INVESTIGATION OF REGULATORY MECHANISMS OF CHEMICAL-MEDIATED FRUIT THINNING IN APPLE (*Malus x domestica* Borkh.)

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ABSTRACT

Fruit thinning is critical to the success of apple industry because most apple trees are prone to biennial bearing characterized by heavy bloom and over-cropping in the "on" year and low or no cropping in the "off" year. Fruit thinning can improve fruit size, increase return bloom, and reduce alternate bearing habit of apple trees. Chemical thinning has been widely used as a horticultural practice by growers for years in apple and other fruit production. However, its thinning results are often variable and very difficult to predict since parameters as well as their interactions (e.g. concentration, environmental condition, cultivar response) that affect thinning effectiveness have been poorly defined and the regulatory mechanisms at molecular level remain to be illustrated. Therefore, the purpose of this study is to characterize the physiological and molecular responses of the apple trees to the thinning treatments, and then use key genes as molecular markers for screening potential thinning agents. The long-term goal is to understand how the applied chemicals and environmental factors interact and regulate key regulatory genes as well as the thinning effectiveness during thinning process, and establish a predictable model for the improvement of fruit thinning consistency and effectiveness in apple and other fruit trees.

Effects of naphthaleneacetic acid (NAA), shading, amimoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP) on young apple fruit abscission, leaf and fruit ethylene production, and the expression of genes related to ethylene biosynthesis, perception and cell wall degradation were examined in 'Golden Delicious' apples (*Malus x domestica* borkh.). NAA at 15 $mg \cdot L^{-1}$ and

shading increased fruit abscission and ethylene production of leaves and fruit when applied at a 12-mm stage of fruit development, whereas AVG, an inhibitor of ethylene biosynthesis, at 250 $mg \cdot L^{-1}$ reduced NAA-induced fruit abscission and ethylene production of leaves and fruit. 1-MCP at 160 $mg \cdot L^{-1}$ had no effect on fruit abscission but induced ethylene production by both leaves and fruit.

Changes in the gene expression pattern responding to each treatment were analyzed by real-time quantitative PCR. NAA treatment was found to enhance the expression of genes related to ethylene biosynthesis (*MdACS5A*, *MdACS5B* and *MdACO1*) and perception (*MdETR1*, *MdETR1b*, *MdETR2*, *MdERS1* and *MdERS2*). AVG reduced NAA-induced expression of these genes except for *MdERS2* in the fruit abscission zone (FAZ). NAA increased the expression of a polygalacturonase gene (*MdPG2*) in the FAZ but not in the fruit cortex (FC), whereas AVG reduced NAA-enhanced expression of *MdPG2* in the FAZ. These results suggest that ethylene biosynthesis, ethylene perception, and *MdPG2* gene are involved in young fruit abscission caused by NAA. On the other hand, 1-MCP did not affect the expression of *MdACS5A* and *MdACS5B* in the FAZ, although it enhanced the expression of these two genes in the FC from 6 to 24 hours post-treatment. The expression of *MdACO1* in both tissues was increased by 1-MCP after 3 or more days post-treatment. 1-MCP had only a small influence on the expression of most ethylene receptor genes, with the exception of *MdETR1*, which was up-regulated in the FC to a level similar to that observed for NAA treatment. In response to 1-MCP, in the FAZ, the expression of *MdCel1* and *MdPG2* was up-regulated at the beginning and the end, respectively, of the experiment, but otherwise remained at or below control levels. 1-MCP did not inhibit NAA-induced abscission of young apple fruit, suggesting that abscission does not solely depend

on ethylene signal transduction, or that the periods of effectiveness for 1-MCP and ethylene were asynchronous in this study. Gene expression analysis also revealed that both NAA and shading enhanced the expression of gene related to ABA biosynthesis in the FAZ, which suggested the involvement of ABA in young fruit abscission.

Global gene expression profile during young fruit abscission was analyzed using an apple oligonucleotide microarray. More than 700 genes were identified with reproducible changes in transcript abundance in the FAZ after NAA treatment. Genes associated with abscission, ethylene, ABA, cell wall degradation, mitochondrial activity, glycolysis, lipid catabolism, secondary metabolism, abiotic stress, and apoptosis were upregulated, while genes involved in regulation of cell cycle, cell wall biosynthesis, photosynthesis, carbon fixation, chromatin assembly, auxin transport/efflux, cytoskeleton function, and flower development were generally downregulated. Comparison of changes in the gene expression patterns during NAA-induced fruit abscission with shading-induced fruit abscission revealed similarities, but also considerable differences. The data suggested that young fruit were shutting down growth and energy production and undergoing a certain kind of programmed cell death (PCD) induced by these different stimuli. Gene expression analysis in abscising young fruit revealed that photosynthesis, carbon utilization, and ABA/ethylene pathways appeared to operate in both NAA- and shading-induced fruit abscission. However, more sugar signaling genes were regulated in shading-induced fruit abscission, compared to NAA-induced fruit abscission.

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Dedication

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Chapter 1

Introduction and Literature Review

History of Apple Fruit Thinning

Many fruit tree species bear an abundance of flowers which produce excessive fruit that the tree is unable to support. For the sake of self benefit and survival in nature, many fruit tress have evolved a self regulatory mechanism whereby they shed part of their fruit load at a certain early period (Dennis, 2000). This ensures that more fruit are not retained by the tree than can be supported under prevailing environmental conditions. From a horticultural point of view, with most of the temperature fruit group, as e.g. apples, this self-regulatory mechanism is entirely insufficient to guarantee the required quality standards. Too many fruits per tree can result in small fruit size and poor quality, breakage of limbs, exhaustion of tree reserves, and reduced cold hardiness. In some cultivars, heavy fruiting can also partially or completely inhibit flower bud initiation (Bangerth, 2000). The growers have recognized these as major problem for fruit production and tree longevity, and adopted fruit thinning practice usually by hand for thousands of years.

Mechanical and chemical thinning

Over 2000 years ago, Theophrastus reported that farmers recognized the tendency of fruit trees to overcrop and described the custom of partial crop removal. Since then, hand thinning remains an important tool for the fruit grower where they thin on purpose, taking away the least promising ones and preserving the best. Hand thinning at the “June drop” had been recommended because this would improve fruit size more than would later thinning, prevent the draining of the energy of the tree by seeds, and avoid removal of fruits that fall of their own

accord (Gourley, 1922). Batjer et al. (1957) developed a system to predict the effect of hand thinning at any given time during fruit development on final fruit sized of apples. It had been suggested that thinning fruit within clusters was most effective in increasing fruit size, indicating that fruit distribution, as well as total fruit number, is crucial in determining the size. However most results showed little effect of hand thinning on annual bearing of apple though fruit size was largely increased. It had been noted that spurs that set fruit generally did not flower the next year, whereas those that blossomed but did not set fruit might or might not flower, and that blossom thinning would induce many spurs to form flower buds, so blossom removal usually increased spur growth and leaf number (Auchter, 1920). Bobb and Blake (1938) hand-thinned trees to 10-12 inches apart at early pink bud for four consecutive years, and they observed that not only did this increase fruit size and promote annual cropping, but the total yield per tree was increased. Despite many advances in knowledge concerning the process, the flower initiation in apple remains difficult to predict, even when conditions (crop load, weather, etc.) appear favorable. The timing of thinning may be more important in some years than in others, but few studies are done where this factor has been critically examined.

In general, mechanical thinning methods such as tree shakers or heavy ropes are not recommended for apple trees because many spurs would be knocked off and the foliage damaged. Also the fruit can be easily bruised, and the damage detracts from the appearance of the mature fruit. Therefore, although some machines are being used in Europe, where environmental restrictions on the use of thinning chemicals are rigorously imposed, results to date have not been commercially acceptable.

A major advance in fruit chemical thinning was the report by Burkholder and McCown (1941) that naphthaleneacetic acid (NAA) applied at full bloom reduced fruit set of apple. This was unexpected since NAA had been reported (Gardner et al., 1939) to delay pre-harvest drop so they had hoped to find a way to increase fruit set. Until the mid-1940s, NAA had been applied only at bloom, but after that NAA has been used primarily as a post-bloom thinner. Further evaluation of timing effects demonstrated that NAA was not effective after the "June drop". On the other hand, considerable variability was observed, probably because of impact of the environmental conditions during treatment. Under some conditions, NAA can induce the formation of "pygmy" fruit. Position of the fruit in the cluster can also affect response to NAA (Black et al., 1995).

The thinning activity of ethephon (2-chloroethylphosphonic acid) which releases ethylene on hydrolysis within the treated tissues had been evaluated (Edgerton and Greenhalgh, 1969), but one of the major problems with this chemical is the marked effect of temperature on the rate of release of ethylene in the tissue. High temperatures following application could result in severe overthinning (Olien and Bukovac, 1978).

Early experiments with benzyladenine (N^6-benzylaminopurine, BA) indicated that it would stimulate cell division in young apple flowers and fruits, with early applications being most effective (Unrath, 1974). Benzyladenine is considered to be a mild thinning agent, and combinations of BA with NAA or carbaryl are often used for maximum effect. Under some conditions, BA alone can stimulate fruit growth more than would be expected from its thinning effect.

The thinning activity of the insecticide carbaryl (1-naphthyl-N-methylcarbomate) was first reported by Batjer et al. (1960, 1962). Since much higher concentrations of carbaryl were required but less overthinning resulted, carbaryl is considered to be a mild thinning agent, and is best used with other compounds, such as NAA or BA, for difficult-to-thin cultivars such as 'Golden Delicious' and spur-type strains of 'Delicious'. However, carbaryl is an insecticide that can kill beneficial insects, including honeybees, so a flowable formulation (Sevin^{R} XLR) is less harmful to bees than is the powder formulation.

As expected, fruit abscission can be induced by shading individual limbs or entire trees, as fruit growth is dependent upon carbohydrates supplied by the leaves (Byers et al., 1985, 1990b). Chemicals that inhibit photosynthesis can also serve as thinning agents for apple. Treatment of the fruit alone is ineffective in inducing abscission, indicating an indirect effect. Stopar et al. (1997) reported that NAA application inhibited carbon assimilation by as much as 25% for up to 48 h in 'Delicious' and 'Empire' leaves, and inhibition continued for 2 weeks. Given the low concentrations of NAA that are effective in thinning under some conditions, inhibition of photosynthesis might only play a minor role in fruit abscission response (Marini, 1997).

Changes Associated with Young Apple Fruit Abscission

Cell separation in the vast majority of plant organs, including young fruit abscission in apple, is highly predictable and reproducible within the same plant species, which may be part of the highly programmed development of a plant, and/or in response to environmental stresses. As we investigate the mechanism by which plant growth regulator signals are perceived and transduced, we can better understand how the process of abscission itself may be induced and regulated.

Anatomical Changes

In most cases, young apple fruit form a swollen abscission zone (AZ) at the base of the pedicle before they shed. The cells that comprise this zone are often morphologically distinguishable before the onset of abscission. They can be considered as multicellular structures in which cell to cell separation occurs between a subset of cells within the zone. Typically, AZ cells are smaller and more densely filled with cytoplasm than are the cells in adjacent regions (Sexton and Roberts, 1982). Tracheary elements are less well developed, and have less lignin deposition (Addicott, 1982), so AZ is a region of arrested development. The number of cells that make up an AZ appears to be fixed for a particular organ in a species, but varies between species.

The cells that comprise a separation layer have been termed abscission zone target cells (Osborne, 1976). Osborne and Sargent (1976b) showed that until these target cells were present, abscission could not be induced even by prolonged exposure of high concentrations of applied ethylene. Also, previous studies have raised the conflict between the idea of predetermination in AZ cells, and the notion that any cell might dedifferentiate to form an AZ cell in response to appropriate signals. However, the anatomical examination of AZ left unanswered the important question as to how abscission is programmed. It is believed that certain chemical changes in the young fruit initiate the abscission in the AZ. The nature of the chemical stimuli and the mechanism by which those signals are perceived at the AZ are crucial questions to be answered.

Physiological and Biochemical Changes

Following abscission signal perception, an ordered series of events might occur in abscising young fruit, involving disintegration of chloroplasts, breakdown of proteins, loss of chlorophyll

and removal of amino acids. Endogenous levels of auxin, cytokinins and gibberellins decline before and with the onset of senescence symptoms (Aharoni and Richmond, 1978; Nooden and Leopold, 1978; Thimann, 1980). It has been proved that the sensitivity of a leaf to ethylene increases with age and in response to ABA (Ronen and Mayak, 1981), but whether this correlation exists in abscising young fruit needs more experimental evidence. Protein determinants have also been shown to be present both in AZ cells before or during abscission (McManus and Osborne, 1990, 1991). The identity of these proteins remains unknown but suggests that AZ cells are biochemically distinct before the separation process commences (Roberts et al., 2000).

Carns (1951) tested the effect of a selected series of inhibitors of respiratory enzymes and found that the explants that abscised showed a "climacteric" rise in respiration, while those that did not abscise showed a slow decline in respiration. These results suggest that oxidative respiration is essential to abscission and abscission involved active physiological processes requiring energy and the functioning of the full machinery of oxidative respiration.

Rabey and Bate (1978) suggested that the abscission was correlated with greatly reduced translocation of carbohydrate into the young fruit. It is possible that the abscission zone is sensitive to the amount of carbohydrate passing through it. High levels of carbohydrate could be a signal indicating that the young fruit is a vigorous sink. Abscission is also related to nitrogen metabolism. A common observation is that plants supplied with abundant of nitrogen retain their leaves much longer and set more fruit than do plants deficient in nitrogen. Additionally, the involvement of calcium in abscission has been proposed by Jones and Lunt (1967) as Ca-pectate

is a major constituent of the cell wall, particularly of the middle lamella. Poovaiah and Leopold (1973) also found that during abscission, calcium essentially disappears from the separation layers and Ca^{2+} can retard or inhibit abscission.

Molecular Changes

Other researchers have identified molecular features that mark particular cells as predetermined to undergo separation. Wong and Osborne (1978) observed that the AZ cells at the base of ovary of female flowers of *Ecballium elaterium* could be distinguished by their capacity to undergo endoreduplication of their nuclear DNA before they gained the competence to respond to ethylene. With the identification of *JOINTLESS* and *SHATTERPROOF* gene products, it has been suggested that such MADS-box genes have a clear role to play in the formation of zones of cells that will at some point undergo separation. Another MADS domain factor, AGL15, has been shown to delay abscission in *Arabidopsis* petals and sepals (Fernandez et al., 2000). Overexpression of AGL15 appeared to maintain the AZ cells in a more juvenile state. These events are not the result of a lack of response to ethylene, but the result of a delay in the release and action of hydrolytic enzymes, possibly polygalacturonase (PG) and/or cellulase.

The separation process itself may be directional in nature. A key step in loss of adhesion between cells within a separation layer is the induction of cell wall-degrading enzymes such as PG. This enzyme breaks down the pectin rich middle lamellae which lead to cell separation. It has been shown that there are abscission-specific PGs that are distinct from PGs associated with other developmental events that require cell separation, such as fruit ripening (Kalaitzis et al., 1997, Li

and Yuan, 2008). The genes encoding these enzymes have been isolated and shown to belong to a multigene family (Roberts et al., 2000).

Thompson and Osborne (1994) suggested that the primary signal to initiate separation could be a diffusible cell wall oligosaccharide arising from cell wall hydrolysis. Subsequent research that used chimeric constructs of *jointless* and wild type tomato (Szymkowiak and Irish, 1999) indicated that the first cells to respond to an abscission signal are situated close to the vascular tissue. The identification of transcription factors that regulate the differentiation of the AZ, or the process of cell separation, suggests that these two events have the capacity for independent control.

Environmental Factors that Contribute to Induction of Young Apple Fruit Abscission

Natural abscission of young fruit and its regulation by plant hormones is considered and compared to the generally accepted model of "senescence triggered" abscission of, for example, leaves or mature fruit. When under developmental control, abscission of plant parts can proceed even under favorable growing conditions. It has been suggested that abscission of young fruit cannot not explained by this model. Alternatively, it is suggested that it should be replaced by a "correlatively triggered" model, where more environmental signals are responsible for the abscission of young fruit (Taylor and Whitelaw, 2001).

Dark and low-light treatments have been shown to increase abscission of flowers, flower buds, leaves and fruits. This effect appears to be mediated by auxin (Mao et al., 1989) and specific to auxin. Wien and Turner (1989) observed that the shade-induced abscission of reproductive

structures in pepper was correlated with increased ethylene production in floral structures, and could be reversed by application of auxin. On the other hand, insufficient nutrition supply and intensified competition for nutrients among the young fruit are other important factors that induce abscission.

Drought can also promote abscission as a result of a decline in the growth and vigor of the plant. As drought develops, there is a reduction in the rate of photosynthesis mainly due to stomatal closure (Chaves, 1991). Drought stress and other water-related stresses have been reported to induce stress ethylene and its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Tudella and Primo-Millo, 1992). The interpretation of these results led to a hypothesis whereby water stress causes an accumulation of ACC in the roots that is translocated to the shoots and oxidized to form ethylene, which in turn promotes leaf abscission. With other stresses, water stress-induced abscission may occur as a result of a tissue-specific increase in ethylene sensitivity.

Wounding and pathogen attack can induce plant defense response involving substantial alteration in gene expression. If the defense response is unsuccessful, then the plant would shed the infected organ, in order to prevent the spread of infection throughout the affected plant. Ethylene may be involved in the transmission of wound/pathogen-induced stimuli, since endogenous ethylene level increases upon injury. In addition, ethylene is known to play a regulatory role in the accumulation of endogenous jasmonates in the wounded leaves (O'Donnell et al., 1996). Jasmonates in turn initiate the defense-related gene expression program in plants (Reinbothe et al., 1994). The involvement of ABA in plant defense response is supported by the fact that

endogenous ABA level increases significantly upon wounding/infection and the increased ABA level has been suggested to be responsible for leaf abscission in cotton (Wiese and DeVay, 1970).

Interacting Signals during Young Apple Fruit Abscission

The level of auxin is regulated by its biosynthesis and degradation, and the auxin status of the abscission zone controls the sensitivity to ethylene (Davies et al., 1999). Abscission does not occur when polar basipetal IAA transport to the abscission zone is maintained (Sexton et al., 1985). On the other hand, ethylene is a potent inhibitor of auxin transport and may enhance the sensitivity of the zone to itself through increasing auxin conjugation (Beyer, 1973). However, a common but contradictory finding is that auxin itself can also stimulate ethylene production and accelerate abscission, suggesting that there is actually no single, key abscission regulator. If so, the induction of abscission might be dependent upon a complex interplay of hormones in addition to the responsiveness of the tissue (Morgan and Hall, 1964; Brown, 1997).

The roles of nonhormonal signals in abscission have also been proposed. Once the abscission signal is recognized by a predetermined competent abscission zone target cell, it needs to be transferred to the surrounding cells in order to initiate gene activation within the separation layer. It has been proposed by Thompson and Osborne (1994) that such transferable signals may be cell wall-derived oligogalacturonides. Oligosaccharides are generated from the plant cell wall due to loss of compartmentalization by mechanical disruption and are found to interact with auxin-binding sites in plant membrane (Farmer et al., 1991; Filippini et al., 1992). So in abscission, it is possible that the oligogalacturonide act by blocking auxin binding sites in the cells of the

separation layer, thus increasing their sensitivity to ethylene and reducing the inhibitory effect of auxin (Taylor and Whitelaw, 2001).

Polyamines have been shown to be associated with the cell wall, stabilizing membrane structures. It also has been suggested to modulate signal transduction by interacting with oligogalacturonides (Messiaen and Van Cutsem, 1999). The level of polyamines usually decreases with senescence, partly as a result of the switch of ethylene biosynthesis and such a reduced level of polyamines might result in increased levels of free oligogalacturonides for signal transduction in the separation layer (Taylor and Whitelaw, 2001).

Proposed Modes of Action of Apple Thinning Chemicals

The mechanisms involved in fruit thinning appear to be complex and researchers continue to debate the effects of applied chemicals on endogenous hormone biosynthesis, photosynthesis, carbon utilization, phloem transport and many other physiological processes. Several mechanisms have been proposed to explain the thinning action of applied chemicals and they are not identical for all the chemicals (Williams, 1979; Bangerth, 2000; Dennis, 2002). Additionally, evidence for the site of action of thinning chemicals suggest various responses of leaf or fruit to different chemicals in terms of thinning effectiveness (Schneider and Lasheen, 1973; Greene et al., 1992).

1) Abortion of seeds or inhibition of seed/embryo development

Some early reports showed that NAA induced seed abortion or inhibited embryo growth, which presumably could reduce the ability of the fruits to compete for nutrients, leading to their

abscission (Luckwill, 1953; Murneek and Teubner, 1955). Recent research demonstrated that BA treatment would block the growth of fruitlet cortex by inhibiting the ovary growth, followed by the activation of the abscission zone (Botton et al., 2010). But NAA can also thin apple cultivars without affecting seed number or even thin seedless fruit in other cases, suggesting that the thinning action of NAA is not always directly associated with embryo abortion (Marsh et al., 1960; Batjer and Thomson, 1962).

2) Inhibition of nutrient transport to fruit

Carbohydrates are essential for fruit set and subsequent fruit growth. Early fruit abscission is a self-regulatory mechanism within the tree that adjusts crop load to ensure that carbohydrate and metabolite supply by the tree could be in balance (Goldschmidt and Monselise, 1977). Guardiola et al. (1984) and Schaffer et al. (1985) also concluded that the final set of fruit appeared to be controlled by the capacity of the tree to supply carbohydrates to the developing fruitlets during the post-anthesis period. Apple fruit abscission after fertilization and during the "June drop" may be caused by competition for essential metabolites among individual fruitlets and between fruitlets and vegetative shoots. The "June drop" is heavier when the number of flowers setting fruit is high, and partial removal of either flower or fruit increases the percentage of flowers setting fruit and decreases fruit drop (Knight, 1980). Competition for assimilates was also suggested as a major factor resulting in the premature abscission of flowers or/and young fruits in soybean, litchi and peach (Stembridge and Gambrell, 1972; Brun and Betts, 1984; Roe et al., 1997).

It had been observed that application of NAA to 'Golden Delicious' reduced the transport of sugars from leaf to fruit and lowered the content of reducing sugars in the fruit (Schneider and Lasheen, 1973; Schneider, 1975). However, Yuan and Greene (2000a) reported that BA application to the fruit stimulated the import of ^{14}C-sorbitol from the leaf but similar treatment to the leaf did not affect the movement of ^{14}C-sorbitol to the fruit, so they concluded that BA does not appear to block the sugar transport.

3) Inhibition of auxin synthesis and transport from fruit

It had been indicated that NAA reduced auxin synthesis and/or transport from the fruit to the abscission zone (Crowe, 1965). Ebert and Bangerth (1982) measured auxin in fruits following application of various thinners and the most consistent effect was a reduction in the amount of diffusible auxin recovered. However, contradictory result also existed (Schneider, 1973). A recent microarray analysis of abscission-related transcriptome in tomato flower abscission zone showed that acquisition of ethylene sensitivity in the AZ is associated with altered expression of auxin-regulated genes, resulting from auxin depletion (Meir et al., 2010).

4) Stimulation of Ethylene Biosynthesis

The role of ethylene in the mechanism of action of chemical thinners has been controversial. Some experiments showed that ethylene is not the primary factor controlling the "June drop" and that thinning response did not parallel fruit ethylene content (Murneek and Teubner, 1955; Schneider, 1974). However, more recent studies reported a strong correlation between ethylene evolution and abscission, which was stimulated by the thinners (McArtney, 2002; Dal Cin et al., 2005; Zhu et al., 2008).

5) Inhibition of Photosynthesis / Stimulation of Dark Respiration

Both shading and application of chemicals that inhibit photosynthesis, stimulate apple fruitlet abscission, suggesting that thinning chemicals may intervene with photosynthesis, leading to a deficiency of carbohydrates (Byers et al., 1985, 1990b). Stoper et al. (1997) observed that NAA application inhibited carbon assimilation by 25% in 'Delicious' and 'Empire' apple leaves; some inhibition lasted for weeks. Also, Yuan and Greene (2000a) reported that net photosynthesis was reduced and dark respiration was stimulated following BA treatment, which might reduce carbohydrate supply to the fruit, leading to the abscission.

Justification for Research

Virginia annually produces about 7 million bushels of apples in 15,000 acres of apple orchard, and ranks sixth in the nation in apple production (USDA-NASS, 2006). The adjoining states of West Virginia, Maryland, Pennsylvania, and North Carolina collectively grow another 50 million bushels. Approximately 30% of Virginia apples are sold as fresh fruit, and 70% are used for processing of apple juice, sauce and cider. The Virginia apple industry contributes an estimated $200 million annually to the state's economy. However, profitable apple production is still a challenge, requiring the maximizing of yields of high quality apples and minimizing of labor and other production costs.

Apples are prone to biennial bearing characterized by heavy bloom and over-cropping in the "on" year and low or no cropping in the "off" year (Childers et al., 1995; Byers, 2003). Fruitlet thinning removes excessive fruit from trees at early stage of fruit development, which can improve fruit size, color and quality, increase return bloom, and reduce alternate bearing habit of

apple trees, thereby increasing growers' return (Childers et al., 1995; Yuan and Greene, 2000a; Byers, 2003). Fruitlet thinning is usually conducted by applications of chemicals since labor is costly, ranging from $500 to $1500/acre. Compared to hand thinning, chemical thinning could be done earlier and timely in the season to achieve better effects (Childers et al., 1995). However, most chemical thinners could not achieve either satisfactory or reliable thinning efficiency, since little is known about the action mechanisms of the chemical thinners, impact of environmental factors on thinning effectiveness and differential response of various genotypes of cultivars (Greene, 2002). To improve competiveness of US products on global markets, it is imperative to establish a reliable and predictable thinning program to reduce production cost and increase growers' return. Understanding the optimum temperature range for chemical thinners will assist in using chemical thinners efficiently. Understanding the modes of action of chemical thinners at the molecular level will benefit the development of fruit thinning technologies and allow initial screening of new potential thinning chemicals without extensive field trials.

Research Objectives

1) To investigate the effects of major postbloom chemical thinner, NAA and other chemicals (or combinations of chemicals) on fruit thinning response, leaf and fruit ethylene production. Hypothesis #1: Chemical thinner NAA reduces canopy photosynthesis, causes sugar starvation, enhances ABA and ethylene production, and results in fruit thinning. Hypothesis #2: Chemical thinner NAA blocks endogenous auxin polar transport and reduces the fruit sink strength, resulting in abscission.
2) To identify key regulatory genes related to fruit thinning. Hypothesis #3: Shading and NAA down-regulate genes associated with photosynthesis and carbon utilization, causing a

stringent carbohydrate level. In the meantime, genes related to sugar metabolism/transport are inhibited, resulting in reduced fruit sink strength. Hypothesis #4: NAA inhibits auxin transporter genes and meanwhile enhances genes involved with ethylene/ABA biosynthesis and signaling, thus promoting fruit abscission through altered balance of these hormones at fruit abscission zone.

3) To profile the gene expression pattern during young apple fruit abscission, using apple oligonucleotide microarray. Hypothesis #5: No single key regulator explains the induction of abscission, which is dependent upon a complex interplay of metabolic and hormonal pathways coordinated in NAA-/shading-induced young fruit abscission.

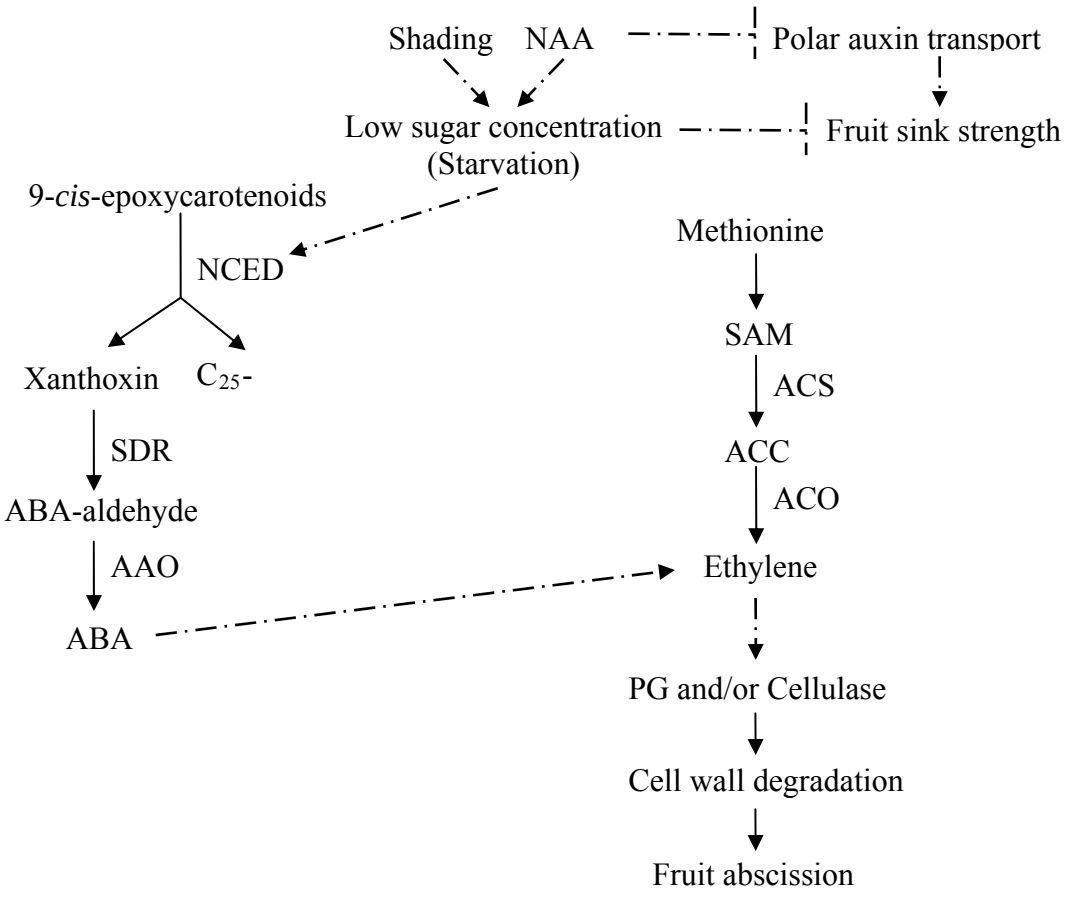

Figure 1.1. A hypothetic mode of action of shading and chemical thinner NAA on fruit abscission in apples.

References

Addicott FT. 1982. Abscission. Berkeley, CA, USA: California University Press.

Aharoni N, Richmond AE. 1978. Endogenous gibberellin and abscisic acid content as related to senescence of detached lettuce leaves. Plant Physiol. 62: 224-228.

Auchter EC. 1920. Some influences of thinning, pollination and fruit-spur growth on the yearly performance record of fruit-spurs and on the size of fruit produced. Proc. Amer. Soc. Hort. Sci. 16: 118-131.

Bangerth F. 2000. Abscission and thinning of young fruit and their regulation by plant hormones and bioregulators. Plant Growth Regul. 31: 43-50.

Batjer LP, Billingsley HD, Westwood MN, Rogers BL. 1957. Predicting harvest size of apples at different times during the growing season. Proc. Amer. Soc. Hort. Sci. 70: 46-57.

Batjer LP, Thomson BJ. 1962. Effect of 1-naphthyl N-methylcarbamate (Sevin) on thinning apples. Proc. Amer. Soc. Hort. Sci. 77: 1-8.

Batjer LP, Westwood MN. 1960. 1-naphthyl N-methylcarbamate, a new chemical for thinning apples. Proc. Amer. Soc. Hort. Sci. 75: 1-4.

Beyer EM. 1973. Abscission: support for a role of ethylene modification of auxin transport. Plant Physiol. 52: 1-5.

Black BL, Bukovac MJ, Hull J. 1995. Effect of spray volume and time of NAA application on fruit size and cropping of Redchief 'Delicious' apple. Scientia Hort. 64: 253-264.

Bobb AC, Blake MA. 1938. Annual bearing in the wealthy apple was induced by blossom thinning. Proc. Amer. Soc. Hort. Sci. 36: 321-327.

Botton A, Eccher G, Forcato C, Ferrarini A, Begheldo M, Zermiani M, Moscatello S, Battistelli A, Velasco R, Ruperti B, Ramina A. 2010. Signalling pathways mediating the induction of apple fruitlet abscission. Plant Physiol. Published on October 29, 2010, as DOI:10.1104/pp.110.165779.

Brown KM. 1997. Ethylene and abscission. Physiol. Plant. 100: 567-576.

Brun WA, Betts KJ. 1984. Source-sink relations of abscising and non-abscising soybean flowers. Plant Physiol. 75: 187-196.

Burkholder CL, McCown M. 1941. Effect of scoring and of A-naphthyl acetic acid and amide spray upon fruit set and of the spray upon pre-harvest drop. Proc. Amer. Soc. Hort. Sci. 38: 117-120.

Byers RE. 2003. Flower and fruit thinning and vegetative: Fruiting balance. In: D.C. Ferree and I.J. Warrington (eds.). Apples: Botany, production and uses. CABI Publ., Cambridge, MA, USA, pp. 409-436.

Byers RE, Barden JA, Polomski RF, Young RW, Carbaugh DH. 1990b. Apple thinning by photosynthetic inhibition. J. Amer. Soc. Hort. Sci. 115: 14-19.

Byers RE, Lyons CG, Yoder KS, Barden JA, Young RW. 1985. Peach and apple thinning by shading and photosynthetic inhibition. J. Hort. Sci. 60: 465-472.

Carns HR. 1951. Oxygen, respiration and other critical factors in abscission. Ph.D dissertation. Univ. Calif., Los Angeles.

Chaves MM. 1991. Effects of water deficits on carbon assimilation. J. Expt. Bot. 42: 1-16.

Childers NF, Morris JR, Sibbett GS. 1995. Modern fruit science. 10th ed. Hort. Publ. Gainsville, FL, USA.

Crowe AD. 1965. Effect of thinning sprays on metabolism of growth substances in the apple. Proc. Amer. Soc. Hort. Sci. 86: 23-27.

Dal Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A. 2005. Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus domestica* L. Borkh). J. Exp. Bot. 56: 2995-3005.

Davies RT, Goetz DH, Lasswell J, Anderson MN, Bartel B. 1999. *IAR3* encodes an auxin conjugate hydrolase from Arabidopsis. Plant Cell 11: 365-376.

Dennis FG. 2000. The history of fruit thinning. Plant Growth Regul. 31: 1-16.

Dennis FG. 2002. Mechanisms of action of apple thinning chemicals. HortScience 37: 471-474.

Ebert A, Bangerth F. 1982. Possible hormonal modes of action of three apple thinning agents. Scientia Hort. 16: 343-356.

Edgerton LJ, Greenhalgh WJ. 1969. Regulation of growth, flowering and fruit abscission with 2-choroethane-phosphonic acid. J. Amer. Soc. Hort. Sci. 94: 11-12.

Farmer EE, Moloshok TD, Saxton MJ, Ryan CA. 1991. Oligosaccharide signaling in plants. Specificity of oligouronide-enhanced plasma membrane protein phosphorylation. J. Biol. Chem. 266: 3140-3145.

Fernandez DE, Heck GR, Perry SE, Patterson SE, Bleecker AB, Fang SC. 2000. The embryo MADS domain factor AGL15 acts postembryonically: Inhibition of perianth senescence and abscission via constitutive expression. Plant Cell 12: 183-197.

Filippini F, LoSchiavo F, Terzi M, Branca C, Bellincampi D, Salvi G, De Desiderio A, Lorenzo G, Cervone F. 1992. Phytoalexin elicitor-active α-1,4-D-oligogalaturonides reduce auxin perception by plant cells and tissues. In: Carssen CM, Van Loon LC, Vreugdenhil D, eds. Progress in plant growth regulation. Dordrecht, The Netherlands: Kluwer Academic Publishers, 517-521.

Gardner FE, Marth PC, Batjer LP. 1939. Spraying with plant growth substances to prevent apple fruit dropping. Science 90: 208-209.

Goldschmidt EE, Monselise SP. 1977. Physiological assumptions toward the development of a citrus fruiting model. Proc. Intl. Soc. Citriculture 2: 668-672.

Gourley JH. 1922. Text-Book of Pomology. New York: MacMillan.

Greene DW. 2002. Chemicals, timing, and environmental factors involved in thinner efficacy on apple. HortScience 37: 477-481.

Greene DW, Autio WR, Erf JA, Mao ZY. 1992. Mode of action of benzyladenine when used as a chemical thinner on apples. J. Amer. Soc. Hort. Sci. 117: 775-779.

Guardiola JL, Garcia-Mari F, Agusti M. 1984. Competition and fruit set in the Washington navel orange. Physiol. Plant. 62: 297-302.

Jones RGW, Lunt OR. 1967. The function of calcium in plants. Bot. Rev. 33: 407-426.

Kalaitzis P, Solomos T, Tucker ML. 1997. Three different polygalaturonases are expressed in tomato leaf and flower abscission each with a different temporal expression pattern. Plant Physiol. 113: 1303-1308.

Knight JN. 1980. Fruit thinning of the apple cultivar Cox's Orange Pippin. J. Hort. Sci. 55: 267-273.

Li JG, Yuan RC. 2008. NAA and ethylene regulate expression of genes related to ethylene biosynthesis, perception and cell wall degradation during fruit abscission and ripening in 'Delicious' apples. J. Plant Growth Regul. 27: 283-295.

Luckwill LC. 1953. Studies of fruit development in relation to plant hormones. II. The effect of naphthaleneacetic acid on fruit set and fruit development in apples. J. Hort. Sci. 28: 25-40.

Mao Z, Craker LE, Decoteau DR. 1989. Abscission in Coleus: light and phytohormone control. J. Expt. Bot. 40: 1273-1277.

Marini R. 1997. Oxamyl is an effective apple fruit thinner when used alone or when combined with other thinners. HortScience 7: 253-258.

Marsh HV, Southwick FW, Weeks WD. 1960. The influence of chemical thinners on fruit set and size, seed development, and pre-harvest drop of apples. Proc. Amer. Soc. Hort. Sci. 75: 5-21.

McArtney SJ. 2002. Ethylene evolution from detached apple spurs in response to chemical thinners. HortScience 37: 662-665.

McManus MT, Osborne DJ. 1990. Evidence for the preferential expression of particular polypeptides in leaf abscission zones of the bean *Phaseolus vulgaris*. J. Plant Physiol. 136: 391-397.

McManus MT, Osborne DJ. 1991. Identification and characterization of an ionically-bound cell wall glycoprotein expression preferentially in the leaf rachis abscission zone of *Sambucus nigra* L. J. Plant Physiol. 138: 63-67.

Meir S, Philosoph-Hadas S, Sundaresan KSS, Selvaraj V, Burd S, Ophir R, Kochanek B, Reid MS, Jiang CZ, Lers A. 2010. Microarray analysis of the abscission-related transcriptome in tomato flower abscission zone in response to auxin depletion. Plant Physiol. 154: 1929-1956.

Messiaen J, Van Custem P. 1999. Polyamines and pectins. II. Modulation of the pectic signal transduction. Planta 208: 247-256.

Morgan PW, Hall DC. 1964. Accelerated release of ethylene by cotton following application of indolyl-3-acetic acid. Nature 201: 99.

Murneek AE, Teubner FG. 1955. The dual action of naphthaleneacetic acid in thinning of apples. Proc. Amer. Soc. Hort. Sci. 61: 149-154.

Nooden LD, Leopold AC. 1978. Phytohormones and the endogenous regulation of senescence and abscission. In: Latham DS, Goodwin PB, Higgins TJV, eds., Phytohormones and related compounds: a comprehensive treatise. Amsterdam, The Netherlands: Elsevier/Northern-Holland, 329-369.

O'Donnell PJ, Calvert CM, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ. 1996. Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914-1917.

Olien W, Bukovac MJ. 1978. The effect of temperature on rate of ethylene evolution from ethephon and from ethephon-treated leaves of sour cherry. J. Amer. Soc. Hort. Sci. 103: 199-202.

Osborne DJ. 1976. Auxin and ethylene and the control of cell growth. Identification of three classes of target cells. In: Pilet P, ed., Plant growth regulation. Berlin, Germany: Springer-Verlag, 161-171.

Osborne DJ, Sargent JA. 1976b. The positional differentiation of abscission zones during development of leaves of Sambucus nigra and the response of the cells to auxin and ethylene. Planta 132: 197-204.

Poovaiah BW, Leopold AC. 1973. Inhibition of abscission by calcium. Plant Physiol. 51: 848-851.

Rabey GG, Bate GC. 1978. The effect of a period of darkness on the translocation of ^{14}C-labelled assimilates from leaves subtending five- to ten-day-old cotton bolls (*Gossypium hirsutum* L). Rhod. J. Agric. Res. 16: 61-71.

Reinbothe S, Mollenhauer B, Reinbothe C. 1994. JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. Plant Cell 6: 1197-1209.

Roberts JA, Whitelaw CA, Gonzalez-Carranza Z, McManus MT. 2000. Cell separation processes in plants-models, mechanisms and manipulation. Ann. Bot. 86: 223-235.

Roe DJ, Menzel CM, Oosthuizen JH, Doogan VJ. 1997. Effects of current CO_2 assimilation and stored reserves on lychee fruit growth. J. Hort. Sci. 72: 397-405.

Ronen M, Mayak S. 1981. Interrelationship between abscisic acid and ethylene in control of senescence in carnation flowers. J. Expt. Bot. 32: 262-267.

Schaffer AA, Goldschmidt EE, Goren R, Galili D. 1985. Fruit set and carbohydrate status in alternate and nonalternate bearing citrus cultivars. J. Amer. Soc. Hort. Sci. 110:574-578.

Schneider GW. 1973. Translocation of ^{14}C-indoleacetic acid and sucrose in excised apple pedicels. J. Amer. Soc. Hort. Sci. 98: 278-281.

Schneider GW. 1974. Ethylene evolution and apple fruit thinning. J. Amer. Soc. Hort. Sci. 100: 356-359.

Schneider GW. 1975. ^{14}C-Sucrose translocation in apple. J. Amer. Soc. Hort. Sci. 100: 22-24.

Schneider GW, Lasheen AM. 1973. NAA and Sevin on composition, development and abscission of apple fruit. HortScience 8: 103-104.

Sexton R, Lewis LN, Trewavas AJ, Kelly P. 1985. Ethylene and abscission. In: Roberts JA, Tucker GA, eds. Ethylene and plant development. London, UK: Butterworths, 173-196.

Sexton R, Roberts JA. 1982. Cell biology of abscission. Ann. Rev. Plant Physiol. 33: 133-162.

Stembridge GE, Gambrell CE. 1972. Peach fruit abscission as influenced by applied gibberellin and seed development. J. Amer. Soc. Hort. Sci. 97: 708-711.

Stopar M, Black BL, Bukovac MJ. 1997. The effect of NAA and BA on carbon dioxide assimilation by shoot leaves of spur-type ‘Delicious’ and ‘Empire’ apple trees. J. Amer. Soc. Hort. Sci. 122: 837-840.

Szymkowiak EJ, Irish EE. 1999. Interactions between jointless and wild-type tomato tissue during development of the pedicel abscission zone and the inflorescence meristem. Plant Cell 11: 156-160.

Taylor JE, Whitelaw CA. 2001. Signal in abscission. New Phytologist 151: 323-339.

Thimann KV. 1980. Senescence in plants. Baco Raton, FL, USA: CRC Press. Syndrome of leaf senescence. In: Wareing PF, ed., plant growth substances. London, UK: Academic Press, 539-548.

Thompson DS, Osborne DJ. 1994. A role for the stele in intertissue signaling in initiation of abscission in bean leaves. Plant Physiol. 105: 341-347.

Tudella D, Primo-Millo E. 1992. 1-aminocyclopropane-1-carboxylic acid transported from roots to shoots promotes leaf abscission in Cleopatra Mandarin (*Citrus reshni* Hort. Ex Tan.) seedlings rehydrated after water stress. Plant Physiol. 100: 131-137.

Unrath CR. 1974. The commercial implications of gibberellins A4+7 plus benzyladenine for improving fruit shape and yield of 'Delicious' apples. J. Amer. Soc. Hort. Sci. 99: 381-384.

USDA-NASS. 2006. Noncitrus Fruits and Nuts 2005 Summary. USDA-NASS Agricultural Statistics 2005.

Wien HC, Turner AD. 1989. Hormonal basis for low light intensity-induced flower bud abscission of pepper. J. Amer. Soc. Hort. Sci. 114: 981-985.

Wiese MV, DeVay JE. 1970. Growth regulator changes in cotton associated with defoliation caused by Verticillium Albo-Atrum. Plant Physiol. 45: 304-309.

Williams MW. 1979. Chemical thinning of apples. Hort. Rev. 1: 270-300.

Wong CH, Osborne DJ. 1978. The ethylene-induced enlargement of target cells in flower buds of *Ecballium elaterium* L. and their identification by the content of endoreduplicated nuclear DNA. Planta 139: 103-111.

Yuan R, Greene DW. 2000a. Benzyladenine as a chemical fruit thinner for 'McIntosh' apples. I. Fruit thinning effects and associated relationships with photosynthesis, assimilate translocation, and nonstructural carbohydrates. J. Amer. Soc. Hort. Sci. 125: 169-176.

Zhu H, Beers EP, Yuan R. 2008. Aminoethoxyvinylglycine inhibits fruit abscission induced by naphthaleneacetic acid and associated relationships with expression of genes for ethylene biosynthesis, perception, and cell wall degradation in 'Delicious' apples. J. Amer. Soc. Hort. Sci. 133: 727-734.

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Aminoethoxyvinylglycine Inhibits Fruit Abscission Induced by Naphthaleneacetic Acid and Associated Relationships with Expression of Genes for Ethylene Biosynthesis, Perception, and Cell Wall Degradation in 'Delicious' Apples

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Abstract. Effects of naphthaleneacetic acid (NAA) and aminoethoxyvinylglycine (AVG) on young fruit abscission, leaf and fruit ethylene production, and expression of genes related to ethylene biosynthesis and cell wall degradation were examined in 'Delicious' apples (*Malus ×domestica* Borkh.). NAA at 15 mg·L^{-1} increased fruit abscission and ethylene production of leaves and fruit when applied at the 11-mm stage of fruit development, whereas AVG, an inhibitor of ethylene biosynthesis, at 250 mg·L^{-1} reduced NAA-induced fruit abscission and ethylene production of leaves and fruit. NAA also increased expression of 1-aminocyclopropane-1-carboxylate (ACC) synthase genes (*MdACS5A* and *MdACS5B*), ACC oxidase gene (*MdACO1*), and ethylene receptor genes (*MdETR1a, MdETR1b, MdETR2, MdERS1*, and *MdERS2*) in fruit cortex and fruit abscission zones. However, AVG reduced NAA-induced expression of these genes except for *MdERS2* in fruit abscission zones. NAA increased expression of the polygalacturonase gene *MdPG2* in fruit abscission zones but not in fruit cortex, whereas AVG reduced NAA-enhanced expression of *MdPG2* in fruit abscission zones. The expression of β-1,4-glucanase gene *MdCel1* in fruit abscission zones was decreased by NAA but was unaffected by AVG. Our results suggest that ethylene biosynthesis, ethylene perception, and the *MdPG2* gene are involved in young fruit abscission caused by NAA.

Fruit thinning, which removes excessive fruit from trees at an early stage of fruit development, can improve fruit size, color, and quality; increase return bloom; and reduce alternate bearing of apple trees, thereby increasing growers' return (Byers, 2003; Childers et al., 1995; Yuan and Greene, 2000a). Because labor is very expensive, fruit thinning is usually conducted by application of chemicals. Compared with hand thinning, chemical thinning also can be done earlier in the season and more effectively increases fruit size, color, and quality (Childers et al., 1995). However, chemical thinning results are extremely variable and very difficult to predict or control because we have an incomplete understanding of the modes of action of chemical thinners (Byers, 2003).

Apple fruitlet abscission after fertilization and during "June drop" has been, at least in part, attributed to competition for carbohydrates among individual fruitlets and between fruitlets and vegetative shoots (Quilan and Preston, 1971; Yuan and Greene, 2000b). Shading or removal of spur and shoot leaves, which affects leaf photosynthesis and thereby reduces carbohydrates available to young fruit, causes extensive apple fruit abscission (Byers, 2003; Ferree and Palmer, 1982; Yuan and Greene, 2000b). Some researchers reported that the primary mechanism of fruit thinning by chemical thinners such as naphthaleneacetic acid (NAA) and 6-benzylaminopurine (6-BA) is the result of reduced carbohydrates available to developing fruit either by interference with photosynthesis (Stopar et al., 1997; Yuan and Greene, 2000a) or by reduced translocation of metabolites, including photosynthates, from leaves to the fruit (Schneider, 1978).

On the other hand, it has been suggested that chemical thinners such as NAA and 6-BA enhance apple fruitlet abscission through increased ethylene production (Curry, 1991; Dal Cin et al., 2005; McArtney, 2002; Walsh et al., 1979). The pathway of ethylene synthesis has been established in higher plants (Yang and Hoffman, 1984). Ethylene is formed from methionine through S-adenosyl-L-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (Yang and Hoffman, 1984). The conversion of SAM to 1-aminocyclopropane-1-carboxylate (ACC) and ACC to ethylene are the

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Table 1. Gene-specific primers used for expression analysis of genes related to ethylene biosynthesis, perception, signal transduction, and cell wall degradation.

Gene	Accession no.	Primer left	Primer right
MdActin	CN938023	5′-TGACCGAATGAGCAAGGAAATTACT-3′	5′-TACTCAGCTTTGGCAATCCACATC-3′
MdACS5A	AB034992	5′-GCAATGGTGGTCTTTTCGTATG-3′	5′-TTCGAACGTCTGCTCCTTGA-3′
MdACS5B	AB034993	5′-GAATTTTGAGTGTTGGATACCTTCTTT-3′	5′-GAACCAACATCTAAAATCCCATTGT-3′
MdACO1	AB030859	5′-CAGTCGGATGGGACCAGAA-3′	5′-GCTTGGAATTTCAGGCCAGA-3′
MdETR1a	AF032448	5′-TTGGCCTGTGAAGAGCAGT-3′	5′-TGCAAACCATGTAGAGCCAT-3′
MdETR1b	AY821544	5′-GCACTGTGTTTGCTTGCGATT-3′	5′-CGGGACGGCAGGTCAA-3′
MdETR2	DQ847145	5′-GTTGTGACGCGGAAAATGC-3′	5′-AATCCAGATGAAACGGCAGTTAC-3′
MdERS1	AY083169	5′-CAACTAGGGATATGCGAC-3′	5′-CACTGGCATCCAAAGACTTC-3′
MdERS2	AB213028	5′-GCTTGTTAAGGTTGGAAGAAATCTG-3′	5′-CGGCATCGTTGAGTGTTACATT-3′
MdCTR1	AY670703	5′-ACAAGATTTTCATGCCGAAC-3′	5′-TATGGACAAGTTTGGAGGCT-3′
MdPG2	AB210897	5′-CGGTTCAGCCGACAAGTTG-3′	5′-TACGAGTGAGGAGGAGTAGATGGA-3′
MdCel1	AY350734	5′-ACCAGAACGATGGATTTCCAGAT-3′	5′-GTACGTTGCAGGCTCCGAAT-3′

rate-limiting steps in ethylene biosynthesis and are catalyzed by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Alexander and Grierson, 2002; Wang et al., 2002). Genes encoding ACS and ACO are members of multigene families, and their expression is differentially regulated by a variety of biotic and abiotic factors (Kende, 1993; Wang et al., 2002). In apples, five ACS genes, *MdACS1*, *MdACS2*, *MdACS3*, *MdACS5A*, and *MdACS5B*, and one ACO gene, *MdACO1*, have been isolated and characterized (Dal Cin et al., 2005; Li and Yuan, 2008). *MdACS1* and *MdACO1* are related to the burst of fruit ethylene production during fruit ripening in apples, whereas *MdACS5B* and *MdACO1* are associated with young fruit ethylene production (Dal Cin et al., 2005; Li and Yuan, 2008). Aminoethoxyvinylglycine is a potent inhibitor of ethylene biosynthesis through inhibiting ACS enzyme activity (Boller et al., 1979). Application of aminoethoxyvinylglycine (AVG) inhibits fruit ethylene production and expression of *MdACS1*, *MdACS5A*, and *MdACO1* and delays fruit ripening and preharvest fruit abscission in apples (Li and Yuan, 2008; Schupp and Greene, 2004; Yuan and Carbaugh, 2007; Yuan and Li, 2008).

After synthesis, ethylene is perceived by a family of membrane-localized receptors. In arabidopsis [*Arabidopsis thaliana* (L.) Heynh], there are five known ethylene receptors, *ETR1, ETR2, ERS1, ERS2*, and *EIN4* (Wang et al., 2002). These receptors seem to undergo conformational changes on the binding of ethylene and then interact with the Raf-like serine/threonine kinase CTR1, a negative regulator of ethylene signal transduction. The signal then passes down a partially elucidated cascade that ultimately controls a myriad of ethylene-associated plant growth and development processes (Klee, 2004; Wang et al., 2002). In apples, it has been reported that ethylene receptor genes, *MdETR1*, *MdETR2*, *MdERS1*, and *MdERS2*, and ethylene signal transduction gene, *MdCTR1*, are involved in fruit ripening and young fruit abscission (Dal Cin et al., 2005; Li and Yuan, 2008).

It has been reported that concomitant with increased ethylene production is increased expression of genes and activity of enzymes associated with cell wall degradation such as β-1,4-glucanase (cellulase or EG) and polygalacturonase (PG) (Bonghi et al., 2000; Roberts et al., 2002), which causes the middle lamellae of abscission zone cells to dissolve and, ultimately, the organ to abscise. Other genes such as pathogenesis-related genes and those involved in secondary metabolism and signal transduction are also enhanced during the abscission process (Roberts et al., 2002).

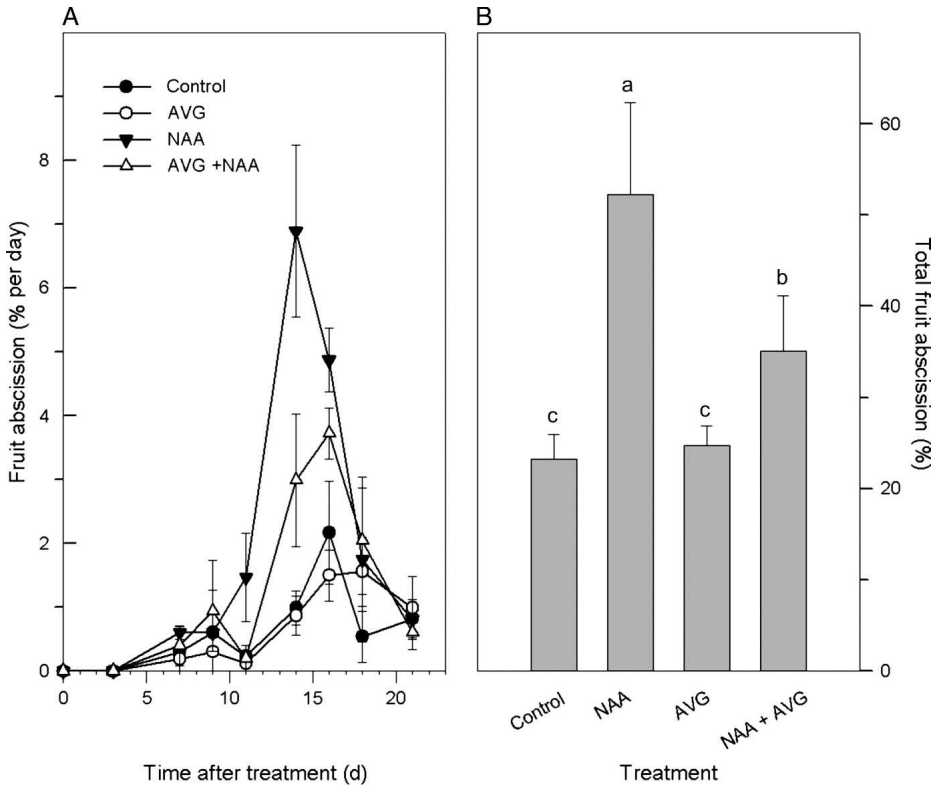

Fig. 1. Effects of NAA and AVG on (**A**) fruit abscission pattern and (**B**) total fruit abscission in 'Delicious' apples in 2007. Data are means ± SE (n = 4). Different letters indicate significant differences among means according to Duncan's multiple range test ($P < 0.05$). NAA = naphthaleneacetic acid; AVG = aminoethoxyvinylglycine.

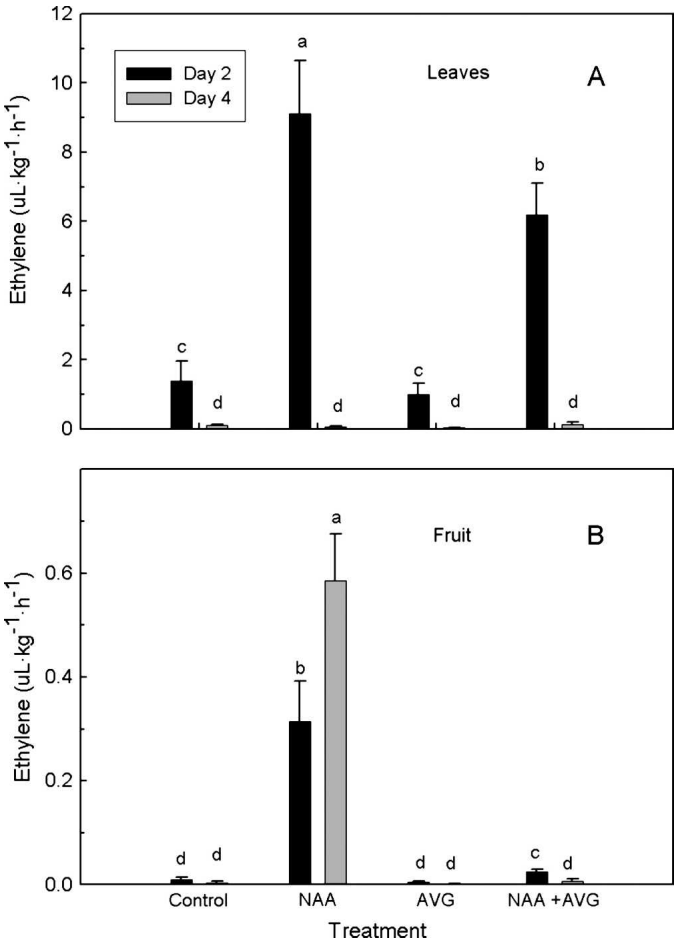

Fig. 2. Effects of NAA and AVG on (**A**) leaf ethylene production and (**B**) fruit ethylene production in 'Delicious' apples in 2007. Data are means ± SE (n = 4). Different letters indicate significant differences among means according to Duncan's multiple range test ($P < 0.05$). NAA = naphthaleneacetic acid; AVG = aminoethoxyvinylglycine.

The purpose of this study was to evaluate whether ethylene biosynthesis, ethylene perception, and cell wall degradation were involved in young fruit abscission caused by the chemical thinner NAA in 'Delicious' apples.

Materials and Methods

Plant material and treatments. Sixteen 13-year-old 'Delicious' apple trees grafted on 'M.111' rootstock were selected in an orchard located at Alson H. Smith, Jr. Agricultural Research and Extension Center, Winchester, VA, and blocked into four groups of four trees each. Apple trees had an average of 2.5 m in canopy height and 2.7 m in canopy diameter. A randomized complete block design with four replications was used. One tree from each block received one of the four treatments on 14 May 2007 when fruit size was ≈11 mm in diameter. Treatments consisted of: 1) water, which served as control; 2) NAA (Fruitone N; AMVAC, Newport Beach, CA) at 15 mg·L^{-1}; 3) AVG (ReTain; Valent BioSciences, Libertyville, IL) at 250 mg·L^{-1}; and 4) NAA at 15 mg·L^{-1} + AVG at 250 mg·L^{-1}. All spray solutions contained Silwet-77 silicone surfactant (Loveland Industries, Loveland, CO) at 0.125% to improve dispersion. The surfactant had no effect on fruit and leaf ethylene production. Solutions were applied to the canopy with a low-pressure hand-wand sprayer until runoff. NAA was applied ≈1 h after application of AVG. Leaves and young fruit were dry when NAA was applied. Average daily high and low temperature in the first 3 d after treatment was ≈26/13 °C.

In 'Golden Delicious' apples, we found that NAA, applied at the 11-mm stage of fruit development, markedly increased young fruit ethylene production and enhanced expression of genes related to ethylene biosynthesis, perception, and cell wall degradation 1, 3, and 5 d after treatment (H. Zhu, E. Beers, and R. Yuan, unpublished data). In this study, both leaf and young fruit samples were collected from each 'Delicious' apple tree of three replicate blocks 4 d after treatment (≈26 d after full bloom). The fruit samples were immediately separated into cortex and fruit abscission zones. Fruit abscission zones were collected by cutting 1 mm at each side of the abscission fracture plane. Promptly after separation of fruit, all samples were frozen in liquid nitrogen and stored at –80 °C for extraction of RNA.

Determination of fruit abscission and ethylene production of fruit and leaves. To determine fruit abscission rate, two limbs on each tree were tagged. Fruits on tagged limbs were counted just before treatment, and then fruit remaining on tagged limbs were counted every 2 or 3 d. To determine ethylene production of fruit and leaves, 15 fruit and 20 leaves were collected from each tree 2 and 4 d after treatment, enclosed in 100- and 1000-mL containers, respectively, and incubated for 3 h. One milliliter of gas sample was withdrawn from the sealed container through the rubber septum affixed to lid, and ethylene concentration was measured with a gas chromatograph equipped with an activated alumina column and FID detector (model 3700; Varian, Palo Alto, CA).

Total RNA extraction and real-time quantitative polymerase chain reaction. Total RNA was extracted from fruit abscission zones and fruit cortex as described by Li and Yuan (2008). DNA was removed from each RNA sample using the TURBO DNA-free™ Kit (Ambion, Austin, TX). Reverse transcriptase–polymerase chain reaction was performed using primers that span an intron in *MdACO* to confirm that each RNA sample was free of genomic DNA contamination (Li and Yuan, 2008).

One microgram of total RNA was used to synthesize cDNA in a 20 µL reaction volume using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time quantitative polymerase chain reaction (PCR) was performed using the Power SYBR Green PCR Master Mix Kit (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. Gene-specific primers were designed for nonconserved areas using Primer Expression 3.0 software (Applied Biosystems) and synthesized by Integrated DNA Technologies (Coralville, IA). The primer sequences are listed in Table 1. Real-time samples were run in triplicate and the reaction volumes were 25 µL. Dissociation curves were generated to determine the specificity of the amplification reactions. In addition, the amplified products were sequenced as described by Li and Yuan (2008). After validation tests, normalization to actin was performed using the $\Delta\Delta C_T$ method (Applied Biosystems, 2005).

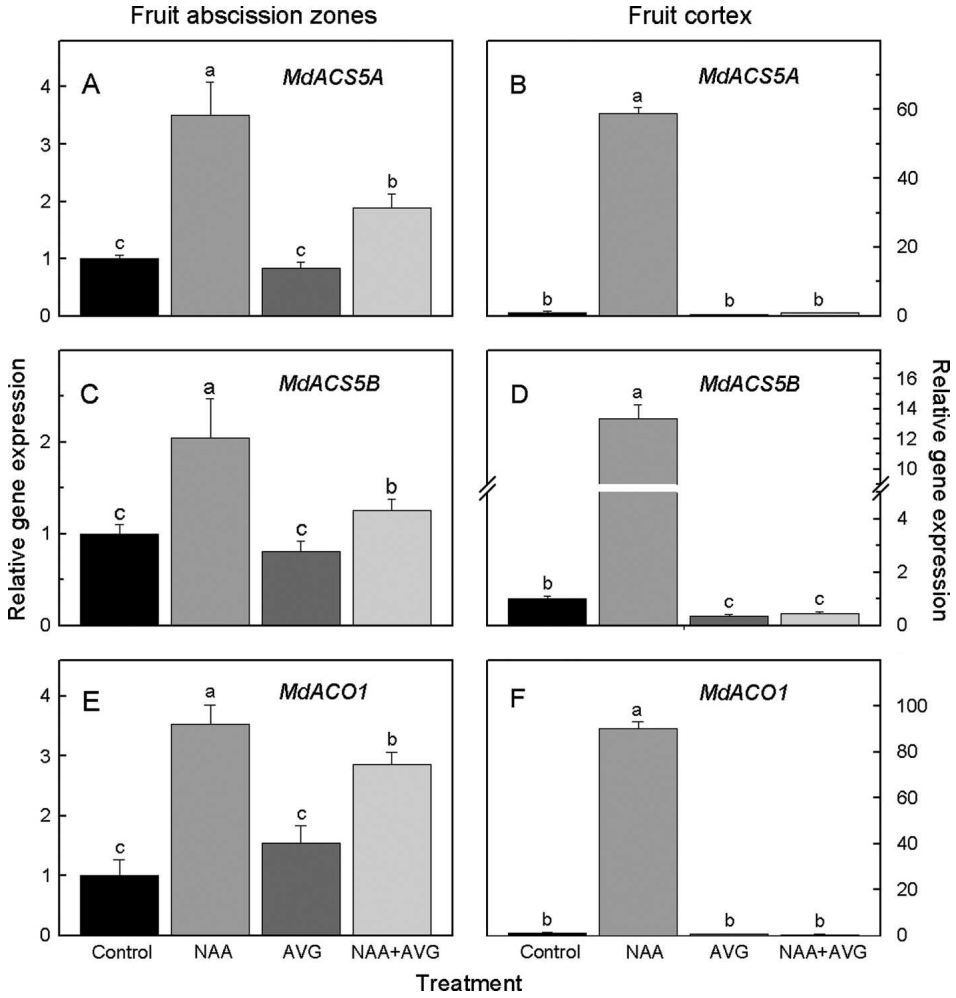

Fig. 3. Real-time quantitative polymerase chain reaction analysis of the expression of *MdACS5A*, *MdACS5B*, and *MdACO1* in (**A**, **C**, and **E**) fruit abscission zones and (**B**, **D**, and **F**) fruit cortex from 'Delicious' apple trees 4 d after application of NAA and AVG. The levels of *MdACS5A*, *MdACS5B*, and *MdACO1* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdACS5A*, *MdACS5B*, and *MdACO1* in fruit abscission zones and fruit cortex from water-treated control 'Delicious' apple trees were arbitrarily set to 1. Different letters indicate significant differences among means according to Duncan's multiple range test ($P < 0.05$). NAA = naphthaleneacetic acid; AVG = aminoethoxyvinylglycine.

Statistical analyses. Statistical analyses included analysis of variance and Duncan's multiple range test. SAS software for PC (SAS Institute, Cary, NC) was used to analyze the results.

Results

Effect of naphthaleneacetic acid and aminoethoxyvinylglycine on fruit abscission and ethylene production of fruit and leaves. NAA at 15 mg·L^{-1} effectively increased fruit abscission, whereas AVG at 250 mg·L^{-1} reduced NAA-enhanced fruit abscission in 'Delicious' apples (Fig. 1A–B). The rate of NAA-induced fruit abscission peaked ≈14 d after treatment. Compared with the water-treated control, ethylene production of NAA-treated leaves increased ≈9-fold 2 d after treatment (Fig. 2A). However, there was no difference in leaf ethylene production between the control and NAA 4 d after treatment. AVG reduced NAA-induced leaf ethylene production 2 d after treatment. Fruit ethylene production was also stimulated by NAA 2 and 4 d after treatment (Fig. 2B). As was observed for leaves, there was no difference in fruit ethylene production between control and the AVG-only treatment. AVG virtually eliminated NAA-induced fruit ethylene production.

Effect of naphthaleneacetic acid and aminoethoxyvinylglycine on expression of genes encoding enzymes involved in ethylene biosynthesis. Very low or no expression of *MdACS1*, *MdACS2*, and *MdACS3* was detected in fruit abscission zones and fruit cortex (data not shown). The expression of *MdACS5A, MdACS5B*, and *MdACO1* in fruit cortex and fruit abscission zones was increased by NAA application (Fig. 3A–F). The cortex of fruit from trees treated with AVG alone had lower levels of *MdACS5B* transcripts than that from water-treated control trees. However, there was no significant difference in the levels of *MdACS5B* in fruit abscission zones between AVG alone and the water-treated control. There was no difference in the levels of *MdACS5A* and *MdACO1* transcripts in fruit cortex and fruit abscission zones between the water-treated control and AVG alone either. NAA-induced expression of *MdACS5A, MdACS5B*, and *MdACO1* in fruit cortex and fruit abscission zones was decreased by AVG.

Effect of naphthaleneacetic acid and aminoethoxyvinylglycine on expression of genes encoding ethylene receptors and ethylene signal transduction kinase CTR1. The levels of *MdETR1a, MdETR1b*, and *MdETR2* transcripts in fruit abscission zones and fruit cortex were increased by NAA (Fig. 4). Expression of *MdETR1a* and *MdETR1b* in fruit cortex and fruit abscission zones was unaffected by AVG alone. AVG alone increased expression of *MdETR2* in fruit abscission zones but not in fruit cortex. NAA-induced expression of *MdETR1a, MdETR1b*, and *MdETR2* in fruit abscission zones and fruit cortex was reduced by AVG.

The levels of *MdERS1, MdERS2*, and *MdCTR1* transcripts in fruit abscission zones and fruit cortex were increased by NAA (Fig. 5). AVG alone increased expression of *MdERS2* in fruit abscission zones, but it had no effect on expression of *MdERS2* in fruit cortex. AVG alone did not affect expression of *MdERS1* and *MdCTR1* in fruit abscission zones and fruit cortex. NAA-induced expression of *MdERS1*, *MdERS2*, and *MdCTR1* in fruit cortex was reduced by AVG. AVG reduced NAA-induced expression of *MdERS1* but not of *MdERS2* or *MdCTR1* in fruit abscission zones.

Effect of naphthaleneacetic acid and aminoethoxyvinylglycine on expression of genes encoding enzymes involved in cell wall degradation in 'Delicious' apples. The expression of *MdCel1* in fruit abscission zones and fruit

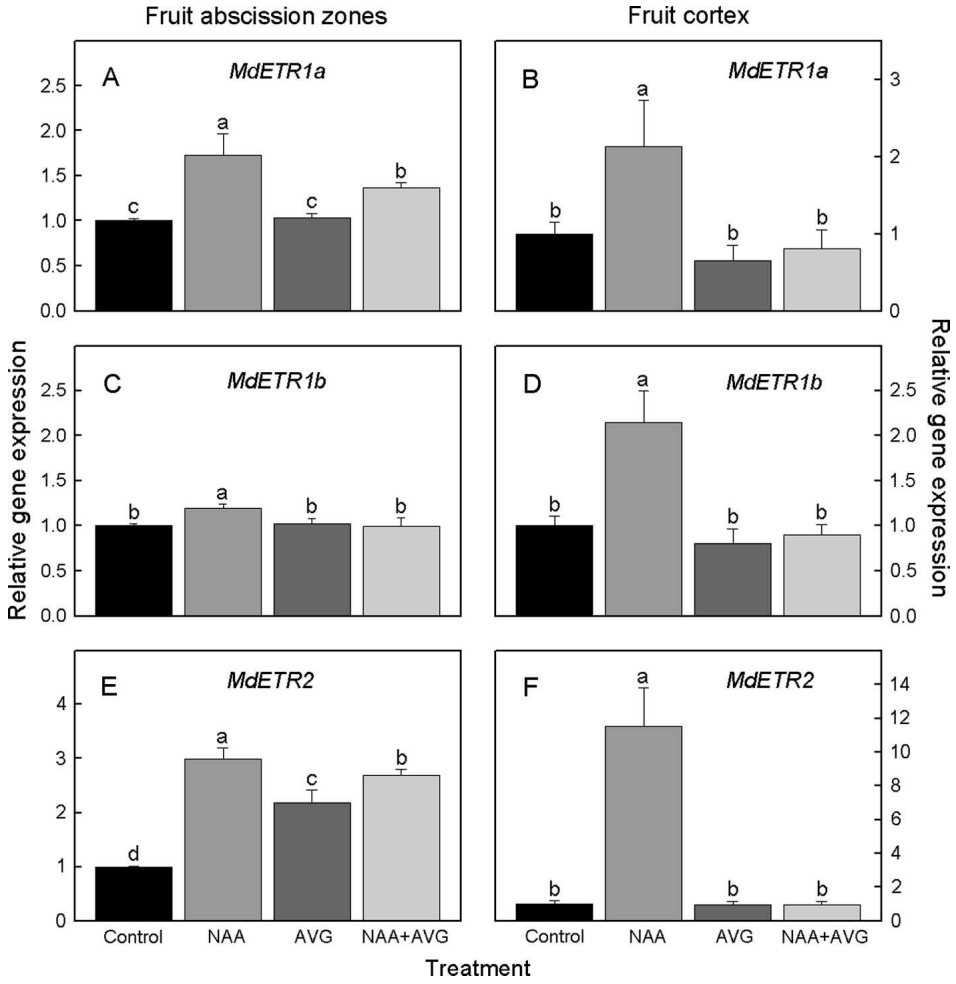

Fig. 4. Real-time quantitative polymerase chain reaction analysis of expression of *MdETR1a*, *MdETR1b*, and *MdETR2* in (**A**, **C**, and **E**) fruit abscission zones and (**B**, **D**, and **F**) fruit cortex from 'Delicious' apple trees 4 d after application of NAA and AVG. The levels of *MdETR1a*, *MdETR1b*, and *MdETR2* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdETR1a*, *MdETR1b*, and *MdETR2* in fruit abscission zones and fruit cortex from water-treated control 'Delicious' apple trees were arbitrarily set to 1. Different letters indicate significant differences among means according to Duncan's multiple range test ($P < 0.05$). NAA = naphthaleneacetic acid; AVG = aminoethoxyvinylglycine.

cortex was decreased by NAA but was unaffected by AVG (Fig. 6A–B). Expression of *MdPG1* was not detected in fruit abscission zones and fruit cortex (data not shown). *MdPG2* expression in fruit abscission zones was increased by NAA but was unaffected by AVG alone (Fig. 6C). AVG reduced NAA-induced expression of *MdPG2* in fruit abscission zones. Expression of *MdPG2* in fruit cortex was unaffected by NAA but reduced by AVG and NAA + AVG.

Discussion

In this study, NAA increased ethylene production of young fruit and leaves and increased young fruit abscission in 'Delicious' apples. This is consistent with previous reports that ethylene production of young fruit and leaves increased rapidly in response to postbloom thinning spray of NAA (Curry, 1991; Dal Cin et al., 2005; McArtney, 2002; Walsh et al., 1979). We expanded on this observation and showed that NAA-induced ethylene production of young fruit and leaves and young fruit abscission were reduced by AVG, a well-known inhibitor of ACS activity (Boller et al., 1979). These results suggest that NAA-induced young fruit abscission is associated with ethylene biosynthesis in apples.

It has been suggested that regulation of ethylene biosynthesis by various stresses and endogenous signals is mainly through the differential expression of ACS and ACO genes (Kende, 1993). Auxin stimulates ethylene production by enhancing ACS expression in various plant species (Abel and Theologis, 1996; Li and Yuan, 2008). Our results showed that expression of *MdACS5A*, *MdACS5B*, and *MdACO1* increased significantly in NAA-treated fruit cortex and fruit abscission zones, whereas very low or no expression of *MdACS1*, *MdACS2*, and *MdACS3* was detected. On the other hand, AVG effectively reduced NAA-enhanced expression of *MdACS5A*, *MdACS5B*, and *MdACO1*. These results suggest that *MdACS5A*, *MdACS5B*, and *MdACO1* but not *MdACS1*, *MdACS2*, or *MdACS3* are related to NAA-induced ethylene production of young fruit and young fruit abscission in 'Delicious' apples.

Our results showed that expression of ethylene receptor genes *MdETR1a, MdETR1b, MdETR2*, and *MdERS1* in fruit abscission zones and fruit cortex and *MdERS2* in fruit cortex was increased by NAA, but AVG reduced NAA-induced expression of these genes, suggesting that NAA-induced expression of these receptors may be dependent on increased ethylene production. Other investigators have also suggested that the increase in the levels of overall receptor mRNA during fruit abscission may be a natural response to increased ethylene biosynthesis (Dal Cin et al., 2005; Kevany et al., 2007; Klee, 2004). Moreover, that the application of AVG reduced NAA-induced expression of *MdETR1a, MdETR1b, MdETR2*, and *MdERS1* genes in fruit abscission zones and NAA-induced fruit abscission is suggestive of a role for these ethylene receptors in NAA-induced young fruit abscission in 'Delicious' apples. A similar correlation between abscission and increased expression of ethylene receptor genes in abscission zones has been reported in flowers of tomato (*Solanum lycopersicum* L.) (Lashbrook et al., 1998; Whitelaw et al., 2002) and young fruit of apples (Dal Cin et al., 2005). Also consistent with our finding is the observation that reduction of *LeETR1* transcript levels by antisense *LeETR1* delayed the abscission of flowers and leaves in tomato (Whitelaw et al., 2002). However, the observed correlation seems contradictory to the model that ethylene receptors negatively

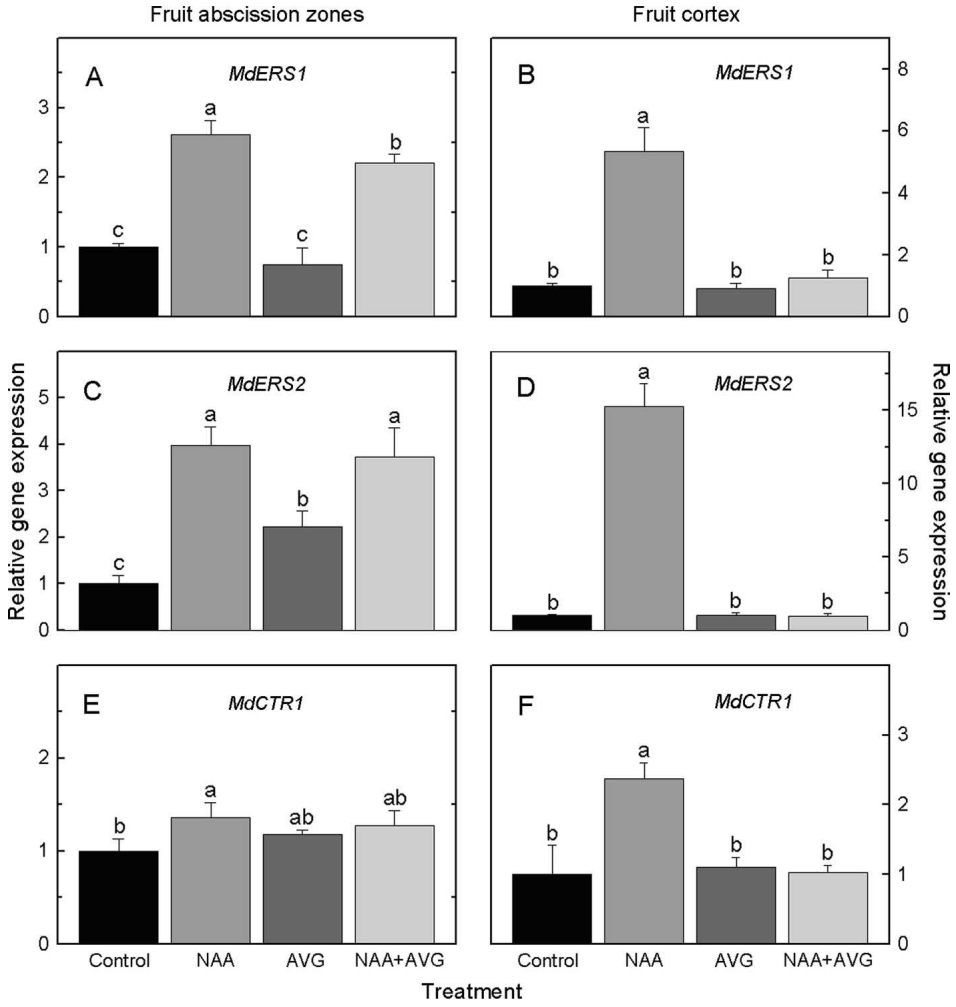

Fig. 5. Real-time quantitative polymerase chain reaction analysis of expression of *MdERS1, MdERS2*, and *MdCTR1* in (**A**, **C**, and **E**) fruit abscission zones and (**B**, **D**, and **F**) fruit cortex from 'Delicious' apple trees 4 d after application of NAA and AVG. The levels of *MdERS1, MdERS2*, and *MdCTR1* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdERS1, MdERS2*, and *MdCTR1* in fruit abscission zones and fruit cortex from water-treated control 'Delicious' apple trees were arbitrarily set to 1. Different letters indicate significant differences among means according to Duncan's multiple range test ($P < 0.05$). NAA = naphthaleneacetic acid; AVG = aminoethoxyvinylglycine.

regulate ethylene responses and there is an inverse relationship between receptor levels and ethylene sensitivity of a tissue (Hua and Meyerowitz, 1998; Klee, 2004). Further work will be necessary to determine the relationship between the levels of ethylene receptor proteins in abscission zones and fruit abscission.

It has been well documented that an increase in PG and cellulase activities is usually associated with fruit abscission (Bonghi et al., 2000; Li and Yuan, 2008). No expression of *MdPG1* was detected in the abscission zones of young 'Delicious' apple fruit regardless of treatment (data not shown), suggesting that *MdPG1* is not related to young fruit abscission in apples. Similarly, Li and Yuan (2008) reported that *MdPG1* is not involved in mature fruit abscission in apples. On the other hand, NAA increased *MdPG2* expression in fruit abscission zones, but the increase was reduced by AVG. These results suggest that *MdPG2* is related to NAA-induced young fruit abscission. Our results also showed that expression *MdCel1*, which encodes cellulase, was significantly decreased by NAA but unaffected by AVG in fruit abscission zones. This indicates that *MdCel1* is unlikely involved in young fruit abscission induced by NAA.

Carbohydrates and fruit ethylene production play a critical role in young fruit abscission in apples (Byers, 2003; Curry, 1991; McArtney, 2002; Stopar et al., 1997; Walsh et al., 1979; Yuan and Greene, 2000a). However, the relationship between carbohydrates and fruit ethylene production is not clear. Recent studies have revealed that sugars not only provide carbon and energy, but also play a pivotal role as signaling molecules in plants that integrate external environment conditions with intrinsic developmental programs modulated by multiple plant hormones (Rolland et al., 2006; Thimm et al., 2004). DNA microarray analysis showed that shading or low sugar concentrations upregulated genes involved in biosynthesis and signaling of abscisic acid (ABA) and ethylene in arabidopsis plants (Cheng et al., 2002; Kim and Arnim, 2006; Thimm et al., 2004), whereas application of glucose downregulated genes upregulated by both shading and ABA (Kim and Arnim, 2006). It also has been reported that defoliation- or shading-induced young fruit abscission was preceded by an increase in the levels of ABA and ACC in citrus [*Citrus unshiu* (Mak.) Marc.] (Gomez-Cadenas et al., 2000; Iglesias et al., 2006). Therefore, it is possible that NAA not only directly increases fruit ethylene production by increasing expression of *MdACS5A* and *MdACS5B*, but also indirectly increases fruit ethylene production through increasing biosynthesis and signaling of ABA and ethylene by reducing photosynthesis and carbohydrate levels. More research work will be necessary to determine the relationship between carbohydrate shortage and young fruit ethylene production.

Unlike the positive effect NAA has on abscission of young apple fruit, NAA reduces mature apple fruit abscission although it increases fruit ethylene production and fruit softening (Li and Yuan, 2008). Using real-time quantitative PCR, Li and Yuan (2008) found that NAA reduced mature apple fruit abscission by inhibiting expression of *MdPG2* in fruit abscission zones, increased mature fruit ethylene production by increasing expression of *MdACS1* and *MdACO1*, and enhanced mature fruit softening by increasing expression of *MdPG1* in fruit cortex. Further efforts are needed to determine how NAA increases expression of *MdPG2* in abscission zones of young fruit but inhibits its expression in mature fruit abscission zones.

In summary, our results showed that NAA increased young apple fruit abscission through increasing expression of

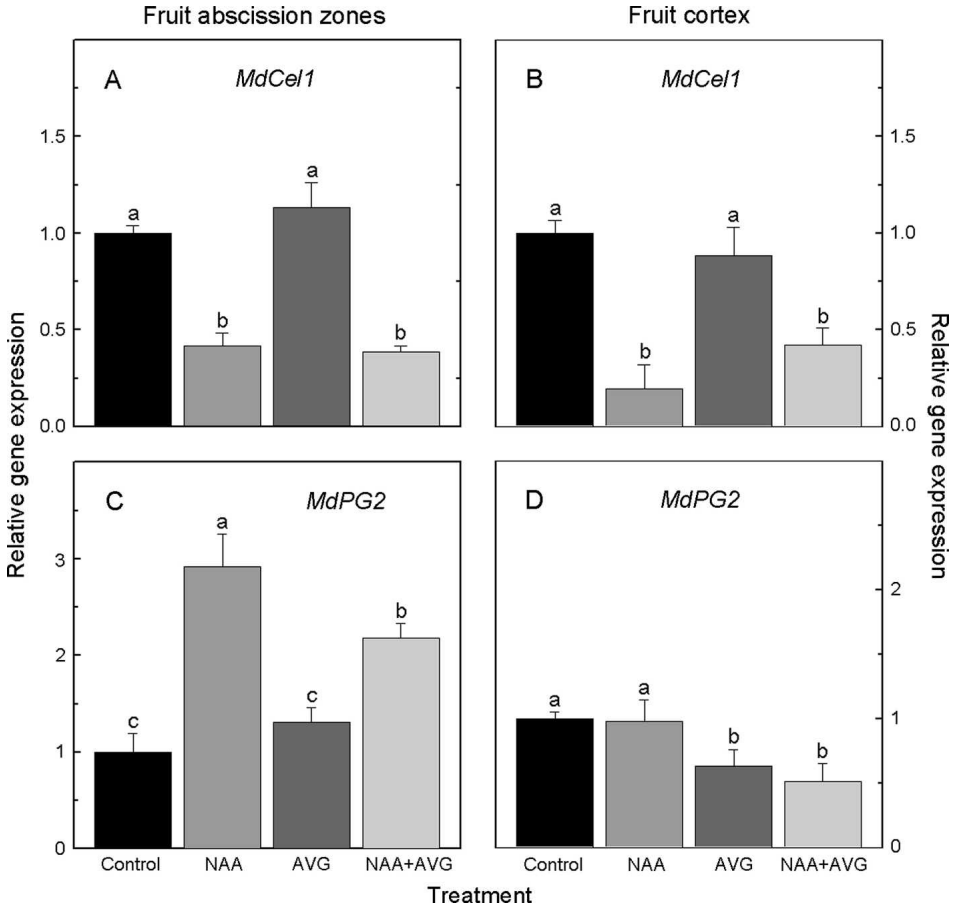

Fig. 6. Real-time quantitative polymerase chain reaction analysis of expression of *MdCel1* and *MdPG2* in (**A** and **C**) fruit abscission zones and (**B** and **D**) fruit cortex from 'Delicious' apple trees 4 d after application of NAA and AVG. The levels of *MdCel1* and *MdPG2* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdCel1* and *MdPG2* in fruit abscission zones and fruit cortex from water-treated control 'Delicious' apple trees were arbitrarily set to 1. Different letters indicate significant differences among means according to Duncan's multiple range test ($P < 0.05$). NAA = naphthaleneacetic acid; AVG = aminoethoxyvinylglycine.

MdACS5A, MdACS5B, and *MdACO1*; fruit ethylene production; and expression of *MdETR1a, MdETR1b, MdETR2, MdERS1*, *MdERS2*, and *MdPG2* in fruit abscission zones. In contrast, AVG reduced NAA-induced young apple fruit abscission by inhibiting fruit ethylene production and expression of *MdACS5A, MdACS5B*, *MdACO1*, *MdETR1a, MdETR1b, MdETR2, MdERS1*, and *MdPG2* in fruit abscission zones.

Literature Cited

Abel, S. and A. Theologis. 1996. Early genes and auxin action. Plant Physiol. 111:9–17.

Alexander, L. and D. Grierson. 2002. Ethylene biosynthesis and action in tomato: A model for climacteric fruit ripening. J. Expt. Bot. 53:2039–2055.

Applied Biosystems. 2005. Guide to performing relative quantitation of gene expression using real-time quantitative PCR. Applied Biosystems, Foster City, CA.

Boller, T., R.C. Herner, and H. Kende. 1979. Assay for and enzymatic formation of an ethylene precursor 1-aminocyclopropane-1-carboxylic acid. Planta 145:293–303.

Bonghi, C., P. Tonutti, and A. Ramina. 2000. Biochemical and molecular aspects of fruitlet abscission. Plant Growth Regulat. 31:35–42.

Byers, R.E. 2003. Flower and fruit thinning and vegetative: Fruiting balance, p. 409–436. In: D.C. Ferree and I.J. Warrington (eds.). Apples: Botany, production and uses. CABI Publishing, Cambridge, MA.

Cheng, W., A. Endo, L. Zhou, J. Penney, H. Chen, A. Arroyo, P. Leon, E. Nambara, T. Asami, M. Seo, T. Koshiba, and J. Sheen. 2002. A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 14:2723–2743.

Childers, N.F., J.R. Morris, and G.S. Sibbett. 1995. Modern fruit science. 10th Ed. Horticultural Publications, Gainseville, FL.

Curry, E.A. 1991. NAA-induced ethylene and ACC in 'Delicious' spur tissues: Changes with temperature and time. J. Amer. Soc. Hort. Sci. 116:846–850.

Dal Cin, V., M. Danesin, A. Boschetti, A. Dorigoni, and A. Ramina. 2005. Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus domestica* L. Borkh). J. Expt. Bot. 56:2995–3005.

Ferree, D.C. and J.W. Palmer. 1982. Effects of spur defoliation and ringing during bloom on fruiting, fruit mineral level, and net photosynthesis of Golden Delicious apples. J. Amer. Soc. Hort. Sci. 107:1182–1186.

Gomez-Cadenas, A., J. Mehouachi, F.R. Tadeo, E. Primo-Millo, and M. Talon. 2000. Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. Planta 210:636–643.

Hua, J. and E.M. Meyerowitz. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94:261–271.

Iglesias, D.J., F.R. Tadeo, E.P. Millo, and M. Talon. 2006. Carbohydrate and ethylene levels related to fruitlet drop through abscission zone A in citrus. Trees (Berl.) 20:348–355.
Kende, H. 1993. Ethylene biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:283–307.
Kevany, B.M., D.M. Tieman, M.G. Taylor, V. Dal Cin, and H.J. Klee. 2007. Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J. 51:458–467.
Kim, B.H. and A.G. Arnim. 2006. The early dark-response in *Arabidopsis thaliana* revealed by cDNA microarray analysis. Plant Mol. Biol. 60:321–342.
Klee, H.J. 2004. Ethylene signal transduction. Moving beyond *Arabidopsis*. Plant Physiol. 135:660–667.
Lashbrook, C.C., D.M. Tieman, and H.J. Klee. 1998. Differential regulation of the tomato *ETR* gene family throughout plant development. Plant J. 15:243–252.
Li, J. and R. Yuan. 2008. NAA and ethylene regulate expression of genes related to ethylene biosynthesis, perception and cell wall degradation during fruit abscission and ripening in 'Delicious' apples. J. Plant Growth Regul. 27:283–295.
McArtney, S.J. 2002. Ethylene evolution from detached apple spurs in response to chemical thinners. HortScience 37:662–665.
Quilan, J.D. and A.P. Preston. 1971. The influence of shoot competition on fruit retention and cropping of apple trees. J. Hort. Sci. 46:525–534.
Roberts, J.A., K.A. Elliott, and Z.H. Gonzalez-Carranza. 2002. Abscission, dehiscence, and other cell separation process. Annu. Rev. Plant Biol. 53:131–158.
Rolland, F., E. Baena-Gonzalez, and J. Sheen. 2006. Sugar sensing and signaling in plants: Conserved and novel mechanisms. Annu. Rev. Plant Biol. 57:675–709.
Schneider, G.W. 1978. Abscission mechanism studies with apple fruitlets. J. Amer. Soc. Hort. Sci. 103:455–458.
Schupp, J.R. and D.W. Greene. 2004. Effect of aminoethoxyvinylglycine (AVG) on preharvest drop, fruit quality, and maturation of 'McIntosh' apples. I. Concentration and timing of dilute applications of AVG. HortScience 39:1030–1035.
Stopar, M., B.L. Black, and M.J. Bukovac. 1997. The effect of NAA and BA on CO_2 assimilation by shoot leaves of spur-type 'Delicious' and 'Empire' apple trees. J. Amer. Soc. Hort. Sci. 122:837–840.
Thimm, O., O. Blasing, Y. Gibon, A. Nagel, S. Meyer, P. Kruger, J. Selbig, L.A. Muller, S.Y. Rhee, and M. Stitt. 2004. Mapman: A user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. Plant J. 37:914–939.
Wang, K.L.C., H. Li, and J.R. Ecker. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14:S131–S151.
Walsh, C.S., H.J. Swartz, and L.J. Edgerton. 1979. Ethylene evolution in apple following post-bloom thinning sprays. HortScience 14:704–706.
Whitelaw, C.A., N.N. Lyssenko, L. Chen, D. Zhou, A.K. Mattoo, and M.L. Tucker. 2002. Delayed abscission and shorter internodes correlate with a reduction in the ethylene receptor LeETR1 transcript in transgenic tomato. Plant Physiol. 128:978–987.
Yang, S.F. and N.E. Hoffman. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35:155–189.
Yuan, R. and D.H. Carbaugh. 2007. Effects of NAA, AVG, and 1-MCP on ethylene biosynthesis, preharvest fruit drop, fruit maturity, and quality of 'Golden Supreme' and 'Golden Delicious' apples. HortScience 42:101–105.
Yuan, R. and D.W. Greene. 2000a. Benzyladenine as a chemical thinner for 'McIntosh' apples. I. Fruit thinning effects and associated relationships with photosynthesis, assimilate translocation, and nonstructural carbohydrates. J. Amer. Soc. Hort. Sci. 125:169–176.
Yuan, R. and D.W. Greene. 2000b. Benzyladenine as a chemical thinner for 'McIntosh' apples. II. Effects of benzyladenine, bourse shoot tip removal, and leaf number of fruit retention. J. Amer. Soc. Hort. Sci. 125:177–182.
Yuan, R. and J. Li. 2008. Effects of sprayable 1-MCP, AVG, and NAA on ethylene biosynthesis, preharvest fruit drop, fruit maturity, and quality of 'Delicious' apples. HortScience 43:1454–1460.

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Effects of 1-Methylcyclopropene and Naphthaleneacetic Acid on Fruit Set and Expression of Genes Related to Ethylene Biosynthesis and Perception and Cell Wall Degradation in Apple

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Additional index words. 1-MCP, ethylene receptor, *Malus* ×*domestica*, NAA, polygalacturonase, abscission

Abstract. **The effects of 1-methylcyclopropene (1-MCP) and naphthaleneacetic acid (NAA) on fruit set and the expression of genes related to ethylene biosynthesis and perception and cell wall degradation in apple (*Malus* ×*domestica* Borkh.) were studied when applied during the normal chemical thinning period. 1-MCP at 209 mg·L^{-1} had a small negative effect or no effect on the final fruit set, depending on the experiment, but could cause a transient delay of June drop when applied at petal fall or the 10-mm stage in 'Pioneer McIntosh' apple. 1-MCP at 160 mg·L^{-1} had no effect on fruit abscission but induced ethylene production by leaves and fruit of 'Golden Delicious' apple. NAA at 6 or 15 mg·L^{-1} effectively increased fruit abscission in both apple cultivars. NAA enhanced the expression of genes related to ethylene biosynthesis (*MdACS5A*, *MdACS5B*, and *MdACO1*) or perception (*MdETR1*, *MdETR1b*, *MdETR2*, *MdERS1*, and *MdERS2*) and cell wall degradation (*MdPG2*). 1-MCP did not affect the expression of *MdACS5A* and *MdACS5B* in the fruit abscission zone (FAZ), although it enhanced the expression of these two genes in the fruit cortex (FC) from 6 hours to 1 day after treatment. The expression of *MdACO1* in both tissues was increased by 1-MCP by 3 days post-treatment and thereafter. 1-MCP had only a small influence on the expression of most ethylene receptor genes, with the exception of *MdETR1*, which was upregulated in the FC to a level similar to that observed for NAA treatment. In response to 1-MCP, in the FAZ, the expression of *MdCel1* and *MdPG2* was upregulated at the beginning and the end, respectively, of the experiment, but otherwise remained at or below control levels. 1-MCP did not inhibit NAA-induced abscission of young apple fruit, suggesting that abscission does not solely depend on ethylene signal transduction, or that the periods of effectiveness for 1-MCP and ethylene were asynchronous.**

Ethylene is involved in young apple fruit abscission (Curry, 1991; Dal Cin et al., 2005; McArtney, 2002; Zhu et al., 2008). Application of ethephon, an ethylene-releasing compound, effectively promoted the abscission of young fruit in apple (Walsh et al., 1979; Yuan, 2007), while aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, reduced fruit ethylene production and the abscission of young fruit in apple (Williams and Flook, 1980; Zhu et al., 2008). Increased abscission of young apple fruit caused by the chemical thinner naphthaleneacetic acid (NAA) is linked with increased ethylene production; hence, NAA may act in part through ethylene signaling (Curry, 1991; Zhu et al., 2008).

The pathway of ethylene synthesis has been well established in higher plants. Ethylene is formed from methionine via S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984). The conversions of SAM to ACC and ACC to ethylene are the two committed steps in ethylene biosynthesis, and are catalyzed by ACC synthase (ACS) and ACC oxidase (ACO), respectively (Alexander and Grierson, 2002). AVG is a competitive inhibitor of ACS and other members of the class of pyridoxal-5′-phosphate-dependent enzymes (Huai et al., 2001). Thus far, five ACS genes and one ACO gene have been isolated and characterized (Dal Cin et al., 2005; Li and Yuan, 2008) in apple. It has been reported that *MdACS1* and *MdACO1* are related to the increase in fruit ethylene during fruit ripening, whereas *MdACS5A*, *MdACS5B*, and *MdACO1* are associated with ethylene production by young fruit (Li and Yuan, 2008).

Ethylene is perceived by a series of receptors that undergo conformational changes upon ethylene binding and then inactivate the Raf-like serine/threonine kinase CTR1, a negative regulator in ethylene signal transduction. This relieves the repression on downstream signaling components, thus allowing for activation of the EIN3/EIL transcription factors and ethylene-inducible genes, which control a myriad of ethylene-associated plant growth and development processes (Chang and

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[1]This paper is dedicated to the memory of Rongcai Yuan. He was a devoted plant scientist, revered colleague and a kind and patient mentor. He is greatly missed by those who were privileged to know him.

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Bleecker, 2004; Chen et al., 2005). Six ethylene receptors have been identified in tomato [*Solanum lycopersicum* L. (Bleecker, 1999)] and five have been isolated and characterized in apple (Dal Cin et al., 2005; Li and Yuan, 2008). Among the ethylene-inducible transcriptional cascade are the genes for hydrolases such as β-1,4-glucanase (cellulase or EG) and polygalacturonase (PG), which are induced in the fruit abscission zone where they catalyze the breakdown of the middle lamellae and cell walls and promote fruit drop (Bonghi et al., 2000; Ward et al., 1999).

1-Methylcyclopropene (1-MCP), a gaseous inhibitor of ethylene action, has been used to delay postharvest ripening of climacteric fruit such as apple (Fan et al., 1999; Sisler and Serek, 1997), peach [*Prunus persica* L. (Kluge and Jacomino, 2002)], avocado [*Persea americana* Mill. (Jeong et al., 2002)], and mango [*Mangifera indica* L. (Jiang and Joyce, 2000)]. 1-MCP also delayed leaf abscission in sweet orange [*Citrus sinensis* L. (Zhong et al., 2001)] and mature fruit abscission in apple (Li and Yuan, 2008; Yuan and Carbaugh, 2007; Yuan and Li, 2008), and has been widely used in the cut flower industry (Blankenship and Dole, 2003). 1-MCP has been formulated as a stable powder that releases the active gaseous form when mixed with water. Recently, a sprayable formulation of 1-MCP became available for experimental use in the field.

The purposes of the present work were to study the effect of a sprayable formulation of 1-MCP on apple fruit set and the effects of 1-MCP and NAA on the expression of genes related to ethylene biosynthesis, perception, and cell wall degradation.

Materials and Methods

Expt. 1: 'Pioneer McIntosh'/M.9, Belchertown, MA. Twenty 18-year-old 'Pioneer McIntosh' apple trees were selected in a non-irrigated block at the University of Massachusetts Horticultural Research Center in 2007. Trees were trained as a central leader and were cared for using normal industry accepted culture and pest management practices. At the pink stage of flower development, two representative limbs per tree were randomly selected and tagged and their circumferences were measured. After counting all blossom clusters on the selected limbs, blossom cluster density was calculated by dividing the number of blossom clusters by the square centimeter of limb cross-sectional area (LCSA). Trees were placed into five groups (replications) based upon similarity in the calculated blossom cluster density. Treatments were randomly assigned among the four trees within each replication. Treatments were sprayable 1-MCP (Rohm and Haas Company, Spring House, PA) applied at three distinct physiological stages: bloom (10 May), petal fall (17 May), and 10-mm-diameter fruit (24 May). One tree in each replication was not sprayed and served as the untreated control. The sprayable formulation of 1-MCP was applied as a dilute handgun application using an 11.4-L backpack sprayer propelled with CO_2 at 276 KPa pressure. In the backpack sprayer, 62.5 g of 1-MCP was placed along with 113.5 mL of summer oil (AFxDR-038, Rohm and Hass) and 6 mL of silicone surfactant (Silwet L-77; Helena Chemical Co., Memphis, TN). This gave a final 1-MCP concentration in the tank of 209 mg·L^{-1} with 1% oil and 0.05% Silwet L-77. The sprayable 1-MCP was mixed in the orchard. The sprayer was filled with water and then silicone surfactant and summer oil were added and mixed using a portable drill equipped with an attached paint mixer. The

Table 1. Effect of 1-methylcyclopropene (1-MCP) application at different physiological stages on fruit set of 'Pioneer McIntosh'/M.9 apple.

1-MCP application[z]		Bloom	Fruit set	
Stage	Date	Blossom clusters (no./cm^2 LCSA)[y]	(no./cm^2 LCSA)	(%)
Control	—	9.9 a[x]	11.0 a	109 a
Bloom	10 May	9.9 a	10.1 a	108 a
Petal fall	17 May	9.9 a	12.3 a	129 a
10 mm	24 May	9.8 a	10.0 a	103 a
Significance				
Treatment		NS	NS	NS

[z]1-MCP was applied with a CO_2 back-pack sprayer at 209 mg·L^{-1} in 1% summer oil (AFxRD-038; Rohm and Hass, Spring House, PA) and 0.05% silicone surfactant (Silwet L-77; Helena Chemical Co., Memphis, TN).
[y]LCSA = limb cross-sectional area.
[x]Mean separation by Duncan's multiple range test at $P \leq 0.05$.
[NS]Not significant.

Table 2. Effect of 1-methylcyclopropene (1-MCP) application at different physiological stages on fruit set of individually tagged spurs on 'Pioneer McIntosh'/M.9 apple.

1-MCP application[z]		Fruit (no./spur)			
Stage	Date	29 May	6 June	13 June	14 Aug.
Control	—	3.4 b[y]	2.7 a	1.9 a	1.5 a
Bloom	10 May	3.3 b	2.6 a	1.9 a	1.5 a
Petal fall	17 May	4.0 a	2.8 a	2.1 a	1.6 a
10 mm	24 May	4.5 a	2.7 a	2.0 a	1.6 a
Significance					
Treatment		**	NS	NS	NS

[z]1-MCP was applied with a CO_2 back-pack sprayer at 209 mg·L^{-1} in 1% summer oil (AFxRD-038; Rohm and Hass, Spring House, PA) and 0.05% silicone surfactant (Silwet L-77; Helena Chemical Co., Memphis, TN).
[y]Mean separation by Duncan's multiple range test at $P \leq 0.05$.
[NS, **]Not significant or significant at $P \leq 0.01$.

Table 3. Effect of 1-methylcyclopropene (1-MCP) application alone and in combination with naphthaleneacetic acid (NAA) at the 10-mm stage on fruit set of 'Pioneer McIntosh'/M.9 apple.

	Bloom	Fruit set	
Treatment[z]	Blossom clusters (no./cm^2 LCSA)[y]	Fruit (no./cm^2 LCSA)	(%)
Control	10.2	10.6	108
NAA	10.2	7.3	89
1-MCP	10.2	8.4	89
1-MCP + NAA	9.7	5.6	60
Significance			
1-MCP	NS	*	*
NAA	NS	**	*
1-MCP × NAA	NS	NS	NS

[z]1-MCP was applied on 24 May with a CO_2 back-pack sprayer at 209 mg·L^{-1} in 1% summer oil (AFxRD-038; Rohm and Hass, Spring House, PA) and 0.05% silicone surfactant (Silwet L-77; Helena Chemical Co., Memphis, TN). NAA was applied at 6 mg·L^{-1} as a dilute hand-gun spray on 25 May, 1 d after 1-MCP treatment.
[y]LCSA = limb cross-sectional area.
[NS, **, *]Not significant or significant at $P \leq 0.01$ or 0.05, respectively.

previously measured 1-MCP was added to the tank, mixed for 30 s, the top was placed on the sprayer, and the tank was then pressurized with CO_2. The contents of the tank were sprayed on trees within 10 min of mixing. About 3 L of spray was applied to each tree.

On 17 May, 20 spurs were randomly selected on the periphery of each tree and tagged. Tagging was done at this time to preclude potential bias when fruit started to enlarge and before fruit size differences within the spur became apparent. The first set count was taken on 29 May when fruit were about 14 mm in diameter and it was possible to get a good indication of initial fruit set. The number of persisting fruit on each spur was counted on 29 May, 6 June, 13 June, and 14 Aug., and the average number of persisting fruit on each spur was calculated. At the end of June drop, in July, all persisting fruit were counted on the tagged portions of each of the two selected limbs and final fruit set was calculated. At the normal time of harvest on 10 Sept., 30 fruit were harvested from each tree. Fruit were weighed and red color was estimated to the nearest 10%. A 10-apple subsample representative of the sample was selected. Flesh firmness was taken on two sides of each fruit using a pressure tester (EPT-1 Econic; Lake City Technical Products, Kelowna, BC, Canada). A juice sample was collected while doing the pressure test and soluble solids were determined using a hand-held refractometer (Fisher Scientific, Waltham, MA). Fruit were then cut at the equator and dipped in a solution containing 8.8 g of potassium iodide and 2.2 g of iodine crystals in 1 L of water for 1 min. The starch distribution pattern was then judged on a scale of 1 to 8 (Blanpied and Silsby, 1992).

Expt. 2: 'Pioneer McIntosh'/M.9, Belchertown, MA. Twenty-four uniform trees were selected in 2007 in the block described above and they were similarly tagged, blossom clusters were counted, and bloom density was calculated. Trees were placed into six groups (replications) based upon similarity of blossom cluster density. Two trees in each replication received a spray of 1-MCP at 209 $mg \cdot L^{-1}$ as described above on 24 May. One day later, one tree in each replication that was previously unsprayed received a spray containing 6 $mg \cdot L^{-1}$ NAA (AMVAC, Newport Beach, CA), while a second tree that was previously sprayed with 1-MCP also received a dilute spray of 6 $mg \cdot L^{-1}$ NAA, leaving one tree that received a spray of 1-MCP only. Spray applications were done similarly to that described in Expt. 1. One tree per replication was unsprayed and served as the untreated control. At the end of June drop, all persisting fruit on the tagged portion of the two selected limbs per tree were counted and final set was calculated. At the normal harvest time in September, a 30-apple sample was randomly harvested from the perimeter of each tree and was subjected to the same evaluation that was described previously.

Expt. 3: 'Golden Delicious'/M.9, Winchester, VA. Sixteen uniform 'Golden Delicious' apple trees were selected from an orchard located at Alson H. Smith, Jr. Agricultural Research and Extension Center in Winchester, VA, and placed into four groups of four trees each. A randomized complete block design with four replications was used. Three replicates were used for gene expression analysis while all four replicates were used to determine the fruit abscission rate and the ethylene production of leaves and fruit. One tree from each block received one of the four treatments on 14 May 2007, when fruit size was about 10 mm in diameter. Treatments consisted of: 1) water, which served as the untreated control; 2) 1-MCP (Rohm and Haas) at 160 $mg \cdot L^{-1}$; 3) NAA (AMVAC) at 15 $mg \cdot L^{-1}$; 4) 1-MCP at 160 $mg \cdot L^{-1}$ + NAA at 15 $mg \cdot L^{-1}$. All spray solutions contained 0.125% silicone surfactant (Silwet L-77) to improve dispersion. To minimize the loss of the active form of 1-MCP, 1-MCP formulation was mixed immediately before spraying. Solutions were applied to the canopy with a low-pressure hand-wand sprayer until runoff. NAA was applied about 1 h after the application of 1-MCP.

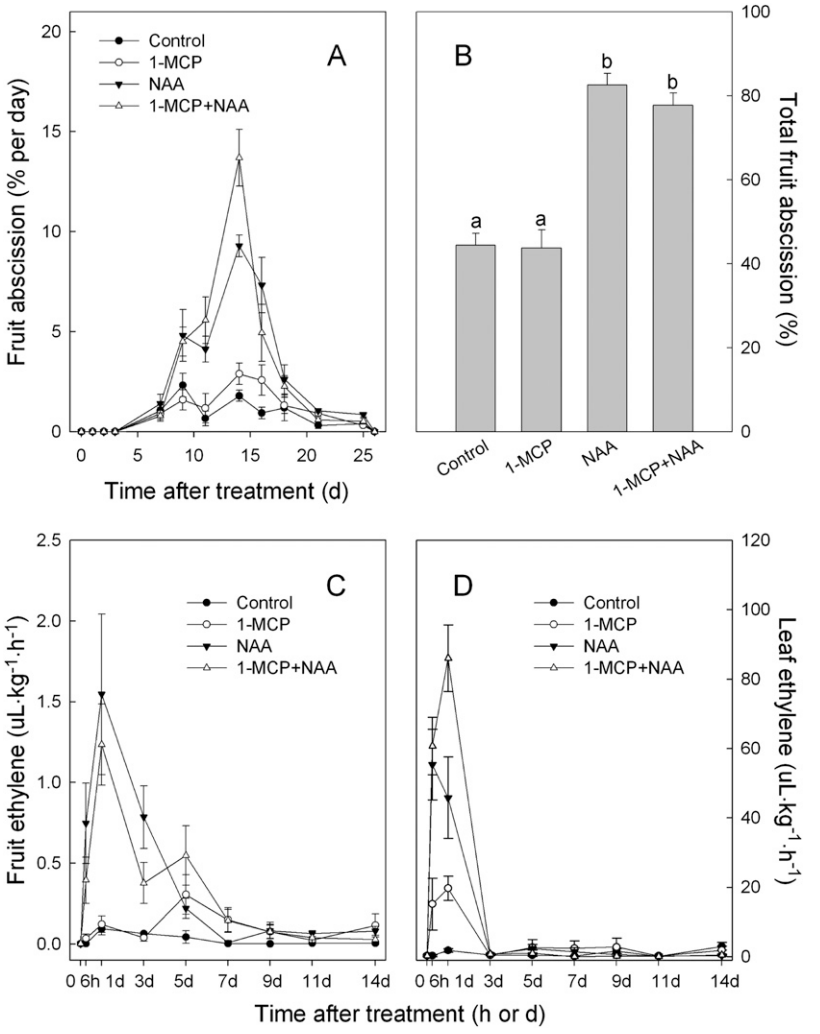


Fig. 1. Effects of 1-methylcyclopropene (1-MCP) and naphthaleneacetic acid (NAA) on fruit abscission pattern (**A**), total fruit abscission (**B**), fruit ethylene production (**C**), and leaf ethylene production (**D**) in 'Golden Delicious' apple in 2007. Data are means ± SE (n = 4). Different letters indicate significant differences among means according to Duncan's multiple range test at $P \leq 0.05$.

About 150 young fruit were randomly collected from each tree of three replicate blocks 0 and 6 h, and 1, 3, 5, and 7 d after treatment. The fruit samples were immediately separated into fruit cortex (FC) and fruit abscission zone (FAZ). For FC collection, skin and seeds were removed. FAZs were collected by cutting 1 mm at each side of the abscission fracture plane at the base of the pedicel. All samples were promptly frozen in liquid nitrogen and stored at –80 °C for RNA extraction.

Determination of fruit abscission and ethylene production of fruit and leaves. To determine the fruit abscission rate, two limbs on each tree were tagged. Fruit on tagged limbs were counted 0, 1, 2, 3, 7, 9, 11, 14, 16, 18, 21, 25, and 26 d after treatment. For ethylene production measurements, 15 fruit and 20 leaves were collected from each tree of four replicate blocks 0 and 6 h and 1, 3, 5, 7, 9, 11 and 14 d after treatment and were enclosed in a 100-mL (for fruit) or 1000-mL (for leaves) container. After a 2-h incubation period, a 1-mL gas sample was withdrawn from the sealed container through the rubber septum affixed to the lid, and the ethylene concentration was measured with a gas chromatograph equipped with an activated alumina column and flame ionization detector (model 3700; Varian, Palo Alto, CA). The ethylene production was calculated and expressed as microliters of C_2H_4 per kilogram per hour.

Total RNA extraction and real-time quantitative polymerase chain reaction (qPCR). Total RNA was extracted from FAZ and FC tissues as described by Li and Yuan (2008), and was purified using a TURBO DNA-free™ Kit (Ambion, Austin, TX). RT-PCR was performed using primers that span an intron in *MdACO* to confirm that each RNA sample was free of genomic DNA contamination (Li and Yuan, 2008).

Purified total RNA (1 µg) from each sample was used to synthesize cDNA in a 20-µL reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Fosters City, CA). Real-time qPCR was performed using the Power SYBR Green PCR Master Mix Kit (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System according to the manufacturer's instructions. Gene-specific primers were designed for non-conserved areas using Primer Expression 3.0 software and synthesized by Integrated DNA Technologies (Coralville, IA) (Zhu et al., 2008). Each reaction was run in triplicate in a 25-µL reaction. Dissociation curves were generated and products were sequenced to determine the specificity of the amplification reaction. After validation tests, normalization to actin was performed and relative gene expression level was calculated using the $\Delta\Delta C_T$ method (Applied Biosystems, 2005).

Statistical analyses. In Expts. 1 and 3, the analyses were for a randomized complete block design, and included analysis of variance and Duncan's multiple range test. We applied GLM procedure in SAS system (SAS 9.1 for Windows; SAS Institute, Cary, NC) to analyze the statistical significance for gene expression by comparing the means of replicates at certain time points from different treatments ($P \leq 0.05$). In Expt. 2, the statistical analysis was based on a 2 × 2 factorial with ± NAA and ± 1-MCP experimental design, using Proc ANOVA in SAS.

Results

Expt. 1: 'Pioneer McIntosh'/M.9, Belchertown, MA

Regardless of the application time, 1-MCP did not affect the final fruit set (Table 1). This was true regardless of whether the set was expressed as fruit per square centimeter of LCSA or as a percentage set. Spurs evaluated on 29 May on trees treated with 1-MCP at petal fall (12 d prior) and at the 10-mm stage (5 d prior) had a higher initial fruit set than the untreated control and spurs treated at bloom (Table 2). However, as June drop proceeded and subsequent counts were made, there was no difference in set among the treatments. Moreover, there were no differences in fruit weight, surface red color, soluble solids, starch rating, or flesh firmness (data not shown).

Expt. 2: 'Pioneer McIntosh'/M.9, Belchertown, MA

NAA treatment was previously shown to cause ethylene production linked with fruit drop (Curry, 1991; Zhu et al., 2008), and 1-MCP interferes with ethylene-dependent processes (Blankenship and Dole, 2003; Sisler and Serek, 1997; Watkins, 2006). Hence, we tested whether 1-MCP would interfere with the abscission induced by NAA. In contrast to our results for

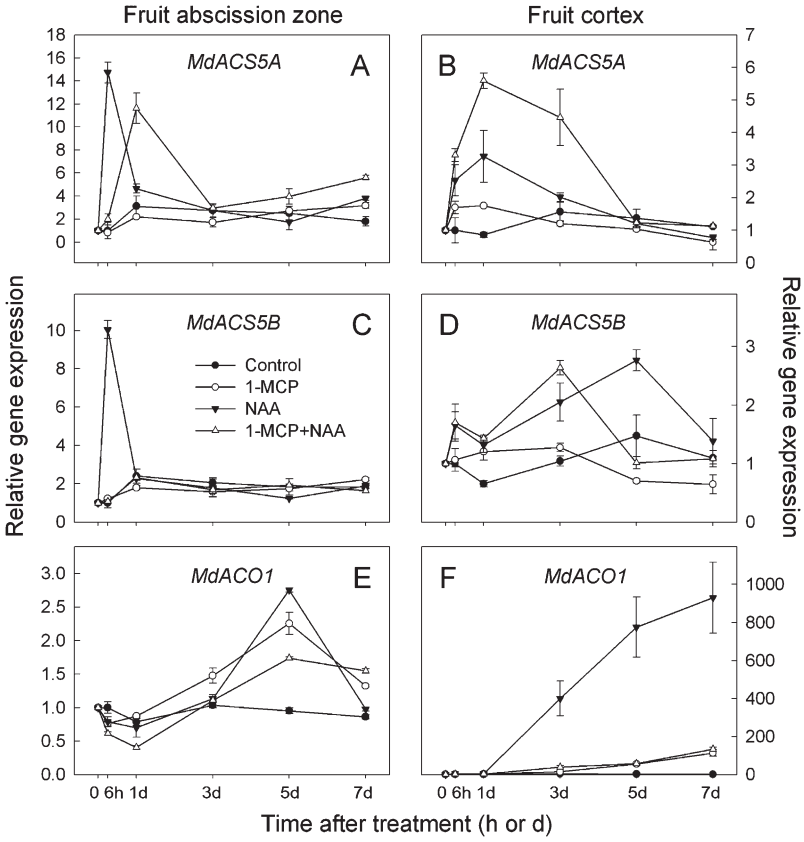


Fig. 2. Real-time quantitative PCR analysis of the expression of *MdACS5A*, *MdACS5B*, and *MdACO1* in the fruit abscission zone (**A**, **C**, and **E**) and the fruit cortex (**B**, **D**, and **F**) from 'Golden Delicious' apple trees after application of 1-methylcyclopropene (1-MCP) and naphthaleneacetic acid (NAA). The levels of *MdACS5A*, *MdACS5B*, and *MdACO1* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdACS5A*, *MdACS5B*, and *MdACO1* in the fruit abscission zone and the fruit cortex from control trees were arbitrarily set to 1.

Expt. 1 (Table 1), application of 1-MCP alone resulted in a significant ($P \leq 0.05$) reduction in fruit set (Table 3). As expected, NAA at 6 mg·L^{-1} also caused significant thinning at $P \leq 0.01$ or 0.05, expressed as LCSA or percentage set, respectively. Although a significant increase in fruit drop was caused by the 1-MCP + NAA treatment, there was no significant interaction between these growth regulators (Table 3). Even though 1-MCP caused some thinning in Expt. 2, most fruit quality characteristics were indistinguishable for control versus 1-MCP-treated fruit, as in Expt. 1 (data not shown).

Expt. 3: 'Golden Delicious'/M.9, Winchester, VA

Effects of 1-MCP and NAA on fruit abscission and ethylene production of fruit and leaves. The fruit abscission rate was significantly increased by NAA at 15 mg·L^{-1} and peaked around 14 d after treatment. While 1-MCP at 160 mg·L^{-1} slightly enhanced the fruit abscission rate from 11 to 14 d, it neither increased total fruit abscission nor reduced NAA-enhanced fruit abscission (Fig. 1, A and B), with the latter being consistent with results from Expt. 2. NAA markedly increased fruit ethylene production, which was detectable at 6 h, peaked at 1 d, and diminished to control levels by 7 d. In contrast with NAA, 1-MCP-induced fruit ethylene increased between 3 and 5 d, and fell to control levels by 9 d (Fig. 1C). The ethylene production of NAA-treated leaves peaked 6 h after treatment, while 1-MCP increased leaf ethylene production at 6 h and 1 d after treatment. By 3 d, leaf ethylene production returned to control levels for all treatments (Fig. 1D).

Effect of 1-MCP and NAA on expression of genes encoding enzymes involved in ethylene biosynthesis. The changes in gene expression discussed below were those exhibiting significant differences ($P \leq 0.05$) relative to the control. 1-MCP alone had no effect on *MdACS5A* or *MdACS5B* expression in the FAZ, but enhanced the expression of these two genes in the FC at 1 d compared with the control (Fig. 2, A–D). In the FAZ, NAA significantly but transiently increased the expression of *MdACS5A* and *MdACS5B*, i.e., the levels for both transcripts reached their observed maximum and returned to control levels within 1 d. However, the expression of these two genes was more persistently enhanced by NAA in the FC, for at least an additional 2 d. Compared with the control, 1-MCP increased the expression of *MdACO1*, which was detectable at 3 d in the FAZ, and the FC and remained above control levels for the duration of the experiment (Fig. 2, E and F). NAA-dependent increases in *MdACO1* expression were noted at 5 and 3 d for the FAZ and FC samples, respectively. 1-MCP reduced the NAA-dependent increase in *MdACO1* expression observed for the FAZ and completely eliminated the NAA-induced expression of *MdACO1* in the FC.

Effects of 1-MCP and NAA on expression of genes encoding ethylene receptors and the ethylene signal transduction kinase CTR1. The level of *MdETR1* transcript in the FAZ was unaffected by 1-MCP except at 5 d, but was significantly enhanced in the FC, maximally at 1 d and remaining above control levels thereafter (Fig. 3, A and B). 1-MCP inhibited *MdETR1b* and *MdETR2* expression in the FAZ and the FC at most time points, although an increase in the expression of both genes was noted in the FC at 7 d (Fig. 3, C–F). In general, the expression of all three *MdETR* genes was significantly increased by NAA in both tissues, whereas the effects of the 1-MCP + NAA treatment relative to NAA alone on the expression of the *MdETR* genes were variable, differing among genes and between tissues for each gene. However, one notable similarity was that 1-MCP treatment resulted in an early transient negative effect on NAA-induced expression of all three *MdETR* genes in the FAZ (Fig. 3, A, C, and E). Similarly, the expression of *MdERS1* and *MdERS2* in the FAZ was inhibited by 1-MCP at an early stage. 1-MCP also reduced the expression of these two genes in the FC, but only from 3 to 5 d (Fig. 4, A–D). NAA caused a sustained increase in the expression of *MdERS1* and *MdERS2* in the FC, whereas both genes exhibited a transient NAA-dependent increase in the FAZ. As was observed for the *MdETR* genes, the effects of the 1-MCP + NAA treatment relative to NAA alone on the expression of the *MdERS* genes were variable. *MdCTR1* expression was not significantly affected by 1-MCP in either tissue, but was continuously enhanced by NAA and 1-MCP + NAA in the FC (Fig. 4, E and F).

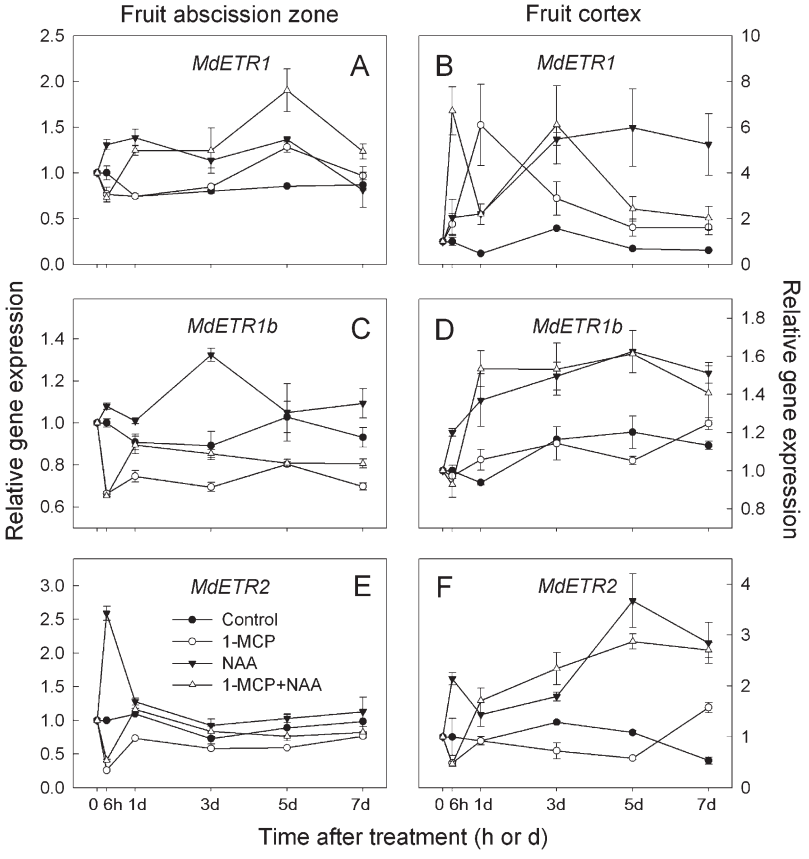


Fig. 3. Real-time quantitative PCR analysis of the expression of *MdETR1*, *MdETR1b*, and *MdETR2* in the fruit abscission zone (**A**, **C**, and **E**) and the fruit cortex (**B**, **D**, and **F**) from 'Golden Delicious' apple trees after application of 1-methylcyclopropene (1-MCP) and naphthaleneacetic acid (NAA). The levels of *MdETR1*, *MdETR1b*, and *MdETR2* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdETR1*, *MdETR1b*, and *MdETR2* in the fruit abscission zone and the fruit cortex from control trees were arbitrarily set to 1.

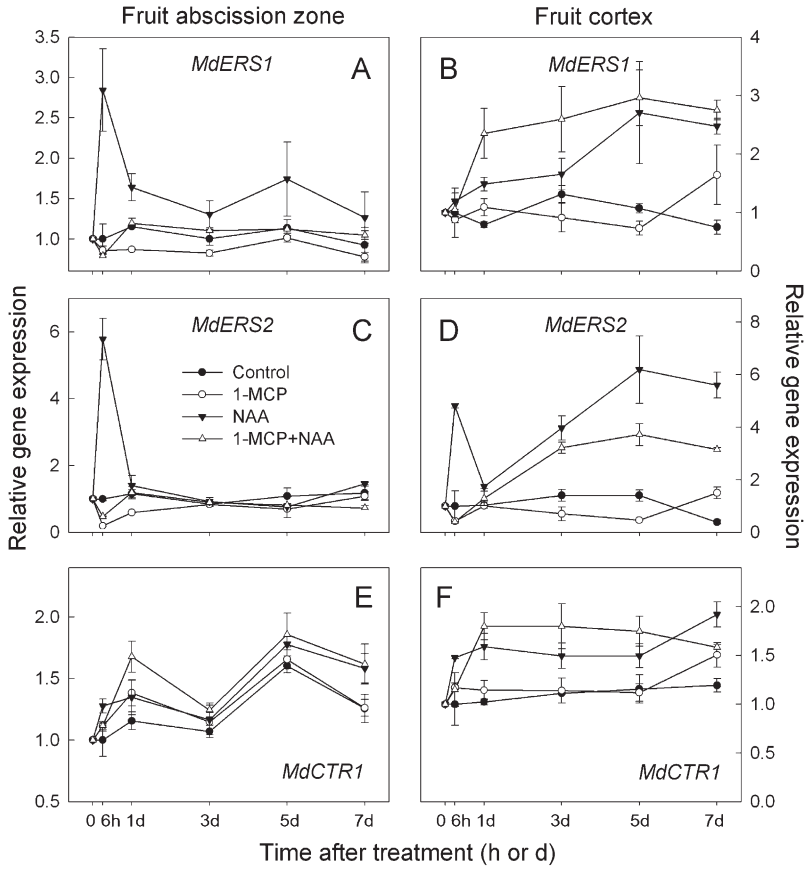


Fig. 4. Real-time quantitative PCR analysis of the expression of *MdERS1*, *MdERS2*, and *MdCTR1* in the fruit abscission zone (**A**, **C**, and **E**) and the fruit cortex (**B**, **D**, and **F**) from 'Golden Delicious' apple trees after application of 1-methylcyclopropene (1-MCP) and naphthaleneacetic acid (NAA). The levels of *MdERS1*, *MdERS2*, and *MdCTR1* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdERS1*, *MdERS2*, and *MdCTR1* in the fruit abscission zone and the fruit cortex from control trees were arbitrarily set to 1.

Effects of 1-MCP and NAA on expression of genes encoding enzymes involved in cell wall degradation. *MdPG2* expression in the FAZ was enhanced by 1-MCP and NAA maximally at 7 d after treatment (Fig. 5, A and B). In the FC, treatment with 1-MCP + NAA mainly increased *MdPG2* expression, while the application of 1-MCP or NAA had little effect. The expression of *MdCel1* was transiently enhanced by 1-MCP or NAA 6 h after treatment, but thereafter was significantly inhibited by either chemical relative to the control, except for an increase in the FC at 7 d caused by NAA (Fig. 5, C and D).

Discussion

Although the interactions between 1-MCP and ethylene physiology have been extensively studied in many fruit and vegetables, questions remain regarding the impact of 1-MCP on the molecular biology of the abscission of young apple fruit. To help address this deficiency and to gain a more detailed fundamental understanding of 1-MCP action, we measured the early and late responses of young apple fruit to a single application of 1-MCP, with and without NAA, focusing on abscission, ethylene production, and the expression of ethylene-related genes. Although a transient increase in fruit set following the application of 1-MCP at petal fall or the 10-mm stage indicated that an early application of 1-MCP has the potential to delay fruit drop, ultimately, 1-MCP applied to 'Pioneer McIntosh' and 'Golden Delicious' apple caused a decrease in fruit set in one experiment and had no effect on final fruit set in two others. These findings indicate that in contrast to its ability to delay preharvest fruit abscission and the ripening of mature fruit (Li and Yuan, 2008), 1-MCP, as used in this study, was not an inhibitor of the abscission of young fruit, and may in some cases promote abscission.

Two systems of ethylene regulation have been proposed to operate in higher plants (Barry et al., 2000; Lelievre et al., 1998; McMurchie, 1972). System I is ethylene auto-inhibitory and is responsible for the low level of ethylene during the preclimacteric stage, while system II is autocatalytic and has positive feedback regulation in which ethylene promotes its own synthesis during the climacteric ripening stage (Yang and Hoffman, 1984). Previous studies have consistently demonstrated that 1-MCP can effectively delay or decrease the ethylene production of climacteric fruit when they enter system II status (Bai et al., 2005; Hershkovitz et al., 2005; Wills and Ku, 2002). On the other hand, 1-MCP does not inhibit and even causes transient ethylene stimulation in other systems, including citrus leaves (Zhong et al., 2001) and fruitlets (Katz et al., 2004) and some non-climacteric fruit and vegetables, including sweet cherry (*Prunus avium* L.), grapefruit (*Citrus paradisi* L.), chinese cabbage (*Brassica campestris* L.), and strawberry (*Fragaria ×ananassa* Duch.) (Gong et al., 2002; Mullins et al., 2000; Porter et al., 2005; Tian et al., 1997). Similarly, in our study, 1-MCP appeared to suppress ethylene feedback inhibition; i.e., 1-MCP caused low-level (relative to the levels induced by NAA) transient increases of ethylene synthesis, although the leaf response appeared to be immediate, while the fruit response was markedly delayed (Fig. 1). The overall low levels of ethylene we observed in the young fruit are reminiscent of a system I-like characteristic and may partly explain why the impact of 1-MCP on the abscission of young fruit diverges from that reported for mature fruit in system II status.

1-MCP treatment led to a decrease in the transcript levels of most of the receptor genes in the FAZ. In contrast, NAA treatment increased the expression of most of the ethylene receptor genes in the FAZ and the FC and of *MdCTR1* in the FC only, perhaps reflecting a signal transduction negative feedback response to elevated fruit ethylene production in NAA-treated trees. These findings are generally consistent with previous reports of a positive correlation between abscission and increased expression of ethylene receptor genes in young apple fruit (Dal Cin et al., 2005; Zhu et al., 2008). In contrast, Yuan and Li (2008) found that despite an NAA-induced increase in the expression of genes encoding some ethylene receptors in mature 'Delicious' apple, preharvest fruit abscission was decreased. Such discrepancies in NAA effects could be related to different fruit developmental stages or other experimental factors differentiating these studies. It is also important to note that paradoxical relationships can exist between receptor

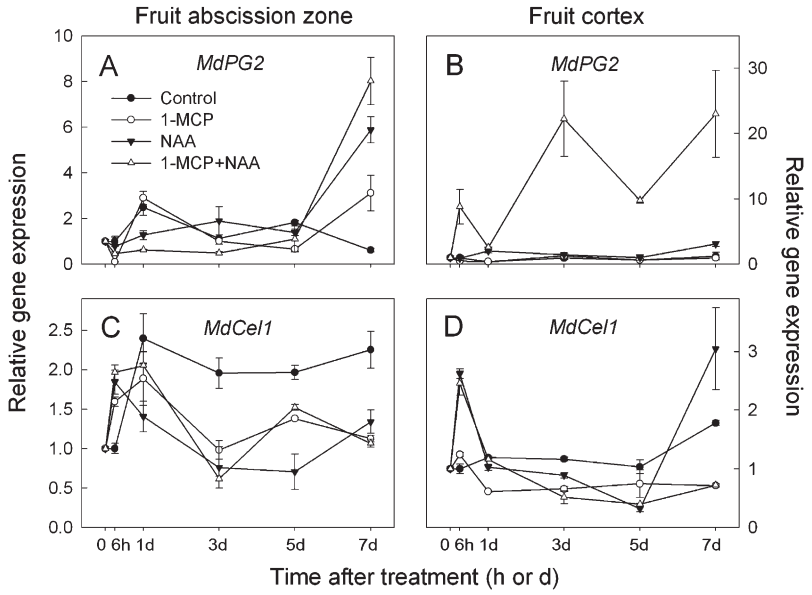


Fig. 5. Real-time quantitative PCR analysis of the expression of *MdPG2* and *MdCel1* in the fruit abscission zone (**A** and **C**) and the fruit cortex (**B** and **D**) from 'Golden Delicious' apple trees after application of 1-methylcyclopropene (1-MCP) and naphthaleneacetic acid (NAA). The levels of *MdPG2* and *MdCel1* transcripts were normalized using actin. Data are means ± SE (n = 3). The values of *MdPG2* and *MdCel1* in the fruit abscission zone and the fruit cortex from control trees were arbitrarily set to 1.

mRNA levels and corresponding protein levels (Kevany et al., 2007), indicating that further work is required to determine the relationship between ethylene receptor mRNA and protein levels in young apple fruit in response to 1-MCP or NAA.

Some reports have indicated that an increase in PG and/or cellulase activities correlates with fruit abscission (Bonghi et al., 2000; Tonutti et al., 1995; Ward et al., 1999), but our study showed that the expression of *MdCel1*, encoding β-1,4-glucanase, was largely inhibited by NAA and 1-MCP in the FAZ, which was consistent with what we found in 'Delicious' apple (Zhu et al., 2008), suggesting that *MdCel1* is not involved in the cell wall degradation induced by NAA. Zhu et al. (2008) did not detect the transcript for *MdPG1* in the FAZ or the FC from 'Golden Delicious' and 'Delicious' apple, which contrasts with other work showing that *MdPG1* was involved in apple fruit softening and that its expression was suppressed by 1-MCP and AVG treatment (Li and Yuan, 2008; Wakasa et al., 2006). The expression of *MdPG2* in the FAZ was significantly induced by 1-MCP and NAA just before the increase in the rate of fruit abscission (Fig. 1), in agreement with our previous work with NAA in 'Delicious' apple (Zhu et al., 2008), and was down-regulated concomitant with an NAA-dependent reduction in preharvest fruit drop (Li and Yuan, 2008). Thus, while it is difficult to draw broad conclusions regarding these genes involved in cell wall hydrolysis, *MdPG2* appears to be the gene most strongly associated with abscission based on current information.

Unlike the ethylene synthesis inhibitor AVG that effectively inhibits NAA-induced young fruit abscission through decreased ethylene production (Zhu et al., 2008), 1-MCP had no effect or a small promotive effect on the abscission of young apple fruit (Tables 1–3). The differences in the efficacy of AVG versus 1-MCP suggest that the ethylene signals can be more easily blocked at the biosynthesis level. However, once ethylene is generated, signaling may not be completely blocked at the receptor level by 1-MCP, due to the influence of changing receptor levels (Kevany et al., 2008), the competition between ethylene and 1-MCP for the ethylene-binding sites on receptors, and/or the changes in levels of ethylene and other ethylene pathway components affected by 1-MCP (Figs. 1–4). Moreover, while our previous work with AVG supports a model where ethylene synthesis is necessary for NAA-induced abscission of young apple fruit (Zhu et al., 2008), those experiments did not rule out a role for other AVG-sensitive, pyridoxal-5′-phosphate-dependent enzymes in the abscission process. This possibility, considered with the results presented here, suggests that in addition to the ethylene-dependent pathway for abscission, a separate AVG-sensitive, 1-MCP-insensitive pathway may also promote the abscission of young apple fruit following NAA treatment. Alternatively, and more simply, the period of effectiveness of 1-MCP following a single application may not have sufficiently overlapped with the period of ethylene effectiveness.

In conclusion, we found that while 1-MCP may have some ability to transiently increase fruit set, it was not effective at increasing final fruit set. In this way, the effects of 1-MCP on the abscission of young fruit are distinct from those reported for mature fruit (Li and Yuan, 2008; Yuan and Carbaugh, 2007; Yuan and Li, 2008). 1-MCP differentially regulated the expression of genes involved in ethylene biosynthesis and perception and cell wall degradation, but the expression patterns appeared to reflect a wide range of temporal and tissue-specific effects of 1-MCP. NAA, as a synthetic auxin, effectively increased the abscission of young apple fruit, possibly through its ability to enhance the expression of nearly all of the ethylene-related genes investigated here in the FAZ and the FC. 1-MCP application did not reduce the level of NAA-induced abscission. Thus, the hypothesis that 1-MCP can inhibit the NAA-induced abscission of young fruit is not supported by this study. Having profiled the short- and long-term ethylene- and abscission-related responses of young fruit to a single application of 1-MCP alone and in combination with NAA, it will be interesting to identify through further research the precise mechanism by which NAA-induced fruit abscission is promoted in the presence of 1-MCP, and to determine the effects of multiple applications of 1-MCP in advance of young fruit abscission in apple.

Literature Cited

Alexander, L. and D. Grierson. 2002. Ethylene biosynthesis and action in tomato: A model for climacteric fruit ripening. J. Expt. Bot. 53: 2039–2055.

Applied Biosystems. 2005. Guide to performing relative quantitation of gene expression using real-time quantitative PCR. Applied Biosystems, Foster City, CA.

Bai, J.H., E.A. Baldwin, and K.L. Goodner. 2005. Response of four apple cultivars to 1-methylcyclopropene treatment and controlled atmosphere storage. HortScience 40:1534–1538.

Barry, C.S., M.I. Llop-Tous, and D. Grierson. 2000. The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant Physiol. 123:979–986.

Blankenship, S.M. and J.M. Dole. 2003. 1-Methylcyclopropene: A review. Postharvest Biol. Technol. 28:1–25.
Blanpied, G.D. and K.J. Silsby. 1992. Prediction of harvest date windows for apples. Cornell Coop. Ext. Bul. 221.
Bleecker, A.B. 1999. Ethylene perception and signaling: An evolutionary perspective. Trends Plant Sci. 4:269–274.
Bonghi, C., P. Tonutti, and A. Ramina. 2000. Biochemical and molecular aspects of fruitlet abscission. Plant Growth Regulat. 31: 35–42.
Chang, C.R. and A.B. Bleecker. 2004. Ethylene biology. More than a gas. Plant Physiol. 136:2895–2899.
Chen, Y.F., N. Etheridge, and G.E. Schaller. 2005. Ethylene signal transduction. Ann. Bot. (Lond.) 95:901–915.
Curry, E.A. 1991. NAA-induced ethylene and ACC in 'Delicious' spur tissues: Changes with temperature and time. J. Amer. Soc. Hort. Sci. 116:846–850.
Dal Cin, V., M. Danesin, A. Boschetti, A. Dorigoni, and A. Ramina. 2005. Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus domestica* L. Borkh). J. Expt. Bot. 56:2995–3005.
Fan, X.T., S.M. Blankenship, and J.P. Mattheis. 1999. 1-Methylcyclopropene inhibits apple ripening. J. Amer. Soc. Hort. Sci. 124:690–695.
Gong, Y.P., X.T. Fan, and J.P. Mattheis. 2002. Responses of 'Bing' and 'Rainier' sweet cherries to ethylene and 1-methylcyclopropene. J. Amer. Soc. Hort. Sci. 127:831–835.
Hershkovitz, V., S.I. Saguy, and E. Pesis. 2005. Postharvest application of 1-MCP to improve the quality of various avocado cultivars. Postharvest Biol. Technol. 37:252–264.
Huai, Q., Y.H. Xia, Y.Q. Chen, B. Callahan, N. Li, and H.M. Ke. 2001. Crystal structure of 1-aminocyclopropane-1-carboxylate (ACC) synthase in complex with aminoethoxyvinylglycine and pyridoxal-5′-phosphate provide new insight into catalytic mechanisms. J. Biol. Chem. 276:38210–38216.
Jeong, J., D.J. Huber, and S.A. Sargent. 2002. Influence of 1-methylcyclopropene (1-MCP) on ripening and cell-wall matrix polysaccharides of avocado (*Persea americana*) fruit. Postharvest Biol. Technol. 25:241–256.
Jiang, Y. and D. Joyce. 2000. Effects of 1-methylcyclopropene alone and in combination with polyethylene bags on the postharvest life of mango fruit. Ann. Appl. Biol. 137:321–327.
Katz, E., P.M. Lagunes, J. Riov, D. Weiss, and E.E. Goldschmidt. 2004. Molecular and physiological evidence suggests the existence of a system II-like pathway of ethylene production in non-climacteric citrus fruit. Planta 219:243–252.
Kevany, B.M., M.G. Taylor, and H.J. Klee. 2008. Fruit-specific suppression of the ethylene receptor LeETR4 results in early-ripening tomato fruit. Plant Biotechnol. J. 6:295–300.
Kevany, B.M., D.M. Tieman, M.G. Taylor, V. Dal Cin, and H.J. Klee. 2007. Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J. 51:458–467.
Kluge, R.A. and A.P. Jacomino. 2002. Shelf life of peaches treated with 1-methylcyclopropene. Scientia Agricola 59:69–72.
Lelievre, J.M., A. Latche, B. Jones, M. Bouzayen, and J.C. Pech. 1998. Ethylene and fruit ripening. Physiol. Plant. 101:727–739.
Li, J. and R. Yuan. 2008. NAA and ethylene regulate expression of genes related to ethylene biosynthesis, perception and cell wall degradation during fruit abscission and ripening in 'Delicious' apples. J. Plant Growth Regul. 27:283–295.
McArtney, S.J. 2002. Ethylene evolution from detached apple spurs in response to chemical thinners. HortScience 37:662–665.
McMurchie, E.J. 1972. Treatment of fruit with propylene gives information about the biogenesis of ethylene. Nature 237:235–236.
Mullins, E.D., T.G. McCollum, and R.E. McDonald. 2000. Consequences on ethylene metabolism of inactivating the ethylene receptor sites in diseased non-climacteric fruit. Postharvest Biol. Technol. 19:155–164.
Porter, K.L., G. Collins, and A. Klieber. 2005. 1-MCP does not improve the shelf-life of chinese cabbage. J. Sci. Food Agr. 85:293–296.
Sisler, E.C. and M. Serek. 1997. Inhibitors of ethylene responses in plants at the receptor level: Recent developments. Physiol. Plant. 100: 577–582.
Tian, M.S., Y.P. Gong, and A.D. Bauchot. 1997. Ethylene biosynthesis and respiration in strawberry fruit treated with diazocyclopentadiene and IAA. Plant Growth Regulat. 23:195–200.
Tonutti, P., L.G. Cass, and R.E. Christoffersen. 1995. The expression of cellulase gene family members during induced avocado fruit abscission and ripening. Plant Cell Environ. 18:709–713.
Wakasa, Y., H. Kudo, R. Ishikawa, S. Akada, M. Senda, M. Niizeki, and T. Harada. 2006. Low expression of an endopolygalacturonase gene in apple fruit with long-term storage potential. Postharvest Biol. Technol. 39:193–198.
Walsh, C.S., H.J. Swartz, and L.J. Edgerton. 1979. Ethylene evolution in apple following post-bloom thinning sprays. HortScience 14:704–706.
Ward, D.L., E.P. Beers, R.E. Byers, and R.P. Marini. 1999. Cutting apple fruits induces cellulose activity in the abscission zone. HortScience 34:601–603.
Watkins, C.B. 2006. The use of 1-methylcyclopropene (1-MCP) on fruits and vegetables. Biotechnol. Adv. 24:389–409.
Williams, R.R. and V.A. Flook. 1980. The mode of action of the hormone apple fruit-setting mixture PP-341B applied to 'Cox's Orange Pippin'. J. Hort. Sci. 55:275–277.
Wills, R.B.H. and V.V.V. Ku. 2002. Use of 1-MCP to extend the time to ripen of green tomatoes and postharvest life of ripe tomatoes. Postharvest Biol. Technol. 26:85–90.
Yang, S.F. and N.E. Hoffman. 1984. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35:155–189.
Yuan, R. 2007. Effects of temperature on fruit thinning with ethephon in 'Golden Delicious' apples. Sci. Hort. 113:8–12.
Yuan, R. and D.H. Carbaugh. 2007. Effect of NAA, AVG, and 1-MCP on ethylene biosynthesis, preharvest fruit drop, fruit maturity, and quality of 'Golden Supreme' and 'Golden Delicious' apples. HortScience 42:101–105.
Yuan, R. and J. Li. 2008. Effects of sprayable 1-MCP, AVG and NAA on ethylene biosynthesis, preharvest fruit drop, fruit maturity, and quality of 'Delicious' apples. HortScience 43:1454–1460.
Zhong, G.Y., M. Huberman, X.Q. Feng, E.C. Sisler, D. Holland, and R. Goren. 2001. Effect of 1-methylcyclopropene on ethylene-induced abscission in citrus. Physiol. Plant. 113:134–141.
Zhu, H., E.P. Beers, and R. Yuan. 2008. Aminoethoxyvinylglycine inhibits fruit abscission induced by naphthaleneacetic acid and associated relationships with expression of genes for ethylene biosynthesis, perception, and cell wall degradation in 'Delicious' apples. J. Amer. Soc. Hort. Sci. 133:727–734.

Chapter 4

Global Gene Expression Profiling for Young Apple Fruit Abscission Using Apple Oligonucleotide Microarray

Abstract

Naphthaleneacetic acid (NAA), a synthetic auxin analogue, is widely used as an effective thinner in apple orchards. When applied shortly after fruit set, excess fruit abscise leading to improved fruit size and quality. However, the thinning results of NAA are inconsistent and difficult to predict, sometimes leading to excess fruit drop or insufficient thinning which is costly to growers. This unpredictability is attributed to our incomplete understanding of how NAA promotes fruit abscission and the influence of other environmental factors, such as light and temperature. In this study, NAA at 15 $mg \cdot L^{-1}$ significantly increased young fruit abscission in 'Golden Delicious' apple (*Malus* × *domestica* Borkh.) when applied at 11-mm stage of fruit development. As a parallel treatment, shading for 5 consecutive days also greatly increased young fruit abscission. To better understand NAA's mode of action, we compared NAA induced fruit drop with that caused by shading via gene expression profiling experiments performed on the fruit abscission zone (FAZ), sampled 1, 3, and 5 d after treatment. More than 700 genes with significant changes in transcript abundance were identified. Genes associated with ethylene, ABA, cell wall degradation, mitochondrial activity, glycolysis, lipid catabolism, secondary metabolism, abiotic stress, and apoptosis were upregulated. On the other hand, there was down-regulation of genes related to photosynthesis, cell cycle, cell wall biosynthesis, carbon fixation, chromatin assembly, auxin transport/efflux, cytoskeleton function, and flower development. While the identified significant gene sets shared only 25% of genes in common, NAA and shading showed substantial similarity with respect to the classes of genes identified. Photosynthesis, carbon

utilization, and ABA/ethylene pathways appeared to operate in both NAA and shading-induced young fruit abscission. The finding that NAA, like shading also repressed photosynthesis and carbon utilization, we tested whether NAA had a direct effect on photosynthesis efficiency. NAA directly interfered with leaf photosynthesis and caused energy shortage, therefore we suggest that NAA may induce the same early response as shading, which preceded hormone signaling pathways and triggered the fruit abscission.

Introduction

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 also lead to branch damage, low quality fruit and drastic reductions in cropping in the following year. Consequently, over-cropping is an undesirable trait. Although a self-thinning process known as the "June drop" can help alleviate the negative impact of excessive fruit bearing, apple growers often find in necessary to apply chemical thinners to remove excess fruit at an early stage of fruit development. Naphthaleneacetic acid (NAA) is one of the most commonly used chemical thinners, but its efficacy varies among different varieties and is affected by environmental conditions following the application.

The physiological mechanisms through which NAA promotes the abscission of young apple fruitlets have been discussed (Hennerty and Forshey, 1972; Dennis, 2002). Principal among these mechanisms is a reduction in photosynthesis (Stopar et al., 1997; Yuan and Greene, 2000a) and carbohydrate availability and translocation from leaf to fruit (Schneider, 1978) that occurs following foliar application of NAA. The importance of photosynthesis and photosynthate

translocation in support of fruit development is further illustrated by experiments involving shading or removal of leaves, two treatments which cause extensive apple fruit abscission (Yuan and Greene, 2000b; Byers, 2003). Moreover, normal apple fruit abscission, which can occur both shortly after anthesis and during the "June drop", has been at least partly attributed to the competition for carbohydrates among young fruits and between fruits and vegetative shoots (Quilan and Preston, 1971; Yuan and Greene, 2000b). Together these findings indicate that photosynthesis is the main source of carbohydrates needed for fruit development and treatments that alter the levels of carbohydrates available for translocation to developing fruits can be used to control fruit set in apple trees. Despite the strong link between naturally occurring or experimentally induced abscission and reductions in carbohydrate levels, little information is available regarding the changes in gene expression that accompany such carbohydrate-linked apple fruit abscission (Weaver et al., 1998; Zhou et al., 2008).

In addition to its effects on carbohydrate levels, NAA application apparently enhances apple fruitlet abscission through increased ethylene production (Curry, 1991; McArtney, 2002; Dal Cin et al., 2005; Zhu et al., 2008). The idea that ethylene is involved in young apple fruit abscission has been supported by previous research (Dal Cin et al., 2005; Zhu et al., 2008). Application of ethephon, which releases ethylene, could effectively promote the abscission of young fruit in apple (Yuan, 2007), while Aminoethoxyvinylglycine (AVG), a strong inhibitor of ethylene biosynthesis, reduced fruit ethylene production and young fruit abscission in apple (Williams and Flook, 1980; Zhu et al., 2008). The NAA-induced increase in ethylene production is positively correlated with changes in expression of ethylene biosynthesis and signal transduction genes, including, five ACC synthase (*MdACS*) genes, one ACC oxidase (*MdACO*) gene, four ethylene

receptor (*MdETR* and *MdERS*) genes and one ethylene signal transduction gene (*MdCTR1*) (Dan Cin et al., 2005; Li and Yuan, 2008; Zhu et al., 2008). Dan Cin et al. (2005) found that apple fruitlet abscission is preceded by a stimulation of ethylene biosynthesis and an acquisition in sensitivity to ethylene. Also, a more recent microarray analysis of the abscission-related transcriptome in tomato flower abscission zone (AZ) further revealed the relationship between the acquisition of ethylene sensitivity in the AZ and altered expression of auxin-regulated genes due to auxin depletion (Meir et al., 2010).

Cell separation process is required within fruit abscission zone (FAZ) for fruit abscission, and the activities of cell wall remodeling and degrading enzymes, including expansin, pectate lyase, polygalacturonase, β-1,4-glucanase, have been shown to markedly increase upon the abscission and concomitant with increased ethylene production (Lashbrook et al., 1994; Belfield et al., 2005; Kim et al., 2006). Most of those genes are induced in the FAZ where they catalyze the loosening and breakdown of the cell wall and promote the fruit drop (Ward et al., 1999; Bonghi et al., 2000; Lashbrook and Cai, 2008).

It is likely that abscission-associated carbohydrate- and ethylene-responsive signaling pathways engage in crosstalk with each other and with other signaling pathways to coordinate wall hydrolysis in the abscission zone. The purpose of this study was to exploit NAA and shading-induced abscission to further reveal through transcriptome analysis the molecular mechanisms controlling abscission of young fruit. Increasing knowledge of changes in gene expression associated with abscission will aid in the development of strategies for more predictable results with existing thinners and set the stage for the development of improved thinners. Moreover, the

identification of regulatory networks that are central to apple fruit abscission will enhance our basic understanding of organ abscission, which is a fundamental aspect of plant development.

Materials and Methods

Treatments and sample collection

Thirty-six uniform 'Golden Delicious' apple trees on M.9 rootstock were selected and divided into three blocks of 12 trees each. Four trees from each block were treated with: 1) Control (water); 2) NAA (Fruitone N; AMVAC, Newport Beach, CA) at 15 $mg \cdot L^{-1}$; 3) Shading (92% polypropylene shade over the entire tree for 5 consecutive days), respectively. Treatments were applied when the fruit size was ~10 mm in diameter. Three biological replicates were conducted independently. Young fruit was collected at 0, 1, 3, 5, 7, 9 d after treatment. At each collection time, around 80 fruit were collected from each tree, with fruit cortex (FC) and fruit abscission zone (FAZ) separated. All samples were promptly frozen in liquid nitrogen and stored at -80 °C for future RNA extraction.

Fruit abscission pattern and leaf/fruit ethylene production

To determine the fruit abscission rate, two limbs on each tree were tagged and fruit on tagged limbs were counted on 0, 1, 2, 3, 7, 9, 11, 14, 16, 18, 21, 25 and 26 d after treatment. For ethylene production measurements, 15 fruit and 20 leaves were collected from each tree of three replicates 0 and 6 h and 1, 3, 5, 7, 9, 11 and 14 d after treatment and enclosed in a 100-mL (for fruit) or 1000-mL (for leaves) container. After a 2-h incubation period, a 1-mL gas sample was withdrawn from the sealed container through the rubber septum affixed to the lid, and the ethylene concentration was measured with a gas chromatograph equipped with an activated

alumina column and FID detector (model 3700; Varian, Palo Alto, CA). The ethylene production was calculated and expressed as microliters of C_2H_4 per kilogram per hour.

Microarray design and construction

A total of 34 cDNA libraries were constructed from different tissues, both vegetative and reproductive (at different stages of development), and under different biotic and abiotic stresses. Tissues used for library construction were collected from either trees grown at the University of Illinois, Urbana, IL or from greenhouse-grown plants subjected to various biotic (Cornell University and USDA-ARS, Geneva, NY) or abiotic (USDA-ARS, Kearneysville, WV) stresses as previously described (Gasic et al., 2009).

Expressed sequence tag (EST) sequences were clustered based on local similarity scores of pairwise comparisons using 88% similarity over 100 nucleotides. Putative functions of the apple unique ESTs were classified according to the Gene Ontology (GO) scheme (Ashburner et al., 2000). The representation of protein families, domains, and functional sites within apple unique sequences was determined using Inter-ProScan. From a total of 34 libraries, 33,825 unique sequences were selected, in conjunction with an additional 6,000 sequences used as positive and negative controls, and used to create a 40,000 Invitrogen 70-mer oligonucleotide-based array.

A total of 40,000 sequences were selected, and oligos were designed and synthesized commercially (Invitrogen). Oligos were resuspended in 150 mM NaPi pH 8.5 to a final concentration of 20 μM and printed on ultragaps coated slides (Corning) using a GeneMachine OmniGrid arrayer (Newport) in a total of 48 blocks. In addition to sample oligos, each block

contains 10 spots with NaPi used as control. Slides were hydrated and UV-crosslinked at 300 mJ/cm^2 (UV Stratalinker 1800, Stratagene) before use.

Experimental design and microarray hybridization

For microarray studies, three time points (D1, 3 and 5) were represented by three biological replicates analyzed in a dye swap design (six hybridizations per time point). A total of 50 pmol of incorporated dye with at least a FOI of 2.0 (calculated using Base/Dye Ratio Calculator from Invitrogen) was used for each sample cDNA and the reference cDNA in the hybridization.

RNA extraction, aRNA amplification and labeling

Total RNA was extracted from the FAZ for each biological replicate as described by Li and Yuan (2008), and purified using TURBO DNA-free™ Kit (Ambion, Austin, TX). RT-PCR was performed using primers that span an intron in *MdACO* to confirm that each RNA sample was free of genomic DNA contamination. The RNA was quantified using the NanoDrop nd-1000 (Thermo Scientific, MA, USA) and the quality checked using the Bioanalyzer 2100 (Agilent, CA, USA) according to manufacturer's directions.

According to the Instruction Manual of Amino Allyl MessageAmp™ II aRNA Amplification Kit, cDNA was synthesized from mRNA in 1 μg of total RNA. Purified cDNA then was in vitro transcribed to synthesize aRNA using IVT Master Mix which contains 5-(3-aminoallyl)-UTP (50 mM), ATP/CTP/GTP Mix (25 mM), UTP (50 mM), T7 10×Reaction Buffer and T7 Enzyme Mix. After purification, aRNA was labeled with either AlexaFluor555 or AlexaFluor647 (Invitrogen, CA, USA) for hybridization. Slides were pre-washed with Pre-hybridization buffer. Labeled

samples were mixed with 1×Slide Hyb Glass Hybridization Buffer (Ambion) and injected into the slide chambers which were heated to 65 °C. The chambers were incubated at 42 °C overnight. The next day, the slides were washed with 1×SSC, 0.2% SDS for 5 min, 0.1×SSC, 0.2% SDS for 5 min and twice with 0.1×SSC.

Data scanning and analysis

Slides were dried and scanned on a GenePix 4000B Scanner (Axon Instruments). Dual channel images from the slides were captured using GenePix4 microarray scanner (Axon Instruments) and automated spot alignment was augmented with manual checking of each slide to remove substandard spots. GenePix Pro software was used to generate gpr files for statistical analysis and data normalization. LIMMA package for R programming environment was used by applying linear model methods.

Each probe was tested for changes in expression over the time points using a moderated F test, which is similar to an ANOVA method for each probe except that the residual standard errors are moderated across genes. The linear models allow for general changes in gene expression between successive time points. The use of dye-swaps in the experimental design eliminated a dye-effect for each probe, which increased the precision with which differential expression could be detected. The computed *P* values were adjusted for multiple testing by the Benjamini and Hochberg method to control the false discovery rate (FDR). Genes were considered significantly expressed if the adjusted *P* values were <0.01 (i.e. expected FDR less than 1%).

Gene clustering and categorization

Hierarchical clustering was performed using the statistic package for R utilizing the Euclidean distance. Figure of merit (FOM) analysis was performed to determine the number of clusters needed for the explanation of the majority of variation in expression patterns. Then a cluster number was assigned for K-means clustering (KMC) analysis to divide the data into distinct expression clusters based on similarities in their expression patterns, using the TM4 package (MultiExperiment Viewer, Version 4.3). Default statistical parameters were used in those analyses. Data were scaled for hierarchical and KMC clustering based on fold-change and $\log_2$ ratio in gene expression.

All significant genes were then categorized using Blast2go application. Annotation was further supplemented with manual BLASTX, conserved domains, and literature searches, mostly based on *Arabidopsis* database. Using this combined information, a functionally driven classification was created manually. Larger categories were further divided into subcategories to cover all the related genes.

Quantitative real-time PCR

Purified total RNA (1 μg) from each sample was used to synthesize cDNA in a 20-μL reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Fosters City, CA). Each qPCR reaction was run in triplicate using 40 ng of cDNA in a 15 μl reaction volume using Power SYBR Green qRT-PCR Kit (Applied Biosystems). Gene-specific primer sets were designed from available apple ESTs sequences using Primer Expression 3.0 and synthesized by Integrated DNA Technologies (Coralville, IA) (Zhu et al., 2008). The reactions were performed

on a 7500 Real-time PCR Cycler (Applied Biosystems, CA, USA). Quantification was achieved using a relative standard curve derived from a standard RNA run in parallel with each primer set. A primer set designed to amplify *Malus* actin RNA was run on all samples and used to normalize the data. A dissociation curve was run to verify that a single desired amplified product was obtained from each reaction (Applied Biosystems, 2005).

Results

Effects of NAA and shading on young fruit abscission and ethylene production of young fruit and leaves

A comparison of the relative effectiveness of NAA and shading as inducers of fruit abscission revealed significant treatment-specific differences in abscission rates and totals. For example, while both treatments promoted detectable increases in abscission rates within the first 7 days post-treatment, the NAA-induced abscission rate remained essentially unchanged from day 7 to day 13, whereas shading resulted in a relatively steady increase in the rate of fruit abscission for the same period (Figure 4.1A). By day 15, however, similar rates of abscission were observed for both treatments and rates were near or below control rates by day 19 for both treatments. Ultimately, shading was significantly more effective at promoting fruit drop, causing 98% of the fruit to abscise within the 19 day period of the study, compared to a 75% loss in the same period following NAA treatment. Interestingly, the pattern of abscission exhibited by controls roughly mirrored that of treated trees but resulted in less that 10% of the fruit being shed, indicating that the NAA and shading treatments were able to act additively or synergistically with a normal self-thinning program.

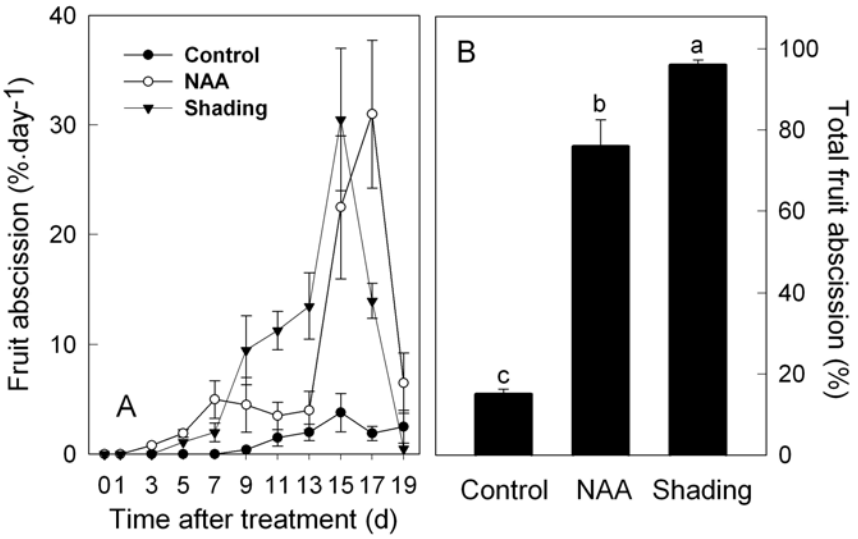


Figure 4.1. Effects of NAA and shading on fruit abscission pattern (**A**) and total fruit abscission (**B**) in 'Golden Delicious' apples in 2006. Data are means ± SE (n = 3). Different letters indicate significant differences among means according to Duncan's multiple range test ($P \leq 0.05$).

Previous studies have documented the link between NAA- and shading-induced abscission and ethylene production. We confirmed that the major increases in ethylene production coincided with the beginning of detectable levels of fruit abscission (Fig, 4.2A compared with Fig. 4.1A). That maximum level of NAA-induced fruit ethylene production occurred 1 d and decreased to control level 7 d after treatment. Shading-treated fruit also had an increased level of ethylene between 1 and 5 d, but lower than NAA-treated fruit (Fig. 4.2A). On the other hand, the ethylene production of both shading- and NAA-treated leaves peaked 1 d, but decreased to control level by 5 and 7 d after treatment (Fig. 4.2B).

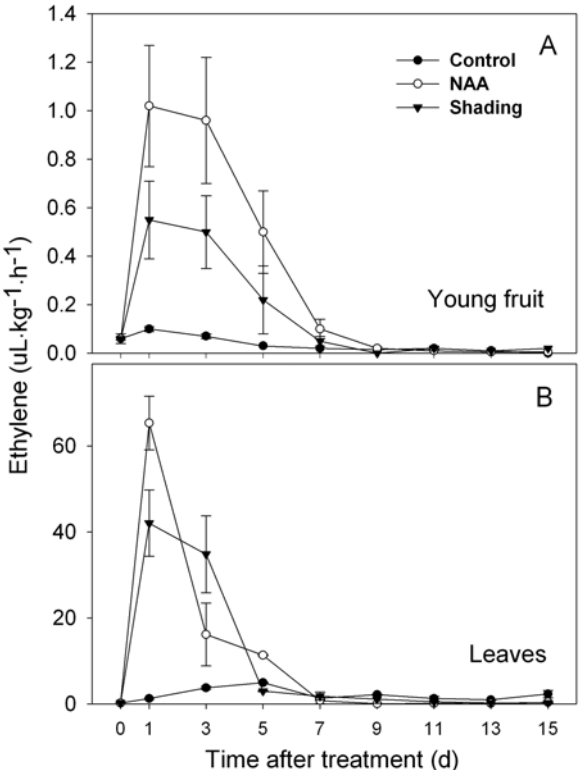


Figure 4.2. Effects of NAA and shading on fruit ethylene production (**A**) and leaf ethylene production (**B**) in 'Golden Delicious' apples in 2006. Data are means ± SE (n = 3). Different letters indicate significant differences among means according to Duncan's multiple range test ($P \leq 0.05$).

Effects of NAA on leaf photosynthesis

To investigate the effect of NAA on leaf photosynthesis, NAA was directly applied to the leaves of both young apple seedlings in growth chamber (21-24 °C, 50% humidity) and mature fruit-bearing apple trees in the field. After treatment, either the whole plant or some leaf samples were collected at intervals (10 min, 1 h and 5 h for young seedlings; 10 min, 4 h and 1 d for mature trees) and the photosystem II efficiency was monitored using IMAGING-PAM Fluorometer with Walz ImagingWin v2.32. NAA at various rates (15, 150, 450 and 900 $mg \cdot L^{-1}$) all caused

photoinhibition in the leaves of young seedlings in growth chamber, and the effects were concentration-dependent (Fig. 4.3A).

NAA at 15 mg·L^{-1}, the working concentration used in the thinning experiment, caused noticeable inhibitory effect of leaf photosynthesis (Fig 4.3B-D). Such inhibition was observed as early as 10 min post-treatment and lasted for hours, but the leaves usually recovered within one day. For the field trial on fruit-bearing trees, more severe effects of NAA at 15 mg·L^{-1} on leaf photosynthesis were found, due to higher temperature and water stress. Also, the effect lasted longer, compared to young seedlings (Fig 4.3E-G).

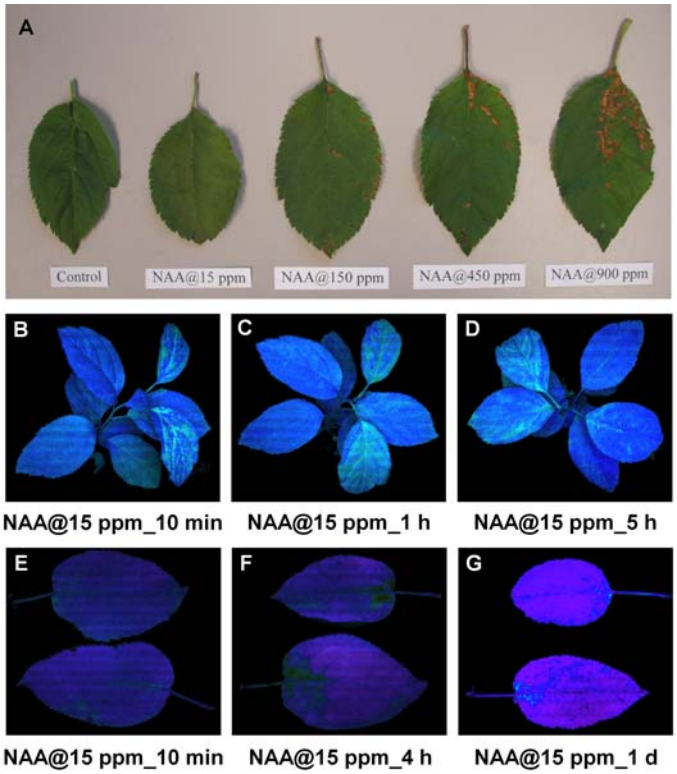


Figure 4.3. Effect of NAA on leaf photosynthesis system II efficiency. (**A**) Effect of NAA at various rates on the leaves of young seedlings in growth chamber. (**B-D**) Effect of NAA at 15 mg·L^{-1}on leaf photosynthesis efficiency of young seedlings in growth chamber. (**E-G**) Effect of NAA at 15 mg·L^{-1} on leaf photosynthesis efficiency of fruit-bearing trees in the field.

Expression profiling of young fruit abscission induced by NAA and shading

In order to identify genes whose expression patterns correlated with the fruit abscission induced by NAA, a gene expression profiling study was performed using the FAZ at three time points (D1, D3 and D5), a period spanning the early stage of abscission induction. The microarray platform used was an apple 70-mer oligonucleotide-based array (Schaffer et al., 2007). For each time point, labeled cDNA from either NAA or shading-treated FAZ was hybridized to reference cDNA from non-treated FAZ. This design used separate reference samples, so as not to confound the changes in gene expression caused by treatments with those occurring during fruit development.

Seven hundred and twenty-two genes were identified from NAA-treated sample hybridizations and 1057 genes from shading-treated sample hybridizations that showed statistically significant changes in gene expression. One hundred and sixty-eight genes from NAA-treated sample were also identified as differentially expressed in the shading-treated sample, and 86% of these shared significant genes (145) displayed highly similar expression patterns, indicating that NAA and shading may share certain mechanisms in regard to fruit abscission.

Both time points and selected genes were grouped according to expression pattern by hierarchical cluster analysis (Fig. 4.4A-B). Most notable changes in gene expression occurred at 3 d, followed by 5 and 1 d after NAA treatment, suggesting that NAA might have caused a transient effect during the abscission induction. In contrast, shading displayed a gradually increasing effect on the overall gene expression, where 3 and 5 d samples both showed more genes with significant expression changes than 1 d sample. For both treatments, there were

approximately equal numbers of genes showing upregulation or downregulation on 1 d, but more genes were induced than repressed later on 3 and 5 d.

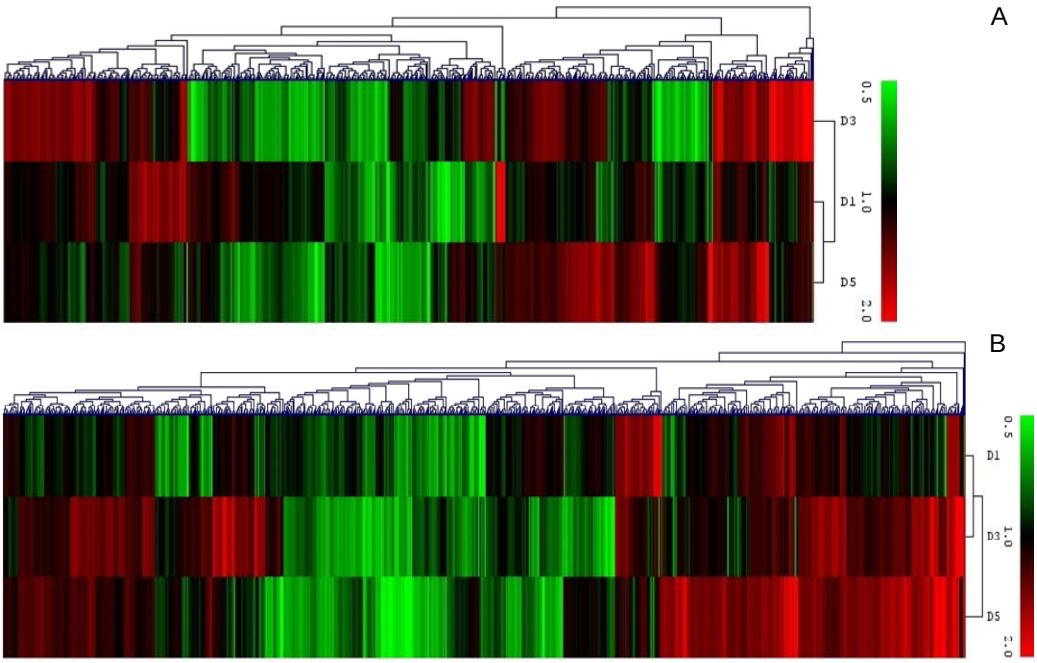
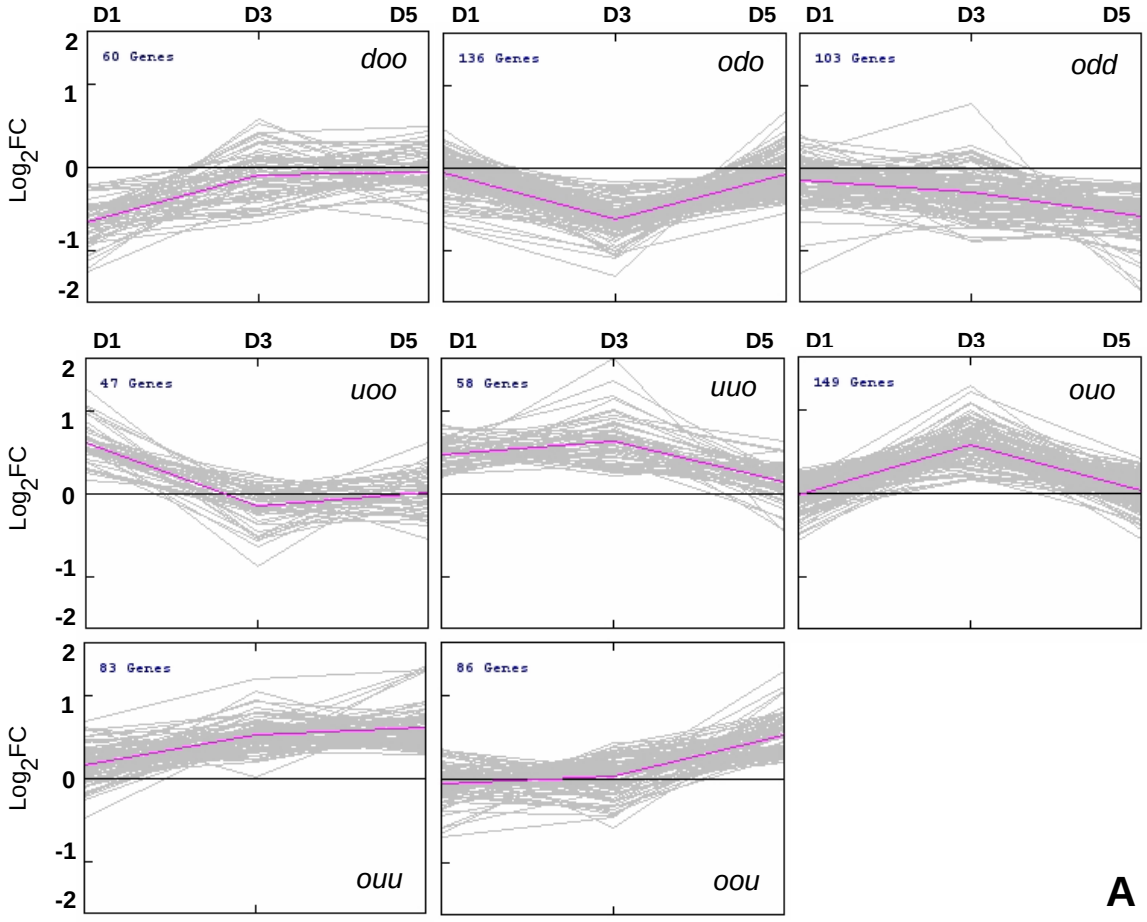


Figure 4.4. Hierarchical cluster of 722 selected genes from NAA-treated FAZ (**A**) and 1057 selected genes from shading-treated FAZ (**B**).

The two array datasets were further analyzed using K-means clustering (KMC) for genes whose expression pattern was concurrent with the induction of fruit abscission. The cluster names were assigned *u*, *o* or *d* for each time point depending on whether expression was upregulated (*u*), unchanged (*o*) or downregulated (*d*). NAA-responsive genes were classified into 8 main clusters, and all the selected genes were shown either up- or down-regulated, but at different time points, confirming that the response to NAA was staged, with a large group of genes responding only at the middle time point (3 d after treatment). Shading-responsive genes were divided into 10 clusters. Similar to NAA dataset, all genes had either upregulation or downregulation, with the exception for cluster #2, where 51 genes showed downregulation 1 d, but upregulation 5 d after

treatment (Fig. 4.5A-B). There were two clusters (cluster #1 and 4) in the shading dataset showing repression and induction, respectively, throughout the three time points, indicating a more persistent effect on the induction of fruit abscission imposed by shading than NAA.



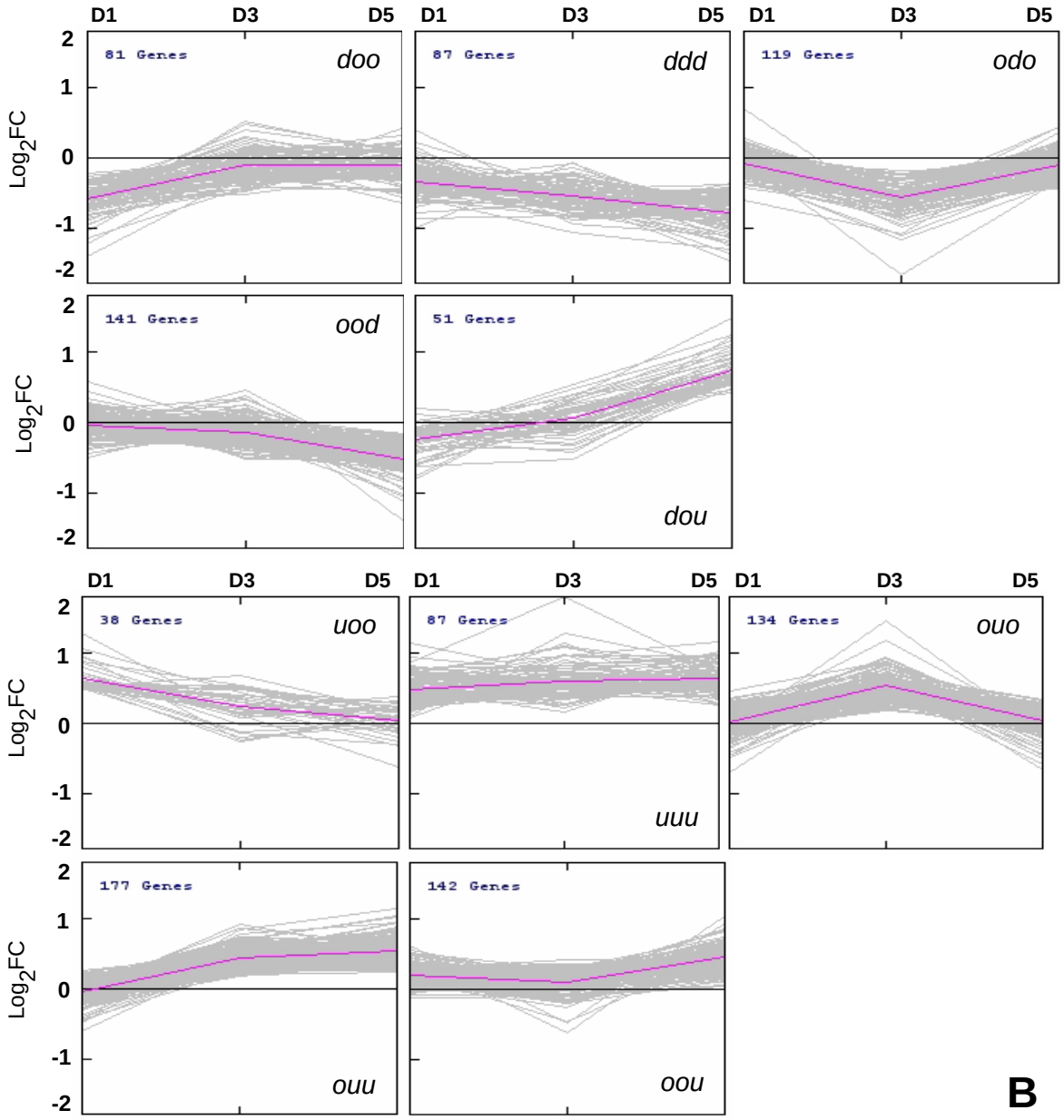


Figure 4.5. Clusters of NAA-responsive genes (**A**) and shading-responsive genes (**B**).

This study focused on expression patterns among different functional categories of genes to identify correlations with the observed fruit abscission. Genes represented on the apple array have both annotation and gene ontology (GO) information, but some annotations and GO categories are too general to provide detailed information on the biological mechanisms, so

additional annotation and literature searches were conducted on the entire significant gene list. Significant genes from both datasets were classified into 15 functional categories, among which eight categories (photosynthesis, metabolism, membrane/cellular trafficking, cell cycle, hormone response, cell wall modification, proteolysis and transcription factor) comprised over 70% of all the significant genes (Fig. 4.6A-B). The resulting classifications were used as context for the subsequent analysis.

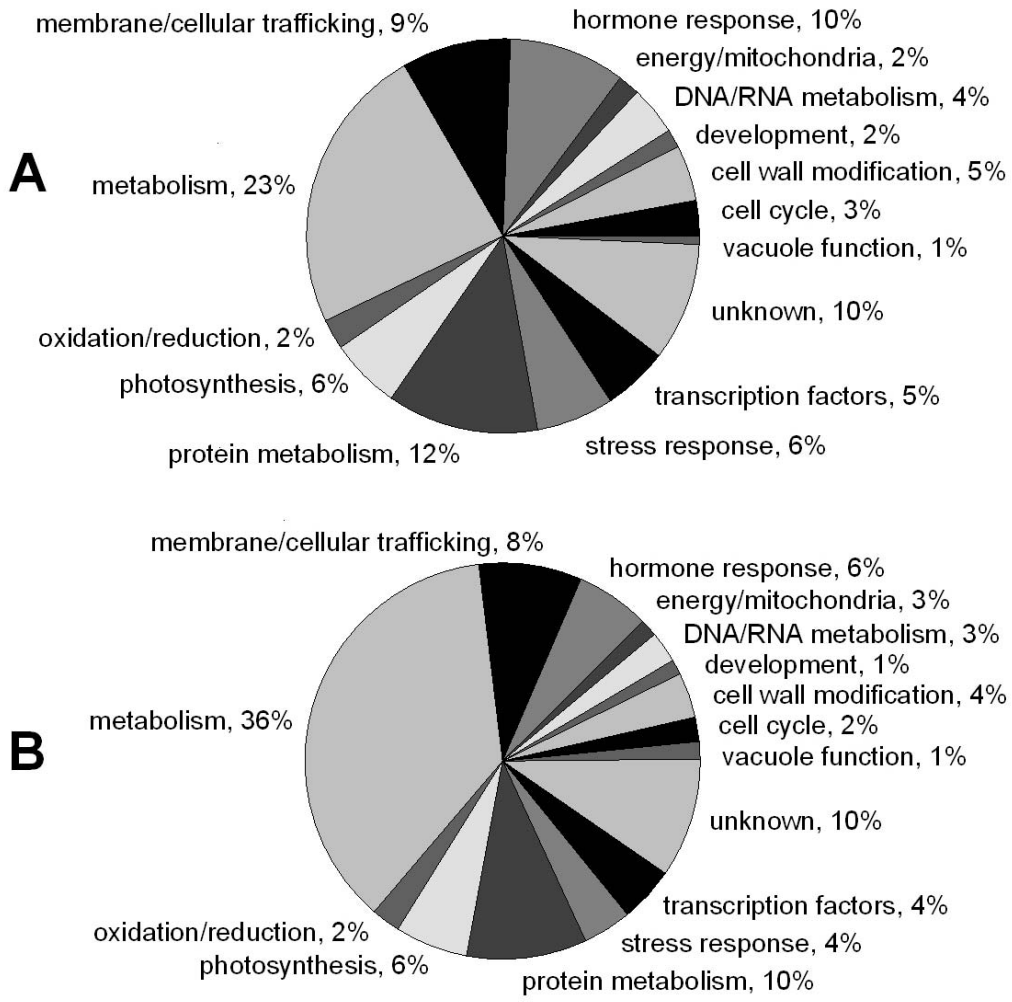


Figure 4.6. Distribution of statistically significant genes for NAA-treated (**A**) and shading-treated (**B**) FAZ. Categories are indicated near each pie slice.

Chloroplast-related genes

Many important metabolic activities take place within the chloroplast, including the energy transduction, the Calvin Cycle phases of photosynthesis and starch metabolism. In both the NAA- and shading-treated datasets, we found that over 90% of the genes related to chloroplast function were repressed. As expected, shading downregulated far more photosynthetic genes compared to NAA. These genes have known functions in light-harvest, oxygen evolvement, electron transport and carbon fixation. Shading-repressed genes were also involved with chlorophyll biosynthesis, chloroplast DNA binding, thylakoid formation and carbon utilization. However, only a small overlap was observed between the repressed genes from two datasets, indicating that NAA and shading may repress photosynthesis through partially distinct mechanisms.

Carbohydrate metabolism and sugar signaling

In this study, the largest set of genes in metabolism category is carbohydrate metabolism, followed by lipid/fatty acid metabolism and secondary metabolism. The carbohydrate metabolism category includes genes that are associated with glycolysis, the breakage of glycosidic bonds, sugar phosphorylation and signal transduction. Thirty-eight genes from the NAA dataset in this category showed altered expression patterns and 149 genes were regulated by shading, around 60% of which were upregulated and 40% downregulated. Among NAA-induced genes, many were involved with glycolysis and starch degradation such as pyruvate kinase, alcohol dehydrogenase, amylase and limit dextrinase. Comparatively, there was a consistent and widespread induction of genes associated with glycolysis and carbohydrate catabolism from the shading dataset, which included various classes of beta-glycosidase,

glycosyltransferase and hydrolase, but a group of alpha-glycosidase was consistently shown repressed. Sorbitol dehydrogenase (SDH) was repressed by both NAA and shading, but other genes related to sucrose metabolism, including sucrose phosphate synthase (SPS), sucrose phosphate phosphatase (SPP) and NADP-dependent D-sorbitol-6-phosphate dehydrogenase (S6PDH) were inversely regulated between the two datasets. Many ADP/UDP-glucose pyrophosphorylase genes that are responsible for starch synthesis were downregulated by shading, but none of them was found in the NAA dataset.

A class of genes that were shown differentially reguated from both datasets is likely to be involved in different sugar signaling pathways. This class includes carbohydrate kinase, SNF1-related protein kinase (SnRK) and two different classes of invertase (Rolland et al., 2006). Various carbohydrate kinases were altered by shading but only hexokinase was shown upregulated by NAA. Shading repressed fructokinase genes but induced galactokinase, hexokinase and pyrophosphate-dependent phosphofructokinase genes. Only one SNF1-related protein kinase gene was found in NAA dataset with an increased expression, but three SnRK genes were consistently shown downregulated by shading. Three putative alkaline/neutral invertase genes showed reduced expression after NAA treatment, which was consistent with the shading result. However, more genes identified as cytosolic and cell wall invertases were induced by shading, but none of those genes was found in the NAA dataset.

Another pathway showing downregulated expression in shading but not in the NAA dataset was that involved in trehalose metabolism. A group of genes encoding trehalose-6-phosphate

synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) were all repressed by shading but not by NAA.

Transport

Genes identified in this category included a large group of transporters for sugar, lipid, amino acid, metal ion, etc. Those genes both altered by NAA and shading associated mainly with sugar, peptide and lipid transport, and all exhibited similar patterns except for two oligopeptide transporter genes. The expression of all sorbitol/sucrose transporter genes was consistently repressed in both datasets, and shading downregulated more genes related to general sugar transport, such as hexose transporters. A class of genes related to membrane and cytoskeleton function, including microtubule, vesicle-mediated membrane transporter and cell adhesion genes, were found exclusively repressed by NAA. In all, twice as many transport-related genes were identified from shading than NAA dataset, among which several metal ion transporters, especially for calcium and potassium, were significantly upregulated by shading while others for water transport were downregulated. Another group of transporters, ATP-binding cassette transporters (ABC transporters), were shown largely induced by both treatments.

Cell cycle-related genes

Another group of genes being differentially expressed were those involved with cell cycle. Approximately the same number of genes was identified from two datasets, including the two classes of regulatory genes, cyclin and cyclin-dependent kinase (CDK), showing largely repressed by NAA and shading. Several cell division control proteins were also downregulated while one CDK inhibitor was upregulated.

Hormone synthesis and signaling

Many genes involved in different hormone synthesis and signaling pathways showed significant expression changes in both datasets. ABA has been implicated in regulating stress-induced senescence (Yang et al., 2003; Weaver et al., 1998). In this study, NAA appeared to have limited effect on ABA-related genes in that it only upregulated three 9-cis-epoxycarotenoid dioxygenase (NCED) genes and a zeaxanthin epoxidase gene, which encode key enzymes in ABA biosynthesis. However, shading totally altered 26 genes and caused a more widespread induction of those genes involved with ABA biosynthesis, including NCED, short chain dehydrogenase/reductase (SDR) and abscisic aldehyde oxidase (AAO). A large group of genes related to ABA signaling, such as ABI1/2 protein phosphatase, was also collectively upregulated by shading.

Conversely, another difference in hormone response between the two datasets was found in auxin. Only two auxin-induced SAUR-like and two auxin transport genes showed significant changes in shading dataset. However, a group of 21 genes were altered by NAA and these genes included IAA-amido synthase, auxin-amidohydrolase, AUX/IAA proteins and various auxin response factors (ARF). Genes related to auxin polar transport were also identified from the NAA dataset. Auxin influx carriers were induced but efflux carriers largely repressed by NAA. This was different from the shading result, where the two identified auxin efflux carrier genes were both induced.

Relatively equal numbers of genes involved with ethylene biosynthesis and signaling pathway were identified from both datasets with a large overlap. Those genes include 1-

aminocyclopropane-1-carboxylate synthase (ACS) and oxidase (ACO) and two kinds of ethylene receptors (ERS and ETR). There were other ethylene response factors (ERF) and ethylene-responsive transcription factors all showing upregulation in expression. Coinciding with the increased ethylene biosynthesis, spermidine synthase, a key gene related to polyamine biosynthesis, was observed with consistently reduced expression in both datasets.

Genes involved with cytokinin and gibberellic acid (GA) signaling pathways were downregulated both by shading and by NAA. Also, shading increased the expression of a GA 2-oxidase gene which is responsible for GA catabolism. Gene expression related to another plant hormone, brassinosteroid, was shown altered by shading. A brassinosteroid oxidase gene was repressed while a BR-signaling kinase gene was induced on 3 d after shading, but neither was identified from NAA dataset.

Cell wall modification

This category included genes associated with cell wall biosynthesis, loosening and degradation. An overlap of 11 genes with altered expression by both treatments was observed and most genes showed the induction in expression on 3 and 5 d after treatment. Several cellulose synthase genes were repressed while other genes related to cell wall loosening and hydrolysis, including β-1,3-glucanase, polygalacturonase and expansin protein, were all induced.

Proteolysis and apoptosis

A number of genes involved in potential ubiquitylation pathways were upregulated. Genes identified from the NAA dataset encode F-box proteins and other members of the ubiquitin

ligase complex, including cullin protein and ubiquitin-conjugating enzyme. In comparison, shading caused a more widespread induction of genes responsible for protein ubiquitination and degradation. Shading also had more effect on a class of subunit proteins for 26S proteasome function. Another group of genes with common induced expression was those related to apoptotic pathway such as clp protease, cysteine protease and autophagy genes. Similar to the cell wall degrading genes, the induction of almost all genes identified in this category occurred on 3 and 5 d, indicating that certain kind of programmed cell death (PCD) became active at later stage of NAA- and shading-induced fruit abscission.

Expression of transcription factors

Transcription factors (TF) are involved in many important cellular functions and biological roles. A large class of transcription factors was identified with significant changes from the array data. Ten TFs were shown altered by both treatments in the same direction, including ERF/AP2 transcription factors, bZIP proteins, MADS-box and MYB domain proteins. Expectedly, many ERF/AP2 TFs displayed a synchronous induction with the biosynthesis and signaling of ethylene and ABA, indicating their roles in those hormone responses. Among those MADS-box TFs, one *JOINTLESS* gene, which is known to control abscission zone formation, was found upregulated in both datasets. Several NAC domain genes showed repressed expression while other NAM-like genes were induced from shading dataset. However, no NACs were present in the NAA dataset. Another difference was for WRKY TFs which were induced by NAA but repressed by shading, and no overlap was found.

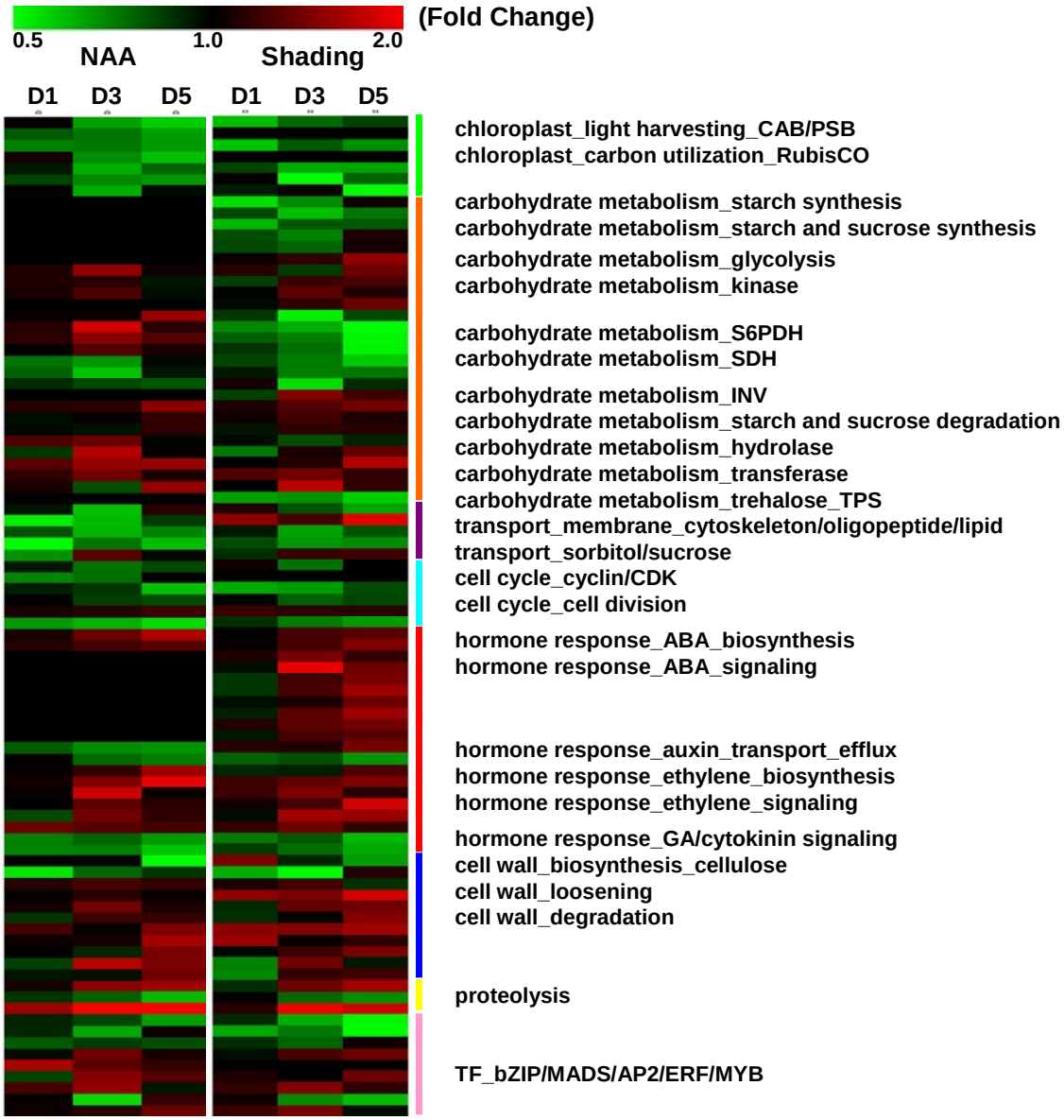


Figure 4.7. A comparative heat map shown for genes involved in different functional categories from the apple microarray data.

Genes selected from these eight categories of both datasets were shown in a comparative heat map (Fig. 4.7). Many of the photosynthetic genes were repressed early during the induction of young fruit abscission, which was common in both datasets. Most genes related to carbohydrate metabolism and metabolite transport appeared to be differentially altered following the

photoinhibition. Cell division was largely inhibited as many cell cycle-related genes were repressed, concurrent with halted fruit growth. Genes associated with hormone response were altered, where some genes related to auxin transport appeared to be first affected while others involved with ethylene and ABA biosynthesis and signaling pathways were upregulated later. Also induced later were those genes related to cell wall degradation and proteolysis. Certain groups of transcription factors exhibited differential expression patterns during the abscission induction, indicating that they may play different roles in the regulation of this process.

Validation of array data via qPCR

Based on Fig. 4.7, subsets of genes from these categories were selected for further validation by real-time quantitative PCR. cDNA derived from three additional time points (D0, D7 and D9 after treatment) and two other tissue types (leaf and fruit cortex) were included to expand the expression pattern data and query tissue-specific expression of these genes (Fig. 4.8-9). The relative expression levels were converted to fold change relative to value obtained from the array data for reference control samples to enable direct comparison to the qPCR results and plotted on a linear time scale. Upon the release of the apple genome (Velasco et al., 2010), we checked to see if our qPCR primers were specific to a single gene or could potentially amplify multiple gene family members using BlastN searches. Generally, the array data from the FAZ samples were consistent with qPCR results in terms of the overall expression pattern but variations were also observed (Fig. 4.10-13). One explanation of such variations could be that many of these genes are members of a gene family, so the array data were likely to represent the collective signal of more than one gene.

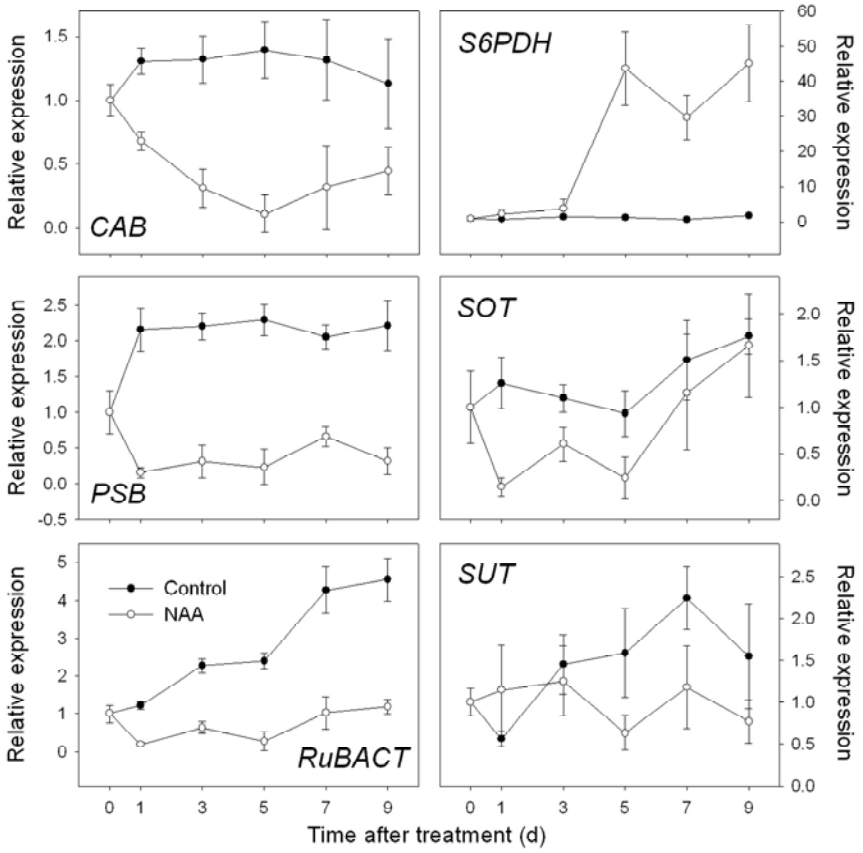


Figure 4.8. Real-time quantitative PCR analysis of the expression of genes related to photosynthesis and sugar metabolism in leaf from 'Golden Delicious' apple trees after application of NAA. The transcript levels were normalized using actin. Data are means ± SE (n = 3). The values of transcript levels in the leaf from control trees were arbitrarily set to 1.

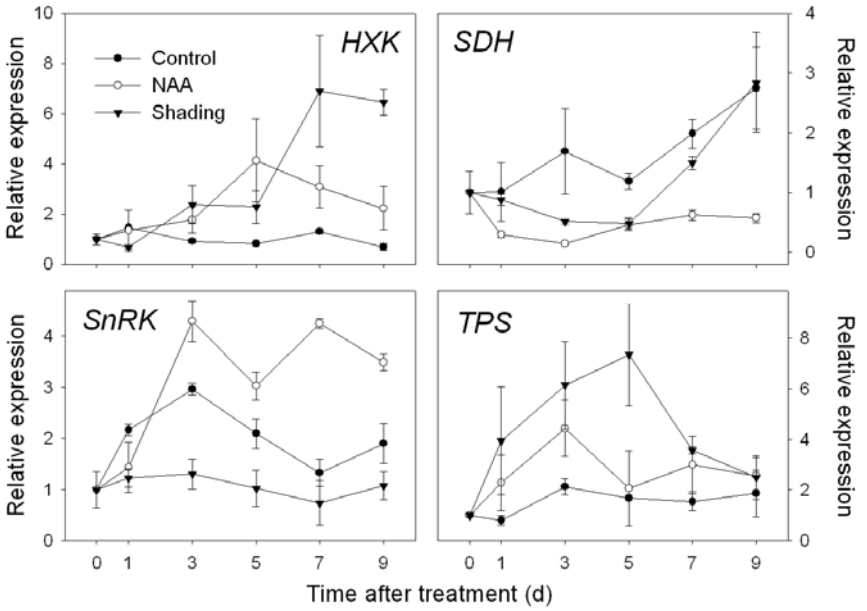


Figure 4.9. Real-time quantitative PCR analysis of the expression of genes related to sugar metabolism and signaling in fruit cortex (FC) from 'Golden Delicious' apple trees after application of NAA and shading. The transcript levels were normalized using actin. Data are means ± SE (n = 3). The values of transcript levels in the FC from control trees were arbitrarily set to 1.

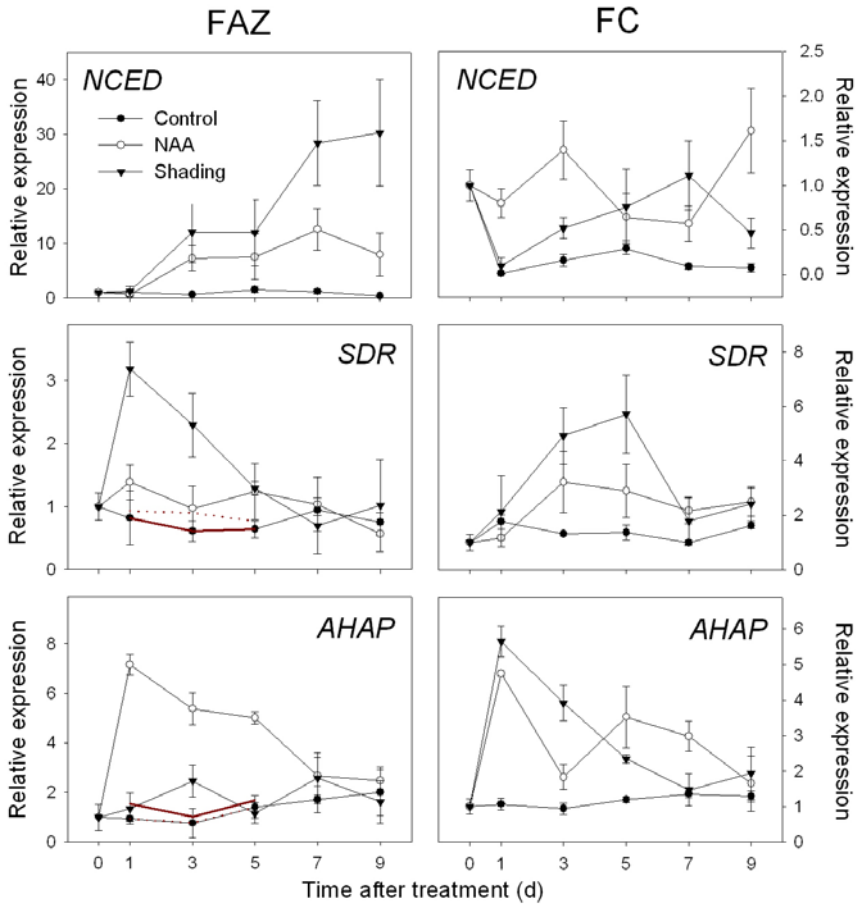


Figure 4.10. Real-time quantitative PCR analysis of the expression of genes related to ABA biosynthesis and signaling in fruit abscission zone (FAZ) and fruit cortex (FC) from 'Golden Delicious' apple trees after application of NAA and shading. The transcript levels were normalized using actin. Data are means ± SE (n = 3). The values of transcript levels in the FAZ and FC from control trees were arbitrarily set to 1. Red lines indicate normalized microarray values (Solid for NAA and dot for shading).

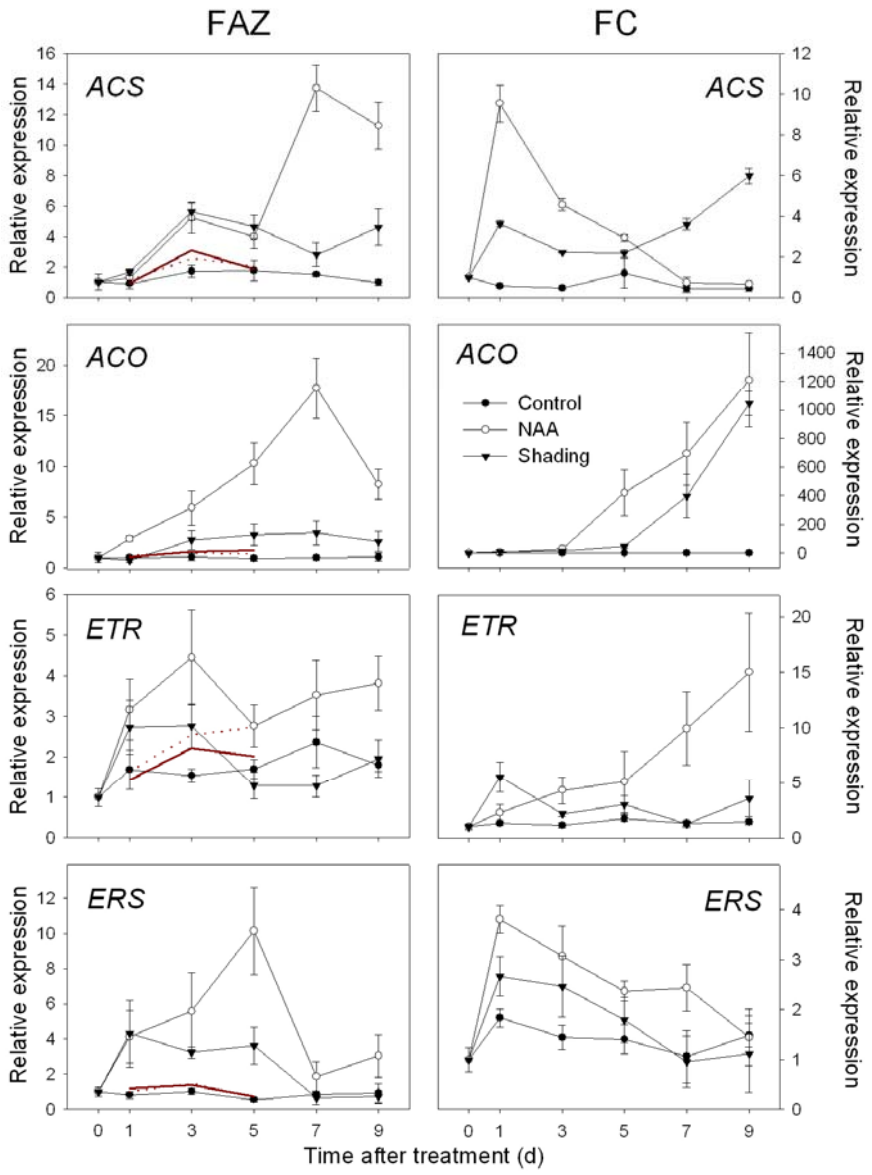


Figure 4.11. Real-time quantitative PCR analysis of the expression of genes related to ethylene biosynthesis and signaling in fruit abscission zone (FAZ) and fruit cortex (FC) from 'Golden Delicious' apple trees after application of NAA and shading. The transcript levels were normalized using actin. Data are means ± SE (n = 3). The values of transcript levels in the FAZ and FC from control trees were arbitrarily set to 1. Red lines indicate normalized microarray values (Solid for NAA and dot for shading).

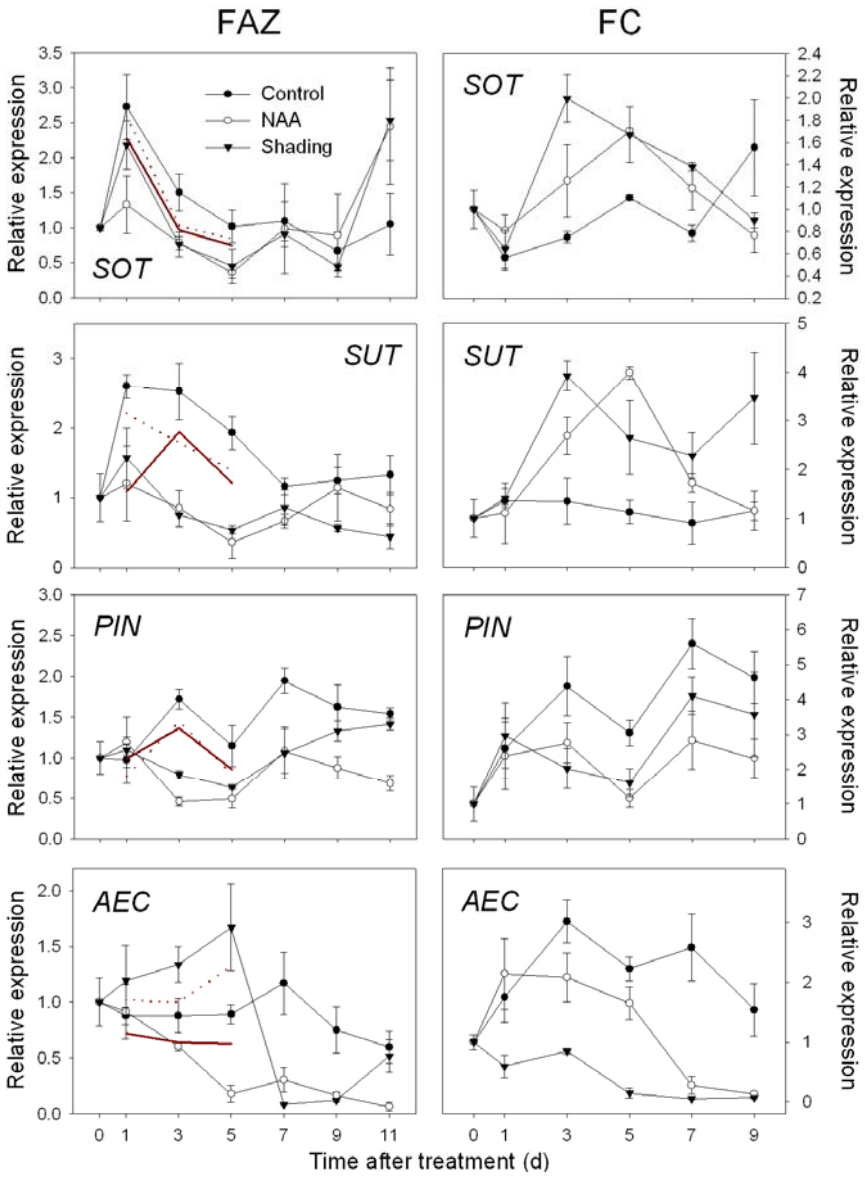


Figure 4.12. Real-time quantitative PCR analysis of the expression of genes related to sugar transport and polar auxin transport in fruit abscission zone (FAZ) and fruit cortex (FC) from 'Golden Delicious' apple trees after application of NAA and shading. The transcript levels were normalized using actin. Data are means ± SE (n = 3). The values of transcript levels in the FAZ and FC from control trees were arbitrarily set to 1. Red lines indicate normalized microarray values (Solid for NAA and dot for shading).

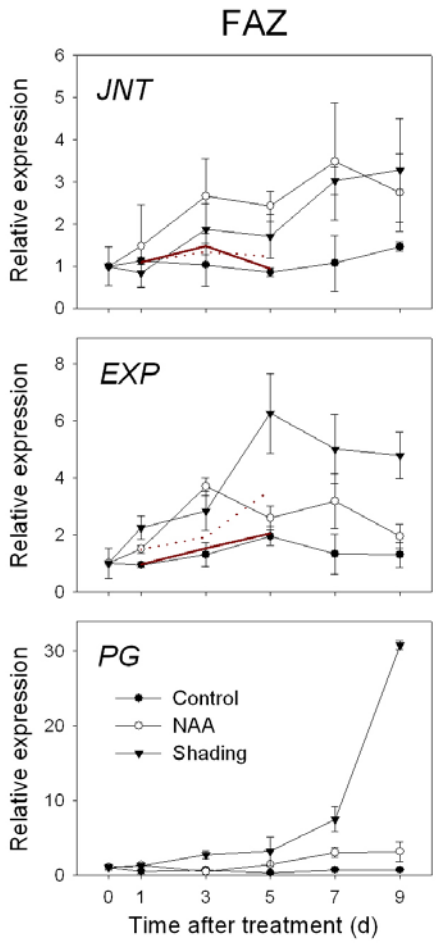


Figure 4.13. Real-time quantitative PCR analysis of the expression of genes related to abscission zone formation and cell wall degradation in fruit abscission zone (FAZ) from 'Golden Delicious' apple trees after application of NAA and shading. The transcript levels were normalized using actin. Data are means ± SE (n = 3). The values of transcript levels in the FAZ from control trees were arbitrarily set to 1. Red lines indicate normalized microarray values (Solid for NAA and dot for shading).

Discussion

The effects of various chemical thinners and the mechanism of fruitlet abscission in apple have been studied for years. Overall, it is a very complex process that responds to many factors (Bangerth, 2000; Dennis, 2000). Genomic studies and transcriptional profile analyses have been reported regarding abscission induced by various factors on model plants (Buchanan-Wollaston

et al., 2005; Wagstaff C et al., 2009) and apple (Lee et al., 2007; Janssen BJ et al., 2008). Those studies collectively show that carbohydrate availability is critical in abscission and various plant hormone signals are involved as either elicitors or concurrent events. Some research groups have exploited dark-induced fruit abscission to characterize many altered metabolic pathways and gene expression during this senescence process (Gomez-Cadenas et al., 2000; Zhou et al., 2008). Although the ability of NAA to interfere with those aspects provides opportunities to decipher the physiological and molecular events associated with its induction of fruit abscission, limited information is available on the global gene expression profiling responding to NAA. Comparing the gene expression profiles of young fruit abscission caused by NAA and shading, we established some general correlations between patterns of gene expression and abscission induction. Although these correlations are circumstantial and not necessarily causative, they overall reveal some possible associations.

The number of genes altered by these two treatments appeared to be consistent with the severity of the ultimate thinning responses. Gene expression data largely reflected such a difference where shading, which induced more fruitlet drop, altered more genes (1057) compared to NAA (722). On the other hand, the observed overlap implied a number of similarities in the tree responses to each treatment. Since the microarray experiments conducted here were limited, (i.e., performed only at three time points using mixtures of fruitlets with different abscission potentials), the observed correlation between numbers of expression changes and final fruitlet drop was likely due to the overall status of the treated trees. Many profound expression changes that occur in specific tissue types or during specific induction stages were likely diluted by the mixed fruitlets of different abscission potentials, so not observed.

Repression of chloroplast-related genes

Chlorosis is one the most common symptoms often associated with reduced photosynthetic capacity (Mathews, 1991). Darkness can inhibit photosynthesis and result in chlorosis and abscission of leaves and fruitlets, which was confirmed in our shading-treated trees. NAA in this study also caused observable chlorosis in leaves and the overall PS II efficiency was largely decreased, which agreed with Untiedt et al. (2001), where they tested various fruit thinning agents and found that NAA consistently reduced the whole tree canopy photosynthesis but increased dark respiration after application. Consistent with the chlorosis, the repression of a group of chloroplast-related genes was observed for NAA-treated leaves (Fig. 4.8), but it was not clear in which cell types such gene repression takes place and whether the repression by NAA is achieved through the same or different mechanisms as the virus-induced repression. Repression of certain plastid-associated genes by viruses has be hypothesized to occur as a consequence of impaired carbon partitioning and increased carbohydrate accumulation in source tissues that leads to subsequent repression of photosynthetic machinery (Herbers et al., 1997). Carbohydrate accumulation was accompanied by reduced levels of Rubisco protein. In our study, a fast and sustained repression was observed for those genes involved with light-harvest, oxygen evolving enhancement and Rubisco activation. Meanwhile, the transporters for both sorbitol and sucrose were found largely repressed in leaves, which might also account for the accumulated carbohydrate level in the source tissue. It remained unclear whether this widespread repression of photosynthetic genes could have resulted directly from NAA-induced photoinhibition or indirectly from impaired carbon allocation. However, the negative effect of NAA on leaf photosynthesis and PS II activity clearly implicated NAA in causing a carbohydrate stress for the sink tissue, the fruitlets.

Impacts on carbon partitioning and fruit growth

It has been reported that dark-induced fruit abscission can be reversed with trunk injection of sorbitol, the primary translocated form of carbohydrate in apple (Loescher et al., 1982), which supports the currently accepted hypothesis that a limitation of assimilate supply at least partly reduces fruit growth and induces fruit drop. However, whether NAA causes a similar carbohydrate shortage through interfering with certain sugar metabolic pathways remains unclear. In this study, we compared NAA- and shading-treated samples in the expression pattern of genes involved with carbohydrate metabolism and found not only differences but also similarities, likely as a consequence of sugar starvation in both cases. Based on the array data, we selected several representative genes and investigated their expression patterns in the FC since young fruit is the most important site for carbohydrate metabolism (Fig. 4.9). Plant hexokinase (HXK) has been implicated in sugar signaling and the regulation of senescence. As a kinase and glucose sensor, HXK has been demonstrated to have dual functions in both glucose metabolism and signaling (Jang et al., 1997; Moore et al., 2003). In this study, we found that a HXK gene in the FC was gradually induced after shading treatment. For NAA, the HXK gene expression increased, peaked on 5 d and remained higher than the control level thereafter. These results suggested a HXK-dependent sugar signaling pathway might be active during the abscission induction. It has been reported that transgenic tomato plants that overexpress *Arabidopsis* HEXOKINASE1 showed inhibited growth and rapid senescence (Dai et al., 1999). Contrarily, Moore et al. (2003) found *Arabidopsis glucose insensitive2* (*gin2*) mutant plants displayed a delayed senescence phenotype. Therefore, the elevated expression level of HXK gene observed in this study could be partly responsible for the inhibited fruit growth and accelerated fruit abscission.

SnRK1 (sucrose non-fermenting-1-related protein kinase 1) plays a key role in metabolic signaling and carbon partitioning, and SnRK1 genes have been identified in many plant species (Halford and Hardie, 1998; Halford et al., 2003). However, the exact nature of the signal that brings about changes in SnRK1 gene expression remains unknown. We found that in the FC, the expression of one SnRK1 gene was largely inhibited by shading while enhanced by NAA from 3 d after treatment. It has been hypothesized that SnRK1 is activated in response to high intracellular sucrose and/or low glucose levels (Halford et al., 2003). From our shading array data, a group of sucrose synthase (SuSy) was repressed, suggesting a reduced level of sucrose, which might in turn result in the reduction in SnRK1 expression. But sucrose might also increase if SuSy as catabolic enzyme decreases. No SuSy genes were identified from NAA array data. On the other hand, Sucrose phosphate synthase (SPS) has been proved to be inactivated by SnRK1 (Su et al., 1996). SPS genes in this study were shown inversely regulated between shading and NAA datasets, which corresponded with their differences in the expression of SnRK1 gene. These contrasting expression patterns reflect the complexity of sugar signaling under the regulation of different stress stimuli, such as shading or NAA treatment. Shading and NAA caused similar induction of fruit abscission, but they were likely to have diverse target genes through different sugar signaling mechanisms. It has been pointed out that ABA, protein kinases and transcription factors are at the interface between metabolic and stress signaling systems (Hey et al., 2010), however, more signaling components need to be identified and integrated into the network.

Sorbitol comprises over 80% of the carbohydrate translocated in the phloem of apple, and thus is the main carbon resource imported by fruit sinks (Loescher et al., 1982). Sorbitol dehydrogenase

(SDH) has been identified as the key enzyme in sorbitol metabolism, converting sorbitol into fructose (Yamaguchi et al., 1996; Beruter et al., 1997). In this study, the expression of SDH gene gradually increased in the control fruit, indicating its role in regulating early fruit development. However, NAA and shading both repressed the expression of SDH in the FC, suggesting that the carbohydrate accumulation was largely inhibited and fruit sink strength was impaired. It has been proposed that the ability of a fruit to persist and grow while competing with other sinks may significantly depend on the ability to utilize the carbohydrates efficiently (Ho, 1988). Therefore, the inhibition of carbon accumulation in the fruitlets caused by NAA might result in their abscission. Another important sorbitol-metabolizing enzyme is sorbitol-6-phosphate dehydrogenase (S6PDH), which synthesizes sorbitol in leaves for the translocation of photosynthates (Sakanishi et al., 1998). Previous studies have reported a relation between an increase in soluble carbohydrates and stress tolerance in some Rosaceae fruit trees (Brown et al., 1985; Lo Bianco et al., 2000), and S6PDH has been revealed as an ABA-inducible gene (Kanayama et al., 2007). We in this study found a strong induction of S6PDH expression in NAA-treated leaves (Fig. 4.8), and that an induced expression of genes related to ABA biosynthesis and signaling preceded the increased S6PDH expression (Fig. 4.10), indicating a likely ABA-mediated stress response.

Trehalose-6-phosphate (T-6-P) has been implicated in the regulation of sugar metabolism (Goddijn and Smeekens, 1998; Goddijn and van Dun, 1999). However, much remains to be ascertained about the physiological role of trehalose synthesis and the identification of cellular targets of T-6-P action. Buchanan-Wollaston et al. (2005) found that both trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) genes were upregulated in dark-

induced senescence in *Arabidopsis*. In contrast, our array data showed a consistent downregulation of both TPS and TPP genes in the FAZ treated by shading and none was identified from the NAA dataset. Such contrasting results were probably due to the tissue-specific gene expression since when we tested the FC, both NAA and shading were shown to cause an early induction of TPS gene. Correspondingly, the increase in the expression of HXK gene occurred later, when the expression of TPS gene decreased, which is consistent with previous reports that TPS and T-6-P are both required for the inhibition of HXK in yeast (Bonini et al., 2000; Noubhani et al., 2000).

Transport regulation

As in leaves, both sorbitol and sucrose transporter genes were consistently repressed in the FAZ by NAA from 1 to 5 d after treatment, which confirmed the array data, indicating that carbon allocation to the fruitlets was significantly hampered (Fig. 4.12). In contrast, the expression of these transporters in fruit cortex was increased from 3 d on after NAA treatment. The array data also showed that many transporter genes were induced by NAA, including a number of ABC transporters and several cation transporters, many of which are associated with metal transport of H^+ exchange. This result was consistent with the finding of Buchanan-Wollaston et al. (2005). Also induced in this study were several nitrate and sulfate transporter genes, implicating their roles in mobilization of N and S from nucleic acid and amino acid degradation. The upregulation of these transporters, especially those involved in sugars, might reflect the shifting function of abscising fruitlets, as they become a source tissue for the mobilization of nutrients to non-abscising fruitlets.

Regulation of hormone pathway genes

Comparative analysis of genes expressed in different hormone signaling pathways allowed us to see their relevance to fruit abscission caused by NAA and shading. ABA has been implicated in the regulation of stress-induced senescence (Yang et al., 2003). From our array data, a more widespread induction of genes involved with ABA biosynthesis and signaling was observed for shading-treated than NAA-treated FAZ. qPCR results confirmed that besides NCED, a SDR family protein and a transcription factor AHAP for the regulation of ABA signaling in the FAZ were induced from 1 to 5 d by shading and NAA, respectively. In the FC the expression of those genes was consistently increased by both treatments, especially on 3 and 5 d after treatment (Fig. 4.10). Those differences indicated that the ABA signaling pathway might be more active in dark-induced than in NAA-induced fruit abscission. Likely as mentioned before, more genes associated with sugar metabolism and signaling were altered by shading than NAA, confirming a close crosstalk between sugar and ABA during the abscission induction.

Ethylene has been regarded as one of the key signals for the coordination of abscission, but is not strictly necessary for abscission (Dal Cin et al., 2005, 2009). In this study, ethylene production increased and peaked in fruitlets and leaves treated by NAA and shading, prior to the onset of fruitlet drop. Also, a common upregulation of genes encoding ethylene biosynthesis and signaling components was found in NAA- and shading-treated FAZ and FC (Fig. 4.11). These results were consistent with previous report that apple fruitlet abscission is preceded by an increase in ethylene biosynthesis and sensitivity (Dal Cin et al., 2005). On the other hand, another research we conducted suggests that apple fruitlet abscission does not merely depend on

ethylene signaling pathway and there should be a very complex interplay of events that controls this process (Zhu et al., 2010).

It has been hypothesized that prevention of abscission requires a constant auxin transport through the abscission zone from the fruit (Taylor and Whitelaw, 2001). Auxin export is mediated by PIN-formed (PIN) proteins and ATP-activated phosphoglycoproteins (PGPs) (Petrasek J et al., 2006; Blakeslee JJ et al., 2007). In this study, a PIN-like auxin transporter gene and an auxin efflux carrier (AEC) gene both showed consistently decreased expression from 3 d in NAA-treated FAZ and FC, indicating that the auxin efflux from the fruitlets was hampered (Fig. 4.12). It has been proposed that the sink strength of organs is related to their ability in producing and exporting auxin (Cline MG, 1991). Therefore, the reduced auxin export from the fruitlets and through the FAZ might serve as a signal of abscission.

Induction of genes associated with AZ formation and cell wall degradation

The cells comprising the abscission zone (AZ) are often morphologically distinguishable before the onset of abscission (Sexton and Roberts, 1982) and abscission could not be induced until those cells are formed (Osborne et al., 1976). *JOINTLESS* (JNT) gene has been identified as a MADS-box gene that plays a key role in controlling abscission zone development (Mao et al., 2000). In this study, the JNT gene expression in the FAZ was increased by both NAA and shading from 3 d after treatment and remained higher than the control, suggesting that the abscission zone formation was largely induced by both treatments.

Abscission involves wall breakdown and several reports have shown that expansins are expressed abundantly in abscission zones (Chen and Bradford, 2000; Belfield et al., 2005). In this study, we observed an increase of expasin (EXP) gene expression in both NAA- and shading-treated FAZ as early as 1 d after treatment, concurring with the burst of fruit ethylene production. This result suggested that EXP gene was associated with cell wall degradation and supported a previous report that certain type of AZ cells enlarge in response to ethylene (Osborne et al., 1976).

Some reports have indicated that an increase in polygalacturonase (PG) activity correlates with fruit abscission (Tonutti et al., 1995; Bonghi et al., 2000). However, *PG1* expression was not detected in the FAZ or the FC from 'Golden Delicious' and 'Delicious' apples (Zhu et al., 2008), which contrasts with other work showing that *PG1* was involved in apple fruit softening and that its expression was suppressed by 1-MCP and AVG treatment (Wakasa et al., 2006; Li and Yuan, 2008). In this study, the expression of *PG2* in the FAZ was induced by NAA and shading on 5 d, occurring before the increase in the rate of fruitlet abscission (Fig. 4.13). This result was in agreement with our previous work with NAA in 'Delicious' apple (Zhu et al., 2008), and another research showing *PG2* downregulation concomitant with an NAA-dependent reduction in preharvest fruit drop (Li and Yuan, 2008). So, based on current evidences, *PG2* appeared to be the gene most strongly associated with fruitlet abscission.

References

Applied Biosystems. 2005. Guide to performing relative quantitation of gene expression using real-time quantitative PCR. Applied Biosystems, Foster City, CA.

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25: 25-29.

Bangerth F. 2000. Abscission and thinning of young fruit and their regulation by plant hormones and bioregulators. Plant Growth Regul. 31: 43-59.

Belfield EJ, Ruperti B, Roberts JA, McQueen-Mason S. 2005. Changes in expansin activity and gene expression during ethylene-promoted leaflet abscission in *Sambucus nigra*. J. Expt. Bot. 56: 817-823.

Beruter J, Feusi MES, Ruedi P. 1997. Sorbitol and sucrose partitioning in the growing apple fruit. J. Plant Physiol. 151: 269-276.

Blakeslee JJ, Bandyopadhyay A, Lee OR, Mravec J, Titapiwatanakun B, Sauer M, Makam SN, Cheng Y, Bouchard R, Adamec J, Geisler M, Nagashima A, Sakai T, Martinoia E, Friml J, Peer WA, Murphy AS. 2007. Interaction among PIN-FORMED and P-glycoprotein auxin transporters in Arabidopsis. Plant Cell 19: 131-147.

Bonghi C, Tonutti P, Ramina A. 2000. Biochemical and molecular aspects of fruitlet abscission. Plant Growth Regul. 31: 35-42.

Bonini BM, Van Vaeck C, Larsson C, Gustafsson L, Ma P, Winderickx J, Van Dijck P, Thevelein JM. 2000. Expression of Escherichia coli otsA in a Saccharomyces cerevisiae tps1 mutant restores trehalose 6-phosphate levels and partly restores growth and fermentation with glucose and control of glucose influx into glycolysis. Biochem. J. 350: 261-268.

Brown CS, Young E, Pharr DM. 1985. Rootstock and scion effects on the seasonal distribution of dry weight and carbohydrates in young apple trees. J. Amer. Soc. Hort. Sci. 110: 696-701.

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signaling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J. 42: 567-585.

Byers RE. 2003. Flower and fruit thinning and vegetative: Fruiting balance. In: D.C. Ferree and I.J. Warrington (eds.). Apples: Botany, production and uses. CABI Publ., Cambridge, MA, USA, pp. 409-436.

Chen F, Bradford KJ. 2000. Expression of an expansin is associated with endosperm weakening during tomato seed germination. Plant Physiol. 124: 1265-1274.

Cline MG. 1991. Apical dominace. Bot. Rev. 57: 318-358.

Curry, E.A. 1991. NAA-induced ethylene and ACC in 'Delicious' spur tissues: Changes with temperature and time. J. Amer. Soc. Hort. Sci. 116:846-850.

Dai N, Schaffer A, Petreikov M, Shahak Y, Giller Y, Ratner K, Levine A, Granot D. 1999. Overexpression of Arabidopsis hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11: 1253-1266.

Dan Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A. 2005. Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus x domestica* L. Borkh). J. Expt. Bot. 56: 2995-3005.

Dan Cin V, Velasco R, Ramina A. 2009. Dominace induction of fruitlet shedding in *Malus x domestica* (L.Borkh): molecular changes associated with polar auxin transport. BMC Plant Biol. 9: 139.

Dennis FG. 2000. The history of fruit thinning. Plant Growth Regul. 31: 1-16.

Dennis FG. 2002. Mechanisms of action of apple thinning chemicals. HortScience 37: 471-474.

Gasic K, Gonzalez DO, Thimmapuram J, Liu L, Malnoy M, Gong G, Han Y, Vodkin LO, Aldwinckle HS, Carroll NJ, Orvis KS, Goldsbrough P, Clifton S, Pape D, Fulton L, Martin J, Theising B, Wisniewski ME, Fazio G, Feltus FA, Korban SS. 2009. Comparative analysis and functional annotation of a large expressed sequence tag collection of apple. Plant Genome 2: 23-38.

Goddijn O, Smeekens S. 1998. Sensing trehalose biosynthesis in plants. Plant J. 14: 143-146.

Goddijn OJM, van Dun K. 1999. Trehalose metabolism in plants. Trends in Plant Sci. 4: 315-319.

Gómez-Cadenas A, Mehouachi J, Tadeo FR, Primo-Millo E, Talon M. 2000. Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. Planta 210: 636-643.

Halford NG, Hardie DG. 1998. SNF1-related protein kinases: global regulators of carbon metabolism in plants. Plant Mol. Biol. 37: 735-748.

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Paul M, Zhang Y. 2003. Metabolic signaling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. J. Expt. Bot. 54: 467-475.

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Zhang Y, Paul M. 2003. Dissection and manipulation of metabolic signalling pathways. Ann. Appl. Biol. 142: 25-31.

Hennerty MJ, Forshey CG. 1972. Tree physiological condition as a source of variation in chemical thinning of apple fruits. HortScience 7: 259-260.

Herbers K, Tacke E, Hazirezaei M, Krause KP, Melzer M, Rohde W, Sonnewald, U. 1997. Expression of a luteoviral movement protein in transgenic plants leads to carbohydrate accumulation and reduced photosynthetic capacity in source leaves. Plant J. 12: 1045-1056.

Ho LC. 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. Annu. Rev. Plant Physiol. 39: 355-378.

Jang JC, Leon P, Zhou L, Sheen J. 1997. Hexokinase as a sugar sensor in higher plants. Plant Cell 9: 5-19.

Janssen BJ, Thodey K, Schaffer RJ, Alba R, Balakrishnan L, Bishop R, Bowen JH, Crowhurst RN, Gleave AP, Ledger S, McArtney S, Pichler FB, Snowden KC, Ward S. 2008. Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. BMC Plant Biol. 8: 16.

Kanayama Y, Moriguchi R, Deguchi M, Kanahama K, Yamaki S. 2007. Effect of environmental stresses and abscisic acid on sorbitol-6-phosphate dehydrogenase expression in Rosaceae fruit trees. Proc. IS on Biotechnol. Temp. Fruit Crops and Trop. Species. Acta Hort. 738. ISHS.

Kim J, Patterson S. 2006. Expression divergence and functional redundancy of polygalacturonases in floral organ abscission. Plant Signaling Behav. 1: 281-283.

Lashbrook CC, Cai S. 2008. Cell wall remodeling in Arabidopsis stamen abscission zones. Plant Signaling Behav. 3: 733-736.

Lashbrook CC, Gonzalez-Bosch C, Bennett AB. 1994. Two divergent endo-β-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers. Plant Cell 6: 1485-1493.

Lee YP, Yu GH, Seo YS, Han SE, Choi YO, Kim D, Mok IG, Kim WT, Sung SK. 2007. Microarray analysis of apple gene expression engaged in early fruit development. Cell Biol. Morphogenesis. 26: 917-926.

Li J, Yuan R. 2008. NAA and ethylene regulate expression of genes related to ethylene biosynthesis, perception and cell wall degradation during fruit abscission and ripening in 'Delicious' apples. J. Plant Growth Regul. 27: 283-295.

Lo Bianco R, Riegera M, Sung SS. 2000. Effect of drought on sorbitol and sucrose metabolism in sinks and sources of peach. Physiol. Plant. 108: 71-78.

Loescher W, Marlow GC, Kennedy RA. 1982. Sorbitol metabolism and source-sink interactions in developing apple leaves. Plant Physiol. 70: 335-339.

Mao L, Begum D, Chuang HW, Budiman MA, Szymkowiak EJ, Irish EE, Wing RA. 2000. *JOINTLESS* is a MADS-box gene controlling tomato flower abscission zone development. Nature 406: 910-913.

Mathews, REF. 1991. Plant virology, 3rd ed. Hartcourt Brace, Javonovich, San Diego, CA, U.S.A.

McArtney SJ. 2002. Ethylene evolution from detached apple spurs in response to chemical thinners. HortScience 37: 662-665.

Meir S, Philosoph-Hadas S, Sundaresan KSS, Selvaraj V, Burd S, Ophir R, Kochanek B, Reid MS, Jiang CZ, Lers A. 2010. Microarray analysis of the abscission-related transcriptome in tomato flower abscission zone in response to auxin depletion. Plant Physiol. 154: 1929-1956.

Moore B, Zhou L, Rolland F, Hall Q, Cheng WH, Liu YX, Hwang I, Jones T, Sheen J. 2003. Role of the Arabidopsis glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science 300: 332-336.

Noubhani A, Bunoust O, Rigoulet M, Thevelein JM. 2000. Reconstitution of ethanolic fermentation in permeabilized spheroplasts ofwildtype and trehalose-6-phosphate synthase mutants of the yeast Saccharomyces cerevisiae. Eur. J. Biochem. 267: 4566-4576.

Osborne DJ. 1976. Auxin and ethylene and the control of cell growth. Identification of three classes of target cells. In: Pilet P, ed. Plant growth regulation. Berlin, Germany: Springer-Verlag, 161-171.

Petrasek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertova D, Wisniewska J, Tadele Z, Kubes M, Covanova M, Dhonukshe P, Skupa P, Benkova E, Perry L, Krecek P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zazimalova E, Friml J. 2006. PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312: 914-918.

Quilan JD, Preston AP. 1971. The influence of shoot competition on fruit retention and cropping of apple tress. J. Hort. Sci. 46: 525-534.

Rolland F. Baena-Gonzalez, Sheen J. 2006. Sugar sensing and signaling in plants: conserved and novel mechanisms. Annu. Rev. Plant. Biol. 57:675-709.

Sakanishi K, Kanayama Y, Mori H, Yamada K, Yamaki S. 1998. Expression of the gene for NADP-dependent sorbitol-6-phosphate dehydrogenase in peach leaves of various developmental stages. Plant Cell Physiol. 39: 1372-1374.

Schaffer, RJ. Friel EN, Souleyre EJF, Bolitho K, Thodey K, Ledger S, Bowen JH, Ma JH, Nain B, Cohen D, Gleave AP, Crowhurst RN, Janssen BJ, Yao JL, Newcomb RD. 2007. A genomics approach reveals that aroma production in apple is controlled by ethylene predominantly at the final step in each biosynthetic pathway. Plant Physiol. 144: 1899-1912.

Schneider GW. 1978. Abscission mechanism studies with apple fruitlets. J. Amer. Soc. Hort. Sci. 103: 455-458.

Sexton R, Roberts JA. 1982. Cell biology of abscission. Ann. Rev. Plant Physiol. 33: 133-162.

Stopar M, Black BL, Bukovac MJ. 1997. The effect of NAA and BA on carbon dioxide assimilation by shoot leaves of spur-type 'Delicious' and 'Empire' apple trees. J. Amer. Soc. Hort. Sci. 122: 837-840.

Su W, Huber SC, Crawford NM. 1996. Identification in vitro of a post-translational regulatory site in the hinge 1 region of Arabidopsis nitrate reductase. Plant Cell 8: 519-527.

Taylor JE, Whitelaw CA. 2001. Signal in abscission. New Phyto. 151: 323-339.

Tonutti P, Cass LG, Christoffersen RE. 1995. The expression of cellulase gene family members during induced avocado fruit abscission and ripening. Plant Cell Environ. 18: 709-713.

Untiedt R, Blanke M. Effects of fruit thinning agents on apple tree canopy photosynthesis and dark respiration. 2001. Plant Growth Regul. 35: 1-9.

Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M, Pruss D, Salvi S, Pindo M, Baldi P, Castelletti S, Cavaiuolo M, Coppola G, Costa F, Cova V, Ri AD, Goremykin V, Komjanc M, Longhi S, Magnago P, Malacarne G, Malnoy M, Micheletti D, Moretto M, Perazzolli M, Si-Ammour A, Vezzulli S, Zini E, Eldredge G, Fitzgerald LM, Gutin N, Lanchbury J, Macalma T, Mitchell JT, Reid J, Wardell B, Kodira C, Chen Z, Desany B, Niazi F, Palmer M, Koepke T, Jiwan D, Schaeffer S, Krishnan V, Wu C, Chu VT, King ST, Vick J, Tao Q, Mraz A, Stormo A, Stormo K, Bogden R, Ederle D, Stella A, Vecchietti A, Kater MM, Masiero S, Lasserre P, Lespinasse Y, Allan AC, Bus V, Chagné D, Crowhurst RN, Gleave AP, Lavezzo E, Fawcett JA, Proost S, Rouzé P, Sterck L, Toppo S, Lazzari B, Hellens RP, Durel C, Gutin A, Bumgarner RE, Gardiner SE, Skolnick M, Egholm M, Peer YVd, Salamini F, Viola R. 2010. The genome of the domesticated apple (*Malus x domestica* Borkh.). Nat. Genet. 42: 833-839.

Wagstaff C, Yang TJW, Stead AD, Buchanan-Wollaston V, Roberts JA. 2009. A molecular and structural characterization of senescing Arabidopsis siliques and comparison of transcriptional profiles with senescing petals and leaves. Plnat J. 57: 690-705.

Wakasa Y, Kudo H, Ishikawa R, Akada S, Senda M, Niizeki M, Harada T. 2006. Low expression of an endopolygalacturonase gene in apple fruit with long-term storage potential. Postharvest Biol. Technol. 39: 193-198.

Ward DL, Beers EP, Byers RE, Marini RP. 1999. Cutting apple fruits induces cellulase activity in the abscission zone. HortScience 34: 601-603.

Weaver LM, Gan S, Quirino B, Amasino RM. 1998. A comparison of the expression patterns of several senescence associated genes in response to stress and hormone treatment. Plant Mol. Biol. 37: 455-469.

Williams RR, Flook VA. 1980. The mode of action of the hormone apple fruit-setting mixture PP-341B applied to 'Cox's Orange Pippin'. J. Hort. Sci. 55:275-277.

Yamaguchi Y, Kanayama Y, Soejima J, Yamaki S. 1996. Changes in the amounts of the NAD+-dependent sorbitol dehydrogenase and its involvement in the development of apple fruit. J. Amer. Soc. Hort. Sci. 121: 848-852.

Yang JC, Zhang JH, Wang ZQ, Zhu QS, Liu LJ. 2003. Involvement of abscisic acid and cytokinins in the senescence and remobilisation of carbon reserves in wheat subjected to water stress during grain filling. Plant Cell Environ. 26: 1621-1631.

Yuan R. 2007. Effects of temperature on fruit thinning with ethephon in 'Golden Delicious' apples. Sci. Hort. 113: 8-12.

Yuan R, Greene DW. 2000a. Benzyladenine as a chemical fruit thinner for ‘McIntosh’ apples. I. Fruit thinning effects and associated relationships with photosynthesis, assimilate translocation, and nonstructural carbohydrates. J. Amer. Soc. Hort. Sci. 125: 169-176.

Yuan R, Greene DW. 2000b. Benzyladenine as a chemical thinner for 'McIntosh' apples. II. Effects of benzyladenine, bourse shoot tip removal, and leaf number on fruit retention. J. Amer. Soc. Hort. Sci. 125: 177-182

Zhou C, Lakso AN, Robison TL, Gan S. 2008. Isolation and characterization of genes associated with shade-induced apple abscission. Mol. Genet. Genomics 280: 83-92.

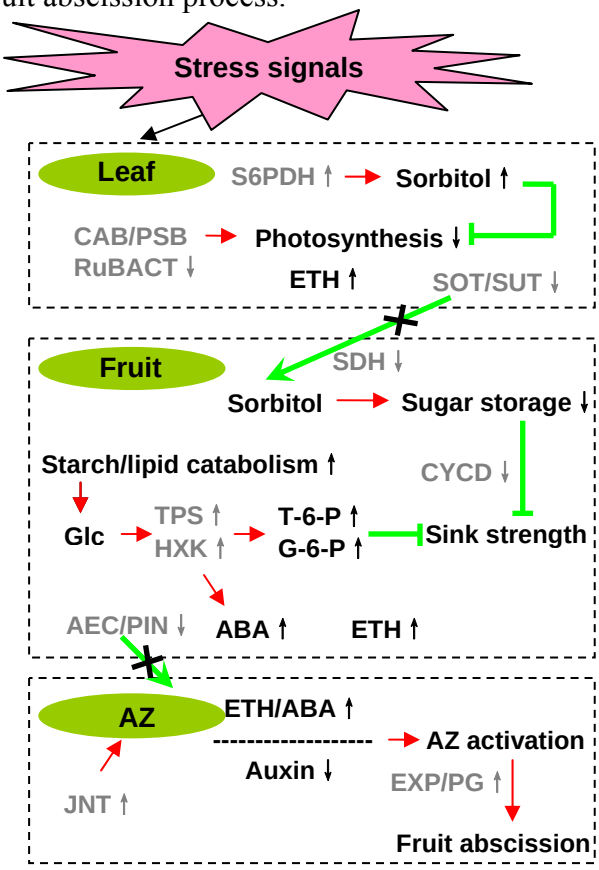
Zhu H, Beers EP, Yuan R. 2008. Aminoethoxyvinylglycine inhibits fruit abscission induced by naphthaleneacetic acid and associated relationships with expression of genes for ethylene biosynthesis, perception, and cell wall degradation in ‘Delicious’ apples. J. Amer. Soc. Hort. Sci. 133: 727-734.

Zhu H, Yuan R, Greene DW, Beers EP. 2010. Effects of 1-methylcyclopropene and napththaleneacetic acid on fruit set and expression of genes related to ethylene biosynthesis and perception and cell wall degradation in apple. J. Amer. Soc. Hort. Sci. 135: 402-409.

Chapter 5

Conclusions and Suggestion for Future Research

NAA, like most chemical thinners, is effective only when applied prior to the "June drop", when fruitlet abscission is occurring spontaneously, suggesting that NAA only stimulate the natural abscission. NAA-induced fruit drop is not accompanied by leaf abscission, indicating that the fruit and leaf abscission are likely to be regulated by different processes at least during the early period of fruit development. Apple trees provide a good model for studying fruit abscission, and we need to gain more insight into the details of the interactions, within various organs of the tree, between different hormones and metabolites that have been implicated in the early events of the fruit abscission process.



http://en.wikipedia.org/wiki/File:Koeh-108.jpg

Figure 5.1. Hypothetical model for young fruit abscission in apple.

The role of ethylene in the thinning action of NAA was confirmed by using AVG to inhibit ethylene production, but 1-MCP did not appear to be functional in blocking ethylene action in this study. Most genes involved with ethylene biosynthesis and perception were determined and found corresponded with the ethylene production in the fruitlet and fruit abscission pattern. Genes related to ABA biosynthesis and signaling also showed upregulation shortly after NAA and shading treatments, indicating a possible involvement of ABA in the early induction of fruit abscission. We identified certain genes related to FAZ formation and cell wall degradation, most being upregulated, as downstream events at later stage of fruit abscission.

The effect of NAA on the global gene expression in fruit abscission zone, in comparison with shading experiment, was investigated by microarray. Many more genes involved in different functional categories were identified and it was revealed that NAA and shading are likely to induce similar hormone signals, mainly ethylene and/or ABA, by first imposing photosynthesis inhibition and through the retrograde signaling pathway. Fruit growth was soon inhibited. Blocking nutrient transport to the fruit and/or diversion of nutrients from the fruit remain possibilities as consistent repression of genes related to carbohydrate translocation from leaves to fruitlet was observed, but more physiological and direct molecular evidence is needed.

Based on our FAZ array data, it appeared that the early induction of fruit abscission had less to do with sugar availability than with hormones. It makes sense that the trees have the ability to directly sense the capacity to produce energy via some kinds of hormone homeostasis which is coupled to photosynthesis. Having profiled the hormone-related responses of the FAZ to the application of NAA, it will be interesting to further identify the precise site at which the NAA-

induced signal is generated and transduced, through directly applying NAA to the FAZ. In addition, future studies will further focus on the signal transduction pathways as to be responsible for the early induction of fruit abscission and more downstream effectors will be investigated in specific type of tissues. Upon the release of the apple genome, it becomes more possible to specifically target certain key genes involved with the induction of apple fruit abscission, using RNA interference or virus-induced gene silencing (VIGS), to see if any modification of those genes would alter the abscission pattern in apple.

On the other hand, growing concerns for food safety have led to limitations on the chemical usage in apple production, so many of the currently used chemical thinners may be withdrawn from the market in the future. For fruit growers to remain competitive and fruit price to remain reasonable for consumers, new environmentally friendly, ideally naturally occurring chemicals need to be found as effective in thinning, and a thorough understanding of the mechanism of apple fruitlet abscission would facilitate the selection of potential chemicals or genetic strategies for apple fruit thinning programs.