid *tac* promoter derived from the *trp* and *lac* promoters and have been shown to have higher efficiency than the parental promoters [21]. Additionally, larger proteins are more likely to have complex folding mechanisms, which render them more vulnerable to the aggregation associated with very high transcription rates at optimal growth temperatures. The approximate molecular mass of MBP-MdANT2 protein is about 114 kDa thus increasing the chances of its mis-folding. Moreover, the purified fusion protein had multiple bands on an SAD-PAGE and as indicated on the western blot. The 40 kDa band displayed highest concentration both on the SDS-PAGE and western blot. This is close to the molecular mass of MBP (42.5 kDa). The protein purification process may have resulted in the degradation of the fusion protein which may explain the presence of these multiple bands. Recombinant proteins may undergo proteolysis by host-cell proteases leading to partial purification of the target protein [22].

Further characterization of the transgenic plants obtained from this study will help in understanding the function of *MdANT1* and *MdANT2* in organ size regulation. Different protein

purification methods may need to be applied to avoid protein degradation issues observed in this study. Increasing the solubility of the fusion protein may also enable better purification of the MdANT2 protein.

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Figure 5.1. *PCR amplifications of DNA extracted from transgenic Arabidopsis plants.*

The PCR products were separated on a 1.2 % agarose gel.

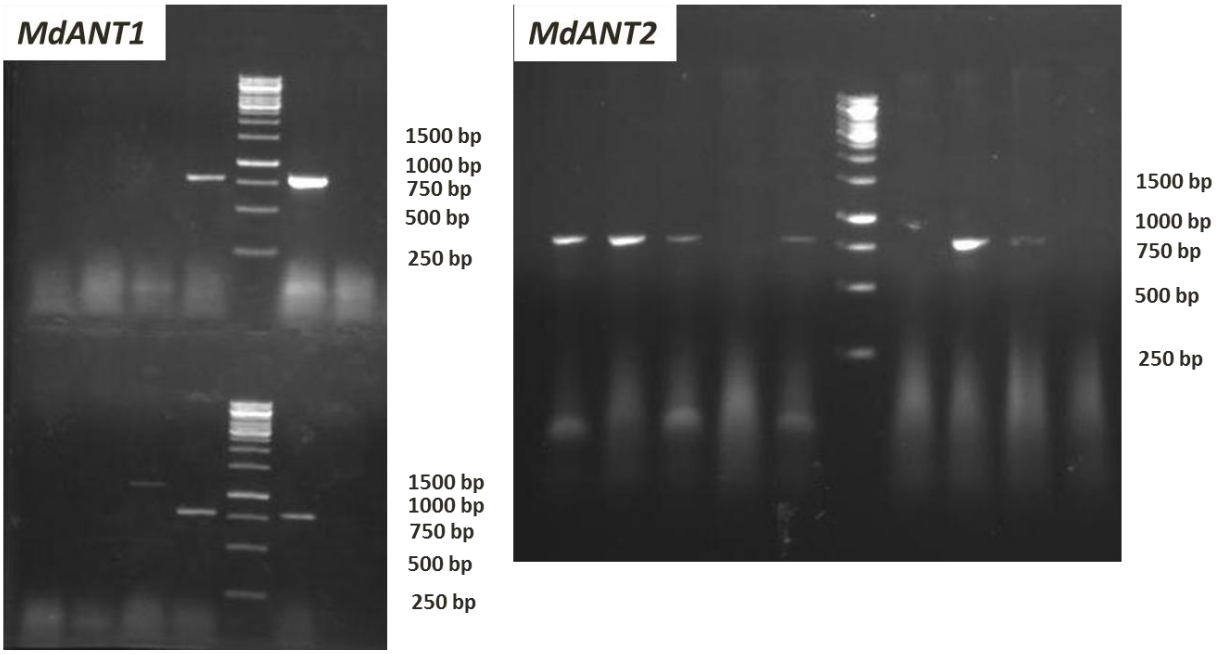

MdANT1
1500 bp
1000 bp
750 bp
500 bp
250 bp
1500 bp
1000 bp
750 bp
500 bp
250 bp
MdANT2
1500 bp
1000 bp
750 bp
500 bp
250 bp

Figure 5.2. *Samples from total intracellular protein fraction analyzed on SDS-PAGE.* Recombinant protein was induced at 25 ^{o}C using 1 M IPTG (P1) and 600 mM IPTG (P2). No IPTG was added to the un-induced protein (Un-Ind) sample.

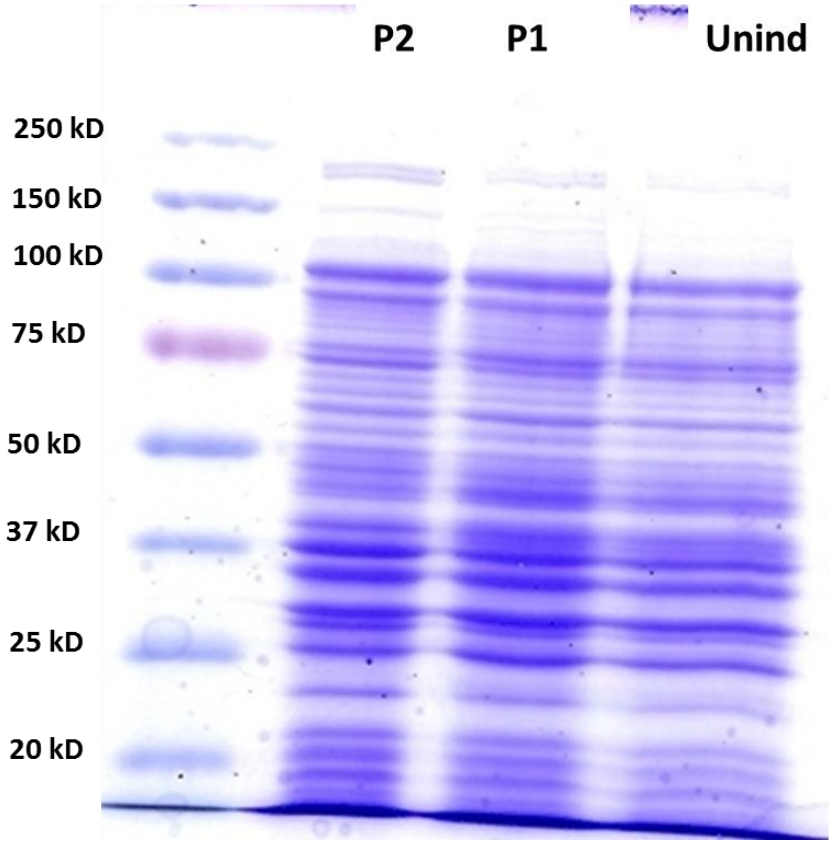

P2
P1
Unind
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD

Figure 5.3. *Samples from soluble (S1 and S2) and pellet (P1 and P2) fraction of fusion protein analyzed on SDS-PAGE.*

Recombinant protein was induced at 37 ^{o}C using 600 mM of IPTG for 24 h (S1 and P1) and 48 h (S2 and P2). No IPTG was added to the un-induced protein (Un-Ind) sample.

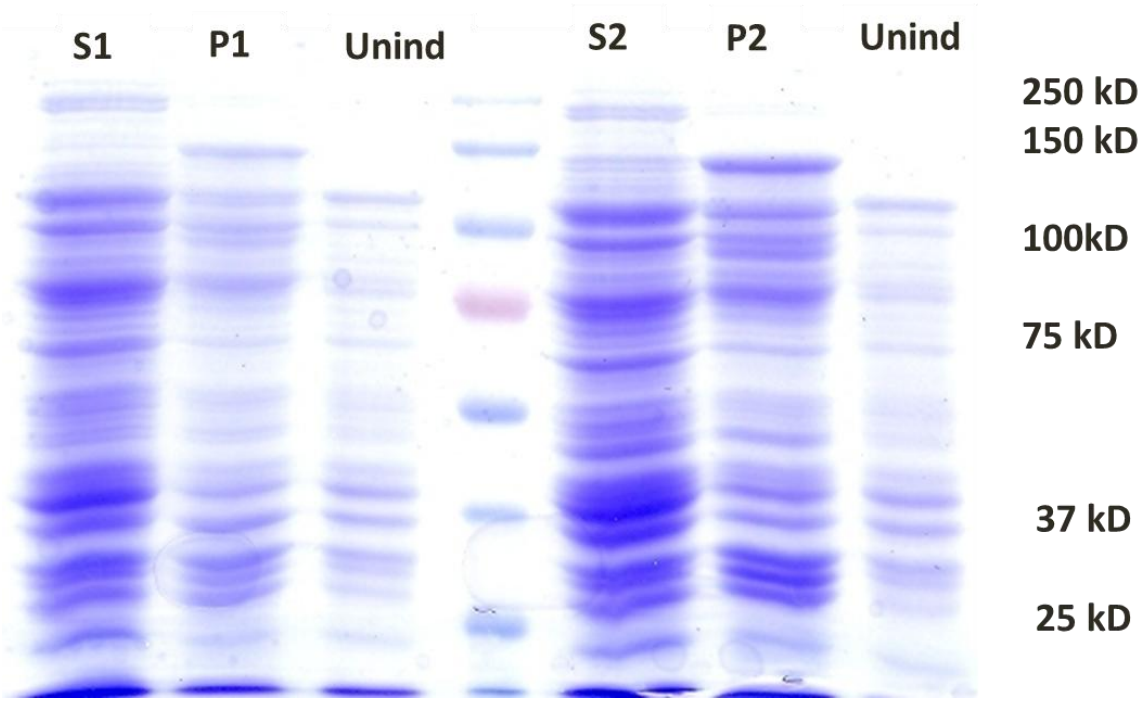

S1
P1
Unind
S2
P2
Unind
250 kD
150 kD
100kD
75 kD
37 kD
25 kD

Figure 5.4. *Samples from protein fractions analyzed on SDS-PAGE.*

Recombinant protein was induced at 37 ^{o}C using 600 mM of IPTG for 48 h. Legend: Ptn = Protein from supernatant; S1 = Supernatant after incubation with amylose beads; W1, W2 and W3 = Supernatants after washing amylose resin with PBS buffer; E1, E2 and E3 = Protein obtained after the 1st, 2nd and 3rd elutions.

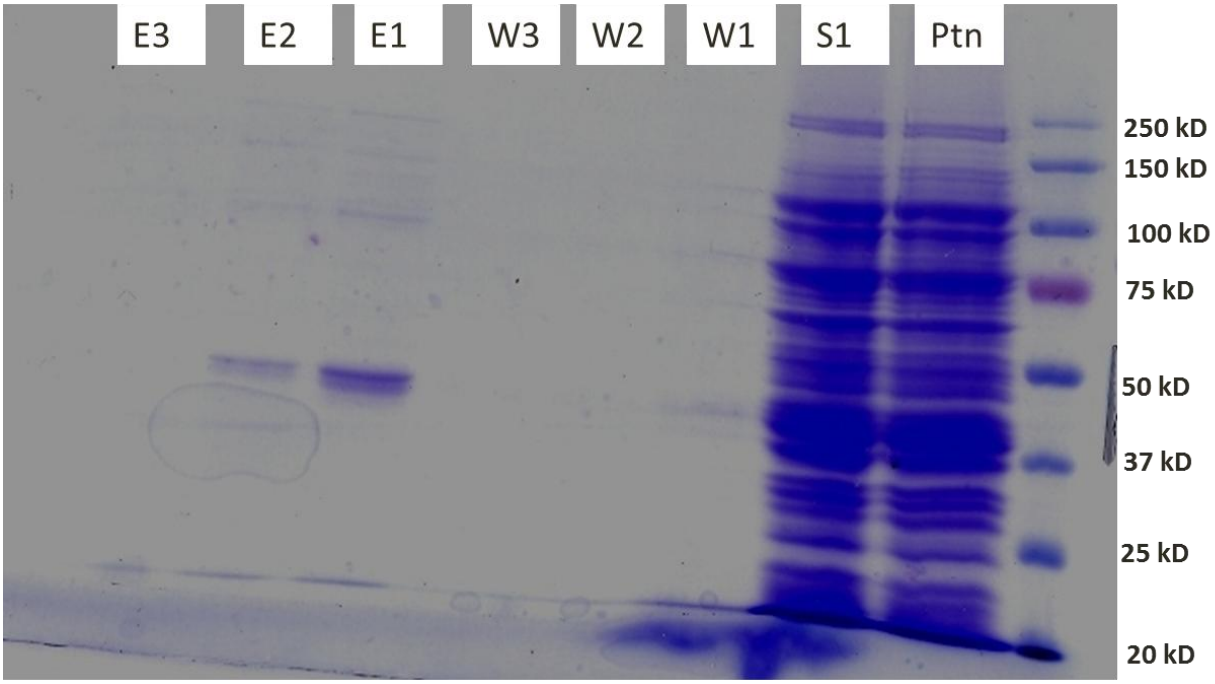
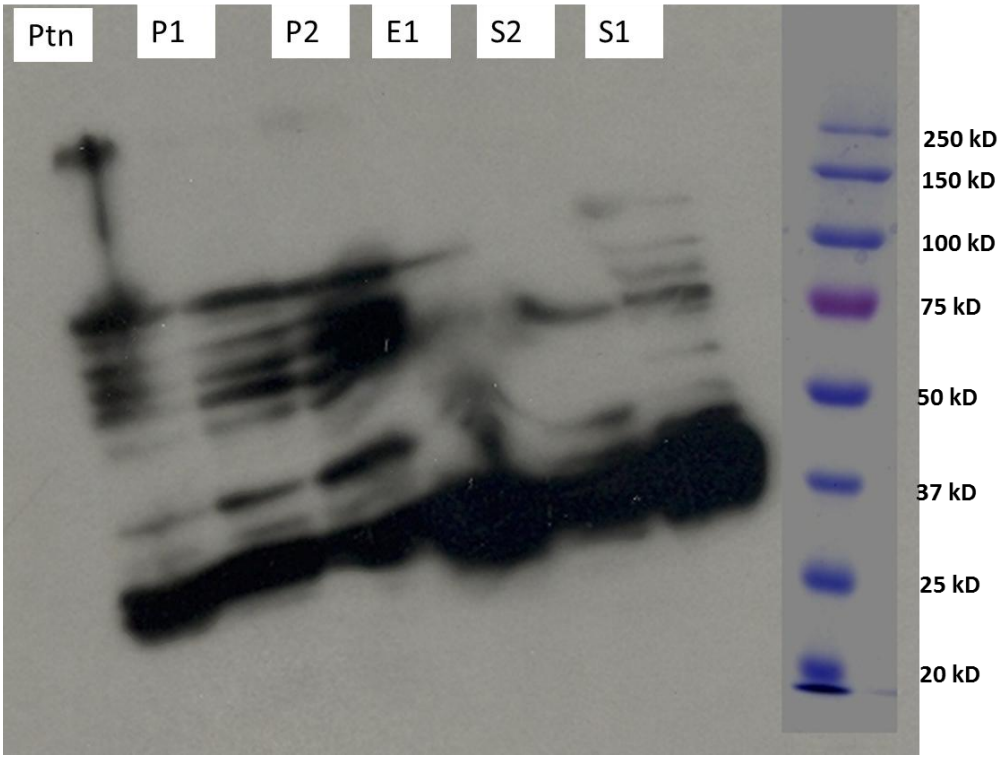

E3
E2
E1
W3
W2
W1
S1
Ptn
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD

Figure 5.5. *Samples from protein fractions analyzed on western blot.*

Recombinant protein was induced at 37 ^{o}C using 600 mM of IPTG for 24 h and 48 h. Protein was purified from the soluble fraction of the protein sample induced for 48 h. Legend: Ptn = Protein from supernatant; P1 = Pellet fraction from protein induced for 48 h; P2 = Pellet fraction from protein induced for 24 h; E1 = Protein obtained after 1st elution; S2 = Soluble fraction from protein induced for 24h; S1 = Soluble fraction from protein induced for 48 h.


Ptn
P1
P2
E1
S2
S1
250 kD
150 kD
100 kD
75 kD
50 kD
37 kD
25 kD
20 kD

CHAPTER 6

CONCLUSIONS

In this study, mechanisms regulating fruit growth in apple were investigated using different approaches. Shade-induced reduction in fruit growth was found to be facilitated by a decrease in cell production and expansion in the fruit cortex. Expression of carbohydrate metabolism-related genes, transcription factors associated with fruit growth, and key genes associated with cell production and expansion was also altered in response to shading. Therefore, coordinated changes in the expression of these genes may induce shade-induced reduction in cell production and expansion subsequently leading to the reduction in fruit growth. The molecular mechanisms mediating thinning induced increase in apple fruit growth were analyzed. Data from this study showed that thinning enhances fruit growth primarily by increasing cell production during early fruit growth and possibly cell expansion at very late stages of fruit development. Thinning also altered the expression of genes associated with carbohydrate metabolism and key genes associated with fruit growth, cell production and expansion. Thinning mediates an increase in cell production which may lead to increased sink activity of the fruits. Therefore, it can be suggested that carbohydrate metabolism and availability is affected in response to change in sink activity due to thinning which might further affect the expression of key genes associated with fruit growth, cell production and cell expansion subsequently leading to increase in fruit growth.

To further identify key genes regulating fruit growth in apple two *AINTEGUMENTA (ANT)* genes, *MdANT1* and *MdANT2*, were isolated from apple. *ANT* is a transcription factor and has

been shown to control organ growth in Arabidopsis by regulating cell production. The two apple *ANT* genes are highly homologous, with 93% homology at the nucleotide level (coding region). *MdANT1* and *MdANT2* expression was analyzed during fruit growth and in response to factors affecting fruit size. The expression of the *ANTs* is closely associated with cell production during fruit development. Additionally, their expression was also correlated with that of several cell cycle genes positively associated with cell production. Sequence comparison of MdANT1 and MdANT2 with the Arabidopsis ANT; suggest that *MdANT1* and *MdANT2* may function as transcription factors in apple. Together, these data suggest that *MdANT1* and *MdANT2* coordinate the expression of cell proliferation genes and thereby regulate cell production during apple fruit development. *MdANT1* and *MdANT2* are a significant addition to the list of candidate regulatory genes regulating fruit growth in apple.

To further determine the function of these genes Arabidopsis plants were transformed with *Act7*::*MdANT1/2* and *Act2*::*GFP:MdANT1/2* constructs. Four transgenic plants for *MdANT1* and six transgenic plants for *MdANT2* were isolated in this study. In order to identify downstream targets for apple *ANT* attempts were made to purify the MdANT2 protein by fusing it to the C-terminal end of the maltose-binding protein (MBP). However, the MBP-MdANT2 fusion protein still had reduced solubility and the purified protein displayed significant degradation.

Future directions

Environmental factors and cultural practices affect fruit growth. These studies indicate that these factors affect fruit growth through coordinated changes in the expression of several genes associated with carbohydrate metabolism, cell production and expansion. However, it is not completely clear how fruit development is modulated in response to the size of available carbohydrate pool. In these studies no significant difference in sugar accumulation between

thinned and control fruit was observed. However, only one stage of fruit growth, towards the period of maximum change in growth, was analyzed in the thinning experiment. Analysis of the earlier period, immediately following the thinning treatment may provide better insights into changes in carbohydrate accumulation. Therefore, further analysis of carbohydrate levels and metabolism in response to shading and thinning is required to better understand the process of carbohydrate mediated regulation of fruit growth.

Data from the above studies strongly suggest that apple *ANT* genes function as transcription factors in apple. Further analysis of GFP-tagged transgenic root seedlings using confocal microscope will help in determining if the proteins are localized to the nucleus. Additionally, transgenic plants expressing the apple *ANT* genes in Arabidopsis have been developed. Seeds obtained from the T1 plants will need to be germinated and plants with the highest expression level for *MdANT1* and *MdANT2* (i.e., homozygous) should be selected for further characterization. Homozygous lines can be selected to further study the effect of expressing *MdANT1* and *MdANT2* on organ growth. This can be achieved by comparing organ growth in vegetative organs such as leaves, as well as floral organs (petals) of the homozygous transgenic plants with the wild type plants. Further kinematic analysis of cell production and cell expansion in developing leaves and petals will also help in determining the effect of *MdANT1* and *MdANT2* expression on cell production and growth. In addition, *MdANT1* and *MdANT2* may be expressed under the native Arabidopsis *ANT* promoter in the Arabidopsis *ant* mutant to examine if the apple *ANT* genes are functional homologs of the Arabidopsis *ANT*.

To identify the genes regulated by the apple ANT, purification of this protein is a critical step. Although attempts were made to purify MdANT2, this was not feasible owing to the low solubility of the recombinant MdANT2 protein. The solubility of a protein can be improved by

using different affinity tags, different expression system like yeast, modifying the cultivation strategies or by co-expression of interaction partners like molecular chaperones promoting proper protein folding [1]. In this study two different affinity tags were used and the solubility was found to be increased by using the MBP tag. Another affinity tag that has been shown to promote solubility is N-utilizing substance A (NusA; [2]). Moreover, protein purification can be attempted using MdANT1. Small differences in the protein sequence can affect solubility as it has been shown that single mutation in a protein can improve its solubility [3,4].

Besides protein purification, synthetic peptides generated using MdANT1 and MdANT2 protein sequences can also be used for antibody production. These antibodies can further be used to identify *in vivo* target genes for MdANT1 and MdANT2 using the technique of chromatin immunoprecipitation (CHIP). Samples from 'Golden Delicious Smoothie' have been collected a week before bloom, during bloom time, and twelve days after bloom in 2011. The samples were cross-linked in 1% formaldehyde, rinsed and frozen using liquid nitrogen. These samples are currently stored at -80^{o}C. Chromatin can be isolated from these fruit samples as described by Bowler et al., 2004. The chromatin can be sheared using sonication. The sheared chromatin can be immunoprecipitated using the antibodies raised against MdANT1 or MdANT2. Fruit samples were also collected at ~10 weeks after bloom to serve as negative controls.

Genome-wide analysis of gene expression, using RNA-seq, during fruit growth may also help in the identification of other regulatory genes controlling fruit growth in apple. Further studies along the lines described above may improve our understanding of the genetic networks regulating fruit size in fleshy fruits like apple. This information has potential implications toward fruit industry since manipulation of these genes may lead to enhanced fruit size not only in apple but also in other fruit crops.

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