ABSTRACT

Title of Dissertation: TRANSCRIPT PROFILING AS A METHOD TO STUDY FRUIT MATURATION, TREE-RIPENING, AND THE ROLE OF "TREE FACTOR" IN 'GALA' AND 'FUJI' APPLES

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'Gala' and 'Fuji' are two high-quality apple (*Malus domestica* Borkh) cultivars. Their fruits mature and tree-ripen over a long period of time, and are resistant to pre-harvest drop. "Tree factor," a putative inhibitor of system 2 ethylene production is hypothesized to account for differences in ethylene production between attached and detached apple fruits. Three years of field data revealed two distinct patterns of maturation and ripening behavior in these two cultivars. 'Gala,' an early cultivar, demonstrated a typical positive "tree factor." Studies of the ripening pattern of 'Fuji' apple, which is a late-maturing cultivar, did not. 'Fuji' data were confounded by cold weather in the late fall. The natural progression of tree-ripening did not lead to the high concentrations of internal

ethylene routinely measured in stored fruits. The stimulation of ethylene found in picked 'Gala' fruits ripened in the orchard might be explained by woundingstress coupled with a loss of nutrients and the water stress. Our alternative explanation for "tree factor" is the effect of continued termination of the phloem and xylem connection. The strength of the "tree factor" declined as 'Gala' fruit maturity progressed. Therefore, the "tree factor" tends to be more obvious in fruits with shorter growing period that mature during warm weather.

To investigate differential gene expression that accompanies maturation and tree ripening, we used cDNA-AFLP (Amplified Fragment Length Polymorphism) to identify changes in transcript profiling during tree-ripening, and in the ripening of harvested fruits. Two hundred differentially-expressed transcript-derived fragments were isolated from 'Gala.' Ripening-related genes including those known to function in the key processes of defense and stress, cell wall degradation, pigment production and aroma biosynthesis were identified. Clones similar to housekeeping genes involved in protein biosynthesis and degradation, intracellular trafficking and sorting, cell structure and mobility, and metabolism-associated genes were also isolated. Expression patterns of these transcript-derived fragments were verified by using a different 'Gala' sample set on microarray and/or Northern blots. Our study supports the hypothesis that many ripening processes are under transcriptional control and that most of these differentially-expressed genes are highly conserved in fruits.

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by

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List of Abbreviations

AAT	Alcohol acyl transferase	MACC	1-malonylamino cyclopropane-l-carboxylic acid
ACC	1-aminocyclopropane-1-carboxylic acid	mRNA	Messenger RNA
ACO	ACC oxidase	MT	Metallothionein
ACS	ACC synthase	PCR	Polymerase chain reaction
AFLP	Amplified fragment length polymorphism	PEX	Peroxisomal biogenesis factor
AP	Aspartic proteinase	PTOX	Plastid-localized terminal oxidase
APRT	Adenine phosphoribosyltransferase	RNA	Ribonucleic acid
BLAST	Basic local alignment searching tool		
CAD	Cinnamyl alchohol dehydrogenase	SAM	S-adenosyl-metalothionine
CaMK	Calcium/calmodulin-dependent protein kinase	SEM	Standard error of the mean
CCR	Cinnamoyl Coa reductase	SDH	Sorbitol dehydrogenase
cDNA	Complemented DNA	SIV	Starch index value
DNA	Deoxyribonucleic acid	SSC	Soluble solids content
eIF3	Eukaryotic initiation factor 3	SurE	Acid phosphatease survival protein E
ER	Endoplasmic reticulum	TCA cycle	Tricarboxylic acid cycle
EST	Expressed sequence tag	TDF	Transcript derived fragment
GC	Gas chromatography	USP	Universal stress protein
GPT	Glucose-6-phosphate translocator	VHS	Vps27, Hrs and STAM
IEC	Internal Ethylene Concentration	α-Afs	α -L-Arabinofuranosidases
LDOX	Leucoanthocyanidin dioxygenase	I_2-KI	Iodine staining solution
LPA	Lysophosphatidic acid		
LPAAT	Lysophosphatidyl acyltransferase		
LRR	Leucine-rich repeat		
LSD	Least significant differences		

Chapter 1: Studies of the "Tree Factor" and its Role in the Maturation and Ripening of 'Fuji' and 'Gala' Apples

ABSTRACT:

'Gala' and 'Fuji' are two apple (*Malus domestica* Borkh) cultivars that are resistant to pre-harvest fruit drop, during maturation, allowing commercial harvest can be delayed until they attain optimum quality. Differences in ethylene production between the attached and detached apple fruits is hypothesized to involved system 2 ethylene production which maybe modulated by a putative inhibitor termed "tree factor."

Three-years of field data revealed two distinct patterns of maturation and ripening behavior between these two cultivars. Field treatments such as girdling plus defoliation were applied to study plant effects in maturation and ripening. 'Gala' apples demonstrated the expected, positive "tree factor," as attached fruits produced lowest ethylene than detached or girdling plus defoliation fruits. The ripening pattern of 'Fuji' apple was confounded by cold weather in the late fall, and did not displaying the expected tree factor. Starch degradation was enhanced in both cultivars by girdling plus defoliation. Fruits from the girdling plus defoliation treatment responded differently than fruit harvested and 'stored' in the tree canopy. Softening of detached and girdled+defoliated 'Fuji' fruits was delayed compared to attached fruit. In contrast to 'Fuji', 'Gala' fruit softened rapidly regardless of treatment.

From studies of the maturity indices, it is inferred that both internal ethylene concentration (IEC) and the starch index value were suitable for the assessment of harvest maturity in these cultivars. The IEC measured during tree-ripe apple occurred

without the expected high ethylene levels as stored-to-ripe apples. This indicated that the natural progression of ripening does not require the high levels of ethylene routinely measured in stored fruits. The greater levels of ethylene found in harvested fruits appear to be a stress response caused by detachments.

An alternative explanation for “tree factor” is the effects of continued phloem and xylem flow. The strength of the “tree factor” was found to decline as fruit maturity progresses. Therefore, the “tree factor” tended to be more obvious in fruits with shorter growing period that grow rapidly and mature during hot weather.

INTRODUCTION:

Fruit Ripening

The ripening of fleshy fruits corresponds to their terminal stage of development. In the natural world, it is facilitated by many progressively-altered biochemical, physiological, and structural changes that attract seed vectors and /or allow the breakdown and decay of the fruit (Giovannoni, 2004). From a horticultural point of view, fruit ripening represents a complex program of genetic, hormonal, and environmental events that leads to dramatic changes in color, texture, flavor, and aroma of the fruit. Many of these changes make fruit desirable for human consumption. One objective of horticultural industries is to harvest fruit at the appropriate maturity and to apply postharvest technologies to control the rates of these changes in order to provide the consumer with an acceptable table-ripe product.

Climacteric vs. Non-Climacteric

Fruits are divided into two categories, climacteric and non-climacteric, based on fundamental differences in their ripening patterns. They are divided into those that demonstrate a peak in respiration with ripening, and those that do not (McMurchie *et al*., 1972). The ripening of climacteric fruit is accompanied by a burst of respiration and a concomitant upsurge in ethylene biosynthesis. Ethylene, a simple gaseous hydrocarbon which is an important plant hormone, is required for ripening climacteric fruits. Climateric fruits include tomato, apple, banana, and most stone fruits. The existence and role of the climacteric rise in respiration in a fruit while it remains attached to the plant is still debated. However, the increase of respiration of detached fruits is broadly accepted to be necessary for providing energy needed for ripening-related processes (Knee, 1983).

Non-climacteric fruits, such as grape, citrus, and strawberry show no dramatic change in respiration during ripening. Ethylene production also remains at a very low level during the ripening of non-climacteric fruits. Some biochemical and molecular events underlying climacteric and non-climacteric ripening appear to be conserved in tomato (Giovannoni, 2004), melon (Lelièvre *et al*., 1997), and strawberry (Alexander and Grierson, 2002) such as the putative common regulators and the MADS box genes in this late stage of floral development.

It appears that climacteric fruit ripening is regulated by developmental factors, and is coordinated by ethylene biosynthesis (Adams-Phillips *et al*., 2004; Giovannoni, 2004). Many physiological events, including the modification of texture, starch degradation and the change in skin color in apple fruits have been reported to be

uncorrelated with the ethylene upsurge (DeEll, *et al*., 2001; Blanpied, 1993; Lau *et al.,* 1986). Instead, they were correlated with development and maturity. The examination of the molecular basis of ethylene biosynthesis and regulation has also demonstrated that ethylene biosynthesis alone is not sufficient for ripening. A developmental competence to respond to ethylene appears to be necessary in tomato for ripening to occur (Giovannoni, 2001 and 2004).

The triggers of ripening in non-climacteric fruits are not as well known . Their maturity and ripening stages are typically based on qualities such as sugar/acid balance, soluble solids concentration, and ground color (Fillion *et al*., 1999). A small amount of ethylene may be required however, for certain processes such as chlorophyll degradation and softening in non-climacteric fruits (Lelièvre *et al*., 1997).

Apple Botany and Cultivation

Apple is an economically important temperate fruit crop. Apples contain beneficial nutrients including vitamins, minerals, and phytochemicals, and have a delightful taste. Commercial apples cultivars are members of the genus *Malus* (Miller) of the subfamily *Maloideae,* and the family *Rosaceae.* The *Maloideae* are characterized by a hypanthium and gynoecium that remain fused to form an inferior ovary with many closely-associated parts. The fleshy, indehiscent fruit tissue derived from the cortex of receptacle is called a 'false' (pseudocarpic) fruit, or pome (Watkins, 2003). Apple (*Malus domestica*), pear (*Pyrus communis*) and melon (*Cucumis melo*) were first classified as climacteric fruits by Kidd and West in their classic research in 1924 (Watkins, 2003). Commercial cultivars are characterized by

having a large diversity in their season and rate of ripening. Global marketing and a long storage life make apple fruits available for consumers throughout the year.

The response of fruit to postharvest handling treatments are affected by cultivar preharvest weather conditions, and production systems Cultivars can influence postharvest management through effects on ripening and fruit quality. For example, early-maturing cultivars usually produce higher levels of ethylene than late-maturing cultivars resulting in short storage life. Conversely, low ethylene producing cultivars generally have a longer storage potential (Watkins, 2003). Several controlled-environment studies have shown that higher temperatures during early fruit growth accelerate fruit-maturity, resulting in changes in starch content, firmness loss, development of a red blush and ethylene production (for review, Dennis, 2003). Late-season temperatures also influence fruit maturation and ripening, and enhancement of the ethylene biosynthetic pathway during the latter stages of development has been reported in 'Granny Smith', 'Fuji', 'Royal Gala' and 'Starking Delicious' apples (Jobling and McGlasson, 1995; Jackson, 2003).

Horticultural management techniques including rootstock/ interstock selection, girdling and plant growth regulators can also affect fruit maturation and ripening. Rootstocks with desirable qualities such as dwarfing, disease and pest resistance, and the adaptation to varied soil and climatic conditions are used commercially (Kaushal and Sharma, 1995). Rootstocks affect tree precocity, fruit soluble solids content, leaf and fruit mineral composition, fruit size, fruit susceptibility to physiological disorders, and the date of fruit ripening (Autio 1991; Schechter *et al*., 1991). Rootstocks can affect apple tree vegetative growth and the partitioning of

photosynthetically produced dry matter between fruit and wood (Autio 1991; Schechter *et al*., 1991). Confounding variables such as growth habit, crop load, light penetration, and environmental stresses have made it difficult to demonstrate the direct effects of rootstock on ripening and storage quality (Autio, 1991; Drake *et al*., 1988).

'Gala' and 'Fuji' Apple Cultivars

'Gala', is a selection from the cross of 'Kidd's Orange Red' x 'Golden Delicious' made in 1934 (Hampson and Kemp, 2003). 'Kidd's Orange Red' was itself a cross of 'Cox's Orange Pippin' x 'Red Delicious'. The apple was named 'Gala' in 1962, released for commercial planting in 1965 (Hampson and Kemp, 2003), and introduced to US in 1970's. This precocious, high-quality cultivar is highly aromatic with a very sweet flavor, and has a firm crisp texture while ripe. Mature fruit is typically yellow with red stripes and it matures from mid- to late- August in Maryland.

'Fuji' is a selection from the cross 'Ralꞌs Janet' x 'Red Delicious' (Hampson and Kemp, 2003). This sweet, flavorful introduction is currently the leading cultivar in Japan and China (). It was introduced into the U.S. in the 1980s. 'Fuji' apples mature horticulturally with a reddish-pink skin color from mid- to late- October in Maryland. Compared to other cultivars, 'Fuji' has a slow rate of firmness loss and a long storage and shelf life, and this may be associated with low ethylene production and low respiration rates (Hampson and Kemp, 2003). Cool temperatures may be needed to stimulate 'Fuji' ripening (Jobling and McGlasson, 1995). 'Fuji' also lacks a classic

climacteric ethylene burst if no cold exposure occurred during maturation (Fellman *et al*., 1997).

It has been well-documented that a cold ripening-requirement exists in pears (Knee *et al*., 1983). Unlike most apples, pears fail to produce significant amounts of ethylene for several months if they are left to ripen naturally. Without chilling, exogenous ethylene fails to induce the expression of ethylene biosynthetic genes in pears (Lelièvre *et al*., 1997). Other apple cultivars besides 'Fuji' have been also postulated to be chilling-sensitive (Jobling and McGlasson, 1995). The long-season cultivars, 'Lady Williams,' and 'Granny Smith' have also been reported to respond to chilling with a climacteric rise in ethylene production (Jobling and McGlasson, 1995). In summary, low temperatures appear to hasten homogeneous ripening and induce the competency to synthesize autocatalytic ethylene in long-season apple cultivars (Knee *et al*., 1983; Jobling and McGlasson, 1995).

'Gala' and 'Fuji' were chosen for this study for their tree-ripening characteristics, lack of preharvest drop, and the need to learn more about the effects of weather and orchard management on their fruit maturation and tree-ripening.

Physiological Changes during the Maturation and Ripening of Apple Fruits

Ethylene evolution and respiration rate

Apple fruit is considered to be a classic climacteric fruit since autocatalytic ethylene production (system 2 ethylene) and increased respiration is associated with ripening. Since a logarithmic change in ethylene evolution occurs, which is far greater than the differences of respiration measured during maturation and ripening, it has been suggested that ethylene production or internal ethylene concentration (IEC) can

be used as a determinant of ripening (Lau, 1985). Alternatively, high ethylene has also been suggested to be the indicator for the end point of harvest, rather than a maturity index *per se* (Watkins, 2003). Ethylene production is greatly affected by cultivar, environment, growing region, and nutritional supply during fruit development. Therefore, site specific ethylene production records would be necessary to use ethylene effectively as a maturity index for predicting harvest.

Starch degradation and increases in soluble carbohydrates

Sorbitol, a sugar-alcohol that is the main translocated photosynthate in apple, is transported from the leaf to the fruit. During early development sorbitol is converted mainly into fructose and starch. Starch that accumulates in apple fruit flesh eventually breaks down into sucrose, glucose and fructose *via* a series of hydrolyses in the latter stages of fruit development (Lau *et al*., 1995; Blanpied, 1993).

Change of skin color

Chlorophyll concentration in the apple fruit peel gives fruits their characteristic immature green ground color. Mature fruit color involves a combination of chlorophyll breakdown by chlorophyllase unmasking carotenoids, and the biosynthesis of phenolic pigments such as anthocyanins to produce a red color. These changes depend on cultivar and clone, nutritional supply and light penetration into the tree canopy (Watkins, 2003).

Aroma biosynthesis

The aromatic profile of apple fruit is quite complex (Plotto *et al*., 1999). Over three hundred compounds have been reported to contribute to the aroma of various

apple cultivars (Plotto *et al*., 1999). These volatile compounds of highly diverse structures (alcohols, aldehydes, esters, ketones, terpenes) participate in our aromatic perception. Butyl acetate, hexylacetate, butanol and 2-methylbutyl acetate are some major volatile substances that emanate from ripe apple fruits, and have been used as subjects for monitoring quality and aroma (Plotto *et al*., 1999). Their synthesis is influenced by factors such as fruit temperature and internal oxygen concentration. The production of aroma volatiles in 'Golden Delicious' and 'Fuji' apple showed that volatile production is maturity-dependent, and is closely related to changes in respiration rate and ethylene production (Song and Bangerth, 1996; Rudell *et al*., 2000).

Firmness loss

The textural characteristics of a fruit are governed by cell size, cell wall thickness and integrity, intercellular volume, turgor pressure and the manner in which cells bind together (Knee, 1993; DeEll *et al*., 2001). Binding is controlled by cell-to-cell adhesion, cell shape and packing (DeEll *et al*., 2001). Thus softening occurs in response to is a series of complex processes that occur during cell wall degradation. DeEll *et al*., (2001) suggested that apple fruit firmness tends to be influenced by a series of preharvest factors, such as genetics, cultural practices and maturity at harvest and completed with postharvest factors, such as cooling, postharvest dips, and storage conditions. Postharvest factors are thought to have a much greater effect on firmness than preharvest factors. Knee (1993) studied cell wall chemistry extensively, and concluded that the decline of flesh firmness during storage or ripening was primarily due to an increase in air space in apple flesh and the climacteric rise in ethylene.

Predicting Optimum Harvest Date

Maturity at harvest is a critical factor determining consumer acceptability and the potential of storage and shelf life of an apple. To increase storage life and reduce yield losses through abscission, senescence, and pathogen , preclimacteric, physiologically mature fruits are recommended for commercial harvest. As they are physiologically mature these fruits are capable of fully ripening. Fruit harvested prior to commercial maturity is incapable of ripening to an acceptable quality. Predicting harvest maturity is still challenging and requires a suitable compromise between two competing factors, fruit quality and storageability (Abdi *et al*., 1997).

From a practical perspective, specific levels of a series of traits may be used to determine maturity needed for different product streams: e.g. fresh pack, long-term storage, export, or processing. Using the calendar as the basis to forecast harvest date and then adjusting the harvest window on the date of bloom and post-bloom temperatures is a simple scheme for predicting harvest date (Jackson, 2003). Monitoring maturity indices is also useful to follow the actual ripening process and then adjust that prediction during harvest. Defining apple maturity involves the measurement of specific physiological and structural traits such as fruit firmness, starch conversion, soluble solids content, chlorophyll degradation, aroma volatile synthesis and ethylene production. An assessment of ground color is also considered a simple and useful harvest index for many bicolor apples, such as 'Gala', 'Braeburn' and 'Fuji' (Watkins, 2003).

The Role of Ethylene in Apple Fruit Ripening

The gaseous hormone ethylene affects a diverse group of processes during the plant's life cycle. These include seed germination, sex determination, stress responses, senescence, and fruit ripening (Yang and Hoffman, 1984; Johnson and Echer, 1998). A review of the role of ethylene biosynthesis inhibitors and perception (Giovannoni, 2001), and its reversibility in antisense fruits during ripening contributed our recent biochemical understanding of ethylene (Oeller *et al*., 1991).

Ethylene is synthesized in plant tissues *via* the conversions of S-adenosyl-metalothionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams and Yang, 1979). This is catalyzed by ACC synthase (ACS) (Adams and Yang, 1979; see appendix 1). 1-aminocyclopropane-1-carboxylic acid synthase is regarded as the rate-limiting enzyme in the ethylene biosynthetic pathway (Yang and Hoffman, 1984). The conversion of ACC to ethylene is then catalyzed by ACC oxidase (ACO) (Yang and Hoffman, 1984). Pre-climacteric and climacteric stages of development are divided by the patterns of ethylene production and regulation that exist. The transition from the ethylene-autoinhibitiory preclimacteric stage to the autocatalytic-climacteric stage of ethylene production is presumably based on changes in expression of the system 2 ACS genes and system 2 ethylene receptors (Oetiker and Yang, 1995). 1-aminocyclopropane-1-carboxylic acid synthase is the critical step in the transition of the ethylene feedback mechanism from negative to positive, and its activity can be the indicator of stage of maturation in climacteric fruits (for review, Oetiker and Yang, 1995).

Both ACO and system 2 ACS are developmentally up-regulated in pre-climacteric fruit (Lelièvre *et al*., 1997), whereas the ACO protein was undetectable in the pre-climacteric period (Lara and Vendrell, 2000a). Later, a rapid, greater increase in ACO transcripts and activity occurred before the climacteric rise than did the surge in ACC content (Yang *et al*. 1986). The sensitivity of fruit tissue to endogenous and exogenous ethylene was shown to increase with physiological age (Lara and Vendrell, 2000b).

Signals that have also been postulated to switch fruits from the pre-climacteric to climacteric stages are abscisic acid (ABA) (Lara and Vendrell, 2000), jasmonate (Fan *et al*., 1998) and auxin (IAA) (Tingwa and Young, 1975; Mousdale and Knee, 1981). Applying a high level of ABA to pre-climacteric 'Granny Smith' apple fruits two-months before commercial maturity could induce the onset of the climacteric at that time, but not when applied to fruits harvested later. A considerable increase was also measured in endogenous ABA levels during ripening (Lara and Vendell, 2000). Exogenous ABA caused the induction of ethylene biosynthesis in both pulp and peel of very immature (2 months before commercial harvest) 'Granny Smith' apple fruit by inducing ACO. This was accompanied by an increase of ethylene production. The same treatment failed to enhance ethylene biosynthesis in more mature fruit (Lara and Vendell, 2000b). Jasmonates have been shown to increase transiently in apple and tomato fruits at the onset of the climacteric ethylene production (Fan *et al*. 1998). Auxin (IAA) concentration also increases 3 to 4-fold in apple fruits just before the onset of ethylene production, but then returns to its original level (Mousdale and Knee, 1981). Tingwa and Young (1975) studied parent-plant effects on ripening in

avocado. They indicated that high concentrations (100 and 1000µM) of exogenous IAA stimulated ripening, *via* auxin-induced ethylene, while lower levels (1 and 10µM) delayed avocado fruit ripening.

During ripening, ethylene response pathway genes including those of ACC biosynthesis: ACS and ACO, ethylene perception: ethylene receptors including *LeETR4* (EThylene Receptor4), *ERS1* (Ethylene Response Sensor1), *Nr* (Never-ripe, similar to *ERS* receptor), signal transduction: signaling kinases(mitogens-activated protein kinase kinase kinase), *CTR1*, (Constitutive Triple Responses1), and downstream components of ethylene signaling transcription factors, *EIN3* (Ethylene INsensitive3) and related *EIL* (*EIN3*-Like) as well as E8 dioxygenase which contributes to the maintenance of ethylene receptor activity are allinduced in tomato (Johnson and Echer, 1998; Ciardi and Klee, 2001; Giovannoni, 2004). Both ACS and ACO belong to multigene families. The differential expression of individual members of the ACS and ACO gene families that occurs with the transition from pre-climacteric to climacteric stages provides the explanation for McMurchie's hypotheses that ethylene mediates that transition (Oetiker and Yang, 1995; Lelièvre *et al*., 1997). ACS genes isolated from pear have also been categorized into system 1, (i.e. *Le ACS6* and *Pc-ACS3*) and system 2 (*Le ACS2*, *Le ACS4* and *Pc-ACS5*, *Pc-ACS4*) ACS alleles (Alexander and Grierson 2002, El-Sharkawy *et al*. 2004). In apple ACS gene family members are also differentially expressed during ripening. The expression of *Md-ACS1* was induced during ripening, while *Md-ACS3* was expressed constitutively irrespective of ripening stage (Sunako *et al*. 1999). The increasing accumulation of ACC in ACO-antisense fruit and in non-ripening *nor*

tomato mutants indicated that system 2 ACS is under developmental control (Lelièvre *et al*., 1997). Ciardi and Klee (2001) suggested that the rapid induction of an ethylene response by a burst of ethylene synthesized during fruit ripening is followed by a dampening of that response with an increase in receptor levels, such as those *LeETR4*.

Respiration vs. IEC

Many investigators have questioned whether the respiratory climacteric is a response that occurs in ripening of detached fruit, but not in attached fruit. Studies using external gas samples in saskatoons (Rogiers and Knowles, 1999), purple passion fruit (Shiomi *et al*., 1996) and charantais melon (Bower *et al*., 2002) found that the increase in climacteric ethylene production was not accompanied by a corresponding rise in respiration in attached fruit. Respiration rate remained constant in saskatoon (*Amelanchier alnifolia* Nutt.), tomato, and purple passion fruit (Rogiers and Knowles, 1999; Saltveit, 1993; Shiomi *et al*., 1996). Employing internal measurements, and then expressing these data on the whole-fruit basis, respiration on the tree (or vine) demonstrated a climacteric increase in both ethylene and respiration (Knee, 1995; Rogiers and Knowles, 1999), the rise in autocatalytic ethylene production is logarithmic, it is of a much greater scale than changes in respiration associated with ripening of climacteric fruits. It has been suggested that the increase in respiration associated with ripening can only be demonstrated on a whole-fruit basis if saskatoon fruit remained attached to the plant (Rogiers and Knowles, 1999). Eliminating possible confounding factors, such as continuing photosynthesis of fruit and difficulties in gas sampling, different patterns of respiration rate also exist between cultivars in the same species. Andrew (1995) postulated that the typical

respiration pattern occurred in detached "indeterminate" salad tomato cultivars 'Counter' and 'Sweet 100.' It did not exist in the "determinate" processing tomato 'Castlemart' (Saltveit, 1993). The actual role of the extremely high levels of ethylene and respiration found in climacteric fruit ripening is still under study.

Tree Factor

Many fruits ripen more rapidly when they are detached from the tree than when they are attached. These include avocado, melon, plum, and apple (Abele, 1973) . It is commonly believed that an inhibitor entering the fruit from the parent plant inhibits ripening through an effect on ethylene biosynthesis (Burg and Burg, 1965; Smock 1972; Abdi *et al*., 1997; Sfakiotakis and Dilley 1973a; Lau *et al*., 1986). Avocado is the classic illustration of parent-plant inhibition of ripening. Avocado fruits fail to ripen or show climacteric ethylene production while attached to trees Apple fruit demonstrates ethylene climacteric one to two months after commercial harvest if the fruit remains attached to the tree ('Red Delicious' of Sfakiotakis and Dilley 1973a; 'Golden Delicious' in Lau *et al*., 1986). Abeles (1973) coined the term "tree factor" to describe the putative inhibitor of autocatalytic, system 2 ethylene production in fleshy fruit tissue. The tree factor is hypothesized to be produced in the leaves and transported *via* the phloem to the fruits. Sfakiotakis and Dilley (1973a) observed that the defoliation and girdling of spurs promoted the onset of climacteric ethylene production in attached apple fruits by nearly a month. Blanpied (1993) reported that this delay in ethylene production was positively correlated with leaf/fruit ratios on ringed limbs. The increase in both ACC and ethylene production occurred earlier in detached, preclimateric 'Golden Delicious' apple fruit, than in attached fruits (Lau *et*

al., 1986). Attached fruit subsequently attained a higher concentration of ACC and a lower level of ethylene than detached fruit. It was suggested by Lau *et al*. (1986) that tree factor delayed both the conversion of ACC to ethylene catalyzed by ACO and the conversion of SAM to ACC catalyzed by ACS. ACO was also more sensitive to inhibition by tree factor than was ACS. Once the climacteric was initiated, the development of ACO preceded the enhancement of ACS.

On the other hand, Knee (1993) suggested that detachment might have little effect on enhancing ethylene biosynthesis. He postulated that cross-diffusion of ethylene among fruits during storagewould explain an earlier upsurge in ethylene found in detached fruits. Additionally, the sensitivity of attached apples to ethylene seems to be no different than the sensitivity of detached fruits. Nevertheless, Knee's suggestion does not explain the incidence of increased ethylene production of attached apples grown on girdled, defoliated spurs (Sfakiotakis and Dilley, 1973a).

An alternate hypothesis to describe this phenomenon is the stimulation of ethylene that occurs with wounding. Wounding coupled with the nutrient and water deficiencies also occur at harvest. Since the sensitivity of fruit tissues to either endogenous or exogenous ethylene increases as maturation progresses (Liu, 1978; Lara and Vendrell, 2000), low amounts of system 1 ethylene caused by wounding might be sufficient to induce system 2 ethylene as fruits mature. The lag period before climacteric ethylene increase was positively correlated with the leaf/fruit ratio on girdled limbs in Blanpied's study (1993). The inhibiting effect of tree factor can be overcome by exogenous ethylene while fruits remained attached in avocado (Bower and Cutting, 1988), apple (Knee, 1993) and plum (Abdi *et al*., 1997).

In conclusion, as the apple fruit approaches physiological maturity, the autocatalytic ethylene biosynthesis and system 2 ethylene perception and signal transduction pathway become competent to respond to the final signal provided by either exogenous or endogenous ethylene.

Objective

The aim of this study was to expand our understanding of the tree factor in apples. We adopted Sfakiotakis and Dilley's (1973a) hypothesis that tree factor, a small molecule, is produced in the leaves and transported through phloem to fruits. To do this we applied treatments to block potential signals from leaves to fruit *via* the phloem over the periods spanning commercial harvest. In this chapter a comparative study of the physiological changes which occurred during the ripening of 'Gala' and 'Fuji' is presented.

MATERIALS & METHODS:

Plant Material and Treatments

Gala :

Experiments were conducted at the Western Maryland Research and Education Center in Keedysville, MD and the USDA-AFRS in Kearneysville, WV. Experiments were conducted in 2002 and 2003. Twelve-year-old 'Gala' trees on their own roots (produced in tissue culture, MD) or budded onto M.9 rootstock (WV) were chosen for this study. Apple fruits *(Malus domestica* Borkh. cv Gala) were harvested and analyzed at 8 to 12 day intervals during development ranged from July 31 to

September 20 in 2002, and August 6 to September 25 in 2003. For detachment studies, three fruits were selected based on uniformity and absence of defects from each of twelve replicate trees during stage I~ III (see Table 1). This corresponded to July 31 to August 19 in 2002 and August 6 to 25 in 2003. Detached apples (one from each replicate tree, 12 in total) were then hungin nylon stockings from limbs in the canopy in attempt to ensure a similar environment to that of attached fruits for 10, 20 and 30 days. One limb of uniform size and crop load in each tree was girdled (bark was removed) and defoliated during stage I~ III in 2003, and fruits from the treated limbs were sampled at the same interval as detached treatment.

Table 1. The defined developmental stages of 'Gala' apples in this study.

Stage	Harvest date	Definition
Stage I	July 31, 2002 August 6, 2003	Mature-green, about 20 days before commercial harvest 100-110 days after full bloom (DAFB)
Stage II	August 8, 2002 August 15, 2003	Early mature, about 10 days before commercial harvest; 110-120 DAFB
Stage III	August 20, 2002 August 25, 2003	Commercially mature, starch index value=3~4; 120-130 DAFB
Stage IV	August 29, 2002 September 4, 2003	Late mature; 130-140 DAFB
Stage V	September 12, 2002 September 15, 2003	Tree ripe; 140-150 DAFB
Stage VI	September 26, 2002 September 25, 2003	Ripe to overripe, starch index value close to 8; 150-160 DAFB

On each sample date, twelve fruits from each treatment were held overnight at room temperature (20 ºC) before determination of the internal ethylene concentration (IEC) and maturity indices. Maturity evaluations included color ratings, fresh weight,

diameter, soluble solids content, flesh firmness, and a visual assessment of the starch index.

Fuji :

Year 1

Twelve treeswere mature, having been planted in 1991 at the Western Maryland Research and Education Center Keedysville, MD and selected for this study. Trees were hand-thinned and one limb of uniform size and crop load in each tree was girdled plus defoliated about one month before commercial harvest on September 7, 2001, 140 days after full bloom (DAFB). Detached apples picked at commercial harvest period on October 9, 170 DAFB, were hung in nylon stockings tied to the control limbs until the end of the experiment on November 19. Stainless needles were inserted into the randomly selected fruits on the treated day and remained in those fruits until the end of the experiment. Internal ethylene concentration, fruit diameter and percent red skin color of the selected fruits were monitored weekly from one week after treatment date to November 19. One apple was picked biweekly from each treated limb for laboratory measurements of soluble solids content, firmness, and visual starch index. Flesh samples were also saved from these fruits (-80°C) for measurement of ACC and MACC contents.

Year 2

A similar experimental design and procedure as ‘Gala’ 2003 was applied to twenty four ‘Fuji’ trees budded onto M9 rootstock. Fruits were harvested and analyzed bi-weekly across this period, ranging from September 12, 2002 until November 21, 2002. These were organized in 6 blocks at Keedysville. Girdling plus

defoliation and detachment treatments were applied four times to each of four individual trees in each block from early mature to commercial mature stages (Sep. 12, Sep. 26, Oct. 10, and Oct. 24, 2002). One fruit from each replicate limb and tree (6 in total) was sampled at subsequent intervals of two, four and six weeks. The same maturity indices used in 2001 were again measured one day after harvest.

Maturity Indices

The IEC was measured by taking a 1 ml gas sample by inserting a stainless hypodermic needle fitted with a serum stopper into the fruit core from the calyx basin. A 20 gauge 50.8 mm stainless hypodermic needle fitted with a serum stopper was inserted into the fruit core in the field studies. A small stainless steel wire plunger was used to remove any enclosed fruit tissue for gas sampling. Ethylene concentration was determined by injection into a GC fitted with a flame ionization detector. The GC (Model 6890, Hewlett Packard Co., Palo Alto, CA) was equipped with activated alumina packed into a stainless steel column. The minimum detectable level of ethylene with this gas chromatograph was about 20 nL/L. Flesh firmness was measured using a penetrometer (QA Systems, Norfolk, VA) after removing a small section of the peel from three locations around the fruit equator.

Starch disappearance was estimated by staining the cut equatorial surfaces with iodine staining (I_2-KI) solution followed by a visual rating. Patterns were compared with Cornell generic starch-iodine index chart for apples (Blanpied and Sisby, 1992) which approximated changes found in ‘Gala’ and ‘Fuji’. This chart describes starch index values (SIV) on a scale from one to eight. An SIV scoreof “one” means that the fruit is filled solidly with starch, while “eight” means that the flesh is devoid of

starch. The concentration of soluble solids content was measured with a temperature-compensated refractometer (Leica Mark II Plus).

About 4 g of pulp was taken per sampling date to analyze for both free ACC and bound (1-malonylamino cyclopropane-l-carboxylic acid; MACC) contents. Samples were extracted with 80% methanol, which was removed under vacuum at 40 °C after centrifuging for 10 min at 10,000g. Free ACC was measured in the aqueous extract according to methods of Lizada and Yang (1979) and Sitrit *et al*. (1988). The ACC concentration was determined on the aqueous extract after hydrolysis with 7.2 M HCl at 100°C for 3 hours, following the procedures described by Atta-Aly*et al*. (2000) . MACC was taken as the difference between total and free ACC contents. ACC results are presented as nmol/g fresh weight.

IEC vs. Ethylene Evolution Rate

IEC and external ethylene evolutions (headspace volatile analyses) in 'Fuji' apple fruits harvested during commercial harvest period in 2001 were monitored at intervals during storage. Internal ethylene assays were performed as described above. The external ethylene evolution rates were obtained by measuring ethylene concentration of gas sample taken from headspace of sealed jars with an individual apple. Total 250 pairs of data were generated from this study. A regression test was taken to analyze their correlation.

Statistical Analyses

'Gala' studies were a randomized complete block design (RCBD). Each tree was treated as a block. Each apple was treated as an experimental unit in the 'Gala' for

both years. For ‘Fuji’ Year 1, the experimental design was a randomized complete block split plot design with repeated measures in time. Experimental structure was a two-rootstock (M 7a and MM 111) by three-treatment factorial. Rootstocks were the main plots with 6 replicates in 6 blocks. Treatments served as subplots with 12 replicates nested within 12 individual trees. Each ‘Fuji’ limb was treated as an experimental unit. For ‘Fuji’ Year 2, the experimental design was also a RCBD. Analysis of variance (ANOVA), and mean separations by least significant differences (LSD) analyses ($P \leq 0.05$) were carried out using the **mixed** procedure of the Statistical Analysis System program package (version 8.2, SAS Institute Inc, NC, USA). For internal ethylene concentration, both original and $\log_{10}$ transformed values were tested for significance. In ‘Fuji’ (2001) the interaction of rootstock and treatment was not significant; the sum of squares for the treatment was pooled within rootstocks.

RESULTS:

General Comparison of attached 'Gala' and 'Fuji' during maturation and ripening

In general, maturity indices in attached 'Gala' and 'Fuji' behaved similarly during maturation and ripening. Besides the significant difference of fruit size, the major difference found between these two cultivars was the relative level of the first plateau of IEC that was measured in apples harvested at commercial maturity, and the fruit softening rate. In both cultivars, the first plateau of IEC in attached fruits appeared to coincide with the local commercial harvest period. The IEC increased dramatically again with later harvests. Similar ethylene production pattern has been reported previously in 'Golden Delicious' (Walsh, 1977) and 'Gala' (Walsh *et al*., 1992). The primary increase of IEC on 'Gala' occurred in August 20 in 2002 and August 25 in 2003. This was ten times the level measured in mature-green fruit and one tenth that measured in tree-ripe fruits. The IEC change during maturation of 'Gala' is shown in Figure 1. There was less noticeable change of IEC during commercial maturity of 'Fuji' (October 9, 2001 and October 10, 2002) (Figure 2). The firmness of tree-ripened 'Gala' apple was about half of that measured in mature-green 'Gala' apple (Figure 3). In contrast, there was less than a 20% loss of firmness in 'Fuji' (Figure 4) harvested from one-month before commercial maturity until about one month afterwards.

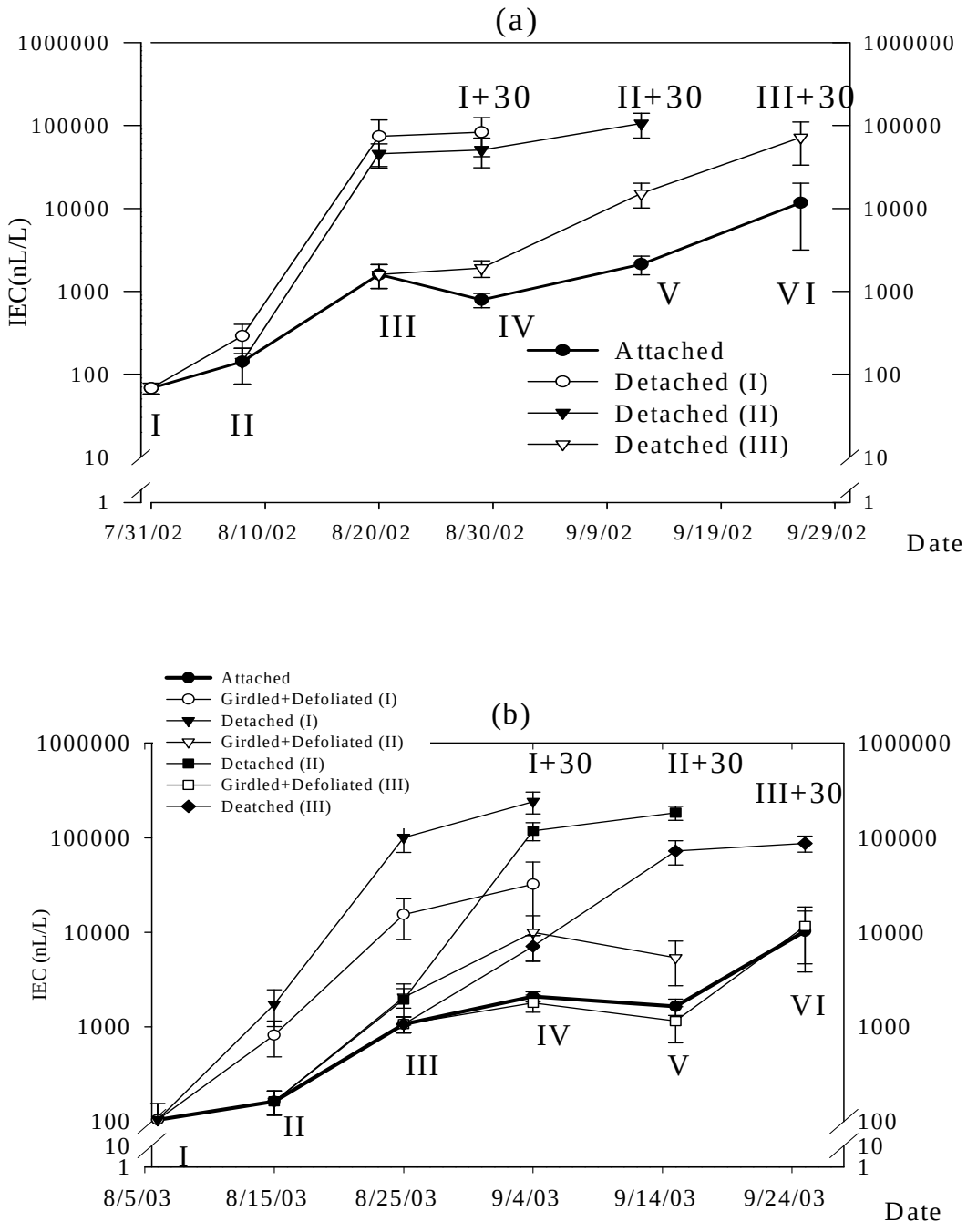


Figure 1. Internal ethylene concentration (IEC) in 'Gala' apple fruit attached to the tree, detached in the orchard (2002 and 2003), and attached to the girdled plus defoliated limbs (2003 only).

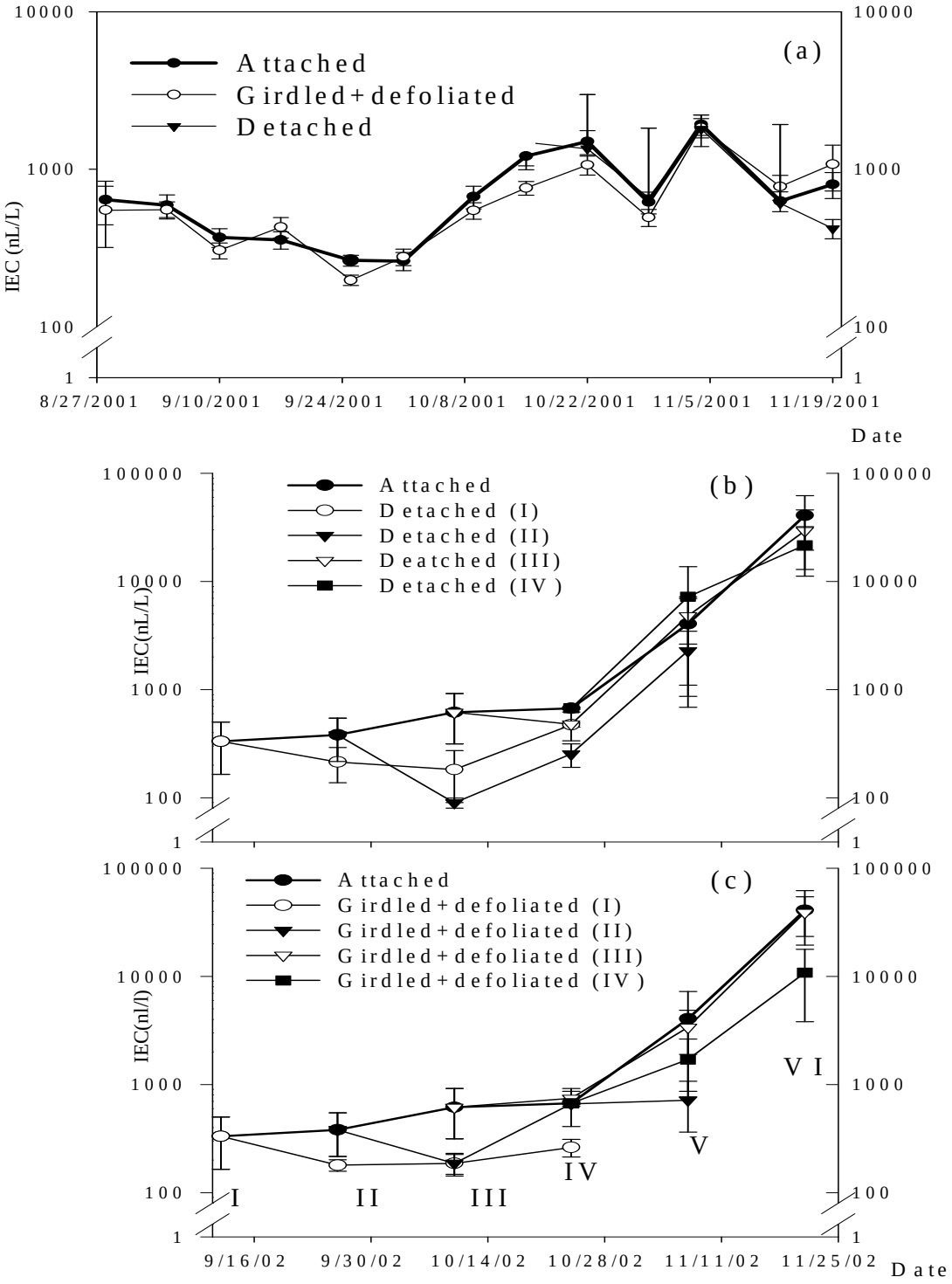
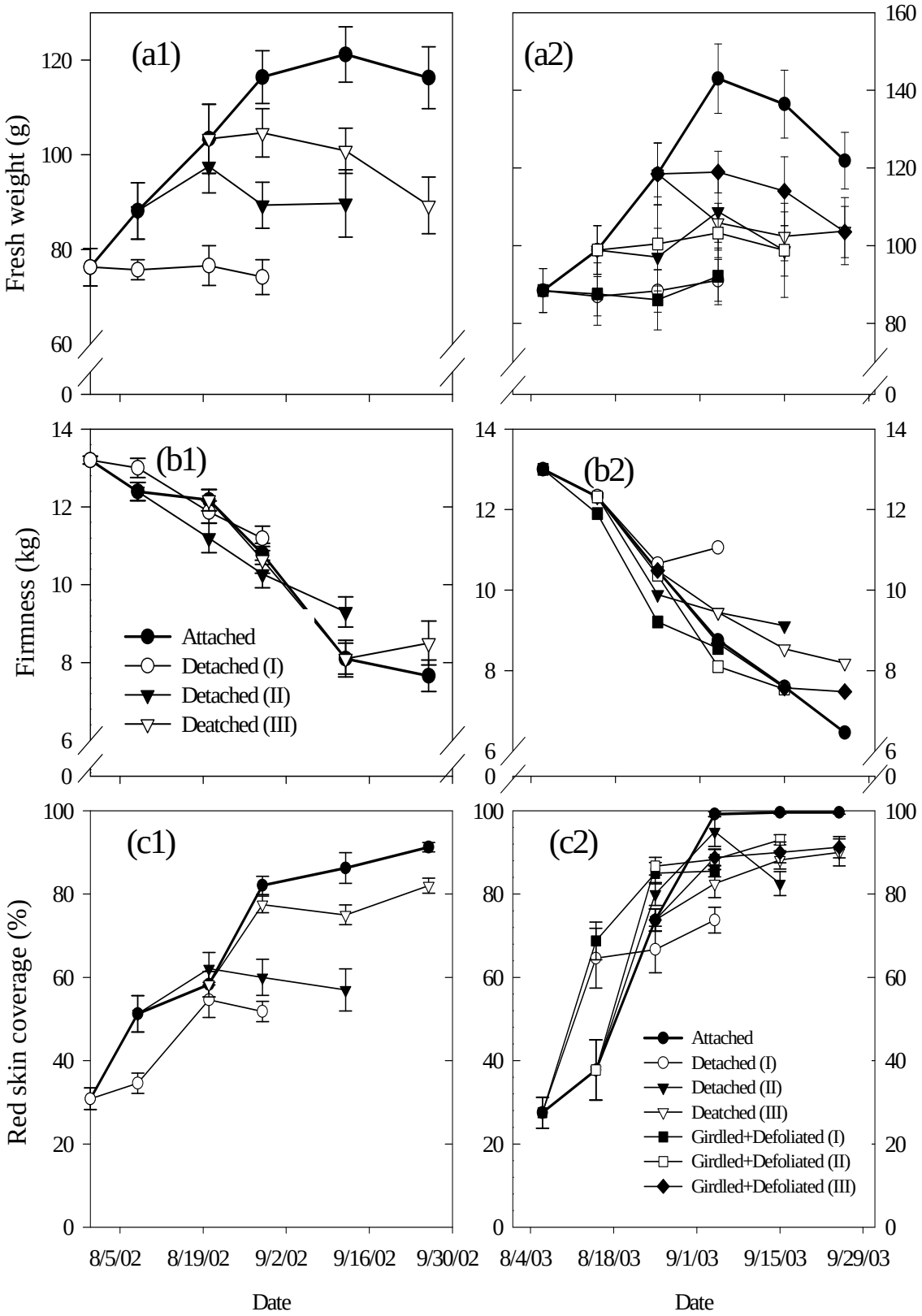


Figure 2. Internal ethylene concentration (IEC) in 'Fuji' apple fruit attached to the tree, detached in the orchard, and attached to the girdled plus defoliated limbs during 2001 (a) and 2002 (b and c).


(a1)
(a2)
(b1)
(b2)
(c1)
(c2)
Fresh weight (g)
Firmness (kg)
Red skin coverage (%)
Attached
Detached (I)
Detached (II)
Deatched (III)
Girdled+Defoliated (I)
Girdled+Defoliated (II)
Girdled+Defoliated (III)
8/5/02
8/19/02
9/2/02
9/16/02
9/30/02
8/4/03
8/18/03
9/1/03
9/15/03
9/29/03
Date
Date

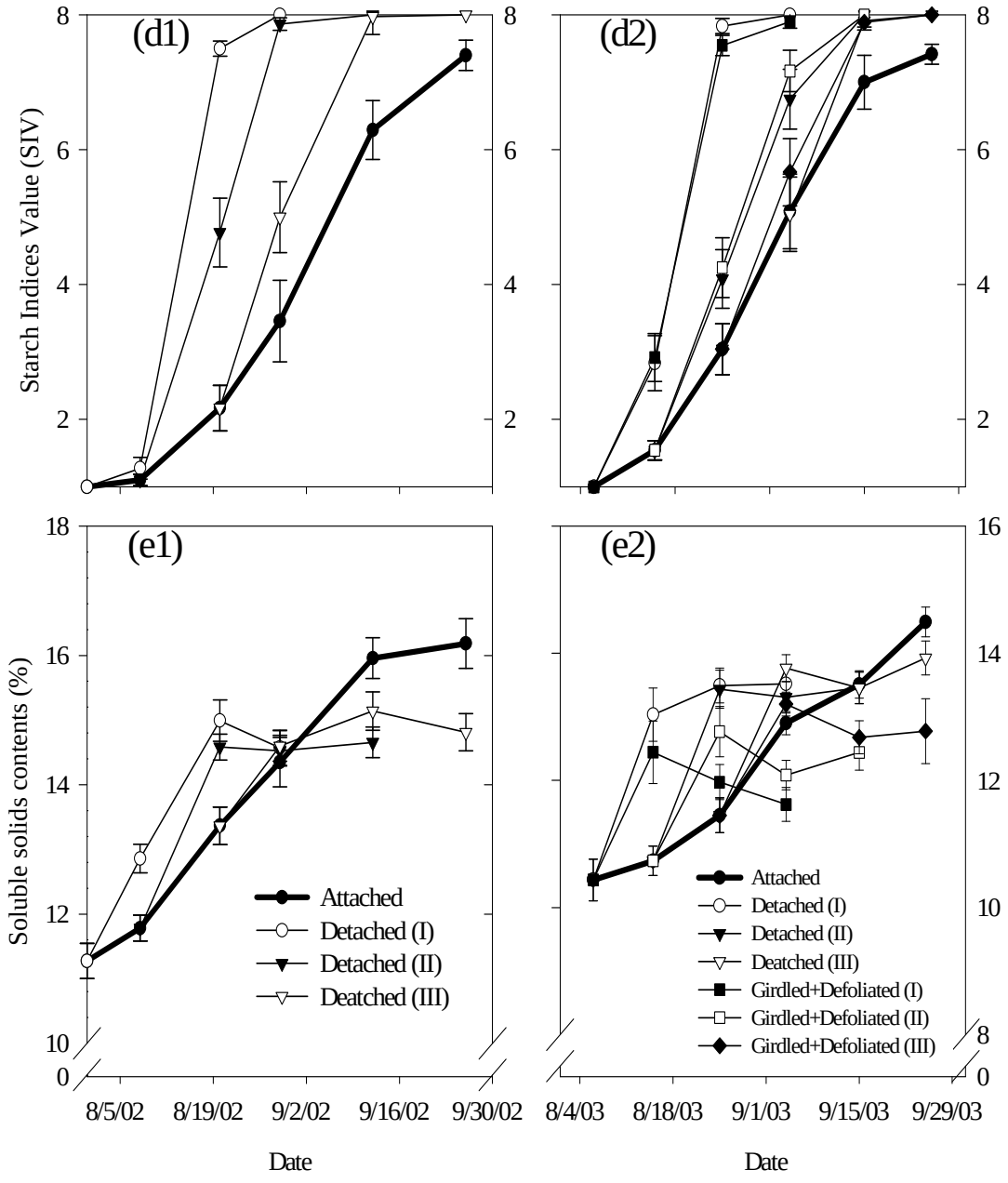


Figure 3. Changes measured in maturity indices of 'Gala' apple fruits in 2002 and 2003.

Fresh weight (a), flesh firmness (b), percent red skin (c), starch indices value (d), and soluble solids contents (e) in 'Gala' apple fruits attached to the tree, detached+hung in the orchard (2002 and 2003), and attached to the girdled +defoliated limbs (2003 only). Vertical bars represent SE (n=12).

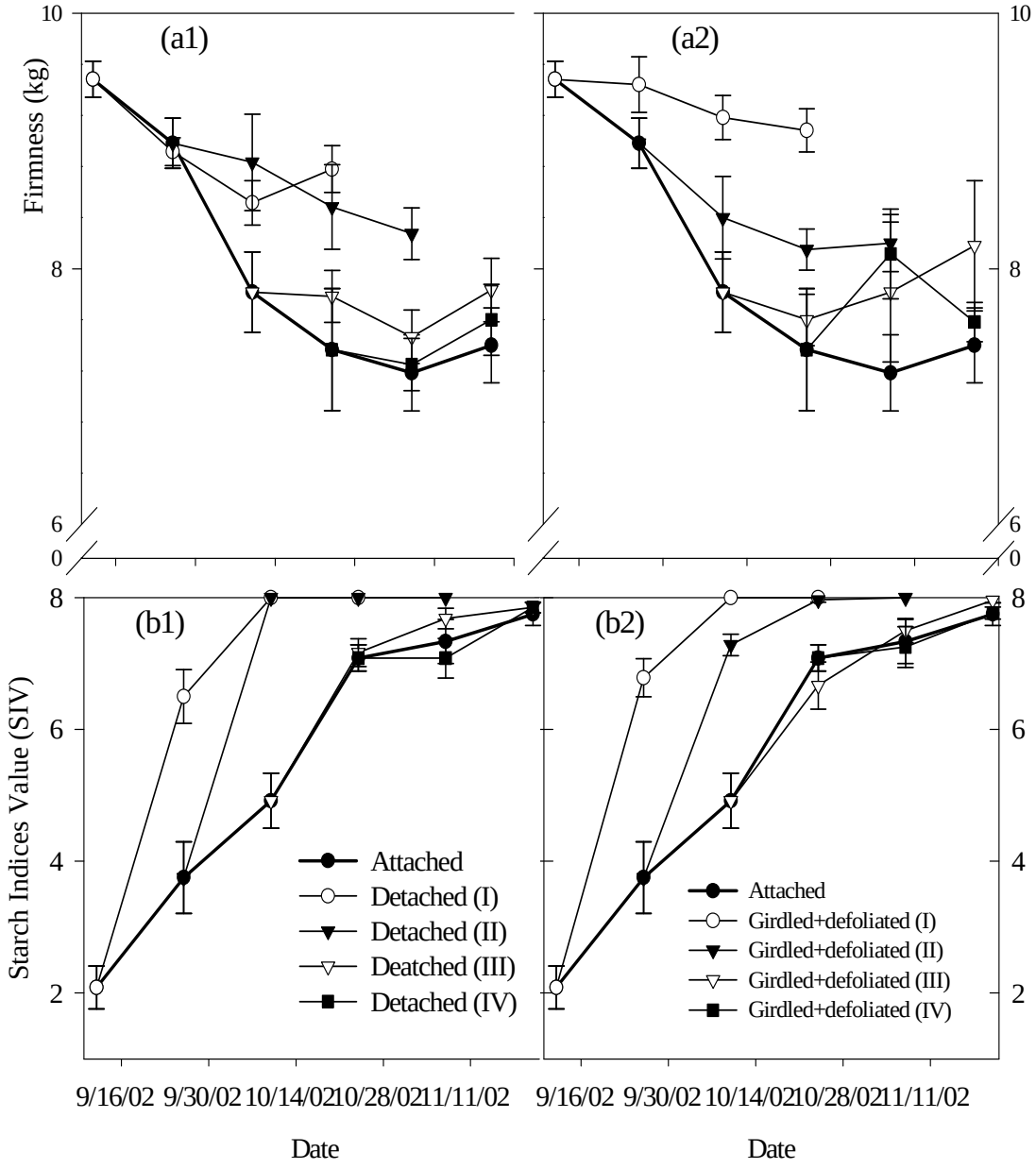


Figure 4. Changes measured in maturity indices of 'Fuji' apples in 2002. Changes of (a) firmness (b) starch indices value in 'Fuji' apple fruit during maturation and ripening in 2002. Vertical bars represent SE (n=6).

The Effects of Detachment and Girdling plus defoliation

Ethylene evolution by detached, girdled +defoliated (2003 only) and attached 'Gala' apple fruits differed (Figure 1). The upsurge of IEC occurred earlier in

detached fruit than in attached fruit. Based on data(Table2) from these two years, at two locations, we concluded that ethylene production is triggered by detachment in 'Gala' apple fruit. The IEC in attached fruit eventually increased, but never to the level measured in detached fruit.

Table 2. ANOVA table of internal ethylene concentration, firmness, and starch index value (SIV) on 'Gala'in 2002 and 2003, in which six treatments dates (stage I to VI) and two treatments (attached and detached) or three treatments (additional girdling plus defoliation) were applied.

Effect	Degree of Freedom (DF)	DF of residue	F value	Pr > F
'Gala' 2002				
Treatment dates on IEC	5	164	0.80	0.5478
Treatment	1	164	11.75	**0.0008**
Date*Treatment (interaction)	4	164	0.69	0.6021
Treatment on firmness	1	169	0.53	0.4674
Treatment on SIV	1	163	44.13	**<0.0001**
'Gala' 2003				
Treatment on IEC	2	252	74.26	**<0.0001**
Treatment on firmness	2	240	2.95	0.054
Treatment on SIV	2	251	24.12	**<0.0001**

Ethylene concentration also differed considerably from fruit to fruit. We could not discern consistent trends on the effect of girdling plus defoliation among harvests in 2003 (P=0.63). Due to the large standard deviation among IEC samples, the effect of

girdling +defoliation was shown to be statistically significant only after logarithmic transformation of IEC data (LSD, P=0.0028). Fellman *et al*. (2003) proposed that the increase of standard error (SE) demonstrates a "breaking point" signaling the onset of climacteric. The effect of girdling plus defoliation on IEC in this study could be seen in Figure 5. The threshold of first two girdling plus defoliation appeared to be broken faster than in the attached control.

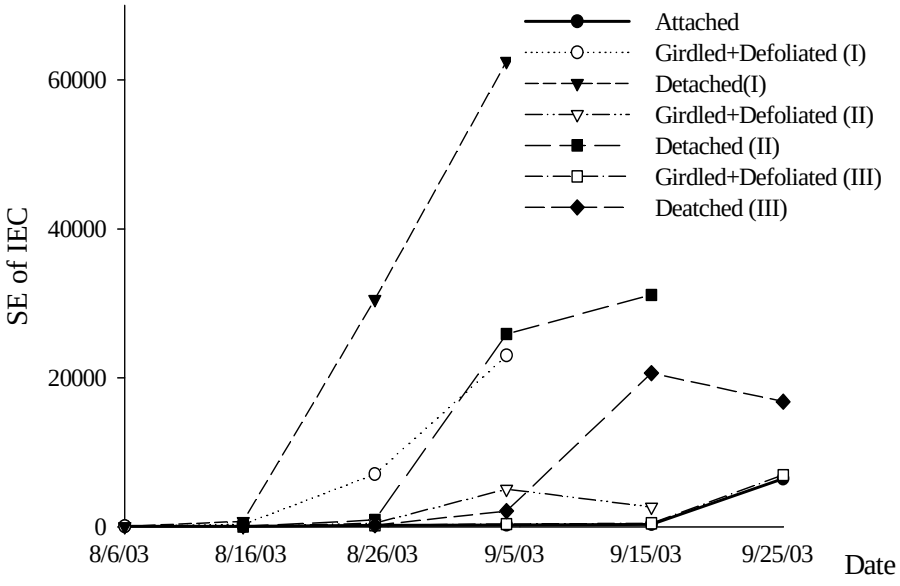


Figure 5. Standard error (SE) of internal ethylene concentration (IEC) in 'Gala' apple fruit attached to the tree, detached on the tree, and attached to the girdled plus defoliated limbs.
The increase in the SE at the date where the "threshold" was broken demonstrates the ethylene climacteric more clearly than it in the IEC.

On the other hand, the IEC remained low until late in fruit development in 'Fuji.' IEC appeared to be produced as fruits tree-ripened (Figure 2). These treatments did not markedly alter the ethylene production during ripening in either year (P=0.69). One exception to that non-significance was observed on October 10, 2002 (P=0.02). On that date, attached fruits had higher IEC levels than all other treatments (LSD, P=0.009 between attached and detached fruits, and P=0.018 for attached and girdled plus defoliated fruits).

The IEC of all tagged fruits in the orchard remained low (<500 nL/L; Figure 2). It increased dramatically one month later, on October 9. The IEC fluctuated between that date and the end of the observations forty days later on November 19 (Table 3). In conclusion, there was no significant difference of IEC taken on site between treatments, rootstock effect or interaction in 'Fuji' (data not shown).

Table 3. Changes of IEC of 'Fuji' apple fruits and ambient temperature in 2001.

Date	IEC (nl/l)	Temperature [Max/ Min; ^{o}C (^{o}F)]
9/17	355.4±44.9	26/ 4 (78/39)
9/25	263.4±21.0	18/ 7 (64/45)
10/01	261.1±34.3	22/ 3 (72/38)
10/09	669.4±110.1	16/ -5 (61/23)
10/15	1211.3±217.2	20/ 6 (68/42)
10/22	1500.1±261.2	26/ 8 (78/47)
10/29	618.3±97.6	15/-6 (59/22)
11/04	1929.3±286.4	21/ 2 (69/34)
11/13	628.2±90.3	14/ -7 (57/19)
11/19	801.7±152.0	19/ -1 (66/31)

A gradual decrease in flesh firmness occurred in both detached and attached 'Gala' apple fruits (Figure 3b). There was no significant difference in softening

among treatments. Soluble solids contents (SSC) increased as ripening progressed in attached and detached 'Gala' (Figure 3e) and in 'Fuji' apples (data not shown).

The detached and girdled plus defoliated 'Fuji' apples showed a rapid loss of starch (Figure 4b1 and 4b2; $P<0.0001$) and a steady increase of red skin color (data not shown). Better firmness retention was found in detached and girdled plus defoliated 'Fuji' apples (Figure 4a; LSD, $P<0.03$ on Oct 10 and Oct 24, 2002). Tree-ripened fruits had a slower rate of starch hydrolysis, flesh softening, accumulation of SSC, and an increased percent of red skin color during on-tree maturation and ripening.

Rootstock affected the fruit size of 'Fuji' apples harvested in 2001 (Figure 6). Fruit size (both fresh weight and diameter) from trees on M.7a was greater than in fruits harvested from trees budded on MM.111. Rootstock had no effect on firmness in this study (data not shown).

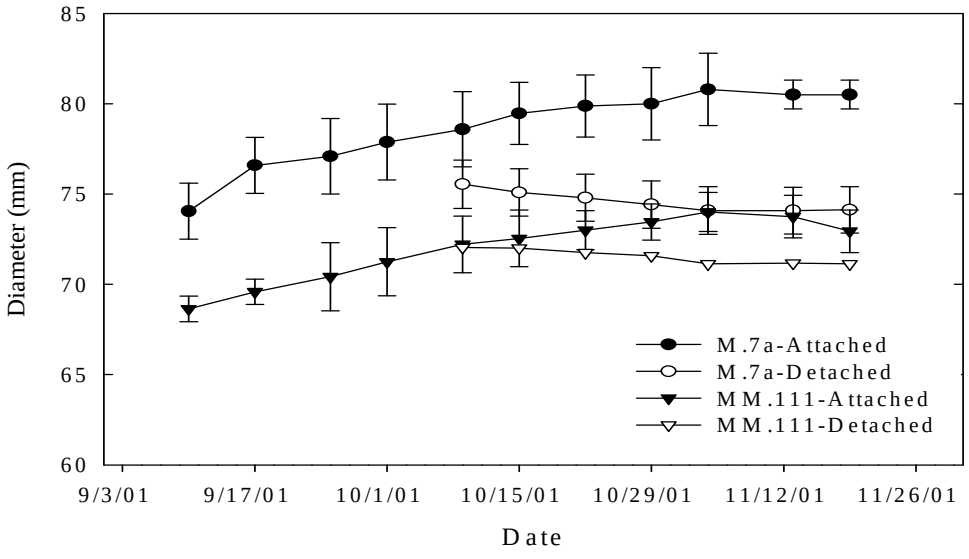


Figure 6. Change in fruit diameter of 'Fuji' apples in response to rootstock and detachment from the tree.

The ACC content measured in attached fruits was consistently greater than that measured in fruits borne on girdled+defoliated 'Fuji' apple limbs (Figure 7). Since a lower IEC was also measured in those samples, this effect on ACC content was not surprising. A statistical separation of ACC content among treatments was only found in early to mid-October in 2001. Taken together, the information of IEC and ACC content indicated that there was no triggering effect of detachment or girdling plus defoliation in 'Fuji' apple. In contrast, there might actually be a requirement of attachment for ripening promotion.

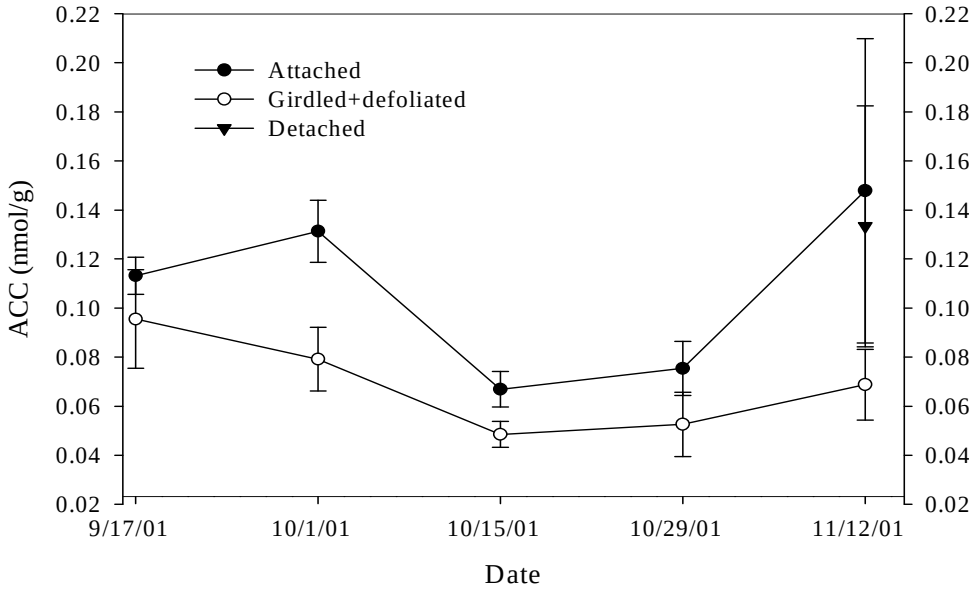


Figure 7. Change of 1-aminocyclopropane-1-carboxylic acid (ACC) in 'Fuji' apples.

Using 250 paired IEC and external ethylene evolution rate data obtained from stored 'Fuji' apples in 2002, a regression equation was calculated to compare ethylene rate and internal concentration: [IEC (nl/l)=$-49.59X^2+6689X$ (X=ethylene rate, nl/g/hr); $R^2=0.9946$] (Figure 8). This correlation appeared to be linear while the

ethylene rate was less than 25 nl/g/hr (IEC less than 140,000 nl/l). Beyond that point, the rate of increase in ethylene rate was higher than the measured change in IEC, so the regression fit a quadratic function.

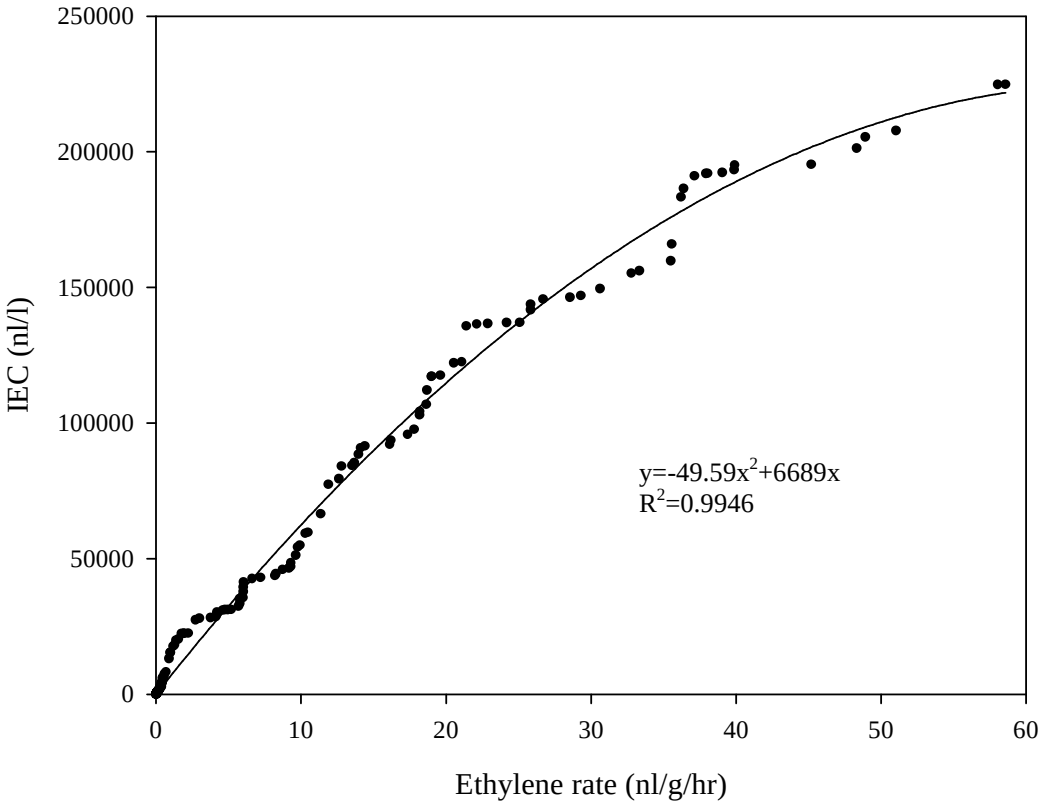


Figure 8. Quadratic regression of internal ethylene concentration (IEC) and ethylene evolution rate.

DISCUSSION:

The Role of IEC in Ripening

Internal ethylene concentration (IEC) has been used as measure of ethylene rate for many years. The IEC data were presented here using a logarithmic transformation in this study, since IEC increases curvi-linearly and a high variance of system 2

ethylene among samples could reduce the significance among treatments. A logarithmic (log_{10}) conversion of ethylene concentration may better enable the comparison to linear changes that are commonly used to gauge fruit quality (Kingston, 1991). Fellman *et al.* (2003) suggested that using the increase in the percentage standard error of the mean (SEM) of IEC at each stage of maturity could clearly demonstrate the onset of the climacteric. With maturity, a shorter lag period exists before the onset of autocatalytic ethylene production (Sfakiotakis and Dilley, 1973b; Knee, 1993). This lag period is thought to involve a progressive increase in receptivity to ethylene, eventually reaching a threshold where endogenous ethylene triggers the climacteric. This may depend on ACC biosynthesis (McGlasson, 1985; Lara & Vendrell, 2000). In addition to the IEC, the lag period before the onset of the ethylene climacteric has also been useful in representing physiological maturity.

In conclusion, using transformed data to normalize the data and minimize the variance is statistically acceptable. Applying the SE to detect the onset of climacteric as suggested by Fellman (2003) is an easy way to handle ethylene data, but might be a risky statistical approach if replications are low. There is currently no uniform threshold available to determine the onset of the climacteric in apple fruits. In this study, we employed the normal logarithmic transformed IEC to represent our data and used the SE of IEC to illustrate the effect of girdling plus defoliation in 'Gala' apples.

There was a good correlation between IEC and the ethylene production rate in 'Fuji' apples (R^2=0.9946, $P<0.05$, n=250). These data confirm that IEC is indicative of the ethylene production rate in this apple cultivar. This was reported previously by

Lau *et al*., (1986). The constant relating IEC and ethylene evolution rate was 3270 nl/l IEC per nl/l/g/hr in 'Golden Delicious' apple. In this study we found a quadratic relationship due to an apparent change in slope that occurred as IEC increased in 'Fuji' apples. A lower constant rate was obtained in fruits with IECgreater than 140,000 nl/l (ethylene evolution rate greater than 25 nL/L/g/hr). The detected external ethylene evolution rate is not dependent on the rate of biosynthesis in fruit tissue and also on gas diffusion rates. The positive correlation between cuticular wax and internal ethylene in 'Delicious' apples has been reported previously (Ju and Bramlage, 2001). However, the reduction in IEC expected found at higher ethylene production rates suggests that the waxy cuticle may not be a barrier to ethylene diffusion.

Tree Factor

The ethylene climacteric in apple fruits has been shown to be delayed for one to two months after commercial harvest if the fruit remains attached to the tree (Sfakiotakis and Dilley 1973a; Lau *et al*., 1986). The tree factor was thought to be produced in the leaves and transported *via* the phloem to the fruits (Sfakiotakis and Dilley, 1973a). Their hypothesis was based on the stimulatory effect of defoliation and girdling of spurs had on ethylene concentration in attached apple fruits. To determine the existence of "tree factor" in apple, ripening processes of two cultivars with girdled+defoliated, detached, and attached treatments were followed throughout the period of maturation and ripening on the tree.

1. The effects of detachment and attachment on fruit ripening

The physiology of an attached fleshy fruit differs from detached fruit in several respects. Continuous import of photosynthate from the plant occurred, though this may decline as ripening proceeds. In addition, plant growth regulators and other substrates are also transported to fruit. Consequently, the ripening behavior of attached fruits might also be mediated *via* a response of the tree to its ambient environment. Detachment from the parent-plant has been suggested to trigger the onset of system 2 ethyleneduring ripening in avocado (Bower and Cutting, 1988), apple fruits (Wilkinson, 1963; Sfakiotakis and Dilley, 1973a; Lau, *et al.,* 1986), purple passionfruit (Shiomi*et al* ., 1996), saskatoon (Rogiers and Knowles, 1999), and plum (Abdi *et al*., 1997).

Our results indicated that the autocatalytic ethylene production of attached 'Gala' apples was delayed, and of lower magnitude than in detached apples. The delay in the onset of massive ethylene production in attached tree-ripening 'Gala' apple supported the existence of a tree factor. Lau *et al*. (1986) reported the same stimulatory effect of detachment occurred in 'Golden Delicious' apples harvested and held in the laboratory at 20^{o}C. Since the field environment was used for all treatments in this study, our results provided solid evidence to support the existence of differences in IEC between attached and detached fruits is not a response to different temperatures.

However, two-year results in 'Fuji' apple did not show similar tree factor results. 'Fuji' demonstrated the slightly-opposite effect to that reported in 'Red Delicious' (Sfakiotakis and Dilley, 1973a), 'Golden Delicious' (Lau *et al*., 1986), 'Jerseymac' (Blanpied, 1993) and 'Gala' (this study). There was no difference in IEC detected

among ‘Fuji’ treatments except on one date at the starting point of commercial harvest. Attached fruits actually yielded the highest measured IEC on that date. A similar negative effect of detachment has been reported on Charantais melon, where detached fruits produced less ethylene than attached fruits (Bower *et al*., 2002).

It is worth mentioning that the detached fruits in this studywere hung in the tree canopy. This would not only to mimic the environmental condition of attachment (Wilkinson, 1963), but also to reduce the potential for cross contamination by ethylene that occurs in storage (Knee, 1993). Severe environmental stresses such as water and heat stresses could still affect detached fruits in the canopy. Consequently, the possibility of stress-induced system 1 ethylene that then stimulated the onset of autocatalytic system 2 ethylene upon detachment can not be eliminated.

2. The effect of girdling plus defoliation on fruit ripening

Smock (1972) pointed out the defoliation and phloem removal of the fruit pedicel caused stimulation in the climacteric respiration rise (as measured by CO_2 production) in one-half of his experiments involving seven cultivars of apple fruits. The dramatic, stimulation of girdling plus defoliation treatment on ethylene production was based on limited data, from only four ‘Red Delicious’ replicate fruits (Sfakiotakis and Dilley, 1973a). Their report of increasing ethylene production in attached apples borne on girdled and defoliated spurs may be suspect due to limited replication. Blanpied (1993) later reported that the lag periods in ethylene caused by limb ringing to >1 ppm IEC or to apple fruit drop were positively related to leaf area per fruit in ‘Jerseymac’ and ‘Paulared’ apple fruits. Blanpied’s research (1993)

supported the existence of tree factor in apple fruits and suggested a positive correlation existed between leaf area and the amount of tree factor. In this study, we applied girdling to whole limbs instead of pedicels or spurs to allow long term observation of treated fruits. Girdling limbs in that manner is easier to perform, and is less likely to cause undetected pedicel damage, but readily removes the phloem.

The reduction of fruit growth and the rapid loss of starch on girdled+defoliated apples of both cultivars suggested that this technique was effective. In theory, the detachment of fruits or the girdling and defoliation of limbs would both block signals transported *via* phloem from the 'parent-tree' to the fruit. Although girdling plus defoliation successfully blocked the carbohydrate supply from parent-trees, its effect on ethylene concentration did not equal the effect of detachment. Only a partial enhancement of girdling plus defoliation on IEC was found in 'Gala' and no effect (or perhaps an inhibitory effect) was observed in 'Fuji.' Eventually, all fruits initiated the climacteric, regardless of treatment. From these results we inferred that other variables, such as environmental conditions, may be more important in triggering 'Fuji' apple ripening.

3. Conclusion

Our results revealed that the difference in ethylene production between the fruits on and off the tree may be related to a tree factor. As no other ripening-related processes such as firmness and color change stimulated by detachment, attachment may only serve to suppress ethylene production and starch degradation. Similar patterns have been indicated previously in 'Golden Delicious' apple fruits (Lau, *et al.*,

1986), and plum (Abdi *et al*., 1997). Tree factor may also only be more likely to be found to exist in early apple cultivars. This research implies that there may not be a tree factor in 'Fuji' apples, an extremely late-maturing cultivar. The partial stimulatory effect of girdling plus defoliation in 'Fuji' actually suggested that the tree factor might also be transported *via* the xylem as postulated by Smock (1972). It may act to suppress ACO and delay the biosynthesis of ACS (Lau, *et al.,* 1986).

Most hypotheses about the tree factor have emphasized the role of an inhibitor suppressing ethylene production of attached fruits until they mature. However, the modulation of ethylene production as it shifts from system 1 to system 2 is from one of suppression to promotion. The existence of enhancer stimulating system 2 ethylene production in detached or girdled+defoliated fruits in some apple cultivars may be an equally tenable hypothesis. The wounding stress caused by harvest coupled with nutrient and water deficiency shocks inducing system 1 ethylene can not be eliminated (Rogiers and Knowles, 1999). These might be sufficient to promote system 2 ethylene in mature fruits (Liu, 1978; Knee, 1988; Lara and Vendrell, 2000).

With that nutritional shortage, the lag period before climacteric ethylene could be explained by a positive correlation with the leaf/fruit ratio on girdled limbs in Blanpied's study (1993). Detached fruits, like newborn babies, need to establish their own survival mechanism. The upsurge in ethylene, just like the first cry, can not only help fruits produce more energy by increasing their respiratory activities but also coordinate thatripening .

This would complete their fruits' natural mission – becoming attractive to seed vectors. Over-mature attached fruits can be stimulated toward system 2 ethylene production by simple stresses such as insect attacks (personal oberservation) or pathogen invasion (Rogiers and Knowles, 1999). The eventual rise in ethylene production that occurs during tree-ripening may be interrelated to fruit senescence. It occurs at the onset of system 2 ethylene. To fit this into the parent-plant hypothesis phloem and xylem flow would be viewed as two alternative tree factors. As the fruits become more mature, their sink strength could become weaker. As the tree factor also gets weaker, and the climacteric is initiated. A similar concept was suggested by Burg and Burg (1965).

Avocado fruits are the classical parent-plant study of tree factor. Attached avocado fruits have been shown to be equally sensitive to exogenous ethylene as detached fruits. An altered ethylene evolution pattern in attached avocado was also affected by long-term preharvest stresses (Bower and Cutting, 1988). This hypothesis of a stimulatory effect of stress with detachment could also to explain the responses to attachment and detachment in avocado.

Comparing the patterns of detachment and girdling plus defoliation, it is possible to classify apple cultivars into two groups. These were stimulated by the treatment or those had no effect. Most early and mid-season cultivars are stimulated bythese treatments. Late varieties were not. The availability of photosynthates, water and mineral nutrients tends to be more critical for the shorter growing periods in early and

mid-season cultivars. In these cultivars, the sink strength during maturation would be greater during maturation than in late-season cultivars.

Sunako *et al*. (1999) surveyed many apple cultivars and divided them into three categories based on the combination for the ACS1 alleles. The parents of 'Gala' ('Cox's Orange Pippin', 'Delicious' and 'Golden Delicious') were either ACS1-1 homozygous or ACS1-1/ACS1-2 heterozygous. They were able to produce a higher amount of ethylene during ripening than mutated ACS1-2 homozygotes. Late cultivars that had the slowest rates of softening and good, long-term storage properties and/or produced little system 2 ethylene were homozygous for ACS1-2. 'Fuji' and its female parent, 'Rall's Janet' are examples of that genotype (Harada *et al*., 2000; Oraguzie *et al*., 2004). It was also concluded that ACS1-2 homozygous fruits produced lower levels of ethylene at the climacteric stage and had less fruit drop than ACS1-1/1 or ACS1-1/2 genotypes (Sunako *et al*., 1999; Sato *et al*., 2004). Oraguzie *et al*. (2004) indicated that 'Gala' was also one of ACS1-2 homozygous cultivars according to its PCR banding profile.

Our IEC data revealed that 'Gala' and 'Fuji' were not likely to be classified into the same category except in one valuable horticultural characteristic: reduced preharvest fruit drop. Therefore, sorting differential IEC among cultivars solely by ACS1 alleles may not always be appropriate. There is a need for additional information about ACS alleles in apples and how this affects differences in quality and abscission between 'Gala' and 'Fuji'.

Other Changes that Occurred during Ripening

Ripening processes took place gradually in tree-ripening apples. Though the higher ethylene concentration of early-detached fruit might coordinate some ripening processes, the quality of these fruits has been decided before harvest. Ethylene does not act equally on all ripening processes. The onset of starch hydrolysis preceded the large increase seen in ethylene concentration. Similar findings were reported for 'Golden Delicious' (Lau *et al*., 1986), 'Jerseymac' (Blanpied, 1993) and in cultivars such as 'Delicious', 'R.I. Greening', and 'MacIntosh' (Blanpied, 1993). Flesh softening is not necessarily associated with the onset of climacteric ethylene either (DeEll *et al*., 2001). Red skin color does not depend solely on ethylene but also is under developmental control, and is affected by solar radiation and temperature. On the other hand, Wang and Dilley (2001) reported that application of aminoethoxyvinylglycine (AVG, applied as ReTain) significantly delayed red color development, starch conversion and slightly reduced firmness loss in 'Gala' apple. In conclusion, the progress of maturity indices of naturally ripened fruits does not appear to be mediated by the final (climacteric) rise in ethylene synthesis, but by a lower critical level of ethylene that is present earlier in maturation. Detachment and girdling plus defoliation stimulated starch degradation, whether or not IEC was affected. 'Gala' apples also softened equally among treatments. Conversely, detached and girdled+defoliated 'Fuji' apples were firmer in comparison to attached fruits although no significant IEC differences were measured among treatments. Tree-ripening 'Gala' apples had the highest percentage of red skin color. No such response was found in 'Fuji'.

Soluble solids content (SSC) increased and starch content decreased steadily as natural ripening progressed in both cultivars. Detachment and girdling plus defoliation triggered a dramatic increase in SSC in the early stage of maturity, but had only a minimal effect at end of the season. In 'Fuji,' the first detached and girdled+defoliated treatments showed a transiently higher level of SSC than attached fruits. The stimulation of starch hydrolysis would be the primary sourcefor increased SSC in detached and girdled+defoliated apple fruits. Since respiration also uses carbohydrate as substrates, it also affects SSC. The highest SSC was measured in tree-ripened 'Gala' apples. The continued transport of photosynthate from the parent-tree to the fruit coupled with starch hydrolysis contributed to this high levelof SSC. Detachment and girdling plus defoliation treatments affected starch patterns similarly in both cultivars. Starch hydrolysis proceeds from the core toward the skin based on the staining pattern found by I_2-KI. Fan *et al*. (1995) postulated that amylose reacts more intensely with I_2-KI solutions than does amylopectin. Therefore, it is possible that a change instarch index may indicate a change in the relative proportion of amylase hydrolysis or in total starch level. This might be the case of 'Fuji.' Fan's group (1995) reported that most of the starch is found as amylopectin during late maturation period. The higher portion of amylopectin in 'Fuji' apple might explain the lack of perfect correlation between the starch index values and SSC readings. Since residual amylopectin exists in ripening 'Fuji' apple, the values of starch degradation measured by I_2-KI wouldnot account for total increase in SSC. In conclusion, starch pattern and SSC in apple fruits during maturation and ripening are associated with basic carbohydrate metabolism, but they can differ among cultivars.

Acceleration of the onset of ethylene production by detachment was cultivar-dependent. The difference of ethylene production between 'Gala' and 'Fuji' fruits did not affect starch (or amylose) hydrolysis. In both cultivars, starch was degraded more rapidly in detached and girdled+defoliated treatments than in attached fruit. These data indicated that starch degradation cannot be attributed solely to the increase in ethylene production that occurs during maturation and ripening.

Field data measuring firmness loss during ripening indicated that fruit firmness may to be under genetic and environmental controls. Although ethylene concentration in detached 'Gala' apple fruits was 10 to 100-fold greater than in attached apple fruits, a steady loss of firmness proceeded independently of treatment. On the other hand, attached 'Fuji' fruits appeared to soften more rapidly than did detached fruits. Reports of a similar reduction in softening rate in harvested fruits vs. in attached fruit have been postulated in 'Golden Delicious' (Lau *et al*., 1986). 'Fuji' fruits had a better retention of firmness than 'Gala' fruits. The original flesh firmness measured in 'Fuji' was lower than that measured in 'Gala'. Consequently, 'Gala' fruits showed a much more dramatic change in firmness during maturation and ripening. Lelièvre *et al*. (1997) reviewed the rate of fruit softening in transgenic tomato fruits with different residual ethylene production rates. They concluded that fruit softening was quite insensitive to ethylene. However, those data also revealed that fruits with a low amount of residual ethylene (3-10 % of wild-type production) were able to soften steadily during ripening. Those transgenic tomato fruits were unable to demonstrate the typical rapid decline of firmness when over-ripen. Our research

demonstrates that apple fruit firmness loss appears to occur at a low IEC threshold that initiates softening during maturation and tree-ripening.

The onset of climacteric ethylene did not directly affect red skin color development in these apple cultivars. Similar findings have been reported on purple passion fruit by Shiomi *et al*. (1996). It is well-documented that anthocyanin accumulation is primarily dependent on environmental factors. Warm, sunny days, and cool nights before harvest enhance red color development in the orchard. The degree of red blush is not a suitable maturity indicator as its development can vary between fruit position in the tree, orchard, season, and geographic location.

In this study, we observed that the first girdling plus defoliation treatment on mature-green 'Gala' fruits accelerated flesh softening and increased the percentage of red skin in comparison to detached or attached fruits at 10-20 days after treatment. There was no difference after 30 days. Without the protection of adjacent leaves and evaporative cooling, the exposure to sunlight would increase the fruit temperatures. This transient "side effect" of enhanced red color (other than IEC) found at the earliest girdling plus defoliation treatment was also seen in 'Fuji' apples. The peel at the "mature-green" stage appears to be more sensitive to solar radiation penetration than latter stages of development. Even so, in 'Gala', the percentage of red skin measured in detached and girdled plus defoliated fruits was less than in attached fruits. This did not occur in 'Fuji'. It appears that some cultivar-dependent leaf products are required for anthocyanin biosynthesis and red color development.

Aroma volatile synthesis is quite maturity-dependent. It usually occursas the respiration and ethylene production rates increase in apple fruits (Song and Bangerth,

1996; Rudell *et al.*, 2000). It has been reported that fruits harvested long before attaining optimum maturity produce far fewer flavor-imparting volatiles than those harvested later (Song and Bangerth, 1996; Lalel *et al*., 2003). Rudell *et al.* (2000) reported that the production of twoflavor volatiles, butyl acetate and 2 -methylbutyl acetate, increased as the IEC increased from the low level found in immature fruits to 3000-4000 nl/l in mature 'Fuji' apples. It appears that an increase in autocatalytic ethylene production and respiratory activity may be essential for the optimum increase in aroma volatile production. The upsurge of climacteric ethylene as a coordinator of ripening likely serves to facilitate a rapid and synchronized synthesis of aroma compounds.

Predicting Optimum Harvest Date

Maturity at harvest is the most important factor that determines storage-life, postharvest quality and softening rate during storage. To ensure optimal fruit quality, suitable indicators of harvest maturity are needed. In this brief study, calendar date appeared to be a relatively-reliable guide to selecting the optimum harvest date. Calendar date appeared to be even more appropriate for 'Fuji' than 'Gala.' Considering that environmental and cultural conditions fluctuate between seasons, and that canopy position affects maturity, standardized sampling techniques are recommended for harvest date prediction (Kingston, 1991). He suggested that the best prediction of harvest date should select an optimum number of fruits from each tree. Thirty fruits per tree appeared to be optimal in his study. We detected a very large variance in IEC with only twelve replicationsper treatment . With non-significant differences between treatment times, it was possible to sum all

experimental units (36). Interestingly, the significant level of treatment effect on IEC before and after pooling samples was still similar.

For both ‘Gala’ and ‘Fuji’ apples, IEC could be used as a very suitable maturity indicator for predicting commercial harvest. The primary plateau of the IEC indicated thatcommercial maturity had been achieved in these cultivars. Taken together, the first increase and plateau of IEC could be used as a maturity marker, which apparently predicts the onset of system 2 ethylene biosynthesis in attached apple fruit.

In conclusion, optimum harvest periods of ‘Gala’ and ‘Fuji’ apples in Maryland are readily predicted by calendar date. IEC and starch pattern can be the suitable indices to allow maturity to be determined with greater precision. The first plateau/ increase of IEC may give the best prediction of the approach of commercial maturity and harvest and correlated with Fellman’s hypothesis (2003) of harvesting as IEC variability increased.

The Quality of Tree-Ripened Fruits

During maturation, fruit flavor and aroma increase while their storage potential decreases. Harvest decisions are a compromise between quality and storageability of fruit. “Dessert quality” is used to describe appearance and an integral aspect along with the flavor perception of a fruit and is greatly influenced by the stage of fruit maturity at harvest (Fellman *et al*., 2003). Tree-ripened fruit has a relatively low IEC. It has a superior organoleptic dessert quality including the aroma and texture

needed for immediate consumption. With the potential loss of aroma volatiles that happens during storage, tree-ripening is preferred for direct marketing (Fellman *et al*., 2003; Song and Bangerth, 1996). Quality of tree-ripening fruits is influenced by their communication with parent tree, and environmental conditions. Tree-ripened fruits are exposed to sunlight and day/night fluctuations in temperatures for ten to thirty days longer than fruits harvested at commercial maturity for storage. In the case of tree-ripened 'Gala' and 'Fuji', the full development of red skin color, high SSC with residual starch, and the highest aroma volatiles are desirable.

Is Chilling Required in 'Fuji' Apples?

The IEC measurements made on site at ambient orchard temperature in year 2001 were generally lower than IEC taken from fruits stored overnight at 20°C in year 2002. All maturity indices progressed as expected with the exception of IEC in 2001. That fall, the first frost occurred on October 9. Following that, fluctuations of IEC appeared to correlate positively with ambient temperatures (Table 1). 'Fuji' apple has been reported to be a chill-requiring variety, and consequently IEC would be affected by ambient temperature. In the following year, mature 'Fuji' apples harvested in late August, 2002 were capable of responding to propylene exposure before receiving any chilling in the field (see Appendix).

Jobling and McGlasson's study (1995) reported that 32 days of storage at 0^{o}C in preclimacteric 'Fuji' apples harvested at 3 weeks prior to commercial maturity could induce an increase in IEC on their return to 20^{o}C. They suggested that 'Fuji' apples are susceptible to rapid ripening if stored at temperatures below 10^{o}C. Fruit cultivars

with a chilling requirement for induction of ethylene synthesis can actually remain preclimacteric for several weeks at 20°C (Knee *et al*., 1983). For chill-requiring pears, the cold requirement cannot be replaced by exogenous propylene or ethylene treatment. For intermediate pears, cold treatment is not required for ripening but may be helpful for promoting a rapid and coordinated ripening on their return to a higher temperature (El-Sharkawy *et al*., 2004). El-Sharkawy *et al*. (2004) proposed that the differential expression of alleles in the ACS gene family could be a critical marker differentiating between cold-dependent and cold–independent cultivars.

Our data suggested that 'Fuji' apple may be classified as cold-intermediate cultivar that is capable of ripening without a cold pretreatment. The system 2 ACS genes might have developed as cold- or ethylene-inducible by evolution and by selection. Chilling can promote uniform ripening of 'Fuji' as reported by Jobling and McGlasson (1995).

In the field, fruit size was the only variable influenced by rootstock. Most fruits harvested at commercial maturity from M.7a trees had initiated theethylene climacteric but fruit harvested at the same time from MM.111 trees had not. Differences in fruit quality such as fruit firmness, SSC, and fruit size can be attributed to growth habit, crop loading, and tree size, which were thought to cause changes in mineral composition (Drake *et al*., 1988; Autio, 1991). The onset timing of climacteric ethylene and storability of apple fruits have been affected by rootstock (Drake *et al*., 1988; Autio, 1991; Fallahi *et al*., 1985). Our data demonstrate that rootstock did affect 'Fuji' ripening by delaying the auto-stimulatory effect of ethylene production.

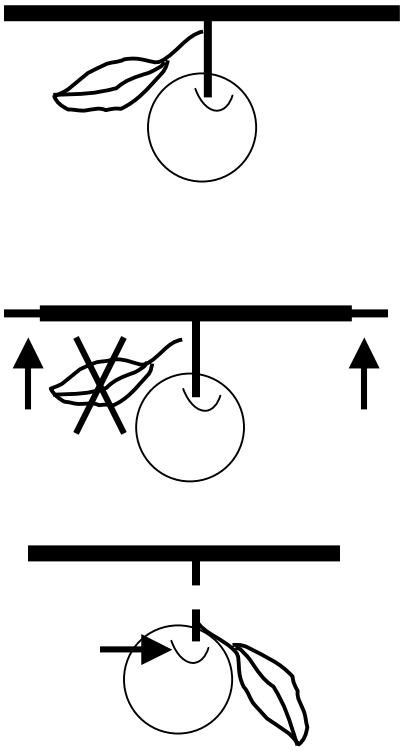
Concluding Remarks

Detachment clearly stimulated ‘Gala’ system 2 ethylene biosynthesis. It was difficult to ascertain whether the loss of “tree factor” influenced IEC directly or indirectly by inducing stresses in these fruits. Plant organs typically maintain a low level of ethylene production throughout their life. Fruit ripening on the tree can occur at a lower ethylene concentration than occursafter the fruit has been picked. The evolutionary function of the fruit is in seed dispersal. Greater ethylene production by fruits removed from the tree may initiate defense systems, and coordinate the ripening processes needed to attract seed-vectors. Unstressed fruits would maintain a lower ethylene production as long as they remained attached to the tree. The diagram in Figure 9 summarizes the treatment effects found in this study.

A ripening summary comparing ethylene production and horticultural maturation among developmental stages ranging from mature-green, commercial maturity and tree-ripened fruits was used to establish a general physiological background between IEC and other maturity indices. A summary of the major developmental changes in ‘Fuji’ and ‘Gala’ apples during the commercial harvest window is shown in Figure 10. These were drawn by summarizing two years of observation at Keedysville using *SigmaPlot* 8.02 (SPSS Inc).

Like pears, late-season apple cultivars such as ‘Fuji’ and ‘Granny Smith’ require a chilling treatment for ripening (Jobling and McGlasson, 1995). This does not appear to affect early and mid-season cultivars, such as ‘Gala,’ ‘Red Delicious’ and ‘Golden Delicious’. These cultivars have no apparent cold requirement. Cold tolerance and an extended developmental period may be needed for the maturation and ripening of

long-season apples. ‘Fuji’ apple and ‘Passe-Crassane’ pear have been found to possess different ACS alleles and differential gene expression in comparison to non-cold-requiring varieties (El-Sharkawy *et al*., 2004).



Attachment:
Fruit continuous growth
Increasing steadily soluble solids and starch slowly hydrolyzed
Ethylene production increased in a sigmoid pattern

Girdling plus defoliation:
Enhancing starch degradation
Partial triggering of ethylene production

Detachment:
Enhancing starch degradation
Triggering ethylene production

Figure 9. **A summary of treatment effects on ‘Gala’ apple fruits.**

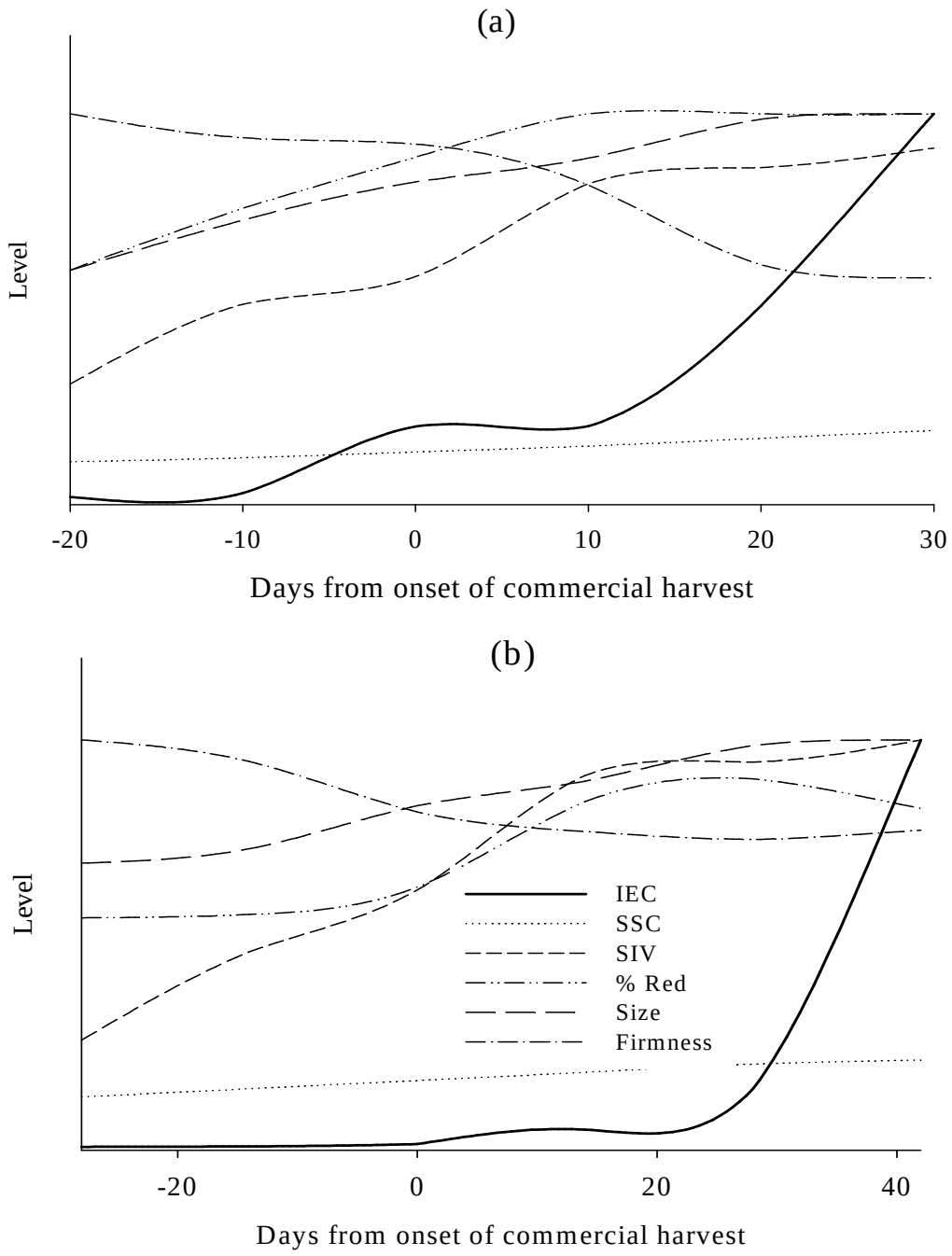


Figure 10. Developmental changes during 'Gala' (a) and 'Fuji' (b) apple fruit maturation and tree-ripening.

Relative changes in internal ethylene concentration (IEC), starch indices value (SIV), percent red color of skin (% red), weight, soluble solids contents (SSC, %), and firmness are shown before, during and after the commercial harvest period. In 'Gala', six time points at 10 day intervals are shown. They represent stage I to stage VI, respectively.

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Chapter2: Transcript Profiling During the Transition from Maturation to Ripening in 'Gala' apples.

ABSTRACT:

To investigate differential gene expression during fruit maturation and ripening, cDNA-AFLP (Amplified Fragment Length Polymorphism) was used for transcript profiling. Thirty five primer combinations were done. Two hundred and four differentially expressed transcript-derived fragments (TDFs) were isolated from 'Gala' apple. One hundred sixteen TDFs represented up-regulated genes, twenty five were down-regulated, and the others were induced or suppressed transiently during fruit development and ripening. The majority of TDFs had significant similarity to genes in other species and included genes identified in defense and stress responses, cell wall hydrolysis and degradation, pigment production and aroma biosynthesis. Additional transcripts associated with protein biosynthesis/ degradation, intracellular trafficking and sorting, cell structure and mobility, and carbohydrate, lipid, and protein metabolism were also isolated. Sixty cDNA-AFLP fragments had similarity with genes of unknown function, novel apple ESTs or represented novel sequences in the public GenBank database. The expression patterns of subsetTDFs wer e verified using microarrays and Northern blots. More than half of differential expression patterns observed by cDNA-AFLP were confirmed by Northern and microarray assays. Apart from providing observation on gene expression profiles and related metabolic pathways during apple fruit maturation and ripening, this study has developed candidate genes for future study.

INTRODUCTION:

Apple (*Malus domestica*) fruits undergo many biochemical, physiological, and structural changes during maturation and ripening. Fruit maturation is defined as the process of transforming from juvenile stage to ripening. It is the adult phase of fruits where enclosed seeds are ripe for the continuing of generations. Apple fruits are classified as climacteric fruits. The ripening process of this annual pome fruit crop is regulated by genetic, developmental, hormonal and environmental signals that lead to dramatic changes in color, texture, flavor, and aroma of the fruit flesh. It is generally accepted that ethylene, "the fruit ripening hormone", regulates climacteric fruit ripening. However, exogenous ethylene did not induce normal ripening in transgenic tomato fruits that lacked the capacity for ethylene biosynthesis or ethylene perception (Giovannoni, 2001 and 2004). A developmental competence to respond to ethylene appears to also be needed for ripening in tomato, another climacteric fruit. It has been suggested that climacteric fruit ripening is regulated by developmental factors which is coordinated byethylene synthesis (Adams-Phillips *et al*., 2004; Giovannoni, 2004).

Changes in gene transcript levels during development, maturation and ripening of a variety of fruit species including tomato, *Arabidopsis,* grape and strawberry are well-documented (Giovannoni, 2001 and 2004; Davies and Robinson, 2000; Aharoni and O'Connell, 2002). Due to the availability of ripening mutants and a short growing cycle, *Arabidopsis* and tomato have been used as model plant species for dry dehiscent fruits and fleshy fruits respectively (Moore *et al*., 2002). The progressive development of genomics tools has lead to an enhanced understanding of the

molecular basis of ethylene-derived ripening. Studies of cell wall hydrolases and wall metabolism (polygalacturonase, pectin methyl esterase, cellulase and β–galactosidase), and the role of light in fruit carotenoid accumulation have been reported (White 2002, Giovannoni, 2004). An examination of the biochemical and molecular changes in climacteric (tomato) and non-climacteric (strawberry) ripening suggests that a common regulatory cascade may operate in these fruits (White 2002; Giovannoni 2000 and 2004). Although some gaps between certain ripening connections remain undiscovered (Giovannoni 2000, 2004), knowledge from tomato has led to the use of differential screening techniques to improve our understanding of ripening in many other plant species.

cDNA-AFLP (Amplified Fragment Length Polymorphism) is an efficient tool for gene discovery, and for the systematic analysis of genes involved in a particular process (Bachem, *et al*. 1996; Breyne and Zabeau 2001; Jones *et al*. 2000; Reijans *et al*., 2003). This PCR-based technique combines high sensitivity and specificity, and has proven useful for genome-wide expression analysis in species where preexisting biological or sequence information does not exist (Bachem, *et al*. 1996; Breyne and Zabeau, 2001; Jones *et al*. 2000). The distinctive feature of cDNA-AFLP is that it ensures referential amplification of a limited number of fragments with fully complementary sequences. Moreover, cDNA-AFLP has been shown to provide polymorphic transcripts as genetic markers specifically targeted to transcriptionally-active regions in mapping (Brugmans *et al*. 2002). The enormous amount of sequence information found in the expressed sequence tags (ESTs) and whole

genome databases are providing researchers with new tools such as cDNA-AFLP to dissect developmental processes.

'Gala' is a precocious, high-quality cultivar with a sweet flavor, and crisp, firm texture while ripe. Fruit ground color is yellow with a red-striped surface color as it matures and ripens. This occurs in mid- to late- August in Maryland. As little fruit abscission occurs between the beginning of harvest and the end of the growing season, commercial harvest can be delayed until fruits attain optimum size and quality. This extremely popular cultivar can be induced to ripen readily and produce high levels of ethylene production by detachment. Since it is not prone to pre-harvest drop, it was selected to study the role of detachment and attachment to the mother-tree during the period of maturation and ripening.

Limited studies have characterized in gene expression as pome fruits proceed from pre-climacteric to post-climacteric development. This is in part due to the lack of genetic mutations that can be studied and the tree's annual fruiting cycle. Lay-Yee *et al.* (1990) first reported that a rapid accumulation of transcripts and proteins took place in the early stages of ripening. During this time the IEC increased from 0-0.1μL/L to 1-5 μL/L. A relationship between fruit ripening and changes at mRNA and protein levels was demonstrated in 'Golden Delicious' apple fruits (Lay-Yee *et al.*, 1990). However, no further studies on maturation were reported. It is worthwhile to construct a ripening model based on identified transcriptional profiles to gain a greater understanding of the mechanisms involved Knowing the associated gene expression pattern is helpful to connect the relationship from transcripts to known physiological changes. Interesting traits obtained can be expected to facilitate

more putative prediction to regulate fruit maturation and ripening, and also provide additional candidates for the fruit quality improvement.

In this project, an investigation of differential gene expression at various stages of fruit development using cDNA-AFLP wasused to study the processes of apple fruit maturation and tree ripening. TDFs identified by usingcDNA AFLP were sequenced and abundance during ripening verified by cDNA microarray.

This chapter describes the application of RNA-fingerprinting, using cDNA-AFLP from ripening apple fruit. Using this method, 204 cDNA gene tags were discovered and characterized. These were derived from genes that were differentially-regulated during 'Gala' fruit maturation and ripening. These techniques revealed a number of novel, ripening-related genes. Most expression patterns of these genes were confirmed by cDNA microarray, and a few by Northern blot analysis. The potential roles for these gene products provide valuable insights in the processes that underpin fruit ripening. Surprisingly, their putative functions revealed that these differentially-expressed genes were highly conserved among species such as grape (Davies and Robinson, 2000), strawberry (Aharoni and O'Connell, 2002) and apple (this study). Functional groups that have been associated with differential screening profiles during ripening, such as cell wall disassembly, production of aroma and flavor compounds, stress responses, and the enhanced turnover of nucleotides, proteins and primary and secondary metabolites were found. It is worth mentioning that many similarities thought to be involved in stress-related responses also accumulated in ripening 'Gala' apples. This may indicate that the ripening process represents a type

of stress, or that genes involved in response to stresses are also function during ripening.

MATERIALS & METHODS:

Plant Material and Treatments

Apple trees *(Malus domestica* Borkh. cv Gala) grown in the Western Maryland Research and Education Center in Keedysville, MD and the USDA-AFRS in Kearneysville, WV were selected for this study. Fruits were harvested and analyzed at 8 to 12 days intervals during development from "mature green" (marketable sized, unripe fruit with mature seeds) to tree ripe and overripe. These are presented as stage I to stage VI ranging from July 31 to September 20, 2002, and from August 6 to September 25, 2003 (see Table 4). Additional fruits of mature-green, early mature to commercial mature stages (stage I-III) were detached and then hungin nylon stockings on the limbs in the canopy in attempt to ensure a similar environment to that of attached fruits for 10, 20, and 30 days which are labeled as I+10, I+20, I+30 and II+10…etc. in this study (for example, see Table 4). These detached and untreated control (attached) fruits were brought back to the laboratory and held overnight at room temperature (20 ºC). Fruit maturity indices including internal ethylene concentration (IEC), visual red color percentage, fresh weight, fruit diameter, soluble solids content, flesh firmness, and a visual assessment of the starch index (Chapter 1) were monitored the day after harvest. Cortical tissue of fruit was frozen and in liquid nitrogen and stored at -80˚C for RNA extraction.

Table 4. The defined developmental stages of 'Gala' apples in this study.

Stage	Harvest date	Definition
Stage I	July 31, 2002 August 6, 2003	Mature-green, about 20 days before commercial harvest; 100-110 days after full bloom (DAFB)
Stage II	August 8, 2002 August 15, 2003	Early mature, about 10 days before commercial harvest; 110-120 DAFB
Stage III	August 20, 2002 August 25, 2003	Commercially mature, starch index value=3~4; 120-130 DAFB
Stage IV	August 29, 2002 September 4, 2003	Late mature; 130-140 DAFB
Stage V	September 12, 2002 September 15, 2003	Tree ripe; 140-150 DAFB
Stage VI	September 26, 2002 September 25, 2003	Ripe to overripe, starch index value close to 8; 150-160 DAFB
Stage I+10	Detached on July 31, 2002; Harvested on August 8 Detached on August 6, 2003; Harvested on August 15	Picked at Mature-green stage and hung in the canopy for ten days before harvested
Stage I+20	Detached on July 31, 2002; Harvested on August 20 Detached on August 6, 2003; Harvested on August 25	Picked at Mature-green stage and hung in the canopy for twenty days before harvested
Stage I+30	Detached on July 31, 2002; Harvested on August 29 Detached on August 6, 2003; Harvested on September 4	Picked at Mature-green stage and hung in the canopy for thirty days before harvested

Extraction of Total RNA

Total RNA was extracted from frozen apple cortical tissue using a modification of the method described by Chang *et al*. (1993). Ten grams of frozen tissue was ground

to a powder in liquid nitrogen with a mortar and pestle, and suspended in 30 ml of suspension solution I (85% ethanol, 0.05% SDS, and 0.05% $Na_2O_5S_2$). Following centrifugation at 14,000 rpm for 15 min, the pellet was re-suspended in 30 ml of suspension solution II (75% ethanol, 0.05% SDS, and 0.05% $Na_2O_5S_2$). After a second centrifugation, the pellet was dissolved at 65˚C in 15 ml extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCl, 25 mM EDTA, 2.0 M NaCl, and 0.5g/l spermidine, Chang *et al*., 1993). An equal volume of chloroform was added and mixed vigorously then centrifuged at 10,000 rpm for 20 min at 20˚C. The aqueous phase was removed, and the chloroform extraction was repeated. RNA was then precipitated by the addition of a quarter volume of 10 M LiCl at 4˚C overnight. Precipitated RNA was recovered by centrifugation at 10,000 rpm at 4˚C for 30 minutes, and the RNA pellet was dissolved in 500 µL SSTE (1.0 M NaCl, 0.5% SDS, 10mM Tris HCl, and 1mM EDTA). After solubilization, the solution was extracted twice using an equal volume of chloroform. Following chloroform extraction, an equal volume of isopropyl alcohol was added to precipitate the RNA overnight at either -20˚C or -80˚C, followed by centrifugation at 14,000 rpm at 4˚C for 30 minutes. The pelleted RNA was then washed twice with 70% ethanol, dried in a speed vacuum and dissolved in 50µl water. RNA concentration was determined spectrophotometrically at 260 nm. RNA purity was determined by measuring the absorption ratio at 260/ 280 nm, and RNA integrity was assessed by electrophoresis in a denaturing 1.4% formaldehyde-agrose gel (Sambrook *et al*., 2001).

mRNA Isolation and cDNA Synthesis

Poly $(A)^+$RNA was isolated from 99 µg of total RNA using Ambion Poly(A)Purist Mag kit (Ambion Inc, TX, USA). First strand cDNA synthesis was carried out with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA) at 50°C for 30-60 min. Second strand cDNA was synthesized using RNase H and DNA polymerase I. After phenol/chloroform extraction (pH= 8.0), the resulting double-stranded cDNA was used for PCR with PCR primers designed for apple ADH (alcohol dehydrogenase) and poplar actin (Figure 11) to verify the suitability of the cDNA for PCR amplification. The cDNA was then subjected to the cDNA-AFLP procedure (Vos *et al*., 1995; Bachem, 2002).

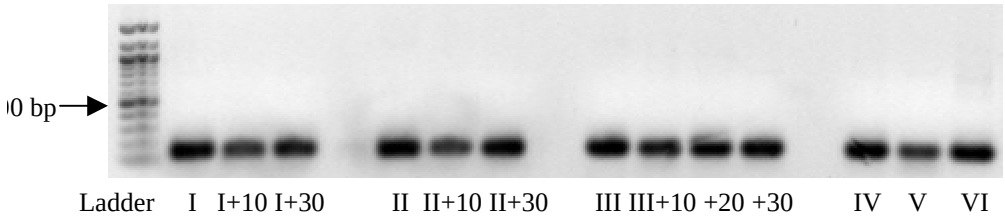


Figure 11. Amplification of apple actin using cDNA synthesized from apple fruit mRNA. Apple fruit cDNA was amplified using primers designed from poplar actin (accession # AB025795) and amplified 25 cycles.

cDNA-AFLP

*Apo*I- *Mse*I combination was chosen to be the optimal enzyme combination for template production based on the *in silico* test results of two restriction enzyme combinations (*Apo*I- *Mse*I and *Ase*I- *Taq*I). These combinations were tested for their genomic coverage and for their possibility to generate a size range of efficient fragments of 100 to 650 nucleotides by performing NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/index.php) on apple gene sequences available in

GenBank in June to July, 2003. 'Gala' apple cDNA was digested with *Mse*I (10 U; New England Biolabs, Beverly, MA) at 37˚Cfor 1 hour in a 40 µl reaction volume. *Apo*I (10U, New England Biolabs) buffer and enzyme were then added to the first digestion mix and incubated at 50˚C for 1 hr. Further amplifying steps to provide a template for cDNA-AFLP were carried out as described previously (Durrant *et al*., 2000). *Apo*I and *Mse*I adaptor primers (50 pmole each, Table 5) were ligated to the digested cDNA in a total volume of 55 µl with T4 DNA ligase (New England Biolabs) at 37˚C for 2 hr. The ligated product was used directly for pre-amplification using primers without selective nucleotides (for sequence, see Table 5). PCR cycle parameters were: 94˚C denaturation, 30 s; 55˚C annealing, 30s; 72˚C extension, 60s. This was repeated for 25 cycles, followed by 5 min at 72 ˚C. The samples were then diluted about 250-fold to a concentration of 1ng/µl. All amplification reactions were carried out on a PE-9600 thermocycler using TaKaRa Ex Taq Hot Start DNA-polymerase (Takara Bio Inc., Shiga, Japan). Selective amplifications were carried out with *Apo*I and *Mse*I primers (for sequence, see Table 5) containing two selective nucleotides. Those two selective nucleotides were chosen for *Apo*I primers containing AG, TC, CA, GT, AA, TT and GA, and for *Mse*I primers containing AT, TG, CA, GC and GT. *Apo*I primers were labeled using [γ -^{33}P] ATP and T4 polynucleotide kinase as suggested by Vos *et al*., 1995 and Bachem, 2002. PCR reactions (10 µl reactions) used 2.5 µl of template with an anneal temperature of 65˚C that was reduced to 56˚C in 0.7˚Csteps for the first 11 cycles (touchdown PCR), and then anneal temperature maintained at 56˚Cfor the additional 24 cycles for a total of 35 cycles.

Table 5. The list of primer sequences

Primer	Sequence
*Apo*I adaptor	5'-CTCGTAGACTGCGTACC-3'
	3'-CATCTGACGCATGGTTAA-5'
*Mse*I adaptor	5'-GACGATGAGTCCTGAG-3'
	3' TACTCAGGACTCAT-5'
Preamplification *Apo*I primer	5'-GACTGCGTACCAATTC-3'
Preamplification *Mse*I primer	5'-GATGAGTCCTGAGTAA-3'
Amplification *Apo*I primer	5'-GACTGCGTACCAATTCNN*-3'
Amplification *Mse*I primer	5'-GATGAGTCCTGAGTAANN*-3'

*N represents selective nucleotide

The amplification products were then denatured at 95˚C for 5 minutes after the addition of equal amount of formamide dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol FF). They were held on ice until loading. Samples were separated on a 5% polyacrylamide sequencing gel (BioRad). Gels were run at 110 W, 50˚C until the bromophenol blue dye front reached the bottom of the gel. Gels were dried directly onto Whatman 3M paper, using a slab gel dryer. A phosphor-imager screen was exposed to the dried gel overnight, and scanned with the phosphor-imager (STORM 860, Amersham) to generate a digital image of the gel. Gels were positionally marked prior to exposing to X-ray films (Kodak Biomax film) for 3-4 days after which they were developed and used for band recovery.

Isolation and Sequencing of Fragments

The developed film and gel were aligned, and the bands that revealed a significant visual-detectable change in expression at one or more time points including differences between attached and detached treatments, and between developmental stages were identified. Bands were cut from the gel by hand using a razor blade. Gel slices were soaked in 50µl water and incubated at 37˚C overnight. An aliquot of the eluted cDNA was reamplified with 30 cycles of PCR under the same conditions as described previously using the same primer combination, and the amplification products were visualized in 1% agrose gels stained with ethidium bromide. Those confirmed amplified products were cloned into a vector with TOPO TA cloning system (Invitrogen Corp., Carlsbad, CA, USA). An aliquot of the cloning reaction was used to transform TOP10 *E. coli* cells flowing the manufacture's recommended conditions for transformation and cloning. Plasmid DNA was extracted with GenElute plasmid miniprep kit (Sigma). To check these clones, an aliquot of the plasmid DNA was digested with *EcoR* (I) endonuclease enzyme (NEB Biolabs). The size of the cloned fragments was determined on a 1% agrose gel stained with ethidium bromide. cDNAs were then selected and sequenced by the Sequencing Facility, at the University of Maryland, using BigDye terminator technology. Sequence similarity was determined by comparison with the all available databases at the National Center for Biotechnology Information (www.ncbi.nlm.nib.gov/BLAST) using Basic Local Alignment Search Tool (BLAST) network service. The databases 'nr' (all GenBank+RefSeq Nucleotides+EMBL+DDBJ+PDB sequences, but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences) and 'est' (database of

GenBank+EMBL+DDBJ sequences from dbEST) were used for Blastn, Blastx and tBlastx (for dbest) analyese. Any similarity with a score of over 46 or an E-value of less than 10^{-5} was considered to be a significant "hit".

cDNA Microarrays

After sequencing, the cloned cDNA-AFLP fragments were reamplified by 30 cycles of PCR using the same primer combination as described above. Two hundred twenty transcripts were diluted to 150 and 250 ng/µl in 3X SSC. They were then spotted onto silane-coated slides with twelve replicates using a GMS 417 arrayer (Affymetrix) by the microarray service provided of UMBI CBR Center (www.umbi.umd.edu/~cbr). Two individual sets of total RNA (25µg) of green maturation (stage I, twenty days before commercial harvest) and tree-ripened apple fruits (stage VI, thirty days after commercial harvest) from 2003 were used as probes to confirm differential expression discovered by cDNA AFLP. In addition, a comparison between tree-ripening (stage VI) and detachment-induced ripening (stage I+30) was also performed using 35µg of total RNA as probes for this second microarray analysis. The microarray analysis of gene expression was analyzed using the TIGR microarray software suite TM4-(http://www.umbi.umd.edu/~cbr/macore/macorestart.htm). The TM4 suite of tools consists of four major applications, Microarray Data Manager (MADAM), TIGR_Spotfinder, Microarray Data Analysis System (MIDAS), and Multiexperiment Viewer (MeV), plus the Minimal Information About a Microarray Experiment (MIAME)-compliantMySQL database. To determine the significance of differential

expression profiles in microarray, the pair-wise t-test comparison on the ratios with the threshold set at 40% (on the basis of signal intensity) between mature-green (stage I) and tree-ripened (stage VI) or between tree-ripened (stage VI) and detachment-induced ripening samples (stage I+30) was applied.

Northern Hybridization

25μg of total RNA from the different stages of ripening apple fruits collected in 2003 were denatured and separated by electrophoresis on a 1.4% formaldehyde-agrose gel. These were transferred onto Zeta Probe nylon membranes (Bio-Rad) by the modified gravity-capillary technique (Ambion). After immobilization by baking for 30 minutes at 80^{o}C, blots were stored at room temperature until used for hybridization. Selected cDNAs with confirmed pattern in cDNA AFLP and in microarrays were labeled with [γ^{32}P]-dCTP using random priming (Ladderman Labeling Kit, Takara Bio Inc., Shiga, Japan). Hybridization was carried out according to the membrane manufacturer's instructions (Takara Bio Inc., Shiga, Japan). Hybridization signals were detected by exposing the membranes to a phosphor screen to obtain digital gel images.

RESULTS:

Genes differentially expressed during apple fruit maturation and ripening were discovered using cDNA-AFLP from the fleshy tissue of 'Gala' apple harvested in

2002. The majority of TDFs were amplified to approximately equal levels among different apple developmental stages (Figure 12).

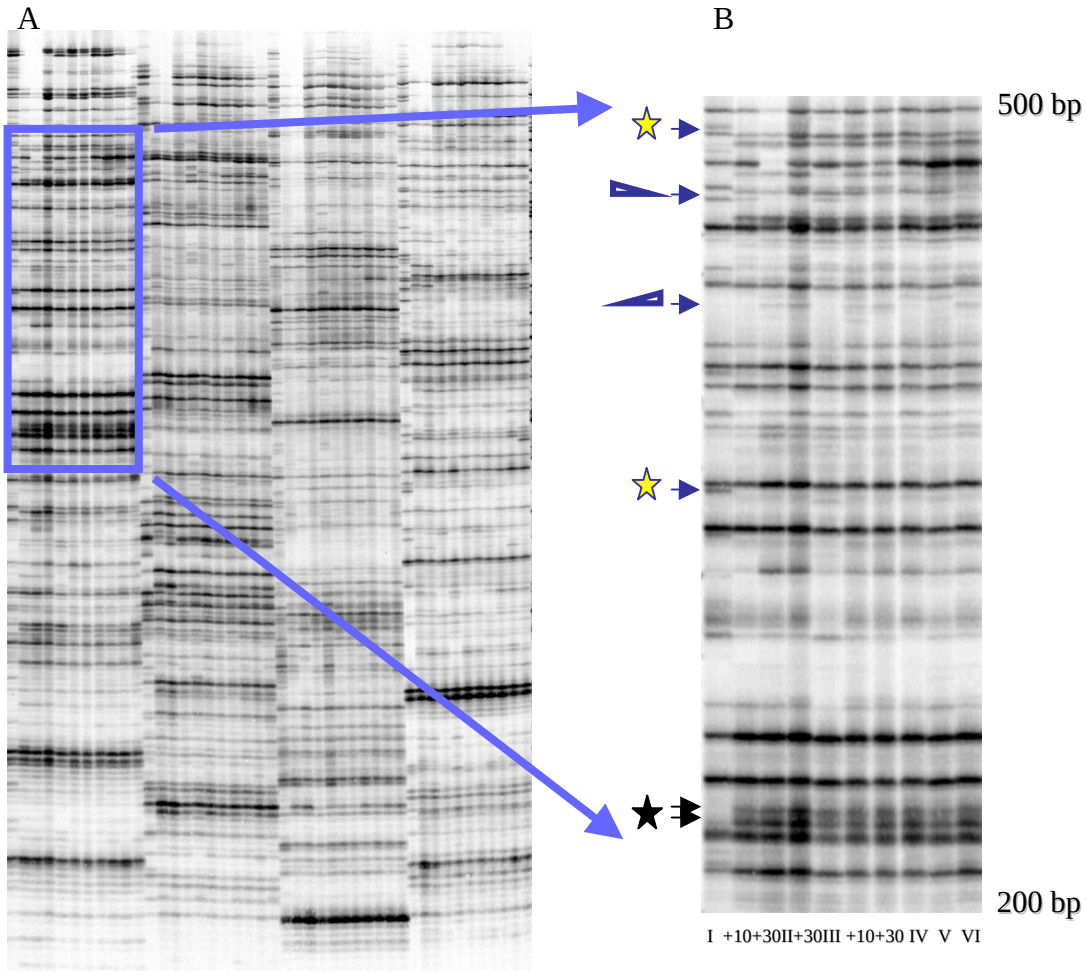


Figure 12. cDNA-AFLP results.
A: Templates were derived from 'Gala' apple harvested at various stages from maturation to ripening. Lanes are in group of 10 samples and total four groups were amplified using different primer combination with two selective nucleotides. B: Enlarged view of the boxed region from left. Different expression patterns can be seen during ripening. From top of B: Empty star mark showing transiently expressed band; triangle indicating decreasing or increasing patterns; solid star pointing transient suppressed bands.

Transcript abundance that differed during natural tree-ripening or detachment-induced ripening were identified by visual inspection and selected for analyses. Two hundred and four TDFs were isolated, cloned and sequenced in this study. Among these clones, one hundred sixteen TDFs increased in abundance during fruit maturation and ripening. Twenty five TDFs abundance declined during fruit maturation and ripening and the abundance of sixty three transcripts varied. Most transient differences happened between mature-green (stage I) and the late r stages, i.e. those TDFs were present in all samples except mature-green stage, or vice versa. These TDFs were subsequently screened using cDNA made from independent 'Gala' apple RNA samples in 2003 from different treesas a probe . Screening was done by microarray and Northern hybridization. Two hundred and four clones were reamplified and then spotted in triplicate onto coated slides for microarray studies. Microarray slides were hybridized with fluorescently labeled probes derived from various ripening stages including mature green (stage I), tree-ripe stage (stage VI), and detachment-induced ripening (stage I+30). Raw hybridization data were statistically analyzed and the threshold for a significant change in expression was set as 40% difference between hybridization intensities ($p \leq 0.05$). We reduced the significant threshold of the array ratio from 2.0 (common ratio) due to only a dozen of TDFs had over than 2-fold difference. The comparison between mature green (stage I) and tree ripe (stage VI) *via* microarray was confirmed the cDNA-AFLP expression patterns of one hundred and fifty five TDFs. The microarray data (listed in the "Array ratio" column in Table 8, 9, 10 and 11) indicated that there was a consistent change in the accumulation of transcripts of seventy two gene fragments during

ripening in both years. A considerable number (eighty five) of these TDFs did not show significant difference in abundance in the microarray analyses, and two clones even exhibited an opposite pattern (Table 6). Only a few differences of transcript level between tree-ripening fruit and detachment-induced ripening were detected by the third microarray (Table 7).

Table 6. Summary of expression profiles of cDNA-AFLP and microarray during maturation and ripening using different 'Gala' apple samples harvested in 2002 and 2003 respectively.

	cDNA-AFLP	Microarray
Total cDNA-AFLP fragments observed/ tend to be confirmed	204	155
Fragments with increasing abundance	116	55
Fragments with declining abundance	25	3
Transiently expressed or suppressed/ Non-significant	63	85
Fragments with reverse pattern	-	2
Non-detectable fragments from cDNA-AFLP	-	10

Gene fragments obtained from cDNA-AFLP were analyzed by BLAST and then submitted to GenBank. The differentially-expressed cDNA sequences were assigned to broad functional categories based on the database similarity search results (*E*-value cutoff = 1*e*-5). Figure 13 shows the breakdown into broad functional groups of differentially-expressed genes identified from all samples.

Table 7. Differentially expressed genes between tree-ripening and detachment-induced ripening in 'Gala' apple fruit using microarray analysis.

cDNA-AFLP Fragment (GenBank accession #)	Annotation	Microarray ratio*
Higher in tree-ripening fruits		
CN544841	Dynamin, cell mobility related	2.01±0.06
CN544882	Metallothionein-like protein, metal metabolism and detoxification, associated with fruit ripening and responses to stresses	4.23±0.13
CN544896	Adenine phosphoribosyltransferase 1, purine salvage	1.62±0.03
CN544901	26S Ribosomal RNA, protein biosynthesis	1.80±0.03
CN544915	28S Ribosomal RNA, protein biosynthesis	1.81±0.08
CV102307	Subtype 25S ribosomal RNA, protein biosynthesis	1.67±0.05
CV102312	Apple EST, unknown function, protein biosynthesis	1.92±0.26
CV102317	1-acyl-sn-glycerol-3-phosphate acyltransferase, lipid metabolism	1.86±0.07
CV102320	subtype b 25S ribosomal RNA, protein biosynthesis	2.08±0.03
Higher in detachment-induced ripening		
CN544902	Ribulose 1,5-bisphosphate carboxylase small subunit, photosynthesis related	0.43±0.01
CN544908	Apple EST, unknown function	0.55±0.04
CN544911	Submergence induced protein, stress related	0.61±0.02
CV102294	Putative pyruvate kinase, glycolytic pathway and carbon metabolism	0.48±0.03

* Array ratio was calculated as hybridization intensity of tree ripening fruit (stage VI)/ detachment-induced ripened fruit (stage I+30). Data represent average of 12 replications± SE.

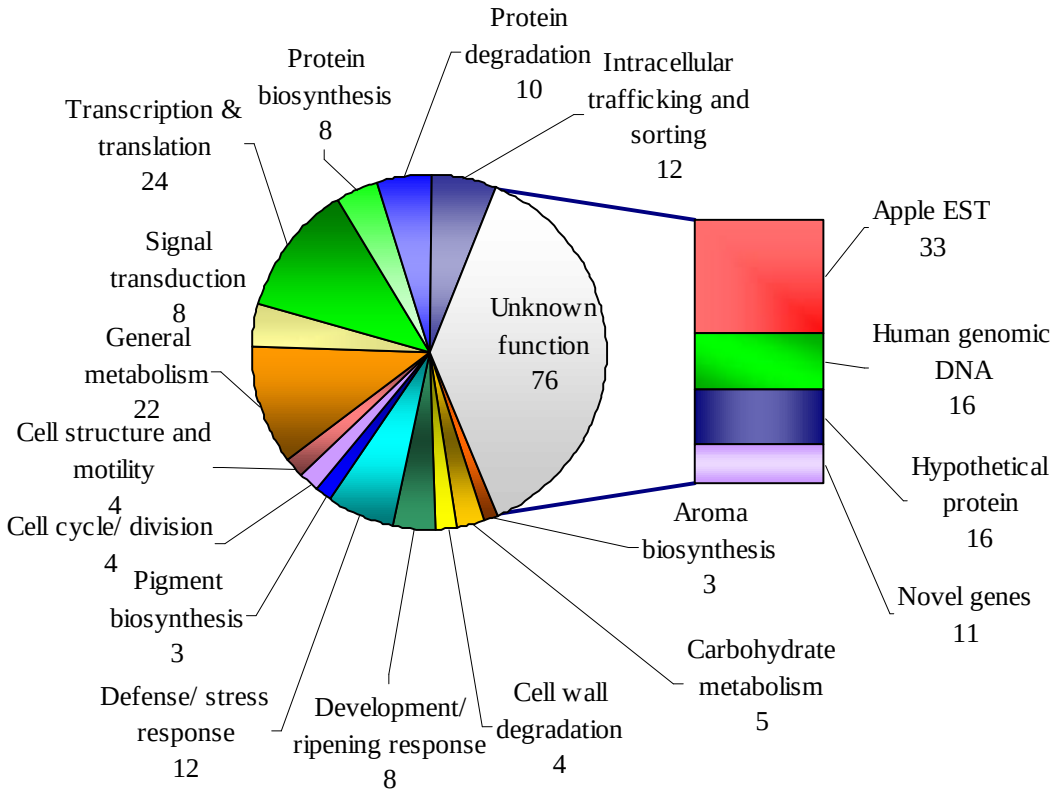


Figure 13. Classification of all the differentially expressed gene fragments to broad functional categories.
The classification of cDNAs associated with different developing stages to functional categories based on their similarity to genes in the GenBank databases.

These include genes involved in protein biosynthesis and degradation, signal transduction, intracellular trafficking and sorting, cell cycle, structure and mobility categories. The second most abundant group was clones with similarity to genes associated with plant stress responses. Many (forty four) genes shared significant similarity to ESTs from an abiotic stress grape database, and have been labeled as potential defense/ stress responses. These are annotated "(DS)" in the "Group" column in the tables. As expected, we also identified a number of clones with similarity reported previously in studies of fruit development and ripening. Seventy six unique clones showing differential expression during ripening could not be

assigned to any functional category because of their similarity to genes with an unknown function, or they were novel genes in the database.

These clones were further divided into three groups on the basis of the relationship with maturation and ripening of their similar genes found in BLASTN and BLASTX databases which include genomic DNA, transcripts (mRNA, cDNA), and protein products. Thirty nine clones were identified as maturation and ripening-associated proteins (Table 8). The putative proteins derived from one hundred and fourteen transcripts were called candidate ripening-related gene fragments. These are shown in Table 9. Some of these clones may not have any recognized relationship to ripening but were similar to many ESTs obtained previously from apricot, peach and grape during development. The remaining gene fragments with unknown functions were similar to novel apple ESTs (Table 10), putative hypothetical proteins (Table 11), human genomic DNA (Table 12), or to unidentified gene fragments (Table 13).

In this present study, we focused our attention on eighty cDNA-AFLP fragments whose expression was altered during ripening. These were selected for their consistent expression pattern in successive years of this study, and/or for their similarity to genes known to be associated with ripening. A summary of these fragments and their functions is summarized on the following pages.

Table 8. Differentially expressed cDNAs found at different stages of ripening as determined by cDNA-AFLP analysis.

Each cDNA was annotated according to the definition and accession of the nucleotide sequence of the BLAST similarity from the NCBI database. The 39 AFLP fragments that matched similar genes associated with growth and development or ripening are listed.

Group[a]	GenBank Accession number	Length (bp)	Expression Pattern[b]	Array Ratio[c]	Annotation[d] (GenBank accession number)	BLAST Score[e]
A	CV102301	122	Up*	1.76	Alcohol acyl transferase from pear, *Pyrus communis* (AY534530)	4e-37
A	CV102255	305	Up	0.96	Palmitoyl protein thioesterase family protein from *Arabidopsis thaliana* (NP_191593)	2e-17
CM	CN544845	398	Up*	1.44	Hexose transporter from grape, *Vitis vinifera* (Y09590)	4e-49
CM	CN544867	126	Up*	1.85	Glucose-6-phosphate/ phosphate translocator-related from *Arabidopsis thaliana* (NM_106409)	7e-17
CM	CN544886	117	Up*	1.73	NAD-dependent sorbitol dehydrogenase (SDH1) from apple, *Malus domestica* (AB016256)	1e-58
CM	CV102275	251	+Detached	1.27	Putative isoamylase from *Arabidopsis thaliana* (AAM98123)	1e-32
CM	CN544859	230	Up	0.80	Sucrose phosphate synthase from mistletoe, *Viscum album* (AY331261)	4e-26
CW	CN544830	97	Up	1.00	Putative cinnamyl alcohol dehydrogenase (CAD) from apple, *Malus domestica* (AF053084)	6e-07

Table 8. Continued.

Group[a]	GenBank Accession number	Length (bp)	Expression Pattern[b]	Array Ratio[c]	Annotation[d] (GenBank accession number)	BLAST Score[e]
CW	CN544874	196	+ I, II, III	0.81	Endo-1,4-beta-D-glucanase, encodes membrane-spanning domain from pear, *Pyrus communis* (AB084463)	2e-27
CW	CN544898	193	Up*	2.96	Putative cinnamoyl-CoA reductase (CCR) from sweet cherry, *Prunus avium* (AF298828)	5e-19
CW	CV102337	160	Up	0.89	Alpha-L-arabinofuranosidase (AFase1) from apple, *Malus domestica* (AY309436)	4e-84
DR/ (DS)	CN544829	257	Up*	1.44	DEM protein from tomato, *Lycopersicon esculentum* (AY323822)	3e-18
DR/TT (DS)	CN544834	319	+Detached	0.72	C2H2 zinc-finger protein (SE1A) precursor from corn, *Zea may* (AF311223)	3e -12
DR	CN544852	459	Up*	1.40	SEU2 protein from snapdragon, *Antirrhinum majus* (CAF18248)	1e-12
DR	CN544909	288	Up	1.05	Small GTP-binding protein from lotus, *L. japonicus* (Z73937)	7e-93
DR	CN544914	170	Up	ND	GAI-like protein from tomato, *Lycopersicon esculentum* (AAP22369)	6e-24
DR	CV102271	145	- IV, V	0.91	Ripening-responsive protein from *Arabidopsis thaliana* (NP_175184)	1e-11
DR/DS	CN544882	355	Down*	0.05	Metallothionein-like protein from sand pear, *Pyrus pyrifolia* (AB021790)	e-130
DR/DS	CV102257	248	Up*	1.50	Serine/threonine protein kinase pk23 from tomato, *Lycopersicon esculentum* (AAL87457)	5e-19

Table 8. Continued.

Group[a]	GenBank Accession number	Length (bp)	Expression Pattern[b]	Array Ratio[c]	Annotation[d] (GenBank accession number)	BLAST Score[e]
DS	CN544889	109	Down	1.12	Phosphoglyceromutase from apple, *Malus domestica* (AJ004915)	2e-44
DS	CN544907	327	Up	1.32	Putative copper/zinc superoxide dismutase copper chaperone precursor from tomato, *Lycopersicon esculentum* (AAK01931)	7e-28
DS/ PD	CV102251	545	Up*	2.17	Aspartic proteinase (apl gene) from cacao, *Theobroma cacao* (AJ313384)	3e-41
GM	CN544875	191	Down	1.01	Hydrolase, alpha/beta fold family protein from *Arabidopsis thaliana* (NM_180484)	2e-15
GM	CN544876	177	+ II, III, IV	3.24	Hydrolase, alpha/beta fold family protein from *Arabidopsis thaliana* (NP_187698)	2e-07
GM	CN544888	78	Up	1.04	Putative 3-beta hydroxysteroid dehydrogenase/ isomerase protein from rice, *Oryza sativa* (AAU44198)	6e-06
GM	CV102294	240	+ III	1.31	Putative pyruvate kinase from rice, *Oryza sativa* (AAP03381)	2e-06
GM	CV102336	474	+III	1.26	Glyoxysomal beta-ketoacyl-thiolase precursor from *Brassica napus* (X93015)	1e-23
GM/ (DS)	CN544835	324	Down in III	0.87	3-ketoacyl-CoA thiolase from cucumber, *Cucumis sativus* (X67696)	7e-10
GM/A	CN544903	425	Up	0.98	Similar to auxin-independent growth promoter from *Arabidopsis thaliana* (BAD37235)	7e-57

Table 8. Continued.

Group[a]	GenBank Accession number	Length (bp)	Expression Pattern[b]	Array Ratio[c]	Annotation[d] (GenBank accession number)	BLAST Score[e]
LM	CN544871	176	Up	0.93	1-acyl-sn-glycerol-3-phosphate	7e-24
	CV102317	190	+Attached	1.00	acyltransferase from almond, *Prunus dulcis* (AF213937)	8e-24
PB	CV102282	288	Up	0.65	Dehydroquinate dehydratase, putative / shikimate dehydrogenase from *Arabidopsis thaliana* (NP_187286)	3e-33
PD/CC	CN544893	146	Up*	1.42	Skp1 from*Medicago sativa* (AF135596)	4e-28
PG	CN544846	659	Up*	1.50	Plastid quinol oxidase from tomato, *Lycopersicon esculentum* (AF302932)	1e-59
PG	CV102284	159	Down	1.20	Glycosyltransferase protein A from peach, *Prunus persica* (AY354512)	1e-59
PG	CV102299	142	Up	ND	Leucoanthocyanidin dioxygenase-like protein from *Arabidopsis thaliana* (T49209)	2e-16
PS	CN544902	256	+ I, III, III+30, IV	0.95	Ribulose 1,5-bisphosphate carboxylase small Subunit from creosote bush, *Larrea tridentata* (AF326774)	4e-14
ST	CN544892	329	Down	1.34	Phosphatidyl glycerol specific phospholipase C-like from pear, *Pyrus communis* (AY436779)	1e-08
ST	CV102316	284	Up	1.28	Seven-transmembrane-domain protein 1, a type of receptor protein from tomato, *Lycopersicon esculentum* (AJ583669)	7e-13

[a] Functional classes: A, Aroma biosynthesis; CC, Cell cycle/ division; CM, Carbohydrate metabolism; CW, Cell wall degradation; DR, Development and ripening;

DS, Defense/ stress response; GM, General metabolism; IT, Intracellular trafficking and sorting; PB, peptide and protein biosynthesis; PD, Protein degradation; PG, Pigment biosynthesis; PS, Photosynthesis; ST, Signal transduction; TT, Transcription & translation; U, Unknown. (DS): similar to ESTs from abiotic stressed grape database.

[b] * Expression pattern was verified by microarray probed by different RNA samples from stage I (mature green) and stage VI (tree-ripening), verified pattern is labeled with a *.

+, Transiently expressed;- , transiently suppressed

[c] Array ratio was hybridization intensity of tree-ripe fruit/ gree mature fruit, the threshold was set as 40%, $p<0.05$; ND: non-detectable

[d] Definition and accession of nucleotide sequences of the best BLASTN or BLASTX similarity.

[e] Scores were either BLASTN or BLASTX corresponding to their representing similarity; sequences with an E value > 1e-05 are not included in this table.

Table 9. Candidate AFLP fragments associated with development and ripening found in other EST databases.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
CC	CN544847	645	Up*	1.50	Putative microtubial binding protein from rice, *Oryza sativa* (BAD05 590)	1e-07
CC	CV102281	304	+Attached	0.96	Microtubule associated protein from carrot, *Daucus carota* (AJ520103)	2e-13
CC	CV102343	280	-I~IV	0.80	Beta-tubulin 6 from Zinnia, *Z. elegans* (D63136)	2e-47
CC	CV102349	212	Up	1.31	Histone 1 (Md-H1) from apple, *Malus domestica* (AB099931)	9e-24
CS	CN544841	126	Down	0.83	Dynamin, putative from *Arabidopsis thaliana* (AAM61645)	7e-12

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
CS	CN544895	81	Down	1.02	Allergen profilin (pf-2 gene) from apple, *Malus domestica* (AJ507458)	4e-11
CS	CV102348	145	- I, III	0.93	Microsatellite from strawberry, *Fragaria x vesca* (AJ508252)	6e-27
CS/ (DS)	CV102277	198	Up	1.23	Myosin heavy chain-like protein from rice, *Oryza sativa* (NP_917041)	1e-09
DS	CN544833	347	+ I, II, III, IV	0.99	Phytochelatin synthetase-like protein from strawberry, *Fragaria x ananassa* (AY642687)	2e-32
DS	CN544851	566	Up	1.22	Universal stress protein (USP) family protein from *Arabidopsis thaliana* (NM_115259)	3e-23
DS	CN544911	195	Up*	1.89	Submergence induced protein 2A from *Arabidopsis thaliana* (AAM63805)	2e-18
DS	CV102279	340	Up	1.21	Na^+/H^+ antiporter from Antarctic hairgrass, *Deschampsia antarctica* (AAM22753)	3e-08
DS	CV102293	291	Up*	1.69	Heat shock transcription factor family protein from *Arabidopsis thaliana* (NM_102966)	9e-34
DS	CV102333	149	Up	1.06	Cytochrome P450 family protein from *Arabidopsis thaliana* (NP_566628)	1e-05

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
DS	CV102344	251	Up in Detachment	1.02	Putative disease resistance protein from *Arabidopsis thaliana* (Q9LRR5)	3e-13
DS	CV102345	246	Up	1.09	Cytochrome P450 family protein from *Arabidopsis thaliana* (NP_198460)	2e-32
DS	CV102347	182	- I	1.15	Acid phosphatase survival protein SurE, putative from *Arabidopsis thaliana* (NP_567449)	5e-06
E	CV102292	296	Up*	1.45	NADH-ubiquinone oxidoreductase, mitochondrial (TYKY) from *Arabidopsis thaliana* (NM_106551)	7e-44
GM	CN544848	624	Up*	1.99	Putative symbiosis-related protein from *Arabidopsis thaliana* (CAB79153)	2e-08
GM	CN544896	500	Up*	35.75	Adenine phosphoribosyltransferase 1, APT from *Arabidopsis thaliana* (BT000370)	5e-12
GM	CV102248	344	Down	1.38	Peroxisomal biogenesis factor 11 family protein/ PEX11 family protein from tomato, *Lycopersicon esculentum* (BT013358)	2e-25
GM	CV102253	385	Down	1.13	UDP-D- xylose 4-epimerase from *Arabidopsis thaliana* (AY195742)	1e-27
GM	CV102267	368	+III	1.60	Type 2 peroxiredoxin (PrxII) from napa, *Brassica rapa* subsp. pekinensis (AF133302)	3e-31

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
GM	CV102270	153	- III-10, 20	1.24	T-complex protein 11 from *Arabidopsis thaliana* (NP_192654)	7e-08
GM	CV102288	303	- III	1.31	Mitochondrial substrate carrier family protein from *Arabidopsis thaliana* (NP_190962)	6e-14
GM	CV102326	217	Up	1.23	Glutaredoxin family protein from *Arabidopsis thaliana* (NP_196885)	3e-20
GM/ DS	CV102332	136	+ I, III	1.19	Putative purple acid phosphatase precursor (or ACP5)from sweet potato, *Ipomoea batatas* (AAF60315)	4e-14
I T	CV102304	362	+ III	1.12	Kinesin light chain-related from *Arabidopsis thaliana* (NP_192822)	1e-12
IT	CN544883	337	Down*	0.38	Vacuolar protein sorting-associated protein 28 family protein (VPS28) from *Arabidopsis thaliana* (NM_202784)	1e-08
IT	CV102254	325	Down	1.37	Kinesin motor protein-related from *Arabidopsis thaliana* (NP_564696)	1e-05
IT	CV102285	404	Up*	1.62	Leucine-rich repeat family protein / protein kinase family protein from *Arabidopsis thaliana* (NP_564904)	5e-44
IT/ (DS)	CV102266	465	+Attached	1.37	ABC transporter from *Arabidopsis thaliana* (BK001010)	1e-34
IT	CV102287	306	Up	0.84	Probable ABC transporter from *Arabidopsis thaliana* (D84680)	6e-15

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
IT	CV102323	314	Up	1.00	Clathrin heavy chain, putative from *Arabidopsis thaliana* (NM_111950)	2e-44
IT/ (DS)	CN544863	466	Up	1.14	Synaptobrevin/vesicle-associated membrane protein from *Arabidopsis thaliana* (NM_121153)	1e-64
IT/ (DS)	CN544884	310	Down	0.86	Proton-dependent oligopeptide transport (POT) family protein from *Arabidopsis thaliana* (NM_105528)	2e-07
IT/ (DS)	CV102273	355	+Detached	1.42	VHS domain containing protein / GAT domain-containing protein from *Arabidopsis thaliana* (NP_187491)	2e-36
IT/ (DS)	CV102308	313	Up	0.91	VHS domain containing protein / GAT domain-containing protein from *Arabidopsis thaliana* (NP_564138)	1e-27
IT/ (DS)	CV102324	302	+ III	ND	Putative nucleoside transporter from *Arabidopsis thaliana* (AAF26446)	1e-14
PB	CN544880	314	Up*	1.93	Aspartate aminotransferase from lotus, *L. corniculatus* (AF029898)	2e-10
PB	CN544897	281	+III-10	1.62	Cysteine protease inhibitor cystatin from apple, *Malus domestica* (AY173139)	e-113
PB	CN544918	132	+Attached	2.23	Putative cleavage and polyadenylation specifity factor from rice, *Oryza sativa* (XP_470435)	7e-11

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
PB	CV102249	579	+ III	1.28	Eukaryotic peptide chain release factor subunit 1-1 (ERF1-1) from *Arabidopsis thaliana* (NM_124162)	1e-09
PB	CV102265	498	- III	0.80	High mobility group (HMG1/2) family protein from *Arabidopsis thaliana* (NP_565788)	2e-28
PB/ES	CV102291	314	+ I	0.94	Pyridoxal-5'-phosphate-dependent enzyme, beta family protein from *Arabidopsis thaliana* (NM_104465)	2e-34
PD	CN544872	144	Up	0.94	Putative cathepsin B-like protease from pea, *Pisum sativum* (AJ251536)	1e-15
PD	CN544919	107	Up	1.12	Zinc metalloproteinase-like from rice, *Oryza sativa* (BAD08898)	1e-06
PD	CV102256	258	Down	1.69	Insulin degrading enzyme from tomato, *Lycopersicon esculentum* (CAC67408)	1e-08
PD	CV102303	577	Up	1.29	Aspartyl protease family protein from *Arabidopsis thaliana* (NM_129222)	1e-09
PD/CC	CN544839	183	Up	1.06	U-box domain-containing protein from *Arabidopsis thaliana* (NP_196542)	3e-14
PD/CC	CN544853	403	Up*	1.97	Ubiquitin-protein ligase 1 from rice, *Oryza sativa* (XP_450304)	8e-09
PD/CC	CN544865	226	Up*	1.56	21D7 antigen from carrot, *Daucus carota* (D13434)	2e-25

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
PD/CC	CN544869	442	+ III, IV	1.87	Polyubiquitin (RUBQ2) from rice, *Oryza sativa* (AF184280)	e-125
PD/CC	CN544917	134	Up	0.93	20S proteasome subunit from soybean, *Glycine max* (AF255338)	2e-11
PS/ (DS)	CV102322	416	+ III-20	1.02	Chloroplast phosphoglycerate kinase from poplar, *Populus nigra* (AB018412)	6e-79
ST	CV102259	194	+ I	1.28	Calmodulin-binding protein (TCB60) from tobacco, *Nicotiana tabacum* (U58971)	8e-27
ST	CV102298	196	Up	1.13	Timing of CAB expression 1-like protein from Chinese cabbage, *Brassica rapa* subsp. pekinensis (AAO27295)	9e-19
ST	CV102309	289	+ I	1.13	Calmodulin-binding family protein from *Arabidopsis thaliana* (NP_193211)	2e-15
ST	CV102316	284	Up	1.28	Seven-transmembrane-domain protein 1, a type of receptor protein from tomato, *Lycopersicon esculentum* (AJ583669)	7e-13
ST	CV102340	361	Up	1.02	Transducin family protein / WD-40 repeat family protein from *Arabidopsis thaliana* (NP_188434)	3e-53
TT	CN544856	209	Up	1.20	Zinc finger family protein from *Arabidopsis thaliana* (NP_175132)	9e-06

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
TT	CN544858	166	Up*	3.09	rRNA processing protein-related from *Arabidopsis thaliana* (Q9LUJ5)	5e-06
TT	CN544890	86	Up*	1.59	Eukaryotic translation initiation factor 3 subunit 11 (eIF3k) from rice, *Oryza sativa* (Q94HF1)	1e-04
TT	CN544891	677	Up	0.79	Putative CRS1 from rice, *Oryza sativa* (XP_481944)	4e-35
TT	CN544901	84	Up	1.17	26S Ribosomal RNA from water speedwell, *Veronica anagallis-aquatica* (AF479169)	1e-38
TT	CN544905	371	+ I	1.11	Putative ER6 protein from rice, *Oryza sativa* (BAD45043)	3e-05
TT	CN544906	324	+ I-10, III	1.18	Ribosomal protein L17-like protein from tobacco, *Nicotiana tabacum* (AB010880)	3e-28
TT	CN544915	168	Up	0.92	28S ribosomal RNA from vampire squid, *Vampyroteuthis infernalis* (AY145422)	2e-11
TT	-	41	Down	1.26	26S rRNA from *Beauveria bassiana* (AB044638)	4e-12
TT	CV102278	194	- III	1.04	G-box-binding protein from fava bean, *Vicia faba* (T12092)	2e-09
TT	CV102295	288	Up	0.95	Subtype g 25S ribosomal RNA gene from Candida, *C. albicans* (AY441789)	4e-14

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
TT	CV102297	202	+ I - I	1.31 1.31	Eukaryotic release factor 1 family protein / eRF1 family protein from *Arabidopsis thaliana* (NM_115701)	2e-24
TT	CV102286	387	+Attached	0.82	Ribosomal protein S28 (rps28.2) from peach, *Prunus persica* (AJ012656)	4e-58
TT	CV102289	254	Up	1.21	Acidic ribosomal protein P0-related from *Arabidopsis thaliana* (NP_564226)	3e-26
TT	CV102290	511	+ I, III	1.89	Homeobox-leucine zipper protein 7 (HB-7)/ HD-ZIP transcription factor 7 from *Arabidopsis thaliana* (AY091364)	4e-22
TT	CV102315	392	Up	0.80	tRNA pseudouridine synthase family protein from *Arabidopsis thaliana* (NP_198390)	1e-21
TT	CV102320	119	Up	1.18	Subtype b 25S ribosomal RNA from *Candida albicans* (AY441784)	4e-12
TT	CV102329	302	Up	0.96	Ribosomal protein PETRP from hot pepper, *Capsicum annuum* (AY496096)	8e-32
TT/ (DS)	CN544862	583	- I, IV, V, VI	0.86	Elongation factor EF-2 from pea, *Pisum sativum* (AB082376)	e-147
TT/ (DS)	CV102302	597	Up	0.77	Transcription elongation factor-related from *Arabidopsis thaliana* (AP003974)	2e-14

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
TT/ (DS)	CV102307	319	Up	0.94	Subtype g 25S ribosomal RNA gene from *Candida albicans* (AY441789)	5e-14
TT/ (DS)	CN544873	429	Up	1.20	Zinc finger (C2H2 type) family protein from *Arabidopsis thaliana* (NP_565271)	1e-39
TT/ (DS)	CV102263	122	+ I, III	1.30	Putative valyl-tRNA synthetase from rice, *Oryza sativa* (BAC83606)	3e-13
TT/ (DS)	CV102274	312	Up	1.21	N-terminal domain-containing protein / zinc finger (C3HC4-type RING finger) family protein from *Arabidopsis thaliana* (NP_974183)	2e-20
U	CN544836	224	Down	1.16	Apple, *Malus domestica* EST (CN862294)	1e-35
U	CN544840	153	Up	1.15	Unknown protein from *Arabidopsis thaliana* (AY142650)	2e-36
U	CN544860	125	Up	1.36	Unknown protein from rice, *Oryza sativa* (NP_922869)	1e-07
U	CN544877	133	Up*	1.52	Apple, *Malus domestica* EST (CV129646)	6e-67
U	CN544900	86	Up	0.87	Peach, *Prunus persica* EST (BU041277)	4e-30
U	CV102327	416	Up*	1.40	Unknown protein from *Arabidopsis thaliana* (BT005503)	1e-06
U	CV102335	503	Up	ND	Expressed protein from *Arabidopsis thaliana* (NP_850197)	1e-44
U	CN544908	290	- II-30	1.25	Apple, *Malus domestica* EST (CN888668)	2e-13

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
U	CN544912	189	+Attached	1.27	Apple, *Malus domestica* EST (CO066940)	9e-98
U	CN544925	76	Up*	1.53	Unknown protein from *Arabidopsis thaliana* (CN920633)	3e-27
U	CV102252	463	+ I, V	1.36	Brassinosteroid-regulated protein from soybean, *Glycine max* (L22162)	8e-57
U	CV102258	238	+ I	1.00	Apple, *Malus domestica* EST (CN934335)	e-126
U	CV102269	153	Up	ND	Apple, *Malus domestica* EST (CN579000)	1e-77
U	CV102321	364	Up	0.95	Expressed protein from *Arabidopsis thaliana* (NP_182051)	5e-46
U	CV102338	304	Up	0.97	Pentatricopeptide (PPR) repeat-containing protein from *Arabidopsis thaliana* (NP_177623)	3e-30
U	CV102341	251	Up	1.37	Far-red impaired responsive family protein (FAR1) from *Arabidopsis thaliana* (NP_567085)	2e-21
U	CV102346	196	Up	0.91	Unknown protein from *Arabidopsis thaliana* (AAN72189)	3e-10
U/ (DS)	CN544910	197	+Attached	1.27	Expressed protein from *Arabidopsis thaliana* (NM_118836)	6e-28
U/ (DS)	CN544916	142	Up	0.94	Apple, *Malus domestica* EST (CO753861)	2e-54
U/ (DS)	CV102283	195	Up	ND	Expressed protein from *Arabidopsis thaliana* (NM_114973)	2e-43

Table 9. Continued.

Group[a]	GenBank Accession Number	Length (bp)	Expression Pattern[b]	Array Ratio	Annotation[c] (GenBank Accession Number)	BLAST Score[d]
U/ (DS)	CV102310	254	Up	1.10	Chloroplast casein kinase II alpha subunit(cpck2a gene) from white mustard, *Sinapis alba* (AJ420786)	5e-26
U/ (DS)	CV102328	314	Up	1.19	Unknown protein from *Arabidopsis thaliana* (AY114086)	2e-08
U/ (DS)	CV102350	237	Up*	1.42	Putative UPF0183 (uncharacterized protein family) protein from *Arabidopsis thaliana* (AAM63377)	2e-31

[a] Functional classes: A, Aroma biosynthesis; CC, Cell cycle/division; CM, Carbohydrate metabolism; CS, Cell structure and motility; CW, Cell wall degradation; DR, Development and ripening; DS, Defense/ stress response; GM, General metabolism; IT, Intracellular trafficking and sorting; PB, peptide and protein biosynthesis; PD, Protein degradation; PG, Pigment biosynthesis; PS, Photosynthesis; ST, Signal transduction; TT, transcription and translation; (DS): similar to ESTs from abiotic stressed grape database.

[b] *Expression pattern was verified by microarray probed by different RNA samples from stage I (mature green) and stage VI (tree-ripening), verified pattern is labeled with a *.; ND: non-detectable

+: Transiently expressed; -, transiently suppressed.

[c] Definition and accession of nucleotide sequences of the best BLASTN or BLASTX similarity.

[d] Sequences with an E value > 1e-05 are not included in this table.

Table 10. Homologies of differentially expressed cDNA-AFLP fragments exclusive to apple (*Malus domestica*) dbEST

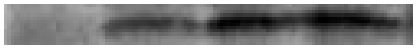
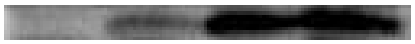
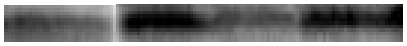
GenBank Accession number	Length (bp)	Expression Pattern[a]	Array ratio[b]	BLAST Hit[c]	BLASTN Score
CN544831	70	 III III+10 III+20	1.00	CN851543	1e-07
CN544837	208	 I I+10 I+20 I+30	1.82*	CO901312	e-108
CN544838	204	 I I+10 I+20 I+30	1.52*	CO576138	e-109
CN544842	104	 I I+10 I+20 I+30  II II+30 III III+10	1.55*	CV082917	2e-26
CN544843	102	 II II+30 III III+10	1.12	CO067953	4e-49
CN544844	75	 I I+10 I+20 I+30	1.51*	CO417498	8e-34
CN544850	176	 II+30 III III+10 III+20	0.99	CN915293	6e-77
CN544854	275	 I I+10 I+30 II	1.41*	CO415699	e-152
CN544866	133	 I I+10 II III IV	1.50*	CO418070	3e-32

Table 10. Continued.

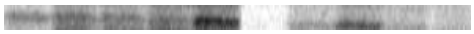
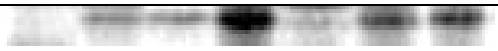
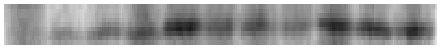
GenBank Accession number	Length (bp)	Expression Pattern[a]	Array ratio[b]	BLAST Hit[c]	BLASTN Score
CN544870	180	 I I+10 II III IV	1.50*	CN874743	2e-92
CN544877	133	 I I+10 I+20 I+30	1.52*	CV129646	6e-67
CN544878	87	 I I+10 I+20 I+30 II	1.14	CN898171	2e-19
CN544879	74	 I I+10 I+20 I+30 II	1.51*	CV129297	1e-29
		 II+30 III III+10 +20 III+30 IV			
CN544887	87	 I I+20 I+30 II II+30	1.70*	CN851679	7e-41
CN544908	290	 II+30 III III+10 +20 +30 IV V	1.25	CN888668	2e-13
CN544912	189	 I I+10+20+30 II +30 III +10 +20	1.27	CO066940	9e-98
CN544916	142	 I +10+20+30 II+30 III +10+20+30 IV V	0.94	CO753861	2e-54
CN544923	92	 I I+10 I+30 II	1.15	CN876558	2e-29
CN544924	84	I, V	1.53	CV083013	6e-38
CV102258	238	 I III IV V VI	0.98	CN934335	e-126
CV102269	153	 I+10 +20 +30 II +10	ND	CN579000	1e-77

Table 10. Continued.

GenBank Accession number	Length (bp)	Expression Pattern[a]	Array ratio[b]	BLAST Hit[c]	BLASTN Score
CV102280	319	 I I+10 I+30 II+30 III III+10 III+20	1.40*	CN996470	e-174
CV102296	178	 I III IV V VI	0.98	CN996633	7e-95
CV102305	237	Up	1.45*	CN488774	1e-04
CV102306	422	Up	1.45*	CN926177	e-126
CV102312	181	Up	1.03	CN880800	3e-32
CV102314	119	Up	1.23	CN919367	8e-60
CV102318	216	- I, I+30	0.90	CO066680	4e-91
CV102325	175	 III +10 +2 +30 IV V VI	1.56	CN881804	3e-88
CV102330	401	 I I+10 I+30 II+30 III III +10 III+30	0.73	CO541186	0
CV102339	193	 I III IV V VI	1.00	CO723632	4e-38

[a] Expression pattern was verified by microarray probed by different RNA samples from stage I (mature green) and stage VI (tree-ripening), verified pattern is labeled with a *.

[b] Array ratio was hybridization intensity of tree-ripe fruit/ gree mature fruit, the threshold was set as 40%, $p<0.05$; ND: Non-detectable

[c] GenBank accession number of the BLASTN similarity.

**Additional similarities of dbEST other than apple were also found

+Transiently expressed; - Transiently suppressed

Table 11. Similarity of cDNA-AFLP fragments to unknown proteins and hypothetical proteins.

GenBank Accession number	Length (bp)	Expression Pattern	Array Ratio	BLAST Hit (GenBank accession number)	BLASTX Score
CN544849	487	Down	1.48	Hypothetical protein from *Sorghum bicolor* (AAL73525)	3e-10
CN544864	380	+ III	1.00	Hypothetical protein from *Arabidopsis thaliana* (AAM61389)	1e-09
CN544885	248	Down*	0.48	Expressed protein from *Arabidopsis thaliana* (NM_124492)	2e-09
CN544904	380	Down	1.20	Expressed protein from *Arabidopsis thaliana* (BAB10284)	2e-11
CN544921	96	Up	1.10	Unknown protein from *Arabidopsis thaliana* (NM_112963)	3e-06
CV102272	138	Up*	1.50	Expressed protein from *Arabidopsis thaliana* (NP_194941)	3e-05
CV102276	266	Up	1.27	Expressed protein from *Arabidopsis thaliana* (NP_190915)	1e-26
CV102311	203	Up	1.30	Unnamed protein product from *Arabidopsis thaliana* (BAB10771)	6e-17
CV102331	254	Up	ND	Hypothetical protein from *Arabidopsis thaliana* (T00919)	6e-15

*Expression pattern was verified by microarray probed by different RNA samples from stage I (mature green) and stage VI (tree-ripening), verified pattern is labeled with a *; ND: non-detectable

+Transiently expressed

Table 12. Differentially expressed cDNA-AFLP fragments with solitary similarity to human (*Homo sapiens*) genomic DNA

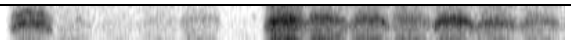
cDNA-AFLP Fragment	Length (bp)	Expression Pattern	Array Ratio	BLAST Hit	BLAST Score
EST0010	238	 I I+10 I+30 II +10 +30 III +10 +20 +30 IV VI	0.65	AC009410	e-126
EST0055	167	 I I+10 I+30 II +10 +30 III +10 +20 +30 IV	0.88	AC009311	1e-83
EST0095	458	 I I+10 I+30 II +10 +30 III +10 +20 +30 IV	0.78	AC011225	0
EST0165	94	I	1.04	AC103740	4e-45
EST0224	164	 I +10 +30 II+30 III +10 +20 +30	2.04	AC007342	1e-86
EST0226	113	 I I+10 I+30 II+30III +10 +20 +30 IV V VI	1.25	AL354803	3e-56
EST0228	119	 I I+10 I+30 II+30 III +10 +20 +30 IV V VI	1.33	AL354803	1e-27
EST0281	363	 I I+10 I+30 II+30	1.98	AL358372	0
EST0367	495	 I I+10 +30 II+30 III +10 +20 +30 IV V VI	0.90	M93287	0
EST0369	243	 I I+10 I+30 II+30 III +10 +20 +30 IV V	1.24	AJ549502	3e-64
EST0382	389	 I others	0.90	AL450425	0
EST0400	237	 I I+10 I+30 II+30 III +10 +20	0.99	AC009410	e-123
EST0410	217	 I others	ND	AL353581	e-105
EST0430	147	 I I+10 I+30 II+30 III III+10	0.87	AC021088	2e-76
EST0440	384	 I I+10 I+30 II+30 III +10 +20 +30 IV V VI	1.33	AC100834	e-149
EST0443	143	 I I+10 I+30 II+30 III +10 +20+30 IV V VI	0.92	AL390920	4e-74

Table 13. Differentially expressed cDNA-AFLP fragments representing novel sequences on the basis of GenBank database.

GenBank Accession number	Length (bp)	Expression Pattern	Array Ratio
CN544855	271	Down	1.23
CN544861	109	Up	1.10
CN544868	81	Up	1.13
CN544894	100	Down	1.35
CV102262	136	Down	0.99
CV102264	160	Down	ND
CV102268	228	Up	ND
CV102300	129	Up*	1.52
CV102313	143	+ I	1.23
CV102319	131	Up	0.91
CV102342	144	+ I	1.11

*Expression pattern was verified by microarray probed by different RNA samples from stage I (mature green) and stage VI (tree-ripening), verified pattern is labeled with a *; ND: non-detectable

+Transiently expressed or suppressed.

DISCUSSION:

Transcriptome analyses were used in this study to determine the temporal expression of ripening-related candidate genes in 'Gala' apple fruit. This analysis significant changes in transcript levels occurred in apple fruits during maturation and ripening. The greatest differences were detected between fruits harvested in mature-green (early mature) stage and later stages, similar to a report by Lay-Yee*et al.* (1990). This indicates that major changes in gene expression occur in apple fruits as the IEC increased from twenty to 1000nL/L. Similar differences in steady state mRNA levels have been observed between pre- and post-ripening states in many other fruits such as tomato (Giovannoni, 2001 and 2004; White, 2002), apple (Lay-Yee *et al.,* 1990), grape (Davies and Robinson, 2000), raspberry (Jones *et al*., 2000), and strawberry (Aharoni and O'Connell, 2002).

Differentially expressed sequence tags (ESTs) obtained from cDNA-AFLP were isolated, cloned, sequenced and compared for similarity using BLAST in the NCBI databases. These ESTs were divided into groups based on the proposed function of similar genes identified by BLAST. These functional groups include roles in aroma biosynthesis, carbohydrate metabolism, cell structure and motility, cell wall degradation, defense/ stress responses, general metabolism, intracellular trafficking and sorting, protein biosynthesis and degradation, pigment biosynthesis, signal transduction, transcription and translation. These diverse functions indicate that during fruit ripening extensive changes in gene expression occur.

Studying these patterns of differential gene expression highlights a common issue in postgenomic science. How does one relate a gene sequence to cellular function and to physiological effects in tissues and organs? Here, we have attempted to connect the relationships from the up-stream precursors to the down-stream products, and from transcripts to known physiological changes investigating genes with differential expression patterns identified during maturation and ripening.

Aroma Biosynthesis

Aroma volatiles are secondary metabolites that play a major role in fruit quality. Volatile esters are major flavor components in manyfruits, including apple (Beekwilder *et al*, 2004). As ester production is closely linked to the onset of the climacteric and ripening, Knee (1993) suggested that the appearance of esters (mainly palmitate and oleate) is one of the earliest events in ripening. In this present study, two differentially-expressed genes involved in aroma biosynthesis were identified. The palmotoyl protein thioesterase (similarity of EST0215 and EST0218; EC 3.1.2.2.) reversibly catalyzes the hydrolysis of palmitoyl-CoA or other long-chain acyl CoA compounds to yield CoA and palmitate or other acyl esters. EST0215 and EST0218 have a nearly identical sequence and 99% similarity. They were initially isolated after noting their increased expression pattern on cDNA-AFLP. That pattern was not verified in the following year using microarray analysis. Whether this result was due to the year to year difference or the variance of sensitivity or specificity between these two genomic tools is not known. Further studies are needed to verify the pattern of changes that occur in this interesting gene affecting aroma.

Of particular interest was the fragment for apple alcohol acyl transferase (AAT; EST0379). This catalyzes the final step in ester formation, by the transacylation of an acyl-CoA into an alcohol. Due to its key role in ester biosynthesis, the activityof AAT has been studied in a variety of fruit species. Using cDNA-AFLP, the greatest expression of AAT was detected in tree-ripened fruit. However, that fruit did not produce the highest level of ethylene in this study. The lowest expression was found in fruits harvested in the mature-green stage, which had the lowest IEC. This pattern was verified by microarray. The expression of EST0379 (having 90% identity to apple AAT2) may be moderated by ethylene production and fruit maturity. This was reported previously by Yahyaoui *et al.* (2002) in melon fruits. The encoded product for this gene also plays a key role in fatty acid biosynthesis, oxidation, and esterification to form triglycerides. Consequently, it may not be solely involved in aroma generation (Beekwilder *et al*, 2004).

A greater expression of 1-acyl-sn-glycerol-3-phosphate acyltransferase (EST0079 and EST0401) was detected in tree-ripening fruit than in detached fruit. This gene is also associated in lipid metabolism. Tree-ripened fruit may have a greater capacity to produce aroma volatiles than harvested fruits. These aroma volatile and ester/ lipid biosynthesis-related gene fragments isolated in this studywere allup- regulated, and their greatest expression was measured in tree-ripening fruits. These expression patterns are in agreement with previous reports that aroma volatile production is maturity-dependent, and related to ethylene production (Song and Bangerth, 1996; Rudell *et al*., 2000).

Carbohydrate Metabolism

The expression of genes involved in carbohydrate biosynthesis, degradation and translocation increased during ripening. These transcripts were expected on the basis of the dramatic changes observed in starch hydrolysis and soluble solids content during apple fruit ripening. The glucose-6-phosphate/phosphate translocator (GPT) related protein (EST0073) has been hypothesized to facilitate both starch and fatty acid biosyntheses as well as the plastidial oxidative pentose phosphate pathway in sink tissues (Kammerer *et al.*, 1998). It also has been shown to have a role in the transition of chloroplasts into chromoplasts during pepper and tomato fruit development (Batz *et al*, 1995; Buker *et al*, 1998). The increasing expression pattern of GPT during ripening may indicate that non-green heterotrophic apple fruits continue accumulating starch or fatty acids in which most carbon is imported from the leaves or plastids in fruits. Monosaccharide (hexose) transporter and sucrose phosphate synthase, similarity of EST0037 and EST0057 respectively, have been suggested to be associated with an autocatalytic sugar accumulation in grape berry (Fillion *et al*, 1999) and the increase in suc rose concentration in muskmelon (Hubbard *et al*, 1989) during ripening.

Isoamylase (EST0287) has been shown to be involved in both amylopectin synthesis as well as debranching in banana fruits. This occurs without any significant change in transcript level (Bierhals *et al*, 2004). Its elevated activity in that study was attributed to the role of isoamylase in the hydrolysis of alpha-1, 6-linkages of amylopectin. Its slight increase in expression detected in this study suggests that different control cascades occur among species. The general kinetic activity of

isoamylase increases during fruit ripening in both species. Sorbitol dehydrogenase 1 (SDH1; EST0101) which catalyzes the first step in the breakdown of transported carbohydrate from the parent-tree plays a crucial role in the latter stages of fruit maturation, but not in early fruit development (Park *et al*, 2002). Knee (1993) indicated that SDH is predominant enzyme needed for the apple fruit to utilize the major translocated carbohydrate, sorbitol, to synthesize fructose. The gene fragment identified as up-regulated during ripening appear to correlate well with observed changes in maturity indices leading to the observed increase in soluble solids concentration and a decrease in starch content.

Firmness Loss

Fruit texture and firmness are determined by cell turgor pressure and by cell wall biochemistry. Modification and turn-over of the primary cell wall is required for growth as well as softening of fruits during maturation and ripening. Enzymes act on the pectin fraction of the cell wall reduce the molecular weight of pectic polymers by depolymerization. This raises the solubility of pectic polysaccharides. Changes in enzymes such as beta-galactosidase, pectin lyase, pectin methylesterase, endo- and exo-polygalacturonase (PGs) have been well-documented.

Another group of enzymes that play a role in softening catalyzes the disassembly of the cellulose -hemicellulose matrix in ripening fruits. These are endo-1,4-beta-D-glucanase (EST0085), expansins, α -L-Arabinofuranosidases (AFase , EST0437), and xyloglucan endo-transglycolase. Transgenic studies on tomato and strawberry indicated that most hemicellulases help to increase enzyme access to the cell wall in

the early stage of fruit development and that pectinases and expansins affect ripening-related softening (DeEll et al., 2001).

The differential role of three isoforms of α -L-Arabinofuranosidases (α-Afs) in fruit softening of tomato has been reported. In tomato, α-Afs is likely to be encoded by a gene family. This is suggested since their functions are not completely mediated by ethylene, and the release of neutral sugars from the cell wall matrix also precedes the ethylene climacteric (Brummell and Harpster, 2001). The existence of more than two members of α-Afs in apple fruits was postulated based on the result of *In Silico* alignments of twelve accessible similar ESTs with AFase. AFase1 is the only α-Af which has been characterized in apple fruits (Brummell and Harpster, 2001). Breyne and Zabeau (2001) reported that microarray has difficulty discriminating among different transcripts from genes belonging to the same gene family. Similar to a report of three homoloues in tomato, α-Af I, α-Af II, and α-Af III, the non-significant difference of hybridization intensity of the up-regulated EST0437 in mature-green and tree ripe 'Gala' apple fruit microarray could be caused by cross hybridization.

Our cDNA-AFLP result with endo-1, 4-beta-D-glucanase (EST0085) was similar to the finding of Salentijn *et al.* (2003). Greater expression levels were observed in firmer cultivars and in firmer fruits. On the other hand, a higher activity of endo-1, 4-beta-D-glucanase was found by Harpster *et al.*(2002) during pepper fruit ripening. These results support the hypothesis that endo-1, 4-beta-D-glucanase is under post-transcriptional control during ripening. The transcript level was higher in the early stage of apple ripening (cDNA-AFLP) but no significant difference was found among stages in the microarray study. Since no sequence similarity to this enzyme has been

found in plants, cross-hybridization may not explain the non-significantly different expression pattern found in our microarray.

Another novel candidate gene associated with cell wall degradation is an up-regulated cinnamoyl Coa reductase (CCR; EST0114). This is involved in lignin metabolism. CCR can influence variations in lignin content and has been postulated to play a major role in the genetic manipulation of strawberry cultivars (Salentijn *et al*., 2003).

Other Developmental and Ripening-Related Processes

Several up-regulated gene fragments associated with other aspects of growth and development were observed in this study. Those were EST0001 with significant similarity to *Defective embryo and meristems* (*Dem*) of tomato, EST0047, similar with SEU2 protein, and EST0217 which encodes a similarity of calcium/calmodulin-dependent protein kinase (CaMK1). CaMK1 is an enzyme in the serine/ threonine protein kinase family. The novel gene, *Dem* has been shown to be essential for the correct organization, and development of shoot apical tissues in developing embryos. It is also required for correct cell division patterns and the maintenance of root meristems (Keddie *et al.,* 1998). *SEUSS* is characterized as a basic fundamental gene in the negative regulation of AGAMOUS and has been proposed to encode a co-regulator of *LEUNIG* (Franks, et al. 2002). This gene is currently assigned to a family of plant regulatory proteins, and given the role of repressing homeotic transformation of floral organs. The extent of organ loss in floral whorls was verified

by *in situ* hybridization, and using double and triple mutant analyses (Franks *et al.*, 2002).

Intensive studies have shown that CaMK is highly regulated, both temporally and spatially. This occurs in both reproductive and vegetative tissues of many species (Zhang and Lu, 2003). A diverse number of responses including host–pathogen interactions, cold stress, gravitropism, light-regulated gene expression, flowering and hypo-osmotic shock have been shown to be mediated by CaMKs (for review, see Zhang and Lu, 2003). The differentially-accumulated transcriptsof CaMK indicated that this gene product is not only involved in development, but also in responses to numerous environmental stresses.

None of the differential expression patterns described above has been reported to occur during fruit development and ripening. The consistent increase of the transcripts detected in this study implicates them as having crucial functions during maturation and ripening. Both EST0001 and EST0020 were highly matched (both 83%) with two ESTs from an abioticly stressed grape library. Based on their functional characteristics, recent data of their involvement with development and ripening, and their responses to environmental cues, it is possible that both stress and ripening can induce similar gene expression (Davies and Robinson, 2000; Aharoni and O'Connell, 2002).

The physiological aspect of adenine phosphoribosyltransferase 1 (APRT; encoded product of EST0112) in recycling of nucleotides has been well documented. This has been studied in mammals, but its up-stream molecular basis remains poorly

understood. APRT was thought to be expressed constitutively, although the enzyme activity which was shown to be higher in the early stages of seed development (Guranowski and Pawelkiewicz, 1978) and breaking dormancy (Robert and Pétel, 2000). A molecular role of APRT has not been reported during fruit ripening. Here, we provide the first evidence of an increasing transcript level of APRT, which is under distinct regulatory control during fruit development and ripening. Its increasing expression pattern may support the suggestion that a rapid turn-over of nucleotide pools occurs as ripening progresses. Further studies of the role of this interesting and important enzyme ARPT are needed to clarify its role in the fruit-ripening processes.

Our results also suggest that the EST0096 (Metallothionein-like gene, MT-like) is a down-regulated gene fragment in either detachment-induced ripening or in attached apple fruit ripening. A similar decrease in the type-2 MT transcript has been reported in ripening banana fruit (Liu *et al.*, 2002, Clendennen and May 1997). However, the closest sequence similarities in sand pear and apple have been reported to be up-regulated as ripening progressed (Itai *et al.*, 2000; Reid and Ross, 1997). An increasing pattern of MT expression during grape berry ripening was implicated in fruit senescence (Davies and Robison, 2000). This enzyme functions to control metal metabolism and detoxification, as well as the cellular redox potential. The physiological implications of this developmentally-specific MT-like protein's function needs additional research.

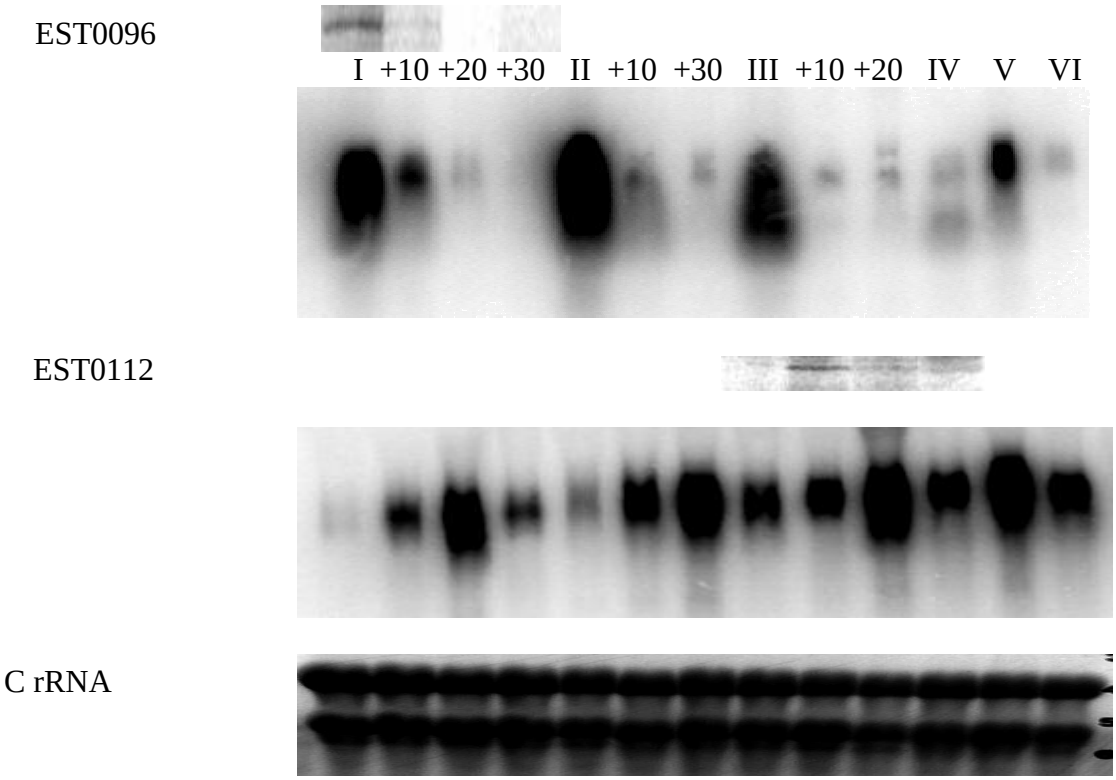


Figure 14. Northern blot analyses confirming differential expressed AFLP fragments, EST0096 and EST0112.

Their expression were suppressed or induced respectively during ripening. Total RNA (25 μg) extracted from different set of apple fruit pulp tissue harvested in succeeding years was used for RNA gel-blot analysis. The bottom panel is a photograph of an ethidium-bromide-stained formaldehyde gel to show the intactness and relative loadings of the RNA samples used in the northern analysis. EST 0096 (A, similarity of a metallothionein-like protein) and EST0112 (B, similar to adenine phosphoribosyltransferase 1) were ^{32}P-radiolabeled and used as probes.

Defense/ Stress-Related Responses

During ripening, a range of transcripts were identified with homologies to detoxification proteins, protection and defense proteins and to stress- related proteins. Many isolated genes have been found to be involved with defense and stress

responses. Genes responsible for normal physiological functions are also prone to play a role in responding to stress. In total, more than 20% of genes identified could be connect to stress responses, based on the putative functions of their similarity. A similar finding regarding the wide-range of stress-related cDNAs with enhanced expression with ripening have also been reported recently in grape and strawberry (Davies and Robinson, 2000; Aharoni and O'Connell, 2002). The differences in stress-related gene expression found here may reflect the possibilitythat attached fruits can be under stress from drought, heat, or pathogens. Osmotic stress also occurs during ripening, in response to the rapid accumulation of sugar accumulation (Davies and Robison, 2000). The activation of stress-related genes is also considered to be a survival mechanism by the fruit to gain more stress tolerance as it matures. Ripening is a series of reactions which change the physiological condition of the fruit as it moves towards senescence. This follows ripening and leads to eventual fruit breakdown. Therefore, the ripening process may lead to the production of various chemicals that induce the expressions of stress-related genes. In turn, those stress-related genes could enhance the tolerance of fruits toward abscission and senescence.

A few up-regulated genes encoding for stress-related products, including a heat shock transcription factor (EST0359), aspartic proteinase (AP, EST0204), Na^+/H^+ antiporter (EST0309), and a putative copper/zinc superoxide dismutase copper chaperone precursor (EST0128) have been shown to mediate various survival responses to stresses caused by senescence, salinity or dangerous levels of superoxide (Wu 1995; Mathew *et al.,* 2001; Simões and Faro, 2004; Shi *et al.,* 2002). Though Na^+/H^+ antiporters have been shown to have roles in salt tolerance, they also function

to regulate cell turgor and hormonal responses (Serrano *et al.*, 1999). Our discovery of a slight rising accumulation of transcripts for the Na^+/H^+ antiporter during ripening may indicate that it may have another feature: firmness retention through maintenance of cell turgor.

Two translated products of EST0428 and EST0456 encoded for cytochrome *P450.* This is utilized in plant biosynthetic and detoxicative pathways, and is particularly crucial in biochemical pathways that respond to a number of chemical, developmental, and environmental cues (Schuler and Werck-Reichhart, 2003). The pathway from activation of *P450* toward ethylene production and then protein degradation mediated by auxin, ACC synthase to ubiquitin was proposed by del Pozo and Estelle (2000). They suggested that a relationship existed between *P450* and ripening. The differential accumulation of cytochrome *P450* transcripts during ripening in this paper supports that hypothesis. A distinctly up-regulated EST0142, encoded a similarity of the submergence-induced protein 2A. Though the function of this encoding product is still poorly understood, its association with stress and ripening can be seen from its name and by the proposed stimuli that enhance the expression of its similar genes in the EST database.

Some defense and stress associated gene products had inconsistent expression patterns. These were an acid phosphatease survival protein, (SurE, similarity of EST0459), a disease resistance protein (EST0455), a phosphoglyceromutase (EST0104), and a phytochelatin synthetase-like protein, EST0019. They have also been proposed to be involved in stress-response, disease resistance or the intracellular detoxification of metals (Mura *et al*, 2003; Guterman *et al*, 2002).

Protein Biosynthesis and Degradation

The accumulation of transcripts related to protein synthesis and turnover during ripening suggests that the fruit cell maintains a progressive and dynamic protein metabolism. Similar findings have been reported previously in the non-climacteric strawberry by Aharoni and O'Connell (2002). Genes putatively encoding biosynthesis-related proteins included transcription factors (EST0020, 0053, 0056, 0082, 0284, 0352), ribosomal proteins (EST0127, 0147, 0170, 0328, 0363, 0405, 0421), an endoreticulum protein (EST0056), an elongation factor (EST0064), a translation factor (EST0105), aminotransferases (EST0094, 0112), and eukaryotic release factors (EST0202, 0371, 0372). These are well-known for their essential roles in transcription, and translation and in synthesis of amino acids, polypeptides, and protein biosynthetic pathways. Several proteins such as the aspartyl protease family of proteins (EST0381), Zinc metalloproteinase-like protein (EST0161), putative cathepsin B-like protease (EST0080) and the large ATP-dependent 26S proteolytic complex (EST0026, 0048, 0071, 0076, 0108, 0153) were found. It is likely that several independent protein degradation pathways exist in apple fruit. The 26S proteasome is known to function in the selective removal of various short-lived proteins. These are first covalently linked to ubiquitin and subsequently degraded by the 26S proteasome complex (Kirschner, 1999). Genes putatively associated with the ubiquitine-26S-proteasome machinery included subunits of the 26S proteasome (21D7 and 20S proteasome subunit), three different types of ubiquitin protein ligase (E3, U-box domain-containing protein, and SKP1) and polyubiquitine (EST0076). These related similarities were up-regulated as ripening progressed. Taken together,

the ripening processes appear to be one of the most intensive stages of protein biosynthesis and degradation in either non-climacteric fruit, such as strawberry (Aharoni and O'Connell, 2002) or in climacteric fruits such as apple.

Among housekeeping genes, eighteen were considered to be metabolism-related, and were induced during ripening. These genes were primarily associated with carbon metabolism (i.e. TCA cycle, glycolysis, pyruvate metabolism) which is known to provide metabolites such as ATP, NAD(P)H. Maintaining ripening-related processes in the fruit requires increased respiration and energy consumption. An elevated expression of these genes was also found in ripening strawberry fruits. They may produce the energy and precursors needed for ripening-related processes such as aroma formation, and amino acid-derived products (Aharoni and O'Connell, 2002).

Calcium Signals: A Central Paradigm in Stimulus–Response Coupling

Certain Ca^{2+} involved gene fragments were observed in this study. These includes calmodulin-binding proteins (EST0222 and EST0390; see Appendix), and CaMK (calmodulin-dependent protein kinase; EST0217). Recent studies implicate free calcium in the cytosol as a secondary message during the transduction of a very wide variety of abiotic signals. These include light, low and high temperatures, touch, hyperosmotic stress, oxidative stress, and biotic stimuli from the plant hormones abscisic acid (ABA) and gibberellins. Fungal elicitors, and nodulation (Nod) factors are also mediated *via* Ca^{2+} (reviewed by Sanders *et al.*, 2002). In plant cells, calmodulin (calcium-binding protein) is considered as one of the primary sensors relaying a signal through changes that occur in cellular free Ca^{2+} levels. Kinases such

as CaMK appear to represent an important pathway by which calcium signals are decoded and then propagated into changes in protein structure and enzyme activity. Interestingly, Aharoni and O'Connell (2002) obtained similar gene clusters associated with the Ca^{2+} molecule transport pathway and a RNA-related small GTP binding protein which were induced in achene maturation process using microarray analysis in strawberry. These authors proposed that these were connected to the ABA signal transduction pathway. Both studies appear to indicate that the change of calcium signal is involved in the fruit ripening process. This may occur *via* the control of transcript level of calmodulin related proteins.

The Role of Ethylene in Apple Fruit Ripening

The changes observed in the mRNA accumulation during ripening are thought to be influenced by changes in the levels of plant hormones, such as ethylene. Other hormones such as ABA (Lara and Vendell, 2000), jasmonate (Fan *et al.*1998) and IAA (Tingwa and Young, 1975; Mousdale and Knee, 1981) have also been implicated in ripening. The effects of ethylene in plant development are mediated by changes in coordinated mechanisms at the transcriptional level and by post-transcriptional and translational acts, such as proteolytic processing or protein phosphorylation/dephosphorylation (Ecker, 1995; Sunako *et al.*, 1999).

We did not make any attempt to isolate known ethylene biosynthesis-related gene families by using the selective primers employed in this study. We did however obtain two gene fragments from the same families as an ethylene receptor, as well as an ACS. The similarity of EST0217, PK23, belongs to a gene family of

serine/threonine protein kinases ethylene receptor CTR (constitutive triple response) which was identified in a mutant that shows the triple response, even in the absence of ethylene. The highest match to EST0355 was a member in the family of pyridoxal-5'-phosphate-dependent enzymes closely related to (and often designated as) putative examples of ACC deaminase or ACC synthase. Members of this family have been shown to be less well conserved. On the other hand, most differential expression patterns of these gene fragments obtained in this study were also reported to occur in non-climacteric fruits such as strawberry (Aharoni and O'Connell, 2002) and grape (Davies and Robinson, 2000).

The tree-ripe fruit (stage VI, attached) and detachment-induced ripe fruit (stage I+30, II+30, and III+30; detached) shared a lot of commonality in gene expression patterns although some physiological differences were observed in this study. The greater IEC, firmer texture, and lower soluble solids contents in detached fruits were not reflected at the transcript level in this study. Our data suggest that the most differential transcriptional levels were not solely related to ethylene.

Human Genomic DNA

Several gene fragments highly identical to human genomic DNA were found in this study, and by Jones *et al.* (2000). In our study, some of these appeared to arise as contamination in cDNA preparation, as their expression was only observed transiently in one specific sample (stage I). However, all of these gene fragments showed hybridizational intensities in the microarray studies with a different set of 'Gala' apple RNA samples. EST0010, EST0055, EST0226, EST0228, and EST0367

are not likely to be contaminated human gene clones since they had multiple expression patterns, and were verified by microarray hybridization data. Does our study provide an evidence for a closer, conserved relation between apple and human genomes? Further studies are needed to clarify this concern.

Technical Issues of These Genomic Tools

RNA isolation constitutes the basis of gene expression studies. Extracting high-quality RNA from apple pulp is a time-consuming complex processes with relatively low yields (see Appendix for details). Both cDNA-AFLP and microarray methods were chosen as they provided the opportunity to rapidly identify changes in gene expression in the developmentally stage-specific pattern, and with a relatively small amount of mRNA.

Specifity and sensitivity are both considered to be important characteristics of expression-detection technologies. Hoheisel and Vingron (2000) pointed out the sensitivity of PCR-based techniques, such as cDNA-AFLP, are considered to be higher than hybridization-based techniques such as microarrays. cDNA-AFLP is thought to be the most powerful tool available because of greater sensitivity than other genomic techniques including microarrays, GeneChip analysis, and Northern blot analysis (Reijans *et al.*, 2003; Bachem *et al*, 1996). Reijans *et al.*(2003) proposed that cDNA-AFLP analysis would allow the identification between genes with a 92% similarity. On the other hand, cross-hybridization may occur to their targets with high degree (>75-80%) of sequence similarity in microarray. Microarray does however, provide a fast way to screen for differential expression of thousands of

genes (Breyne and Zabeau, 2001; Reijans *et al*, 2003). Consequently, some unidentified patterns in our microarray studies were likely in part due to these differences in specifity and sensitivity between these two techniques.

This research was directed at gaining a better understanding of the molecular events associated with apple fruit ripening. Identifying ripening-related transcripts that are differentially expressed in apple pulp was thought to enable a more in-depth understanding of concurrently physiological events. It will be possible to use these isolated cDNA clones in future experiments to investigate patterns of stage-specific gene expression in response to physiological and environmental changes, or to determine which novel genes respond to ethylene. With additional information about their relative abundance and temporal-expression patterns, the potential roles of the translation products can be examined in depth.

Concluding Remarks

The enhanced expression of defense/ stress-induced proteins and DNA, RNA and protein metabolism associated proteins found during ripening in climacteric and non-climacteric species indicates that one highly-conserved genetic mechanism regulating ripening is not totally controlled by ethylene. Moreover, this study demonstrated that the similar biochemical changes of fruit ripening and plant senescence requires the strong support of many housekeeping genes. Most of these are under transcriptional control. A number of genes associated with key ripening traits, such as metallothionein-like protein, endo-1,4- beta-D-glucanase, alcohol acyl transferase have been identified here and studied previously in both climacteric and non-

climacteric fruits. They play a role in stress response, cell wall disassembly, and are major enzymes in the formation of volatile esters affecting fruit qualities such as flavor and aroma.

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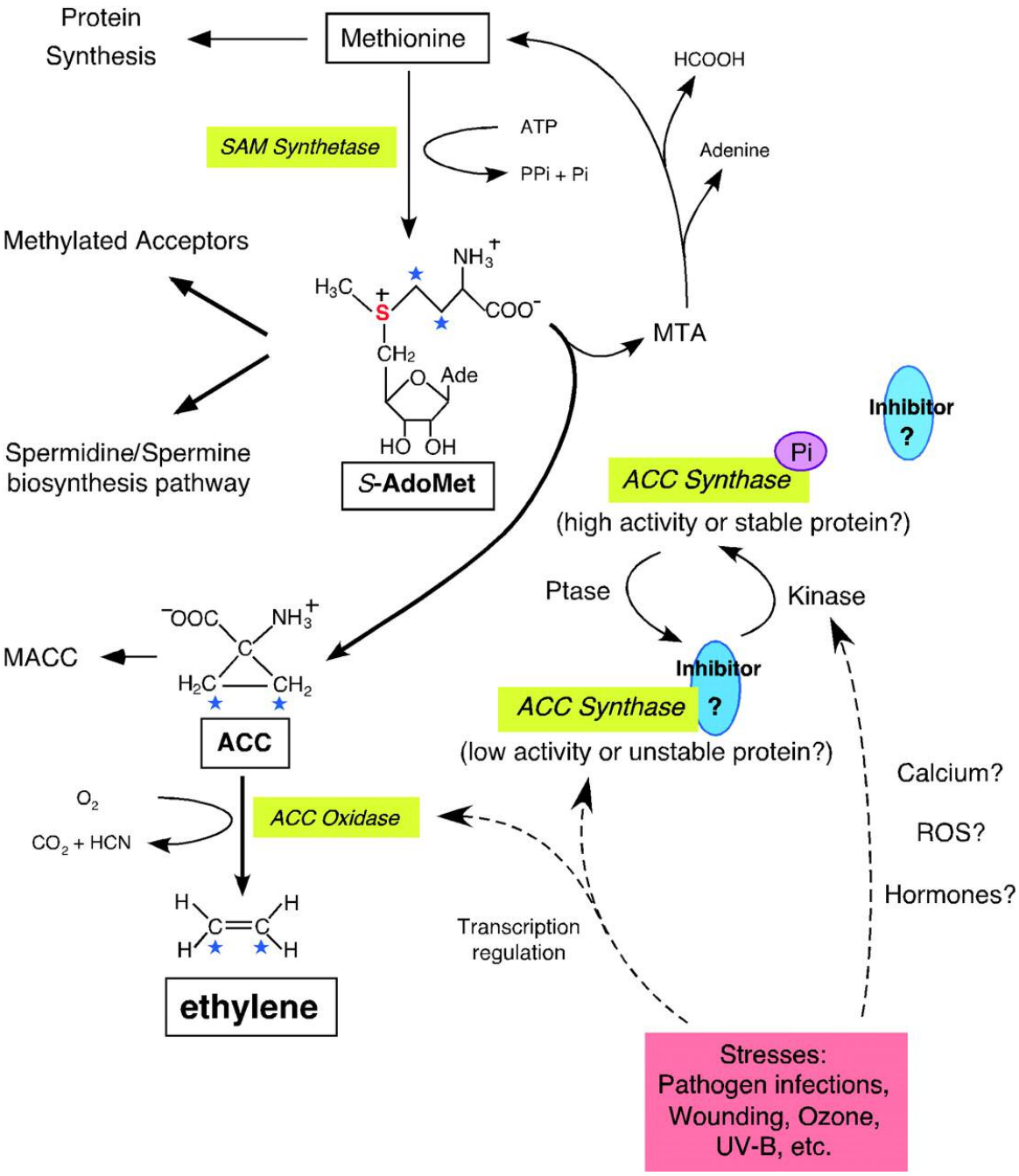
Appendix

Appendix 1: Biosynthetic Pathway and Regulation of Ethylene (Wang et al., 2002).



Appendix 2: Propylene Treatment vs. Fruit Maturity

Propylene, an ethylene analogue, is the next most active compound to show ethylene-like action. It can be readily separated from ethylene by gas chromatography. The equivalent concentration of propylene to cause half-maximum ethylene response was found to be 130 times that of ethylene (Burg and Burg, 1967). The fruits' potential for autocatalysis can be determined by its response to propylene (McMurchie *et al*., 1972). The shorter the lag period to the onset of climacteric the higher the maturity would be (Sfakiotakis and Dilley, 1973a).

To evaluate the role of ethylene and chilling exposure in the ripening processes, storage study with short term application of propylene or cold temperature (0^{o}C) on 'Fuji' apple was achieved.

Materials and Methods for Storage Study of 'Fuji' apple

1. 2001

The experimental design was complete randomized design with 12 replications. Experimental structure was a two-rootstock by three-harvest interval factorial. Six 'Fuji' apple trees were on M 7a or MM 111 and two fruits of each were picked periodically prior to and during fruit ripening (on 1, 15, 29 Oct. 2001) and then held individually in an ethylene-free lab at 20°C. Internal ethylene concentration and external ethylene evolutions (headspace volatile analyses) were measured at intervals for 2 months after harvest. Internal ethylene assays were performed as described above.

2. 2002

'Fuji' apples, obtained from trees budded on rootstock M 7a at the Western Maryland Research and Education Center Keedysville, MD, were harvested on Aug. 20, 29, Sep. 12, 26, and Oct. 10, 24, 2002. Forty similar-sized apples were divided

into five treatments on each harvest date. Two groups of fruits were treated with 500 µL/L propylene for 3 or 7 days in chambers maintained at 20˚C. Another two groups were stored at 0˚C for 3, and 7 days before being transferred and held at 20˚C. Untreated controls were held in an ethylene-free lab at 20˚C. Ethylene and other maturity indices were monitored one day, two weeks and 1 month after harvest. Eight replicates were used in all treatments for each IEC; four replicates were sacrificed for flesh firmness, soluble solids, and starch measurements at each of three intervals during storage.

Results for Storage study

'Fuji' apple fruits of different maturities were held at 20˚C in an ethylene-free lab immediately after harvest showed a change of IEC in succeeding years. Only trace amounts of ethylene were detected in most fruits harvested in early-commercial maturity even after one month at 20˚C (Tables 14 and 15). The number of fruits that underwent climacteric after one month storage decreased in fruits harvested in commercial maturity from trees on MM.111 in 2001 and M.7a in 2002. Later, significant quantities of ethylene were produced by fruits harvested later than commercial maturity in both years and in both rootstocks.

Propylene and cold treatments were applied continuously after harvest to 'Fuji' apple fruits in 2002. With the exception of the three-day propylene treatment to fruit harvested on August 20, 2002, all propylene treatments induced system 2 ethylene productions in two to four weeks after harvest. Not surprisingly, in propylene-treated fruits, the later the harvest dates the shorter the lag periods postharvest before ethylene increased.

Table 14. IEC of stored 'Fuji' apple fruits.
Fruits were picked on 1, 15, 29 Oct. 2001 from trees budded onto M.7a or MM.111 and stored at 20°C for 1 month. a, b= mean separation as determined by Tukey's protected LSD, $p \leq 0.05$.

Harvest Time	Rootstock	IEC (μL/l)
10/1/2001	M. 7a	32.24±28.89 a
10/1/2001	MM. 111	33.42 ±28.89 a
10/15/2001	M. 7a	183.48±31.62 b
10/15/2001	MM. 111	0.69±0.14 a
10/29/2001	M. 7a	157.89±53.13 b
10/29/2001	MM. 111	117.33±48.50 b

Table 15. IEC of stored 'Fuji' apple fruits.
Fruits were picked on 12, 26 September, and 10, 24 October 2002 from trees budded onto M 7a and stored at 0°C for 0 , 3, and 7 days or treated by 500 ppm propylene and then transferred to 20°C for a total duration of 1 month. a, b= mean separation as determined by Tukey's protected $_{LSD}$, $p \leq 0.05$.

Harvest Time (DAFB)	Treatments (days)	IEC (μL/L)
8/20/2002 (120)	Control	0.046±0.006a
8/20/2002 (120)	Propylene 3	0.054±0.005a
8/20/2002 (120)	Propylene 7	60.87±28.09b
8/29/2002 (129)	Control	0.068±0.006a
8/29/2002 (129)	Propylene 3	146.78±55.92b
8/29/2002 (129)	Propylene 7	245.27±91.89b

Table 15. Continued.

Harvest Time (DAFB)	Treatments (days)	IEC (μL/L)
9/12/2002 (143)	Control	0.24±0.065 a
9/12/2002 (143)	Propylene 3	161.24±92.43b
9/12/2002 (143)	Propylene 7	120.58±71.82b
9/12/2002 (143)	Cold 3	103.46±63.22 b
9/12/2002 (143)	Cold 7	0.46±0.14 a
9/26/2002 (157)	Control	36.84±15.86 a
9/26/2002 (157)	Propylene 3	55.51±47.01a
9/26/2002 (157)	Propylene 7	181.89±140.85a
9/26/2002 (157)	Cold 3	28.87±25.54 a
9/26/2002 (157)	Cold 7	53.31±42.04 a
10/10/2002 (171)	Control	2.03±1.46 a
10/10/2002 (171)	Propylene 3	101.02±65.71b
10/10/2002 (171)	Propylene 7	202.53±128.35b
10/10/2002 (171)	Cold 3	68.62±60.67 a
10/10/2002 (171)	Cold 7	295.8±76.9 b
10/24/2002 (185)	Control	82.15±21.38 a
10/24/2002 (185)	Propylene 3	283.87±182.54a
10/24/2002 (185)	Propylene 7	18.92±13.74a
10/24/2002 (185)	Cold 3	68.91±42.30 a
10/24/2002 (185)	Cold 7	15.88±4.52 a

Our data infer that ‘Fuji’ apples grown in Maryland need more than 120 DAFB (April 20, 2002 to August 20, 2002) to attain physiological maturity as fruits harvested in 120 DAFB were not able to respond to 3-day external propylene treatment. No fruits harvested during 120-150 DAFB were found to produce autocatalytic IEC after storage at room temperature for a month although some were capable of responding to ethylene or propylene. The increased IEC in untreated control fruits harvested at 150 DAFB (September 26, 2002) was initially detected after 1 month storage. However, fruits harvested at 160-170 DAFB were not able to ripen after one month storage. Interestingly, both cold and ethylene treatments could induce ripening of fruits harvested at this stage. Finally, fruits harvested at 180 DAFB later ripened rapidly, in less than two weeks. It appears that cold treatment can induce climacteric ethylene production of fruit harvested after 150 DAFB. Three days at 0˚C were generally more effective than 7 days cold exposure in this limited trial.

Most fruits harvested in two weeks before and two weeks after commercial maturity regardless of treatments appeared competent to produce ethylene after one month storage. However, 3-day or 7-day period of chilling completely accelerated ethylene production in ‘Fuji’ apple only at about 165 DAFB.

Discussion for The ripening behavior of postharvest ‘Fuji’ apple fruits

Our storage results indicated that the later the fruits were picked, the shorter the lag period for the climacteric and the higher IEC average. This has been reported by many researchers previously (Lau *et al*., 1986, Blanpied, 1993). Most of early-picked ‘Fuji’ apple fruits that maintained at low levels of ethylene at 20°C were not capable

of self-induced ripening on their own, although they could be triggered by exogenous ethylene and propylene. Fruits harvested after commercial maturity did not respond to propylene, since high level of endogenous ethylene was adequate to induce the logarithmic increase in ethylene production. The ripening behavior of fruits harvested around commercial maturity was unexpected. The ethylene production decreased in fruits harvested from trees on MM.111 (2001) and M.7a (2002) at commercial maturity after 1-month of storage. Together our results indicated that 'Fuji' apple required 120-130 DAFB to be physiological mature in Maryland, but fruits were not competent to accomplish autostimulated ripening if harvested before 150-160 DAFB. The ripening behavior of 'Fuji' apple was also found to be affected by genetic control (maturity), rootstock and environmental factors. To further study this interaction controlling temperatures using a growing chamber could elucidate 'Fuji' apple ripening. Ripening sensitivity of apples to a given ethylene level has been shown to increase as maturation progresses (Liu, 1978).

On the other hand, 'Red Delicious' apples have been shown to respond to external propylene when relatively immature. Based on a 145-days post bloom growth period they responded at only about 75 days after full bloom (Sfakiotakis and Dilley, 1973b). 'Fuji' is a late-maturing cultivar which needed 75-80% maturity based on 150-160 DAFB to respond to exogenous propylene.

Appendix 3: Techniqual issues

Many different protocols including commercial RNA extraction kits were tested (data not shown). The one described by Chang *et al*. (1993) with preceding ethanol

cleaning steps was found to be the most efficient for RNA isolation from apple pulp. The application of ethanol cleaning procedures was needed to remove considerable amounts of polysaccharides, polyphenol and other secondary metabolite compounds that limit RNA extraction yield and purity. The general yield of high quality total RNA from 'Gala' apple was 2-5 μg RNA /g of fleshy tissue.

The restriction enzymes used for cDNA-AFLP were *Apo*I and *Mse*I (Durrant *et al.*, 2000) which provided most appropriate size and number of fragments based on an *in silico* review of the available apple cDNAs in June, 2003 (see Appendix 3). The majority of analyzed cDNAs had *Apo*I and additional *Mse*I cutting sites. This was not the case for the *Ase*I and *Taq*I combination which the rare cutter *Ase*I limit the availability of suitable sized fragments and can cause potentially invisible fragments (two ends of *Taq*I sites).

Among the approximately 3500 fragments inspected in cDNA-AFLP displaying gels, most of the mRNAs appeared to be constitutively expressed. Less than ten percent of total fragments of sizes ranging from 100 to 600 bp were found to be differentially expressed during ripening. Two hundred and twenty clones with altered levels of TDFs were successfully isolated, cloned and sequenced. BLAST results indicated thatsixteen that were cloned were a portion of the same gene fragment or were highly similar. For example, in the case for the two adjacent bands, EST0371 and EST0372, the expression patterns were transiently expressed in stage I and transiently suppressed in stage I respectively (Figure 15). However, they shared exactly the same sequence.

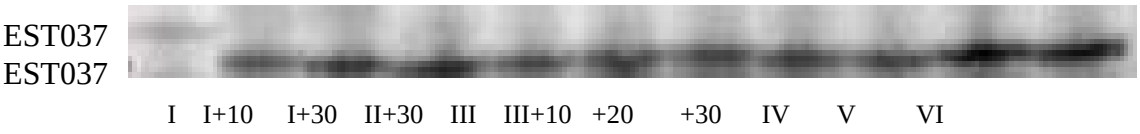


Figure 15. False differential gene expression in cDNA-AFLP.

Some gene fragments are expressed continually. However, we did get few constantly-expressed genes with a differential expression pattern during ripening. One was EST0460, a similarity of wild strawberry microsatellite or SSR (simple sequence repeat) DNA, with repeat motif as $(GTC)_8$ (James *et al*., 2003). In microsatellites or SSRs analysis, short tandem repeats of one to six base pairs have been used in cultivar identification and genetic analyses. These can be used to reveal identities, genetic diversity and relationships in a core subset collection. They are co-dominant, multi-allelic markers which are generally highly polymorphic (Brown and Maloney, 2003).

Appendix 4: Partial results of *In Silico* study (Gene fragments with * are visible on sequencing gels).

GenBank accession #: AY062129

#	Ends	Coordinates	Length (bp)	Ends	Coordinates	Length (bp)
1	*Apo*I-*Apo*I *	2520-2922	403	*Taq*I-*Taq*I	1176-2400	1225
2	*Apo*I-*Mse*I *	894-1194	301	(Left End)-*Ase*I	1-717	717
3	*Apo*I-*Mse*I *	1950-2183	234	*Ase*I-*Taq*I *	718-1175	458
4	*Apo*I-*Mse*I *	3321-3551	231	*Ase*I-(Right End)	5093-5470	378
5	*Mse*I-*Apo*I *	1734-1949	216	*Ase*I-*Taq*I *	3714-4088	375

GenBank accession #: AJ011518

#	Ends	Coordinates	Length (bp)	Ends	Coordinates	Length (bp)
1	*Apo*I-(Right End)	1092-1633	542	*Taq*I-*Taq*I	181-793	613
2	*Mse*I-*Mse*I	352-715	364	*Taq*I-(Right End)	1161-1633	473
3	*Mse*I-*Apo*I *	776-1012	237	*Taq*I-*Taq*I	794-1160	367
4	*Mse*I-*Mse*I	71-219	149	*Ase*I-*Taq*I *	49-180	132
5	*Mse*I-*Mse*I	220-342	123	(Left End)-*Ase*I	1-48	48

GenBank accession #: X98627

#	Ends	Coordinates	Length (bp)	Ends	Coordinates	Length (bp)
1	(Left End)-*Mse*I	1-433	433	*Taq*I-*Taq*I	830-1203	374
2	*Apo*I-*Mse*I *	876-1284	409	*Taq*I-*Taq*I	151-460	310
3	*Mse*I-*Apo*I *	1285-1508	224	*Taq*I-(Right End)	1371-1579	209
4	*Mse*I-*Mse*I	434-595	162	*Taq*I-*Taq*I	1204-1370	167
5	*Apo*I-*Apo*I *	763-875	113	*Taq*I-*Taq*I	671-829	159

GenBank accession #: U89156

#	Ends	Coordinates	Length (bp)	Ends	Coordinates	Length (bp)
1	*Apo*I-*Mse*I *	3410-3977	568	*Taq*I-*Taq*I	2496-3111	616
2	*Mse*I-*Mse*I	4761-5237	477	*Taq*I-*Taq*I	3479-4029	551
3	*Mse*I-*Mse*I	2667-2912	246	*Ase*I-*Ase*I *	1900-2363	464
4	*Mse*I-*Apo*I *	3094-3330	237	*Taq*I-*Taq*I	4030-4447	418
5	*Apo*I-*Mse*I *	127-357	231	*Ase*I-*Taq*I *	520-895	376

GenBank accession #: U73815 ***(single cutting site of AseI without TaqI cutting site)***

#	Ends	Coordinates	Length (bp)
1	*Apo*I-*Mse*I *	146-514	369
2	*Mse*I-*Mse*I	515-799	285
3	*Apo*I-*Apo*I *	838-975	138
4	*Mse*I-*Apo*I *	38-145	108
5	*Mse*I-(Right End)	1035-1094	60

GenBank accession #: L31347 ***(no AseI cutting site)***

#	Ends	Coordinates	Length (bp)
1	*Apo*I-*Mse*I	871-1438	568
2	*Mse*I-*Mse*I	303-554	252
3	*Mse*I-*Apo*I	555-791	237
4	*Mse*I-*Mse*I	92-302	211
5	*Apo*I-*Mse*I	1609-1780	172

GenBank accession #: U03294 ***(no AseI cutting site)***

#	Ends	Coordinates	Length (bp)
1	*Apo*I-*Mse*I	745-1312	568
2	*Mse*I-*Apo*I	429-665	237
3	*Mse*I-*Mse*I	3-176	174
4	*Mse*I-*Mse*I	1313-1472	160
5	*Apo*I-*Mse*I	276-428	153

Appendix 5: Functions of a few gene fragments with non-verified expression patterns

Aroma Biosynthesis-Related Gene Fragments:

EST0379 alcohol acyl transferase (AAT1); up-regulated

The up-regulated EST0379 encodes a key enzyme, alcohol acyl transferase (AAT; 91% identity at the protein level) which is involved in the synthesis of a variety of esters. Aroma volatiles are secondary metabolites that play a major role in fruit quality. Volatile esters form the majority of the flavor components in apple, pear, and banana fruits (Beekwilder *et al*., 2004). AATs link aliphatic, branched and aromatic alcohols to acyl moieties, leading to the formation of a variety of esters in different fruit species (Beekwilder *et al.*, 2004). Yahyaoui *et al.* (2002) reported that the gene encoding AAT1 was increasingly expressed in melon fruit in the early and middle phases of ripening, but did not occur in vegetative tissues. The requirement of ethylene on the capability of AAT appears to be species-dependent as ethylene serves as an inducer in melon but has no effect in strawberry (Flores *et al*, 2002; Aharoni *et al*., 2000). Our data agreed with the finding of Yahyaoui *et al.* (2002) as the highest expression was found in fruit harvested at the tree-ripe stage.

Carbohydrate Metabolism-Related Gene Fragments:

EST0037 Hexose transporter (Hxt); up-regulated

The closest sequence match for EST0037 in the databases is to grape hexose transporter (Hxt). This is a protein encoded by a multigene family of up to 12

members found in various species. It transports glucose across the plasma membrane. The protein deduced from the EST0037 sequence is 87% identical to a series of sugar-carrier proteins. This group includes the glucose transporter, hexose transport protein, monosaccharide-H^+ symporter, and monosaccharide transporter (MST1). MST1 has been reported to be strongly expressed in sink tissues, such as roots, flowers, and young leaves in tobacco (Sauer and Stadler, 1993). The gene encoding for the hexose transporter (Vvht1) in grape berries was shown to have biphasic pattern of expression. An early peak was found shortly after fertilization and followed by a stage-specific increase later in development (Fillion *et al.,* 1999). Since sucrose boxes are found in the promoter sequence of Vvht1, Fillion's group suggested the expression of this gene is induced by sugar level. Consequently, sugar accumulation would occur as an autocatalytic process in grape berry development. The putative function and expression pattern of EST0037 suggested itsmay also play a role in sugar accumulation as 'Gala' apple fruits ripened.

EST0073 Glucose-6-phosphate/phosphate translocator-related; Up-regulated

The low abundance of EST0073 at the early stage of ripening in 'Gala' apple was detected in 2002 and again in 2003. The most closely related database sequence to EST0073, glucose-6-phosphate translocator (GPT), has been shown to occur as chloroplasts differentiate into chromoplasts in developing pepper and tomato fruits (Batz *et al*., 1995; Buker *et al*., 1998). GPT has been postulated as one of the major enzymes that facilitate the supply of carbon skeletons for starch or fatty acid biosynthesis and/or as the substrate for the plastidial oxidative pentose phosphate

pathway in heterotrophic plastids of various sink tissues. This has been reported to occur in different species including maize, oilseed rape embryos, and cauliflower inflorescences (Kammerer *et al*., 1998; Eastmond and Rawsthorne 2000). The diagram below (Figure 16) shows raw starch accumulation in non-photosynthetic sink tissues can occur in response to a variety of soluble carbon sources (sucrose and hexose) imported from source tissues. These are converted to glucose-6-phosphate (G6P) in the cytosol which is transported *via* GPT into the plastid (Kammerer *et al*., 1998).

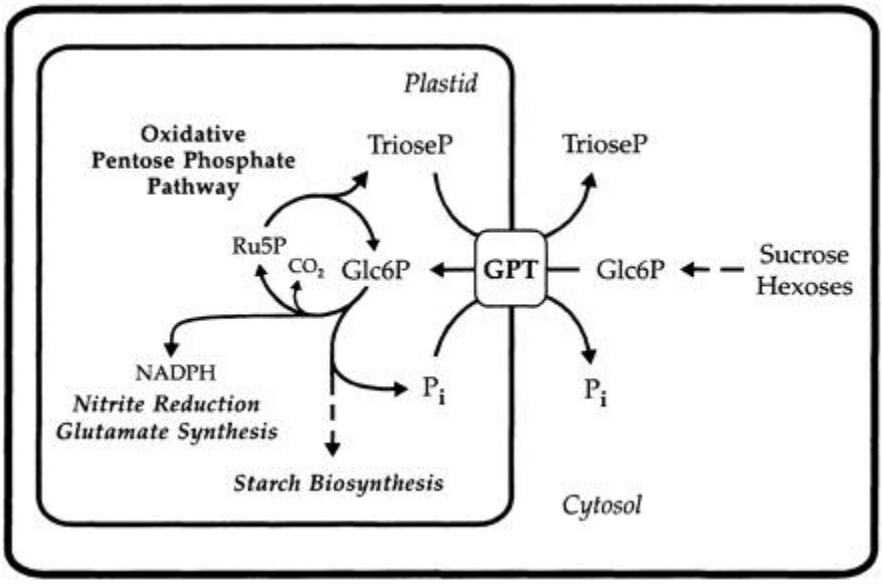


Figure 16. Proposed Function of the GPT Protein in Heterotrophic Tissues (Kammerer *et al*., 1998).

EST0101 NAD-dependent sorbitol dehydrogenase (NAD-SDH, MdSDH1); up-regulated

The transcript derived fragment EST0101 encoding NAD-SDH has considerable similarity to NAD-SDH from reported in human, rat and sheep tissues. It was highly

expressed at the late stages of maturation and ripening of 'Gala' apple. The same pattern in 'Fuji' apple has been reported by Park *et al.* (2002). They found that MdSDH1 and three other isoforms were not detectable in early fruit development (30-60 days after full bloom), but gradually increased as fruit matured. MdSDH1 was also detected in source leaves as well as young sink leaves. SDH activity has been shown to increase with the onset of the climacteric ethylene and respiration peaks (Marlow and Loescher, 1985). However, a different expression pattern was reported by Nosarszewski *et al*. (2004). They reported that SDH was only expressed with significant activity immediately after apple fruit growth was initiated.

Sorbitol was found by Bieleski (1969) to be the primary photosynthetic product and the major translocated form photosynthate in many species of *Rosaceae*. These include apple, pear and peach. Sorbitol comprises about 80% of the total soluble carbohydrate in apple leaves (Loescher *et al*., 1982). NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH, EC 1.1.1.200) is thought to be the major enzyme regulating sorbitol biosynthesis (Loescher *et al*., 1982). In the fruit, sorbitol must be converted to fructose by SDH before any conversion to other compounds can occur. It is likely that SDH plays a critical role in modulating sink strength during the fruit growth since sink organs utilize sorbitol mainly *via* NAD-SDH activity (Park *et al*., 2002). Like many of these tricarboxylic acid cycle (TCA) enzymes, the increase of SDH activity begins prior to the onset of the climacteric. A good relationship was observed by Marlow and Loescher in 1985. Taken together, both transcript level and enzyme activity of SDH appear to increase during fruit ripening.

EST0287 Putative isoamylase; transiently expressed in detached fruits

The EST0287 was only amplified in detached 'Gala' applesamples using cDNA-AFLP. This EST is similar to the deduced debranching enzymes that hydrolyze 1, 6-alpha-glucosidic branch linkages in glycogen, amylopectin, and in their beta-limit dextrins (Hussain *et al*., 2003). Biochemical and molecular evidence have reported that three distinct isoform classes of plant isoamylases exist in potato tubers and in other plant species (Hussain *et al*., 2003). Since starch granules are composed of amylose and amylopectin, several enzymes appear to be involved in starch solubilization during banana ripening. The necessary participation of one starch-debranching enzyme is hypothesized to hydrolyze the alpha-1,6-branches of amylopectin.

The role of isoamylase during ripening has only been reported to occur in banana (Bierhals *et al*., 2004), although it is likely that isoamylase is involved in both amylopectin synthesis and debranching as banana fruit develops. As no significant change in isoamylase gene expression were observed during banana fruit ripening, the authors suggested that the high activity was caused by a pre-existing isoamylase-type debranching enzyme in concert with other amylolitic enzymes (Bierhals *et al*., 2004).

Cell Wall Degradation-Related Gene Fragments:

EST0114 Cinnamoyl CoA reductase (CCR) ; up-regulated

EST0002 cinnamyl alcohol dehydrogenase 2 (CAD2); up-regulated

A similarity of cinnamoyl Coa reductase (CCR; EST0114) encoding an enzyme active in lignin metabolism was consistently up-regulated during 'Gala' ripening. CCR catalyzes the reduction of cinnamic acid CoA esters into their corresponding aldehydes. This is the first step of the phenylpropanoid pathway specifically dedicated to monolignol biosynthesis. The corresponding protein of CAD was localized specifically in immature xylem cells undergoing active lignification (Aharono *et al*, 2002). Another gene, cinnamyl alchohol dehydrogenase (CAD; EST0002), encoding the enzyme which catalyzes the subsequent step in the lignin pathway and the biosynthesis of flavor compounds (Mitchell and Jelenkovic, 1995) was increasingly-expressed in 2002 but was not significantly expressed in 2003. Interestingly, these two genes, CCR and CAD, have been shown to be differentially induced in soft and firm-fruited strawberry cultivars, respectively (Salentijn *et al.* 2003). Thus, CCR has been suggested to be as a novel candidate gene associated with cell wall degradation. Our results are in agreement with their viewpoint that CCR is a possible new candidate gene affecting fruit firmness. CAD may also play a role modulating the flux of hydroxyl-cinnamic acids or aldehydes and have a potential effect on flavor, or on cell-wall bound hydroxycinnamates (Kroon and Williamson, 1999).

EST0437 α -L-Arabinofuranosidases (AFase1); up-regulated

The up-regulated fragment of α -L-Arabinofuranosidase gene (AFase1) is believed to release terminal arabinofuranosyl residues from cell wall matrix polymers and other glycol-conjugates. In tomato, α -L-Arabinofuranosidases (α-Afs) are a divergent family of enzymes of at least three isoforms (Sozzi *et al.*, 2002). Isoforms were reported to have different properties, activity profiles during fruit ontogeny, activities against cell wall fractions, and to respond differentially to plant hormones. Since these arabinosidases overlap during ripening, it has been postulated that a specificity of function on different substrates, or cell wall microstructural domains exists. It is hypothesized that α-Afs I and II could promote discrete modifications of cell wall architecture during growth and expansion, while α-Af III may be involved in the major cell wall breakdown that takes place during ripening. Thus, Brummell and Harpster, (2001) proposed that tomato α-Afs are likely to be encoded by a gene family, similar to other cell wall-modifying enzymes. One tomato α-Af gene, LeARF1 was expressed during tomato fruit development but decreased after the onset of ripening (Itai *et al*, 2003). The rest oration of LeARF1transcripts in ripe fruit by applying 1-methylcyclopropene (1-MCP, an ETR binding inhibitor) supports the concept that LeARF1 expression is negatively regulated by ethylene. There is no reported information in apple about the functional genomics of the gene AFase1.

Developmental/ Ripening-Related Gene Fragments:

EST0096 Metallothionein-like protein; down-regulated

The deduced protein sequence encoded by a 355 bp gene fragment showed a close similarity to the type-2 metallothionein (MT)-like proteins from sand pear. This was down-regulated in both 2002 and 2003 by cDNA-AFLP, microarray hybridization, and byNorthern blot analysis (Figure 14). Members of the MT like group encode small cysteine-rich polypeptides that may be also involved in metal detoxification and in homoestasis. MTs are found in a wide variety of organisms, including animals, plants, and fungi (Robison *et al*., 1993). In addition to their induction by metal ions such as Cu^{2+}, Zn^{2+}, and Cd^{2+}, MT-like proteins are known to be biologically induced. Ethylene evolution during leaf abscission and senescence, wounding stress, virus infection, heat shock, and cold storage of apple can induce MTs (Reid and Ross, 1997). Several studies reported that MTs were associated with fruit development and ripening in banana (Liu *et al*., 2002, Clendennen and May 1997), apple (Reid and Ross, 1997), sand pear (Itai *et al*., 2000), kiwifruit (Ledger and Gadner, 1994), and black currant (Woodhead *et al*., 1998).

A declining expression pattern of a type-2 MT like protein [MT2A, pBAN 3-23(6)] was reported during banana fruit ripening (Liu *et al*., 2002, Clendennen and May 1997). The transcript was barely detectable in the ovary, increased to a high level in young fruits, and then gradually declined (Liu *et al*., 2002). In apple, the transcript of type 2 MT-like protein, AMT1, was abundant in flowers and during the early stages of development but declined while apple fruit matured (Reid and Ross, 1997).

In ripe fruits and leaves, however, its expression was again up-regulated. In sand pear, the expression pattern of the most abundant similarity, PPFRU16 (GenBank accession number: AB021790) was reported to be up-regulated as ripening progressed (Itai *et al*., 2000). They also proposed that the expression of PPFRU16 was subject to ethylene control during fruit ripening. This was based on its reduced expression following treatment with 1-MCP. Although our knowledge about the type-2 MT-like protein is still somewhat fragmented, its connection to ripening was not unexpected.

EST0112 Adenine phosphoribosyltransferase 1; up-regulated

The EST0112 cDNA clone encoded adenine phosphoribosyltransferase 1 (APRT, EC 2.4.2.7). APRT is known to catalyze the Mg^{2+}-dependent transfer of the phosphoribosyl group from 5-phosphorylribosyl 1-pyrophosphate (PRPP) to adenine. This then forms the nucleotide adenine monophosphate (AMP) (Alfonzo *et al*., 1995). APRT was first isolated from yeast in 1955 by Kornberg and his colleagues. It has since been isolated from a wide variety of organisms including bacteria, invertebrates, mammals and higher plants (Schomberg and Stephan 1997). APRT is the key enzyme involved in a one-step salvage pathway where the direct recycling of free adenine into purine nucleotide pools occurs. APRT is encoded by constitutively expressed single-copy genes (Allen, *et al*., 2002). The activity of APRT has been found to be high during the early phase of *Vinca rosea* cell growth, and to increase as strawberry plants increase their capacity to synthesize nucleotides (Robert and Pétel, 2000). These authors also proposed that the change of APRT activity could be assayed as a possible indicator of dormancy release in perennial plants.

In this study, the ripening induced transcript of APRT was verified bycDNA - AFLP, bymicroarray, and then Northern analysis (Figure 14). This protein may play an important role in, or serve as a possible maturity marker during fruit development and ripening. The expression pattern corresponded with measured changes in IEC which infers that a relationship exists between this enzyme and the ripening-related hormone, ethylene.

EST0217 serine/ threonine protein kinase PK23; up-regulated

The up-regulated fragment EST0217 was closely related to a similarity of the protein kinase PK23. This is a novel fruit-ripening and wound-regulated serine/ threonine protein kinase from tomato that is similar to the calcium/calmodulin-dependent protein kinase from tobacco (CaMK1) and maize. CaMK has been shown to be highly regulated temporally and spatially in reproductive and vegetative tissues during development (Zhang and Lu, 2003). This transcript accumulates in tissues undergoing rapid growth and metabolic activity, such as the root apical meristem, flower primordia, sporogenous cells and anther tetrads in flowers, and in developing embryos (Zhang *et al*., 2002). Calcium ion-regulated protein phosphorylation has been implicated in a range of responses including host–pathogen interactions, cold stress, gravitropism, light-regulated gene expression, flowering and hypo-osmotic shock. CaMKs are hypothesized to mediate these responses (for review, see Zhang and Lu, 2003).

Carotenoid Biosynthesis-Related Gene Fragments:

EST0039 Plastid-localized terminal oxidase (PTOX); up-regulated

The similarity of plastid localized terminal oxidase (PTOX) appeared to up-regulated during ripening. It participates in chlororespiration, chromo respiration and carotenoid desaturation. Chlororespiration is defined as an electron transport process from endogenous reductant(s), through parts of the photosynthetic electron transport chain that leads to consumption of oxygen by chloroplasts. Chromorespiration can be defined as a NAD(P)H-dependent redox pathway leading to membrane energization. It is utilized for chemiosmotic ATP synthesis and carotenoid biosynthesis. Carotenoid desaturation consists of a series of dehydrogenation reactions which convert the precursor phytoene into colored carotenoids. The identification of PTOX in pepper chromoplasts, as well as the clear increase in PTOX gene expression during pepper and tomato fruit ripening (Josse *et al.*, 2000) suggested its role in involvement in chromorespiration. Its complex interactions with metabolic and electron-transport pathways matches that of its mitochondrial counterpart, the alternative oxidase (Josse *et al*., 2003).

Stress-Related Gene Fragments:

EST0204 Aspartic proteinase; up-regulated

The increased accumulation of transcripts during fruit development suggested that the encoding product of EST0204, Aspartic proteinase (AP), was involved in apple fruit maturation. Microarray analysis confirmed the same pattern; tree-ripening fruit accumulated higher transcript levels than green-mature fruits. APs, which are widely distributed among prokaryotic and eukaryotic organisms have been extensively studied and characterized. Their biological functions are still unclear in plants, although they are thought to control protein processing and degradation to release nitrogen. APs may be associated with stress responses, leaf and petal senescence, programmed cell death, and reproduction (Simões and Faro, 2004).

EST0045 Universal stress protein (USP); up-regulated

The EST0045 cDNA sequence was found to be 81% identical to the universal stress protein (USP) sequence in *Arabidopsis*. The USP superfamily encompasses an ancient and conserved group of proteins that are found in bacteria, Archea, fungi, flies and plants. Several matching fruit ESTs have been extracted previously from half-ripe apricot fruit, developing peach fruit, and nearly-ripe peach fruit. This was similar to E6 ethylene responsive EST (AB026636). This stress-related TDF could potentially play a role in apple fruit ripening. This TDF was isolated due to its increasing presence in the sequencing gel. However, its up-regulation could not be verified by microarray.

EST0309 Na^+/ H^+ antiporter; up-regulated

The translation product of EST0309 is similar to a stress-related enzyme, the Na^+/H^+ antiporter, reported in Antarctic hairgrass. Plasma membrane exchange glycoprotein transporters catalyze the exchange of Na^+ for H^+ across membranes. As such they serve a variety of functions, regulating intracellular pH, sodium levels, cell turgor and cellular responses to hormones and mitogens (Serrano *et al.*, 1999). Ion transporters selectively transport ions and maintain them at physiologically relevant concentrations. The Na^+/H^+ antiporters also play a crucial role in maintaining cellular ion homeostasis, permit plant survival and growth under saline conditions. Overexpression of the vacuolar Na^+/H^+ antiporter AtNHX1 in *Arabidopsis*, napas and tomato plants promoted their growth and development in potting media irrigated with 200 mM sodium chloride. This salinity tolerance was positively correlated with elevated levels of the AtNHX1 transcript, and with protein and vacuolar Na^+/H^+ antiporter activity (Apse *et al.*, 1999; Zhang *et al*, 2001; Zhang and Blumwald 2001). Although these transgenic tomato leaves accumulated a high sodium concentration, the tomato fruits had a very low sodium content, demonstrating their potential to maintain fruit yield and quality in plants grown in saline conditions. The *Arabidopsis* plasma membrane Na^+/H^+ antiporter, encoded by the SOS1 gene, appeared to be essential for salt tolerance (Shi *et al*, 2002). Shi *et al.* (2003) reported that the overexpression of SOS1 improved salt tolerance in transgenic *Arabidopsis*. These authors also reported that the increased salt tolerance was correlated with a reduced level of sodium in these plants.

EST0359 Heat shock transcription factor family protein; up-regulated

The lower expression of EST0359 was discovered in stage I in 'Gala' apples during both years of this study. Its encoding product closely matched the heat-shock transcription factors (HSF8) in *Arabidopsis* and tomato (CAA47869, Scharf*et al*, 1993). The rapid accumulation of stress-related gene expression products, and the structure and function of the heat-stress proteins is highly conserved in prokaryotic and eukaryotic cells (Parsell and Lindquist, 1993). The heat-shock response serves as a cellular defense against the deleterious effects of stresses from heat, cold and drought. These stresses are mediated by heat-shock transcription factors (HSFs). Upon activation, HSFs trimerize and function as transcriptional activators that bind with conserved specificities to the heat shock element, and manipulate heat-shock gene transcription. This affects the expression of a diverse series ofheat shock proteins and molecular chaperones with many important functions. Not only do they protect proteins against stress damage, but also affect their folding, intracellular distribution and degradation of proteins (Wu 1995; Mathew *et al*., 2001). Other similarity to EST0359 such as that found in 'Chardonnay' grape berries (CB913941) and 'Japanese' rice (NP_921505) have also been induced by abiotic stress and auxin respectively. Overall, the EST0359 appears to encode a protein that modulates stress responses in a variety of plants.

EST0428 and EST0456 cytochrome *P450*; up-regulated

The translated products of EST0428 and EST0456 were both members of a large superfamily of heme-dependent oxidases of cytochrome *P450*. In plants, cytochrome *P450* monooxygenases (*P450*s) constitute the largest group of enzymes associated with the synthesis of secondary metabolites, such as hormones, flavonoids, and lignin. These occur during development or in response to environmental cues affecting wound healing, pest resistance and herbicide tolerance (Bolwell *et al.*, 1994). Plant systems utilize a diverse array of P450s in their biosynthetic and detoxicative pathways. In biosynthetic pathways, *P450*s play critical roles in the synthesis of lignins, UV protectants, pigments, defense compounds, fatty acids, hormones, and signaling molecules. In catabolic pathways *P450*s participate in the breakdown of endogenous compounds as well as toxic compounds from the environment. Due to their roles in many metabolic processes, plant *P450* proteins and transcripts can serve as downstream reporters for many different biochemical pathways (Schuler and Werck-Reichhart, 2003). A banana cytochrome *P450* cDNA (MAP450-1, related phylogenetically to the avocado *P450* CYP71A1) has been reported to have elevated transcripts in peel and pulp during ripening, reaching a maximum in post-climacteric fruits. It was not found in unripe fruit tissue or in roots, leaves, or flowers (Pua and Lee, 2002). Ethylene and sucrose appear to play an up and down-regulatory role in *P450* expression in banana, respectively. Exogenous ethylene application induced transcripts of MAP450-1 but exogenous sucrose decreased its transcription (Pua and Lee, 2002). Although the role of *P450* in plants

has been well documented in defense and stress-responses, function during ripening still remains poorly understood.

EST0459 Acid phosphatase survival protein SurE; transiently expressed at stage I

The closest match to the translated product encoded by EST0459 cDNA sequence was an acid phosphatase survival protein in *Arabidopsis*. The SurE family was discovered by Clarke and his colleagues during the stationary-phase survival of *Escherichi coli* bacteria, and in various repair and stress-response phenotypes of that species (Mura *et al*, 2003). These genes may form a bicistronic operon essential for *E. coli* viability under stresses, such as elevated temperatures, osmotic stress, or high cell density. SurE has also been shown to play a significant physiological role in stress-responses. EST0459 was cloned as it was transiently-expressed in apple fruit harvested in stage I. However, the microarray study did not confirm that transient pattern.

Cell Structure and Mobility (transport)-Related Gene Fragments:

EST0028 Dynamin, putative; down-regulated but expressed at higher levels in attached fruits than in detached ones

The down-regulated EST0028 encoded to dynamin. This has been proposed as a possible surface protein responsible for cell-to-cell interactions. Dynamin interacts directly with several proteins that regulate actin assembly, including profilin and cortactin. The mechanism of dynamin in remodeling membranes has been suggested *via* regulating actin filaments, coordinating its activities during endocytic traffic, cell morphogenesis and cell migration (Schafer *et al.*, 2002). Recent data reviewed by

McFadden and Ralph (2003) suggest that dynamins in plants are involved with chloroplast division and with the mechano-chemical proteins involved with pinching off of vesicles.

EST0293 Myosin heavy chain-like protein; up-regulated

The function of the similarity of EST0293, myosin heavy chain-like protein in plants is poorly understood. The myosin family of molecular motors has been characterized to move cargo on actin filaments. The actin cytoskeleton in plants has been shown to be involved in processes such as transportation, signaling, cell division, cytoplasmic streaming and morphogenesis (Reddy and Day, 2001). The specific role of the myosin heavy chain-like protein with advancing age and physical frailty in humans has been shown to contribute to muscle protein wasting that occurs with advancing age. A similar EST for abiotic stress of 'Chardonnay' grape leaves was obtained in the BLASTN search. This may eventually be found to have a relationship with stress or aging in plants, as was previously shown in animals.

EST0040 Putative microtubial binding protein; up-regulated

EST0313 Microtubule associated protein; transiently expressed at attached fruits

EST0454 Beta-tubulin 6; up-regulated

Tubulin molecules and their bead-like structures are a component of the protofilament. Beta-tubulin (EST0454) may bind with GTP or GDP, and it can hydrolyze its bound GTP to GDP plus P_i, release that P_i, and then exchange the bound GDP for GTP (see Diwan's webpage). An alfa, beta-tubulin heterodimer forms the

basic structural unit of microtubules (EST0040, EST0313). Microtubules function as conveyer belts inside plant cells. They move vesicles, granules, mitochondria, and chromosomes *via* special attached proteins. Microtubules also serve a cytoskeletal role. Structurally, they are linear polymers, so- called protofilaments, of the globular protein tubulin. These three putative microtubule-associated proteins were up-regulated during apple ripening although not all patterns were found in the second year of the study. Similar ESTs isolated from ripening grape may also provide a hint of the differential transcriptional control for these cytoskeleton-related proteins.

Signal Transduction-Related Gene Fragments:

EST 0399 Seven-transmembrane-domain protein; up-regulated

The EST0399 was up-regulated in cDNA-AFLP, and its nearest database match was to a receptor-like protein. Seven- transmembrane-domain proteins are a type of receptor protein containing 7 hydrophobic domains in a single polypeptide chain. These cross the cell membrane lipid bilayer. The characteristic analyses of proteins coupled to 7-transmembrane proteins suggested their function as receptor-like proteins to mediate signal transduction such as the ethylene receptor (ERS1) and the G-proteins. G-proteins are a family of signal-coupling proteins that act as intermediaries between activated cell receptors and effectors. For example, they function to convey hormonal signals from the cell's surface to the cell interior. G-protein is thought to be embedded in the cell membrane with parts exposed on the outside and inside surfaces (for review, see Mombaerts, 1999).

EST similarity of signal transduction proteins have also been reported in fruits. Climacteric peach mesocarp (AJ631553), as well as immature ovaries (CF508161) and developing fruits (CK939616) from field-collected '*Valencia'* sweet orange have been shown to express similar ESTs. Its putative function and presence in fruits implies that EST0399 may be an essential signal transduction-protein related to fruit development and/ or ripening.

Protein Biosynthesis-Related Gene Fragments:

EST0056 Probable rRNA processing protein; up-regulated

The closest match to the translated product of EST0056 using BLASTX was the rRNA processing protein EBP2 similarity found in *Arabidopsis*. The transcript of this TDF increased during apple ripening in our study. The function of its similarity, pre-rRNA processing protein is thought to yield a functional rRNA *via* methylation and ribosome assembly. This includes cleavage as well as other modifications. Since ripening requires new protein synthesis, isolation of this fragment is not unexpected.

EST0094 Aspartate aminotransferase (AAT2); up-regulated

The similarity of the up-regulated EST0094, aspartate aminotransferase (AAT), plays a significant role in plant nitrogen assimilation and transport, and in carbon metabolism. AAT is encoded by a small gene family; only five genes have been reported in *Arabidopsis* to date. It is believed to catalyze the asparagines and methionine biosynthesis catabolism of aspartate and several other essential amino acids such as Asn and Met (Silvente *et al*., 2003). In addition to attaching newly-

formed organic nitrogen to the nitrogen carriers, Glu and Asp, and the conversion of TCA cycle intermediates to amino acids, this enzyme also regulates nitrogen and carbon pools in the cytoplasm and vacuole. One isoform, AAT-2, is involved in the assimilation of nitrogen compounds in bean nodules (Silvente *et al.*, 2003). The mutation study of Miesak and Coruzzi (2002) on cytosolic AAT2 demonstrated a nonredundant role for AAT2 for the bulk assimilation of nitrogen into aspartate and derived amino acids in the cytosol of leaves and dry fruits (siliques).

EST0105 Eukaryotic translation initiation factor 3 subunit 11 (eIF-3 p25) (eIF3k); up-regulated

The up-regulated EST0105 encodes a similarity of eIF3 which may be involved in novel pathways controlling protein homeostasis (Verlhac *et al*., 1997). Protein synthesis plays a major role in cell cycle regulation in eukaryotic cells. Eukaryotic initiation factor 3 (eIF3), a multiprotein complex of at least eight subunits, is the largest factor for the process of protein synthesis initiation (Verlhac *et al.*, 1997). Many roles have been assigned to eIF3 in the translational initiation pathway in mammalian cells. eIF3e/Int6, which might be necessary for the translation of specific transcripts by eIF3, and for the degradation of specific targets by the proteasome, has been shown in regulating protein turnover through binding to the regulatory lid of the 26S proteasome (von Arnim and Chamovitz, 2003). Numerous differentially-expressed sequence tags from various stages of development in apricot (CV049426; CV045422) and peach fruits (BU047076) which appeared to be similar to EST0105 and may suggest thatthe association between EST0105 transcripts and many ripening processes.

EST0352 homeobox-leucine zipper protein 7 (HB-7)/ HD-ZIP transcription factor 7; transiently expressed at stage I and III

Homeodomain proteins are involved in the control of gene expression during morphogenesis and development (NCBI NLM MeSH webpage). Homeodomain proteins are encoded by homeobox genes. These genes exhibit structural similarities to certain prokaryotic and eukaryotic DNA-binding proteins. Reports of apricot (CB820875) and peach (BU040588) EST similarity existing at various stages of fruit development lends support to the idea that homeobox-leucine zipper protein 7 is associated with the regulation of gene expression, or that its gene expression is differentially regulated during fruit development.

Protein Degradation-Related Gene Fragments:

Protein degradation is a key regulatory component of many vital cellular processes that regulate the growth and development of eukaryotic organisms. Protein degradation is involved with processes such as cell cycle control, transcription, receptor desensitization, and antigen processing (Kirschner, 1999). Ubiquitin-26S-proteasom mediated regulatory protein degradation is the predominant form of intracellular proteolysis. In this system, a target protein that is destined to be destroyed is tagged by the covalent attachment with a polyubiquitin chain before degradation by the 26S proteasome.

Ubiquitin attachment involves four successive steps, catalyzed by three enzymes. These are ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and

ubiquitin protein ligase (E3). This process is repeated until several ubiquitin moieties are attached to the target protein. A polyubiquitin chain, formed facilitated by a multiubiquitin chain assembly factor (E4). Generally, the specificity of this pathway is determined by the E3. Many types of E3s appear to be present in an organism, to allow the ubiquitination of their diverse array of proteins (Dharmasiri and Estelle, 2004). We isolated three forms of E3 in this study: EST0048 (ubiquitine-protein ligase), EST0026 (U-box domain containing protein), and EST0108 (Skp1). These differ in their subunit organization and/or their mechanism of ubiquitin transfer (see below). Ubiquitinated proteins are recognized and degraded by the 26S proteasome, a multiprotein complex comprising a 20S core unit (EST0153) and two 19S regulatory particles. The polyubiquitinated substrates might be recognized, and then subsequently degraded by the 26S proteasome. However, the link between polyubiquitination and this proteasome is still poorly understood (Azevedo *et al*, 2001).

EST0108 SKP1; up-regulated

Differential EST reported from developing peach and apricot fruits closely matched EST0108. This was up-regulated during 'Gala' apple ripening. *SKP1* (kinetochore protein), encoded by the similarity of EST0108, is of central importance to a number of cellular processes. SKP1 has been proposed to be involved in cell cycle regulation and the ubiquitin proteolysis process by combining Cullin (or Cdc53) and F-box into a complex SCF. SCF is a type of E3. The regulation of proteolysis in different stages has been suggested by the behaviors of different *skp1* mutant cells

during G1 or G2 stages of the cell cycle (Bai *et al.*, 1996; Azevedo *et al*, 2001). Numerous studies have also shown that SKP1 is capable of binding to several proteins whose common features are the presence of an F-box, or another motif involved in protein-protein interaction. For example, binding *via* WD 40- or Clink or ORE9 to an F-box protein has been shown to regulate leaf senescence (Bai *et al*., 1996; Aronson *et al*, 2000; Woo *et al*, 2001).

Subunits of 26S proteasome complex:

EST0153 20S proteasome subunit; up-regulated

Differentially expressed sequence tag 20S proteasome subunit similarity from developing peach, apricot, and citrus were found in the EST database. The 20S proteasome is one of the subunits of the 26S proteasome complex involved in protein degradation by the ubiquitin pathway. The 20S proteasome is an ATP-independent protease consisting a stack of four seven-membered rings. Each of the two outer rings is formed by seven subunits, and the two inner rings are also formed by seven subunits. Previous research demonstrated that the suppression of gene which encodes for the 20S proteasome subunit expression led to spontaneous programmed cell death in *N. benthamiana.* This was accompanied by reduced proteasome activity and the accumulation of polyubiquitinated proteins (see Kim *et al*., 2003).

EST0071 21D7 antigen; up-regulated

The 21D7 protein is a subunit of the plant 26S proteasome, thought to belong to the proteasome regulatory complex (Smith *et al*., 1997). The 21D7 protein is expressed only in plant tissues, and may participate in the control of cell division by regulating

the activity or specificity of the 26S proteasome in the nucleus. The function of 21D7 is conserved during evolution. It is thought to be essential for cell proliferation, based on its complementary effect in a yeast *sun*2 mutant study (Smith *et al*., 1997). The yeast gene SUN2 (Suppressor of *nin*1-1) encodes a polypeptide with 32% identity to the plant 21D7 protein.

EST0080 Putative cathepsin B-like protease; up-regulated

This has been previously reported as a defense related EST similarity from cacao leaves. It is also present in green apricot fruit. Cathepsin B is from an ancient family of eukaryotic cysteine proteases. Cysteine proteases are a group of enzymes identified in bacteria, yeast, animals, and plants. They play an important role in intracellular protein degradation (Barrett, 1986). These are synthesized as preproteins that are processed autocatalytically or with the aid of a processing enzyme. These proteases are stored in the vacuole, in lysosomes, or are excreted from the cell. Among the cysteine proteases, the H and L cathepsins have been widely studied in mammals, and recently investigated in plants (Martínez *et al*, 2003). The cathepsin B-like cysteine protease (CatB) gene has been reported to be involved in proteolysis during germination of barley seed, and in cold stress response in barley tissues (Martínez *et al*, 2003). It was also induced in *Nicotiana rustica* leaves in response to wounding (Lidgett *et al*, 1995). Since CatB mRNA expression increases during germination, it is thought to play a significant role solublizing amino acids needed for development before that plantlet can survive autotrophicly (Martínez*et al*., 2003). It

is hypothesized to degrade storage proteins in the barley seed aleurone during germination.

Cell Division-Related Gene Fragments:

EST0053 Zinc finger family protein C3HC4-type RING finger; up-regulated

EST0284 N-terminal domain-containing protein / zinc finger (C3HC4-type RING finger) family protein; up-regulated

Both EST0053 and EST0284 encoded to similarity of the zinc finger (C3HC4-type RING finger) family proteins. Interestingly, the reason for their isolation was their transient suppression in stage I. In a subsequent study, their expression patterns were not found to be significantly different between stage I and VI in microarray analysis. The different level of sensitivity between cDNA-AFLP and microarray could provide an explanation for these results. This protein is one of the largest gene families in mammals. It contains a conserved cysteine- and histidine-rich domain essential for the binding of zinc ions. The gene encodes a C3HC4-type zinc finger protein motif (ring finger motif) consistent with a role in pre-meiotic or post-meiotic animal sperm development (Zhang *et al.*, 2001). There are not any reports of the role of the zinc finger family proteins in plants to date.

EST0106 putative CRS1; up-regulated

The closest similarity of the EST0106 translated product was CRS1, a group II intron splicing factor. CRS1 may facilitate the splicing of mitochondrial group I introns by stabilizing an on-pathway folding intermediate, or by stabilizing the fully-assembled intron. It may also function as an RNA chaperone to resolve misfolded

intron RNAs and increase the yield of catalytically active introns (reviewed by Herschlag, 1995). Some members of CRS1 family are also thought to be in mitochondria and chloroplasts. Each of these members of the family would harbor multiple group II introns. Other members of the family may bind to the atpF, a group II intron that is needed to straighten and activate splicing with specificity (for review, Till *et al*., 2001).

EST0391 Chloroplast casein kinase II alpha subunit (cpck2a gene); up-regulated

Casein kinase (CKII) is composed of two subunits, a catalytic alpha subunit and regulatory beta subunit. Studies in animal systems have shown that this enzyme plays a central role in the regulation of cell division. EST0391 had two similarity; one from developing almond seed (BU573036) and another from the abiotic stress grape library (CD712200). It may play a physiological role in both plant development and in plant stress response.

Intracellular Trafficking and Sorting-Related Gene Fragments:

EST0066 Synaptobrevin/vesicle-associated membrane protein; up-regulated

Synaptobrevin/vesicle-associated membrane protein is one of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. It is thought to provide specificity for the targeting and fusion of vesicles with the plasma membrane. It belongs to a class of membrane proteins which lack a signal sequence and contain a single hydrophobic segment close to their C-terminus (for review, see Kutay, 1995). This leaves most of their polypeptide chain tail –anchored in the cytoplasm.

In neuroendocrine PC12 cells, synaptobrevin is not directly incorporated into the target organelle, synaptic-like vesicles. Rather, it is first inserted into the endoplasmic reticulum (ER) membrane and is then transported *via* the Golgi apparatus. Its insertion into the ER membrane in vitro occurs post-translationally, is dependent on ATP and results in a trans-membrane orientation of the hydrophobic tail. Membrane integration requires ER protein(s) different from the translocation components needed for proteins with signal sequences, thus suggesting that a novel mechanism of insertion exists (Kutay, 1995).

EST0214 Kinesin motor protein-related; down-regulated

EST0383 Kinesin light chain-related; transiently expressed at stage III

Both these TDFs were transiently expressed in fruits harvested at commercial maturity in 2002. In our microarray pattern, no differences were found between stages I and stage VI in 2003. The EST database searches revealed matches to a number of ripe and half-ripe apricot ESTs (CB823569; CB821743) as well as an EST from developing peach fruit (BU039253). The putative protein derived from the EST0383 translation product was a kinesin light chain-related protein from *Arabidopsis*. Kinesin is a microtubule-associated mechanical adenosine triphosphatase, which uses the energy of ATP hydrolysis to move organelles along microtubules toward the positive end of the microtubule. The kinesin motor protein, the similarity of EST0214, plays a similar role in intracellular transport along microtubules in many different cells. The kinesin light chain is located in the presumptive cargo-binding domain of kinesin. Its essential function in mediating the

interactions of the kinesin motor with intracellular cargo has been proposed by Gindhart, Jr. *et al*. (1998).

EST0282 & EST0389 VHS domain-containing protein / GAT domain-containing protein; up-regulated

VHS domain-containing proteins, derived from its presence in Vps27, Hrs and STAM have been implicated in intracellular trafficking and sorting (Lohi *et al.*, 2002). The VHS domain is a 140 residue domain present at the NH2-terminus of at least 60 proteins. Based on their functional characteristics and on their involvement of VHS in cargo recognition in trans-Golgi, VHS domains are considered to have a general membrane targeting/cargo recognition role in vesicular trafficking. The VHS domain can be divided into four groups based on their domain/motif entourage. Both EST0282 and EST0389 are encoding to the protein in the third group. These consist of GGA proteins composed of a VHS domain, a GAT (GGA and Tom1) domain with high similarity to Tom1 protein. Tom1 protein is a flexible hinge region that contains clathrin boxes, and the C-terminal GAE (gamma-adaptin ear) domain. GGAs are ARF-binding proteins. Hence the name GGA for Golgi-localizing, gamma-adaptin ear similarity domain. The VHS domains of GGAs interact directly with sorting receptors that traffic and transfer cargo between TGN and the endosomal compartment. However, the interaction of the VHS domain with the receptor of the VHS alone is not sufficient to recruit GGA from the cytosol to the TGN (Lohi *et al.*, 2002).

There are no reports on the changes of transcripts of the VHS family during fruit development. Similarity in the EST database included developing peach fruit

(BU041034), and in an expressed sequence tag database for abiotic stressed grape leaves (BM438070; CD718693) do exist, however.

EST0467 Md-H1 for histone 1; up-regulated

Apple histone 1 (Md-H1), a highly similar similarity of EST0467, is a DNA binding protein which involves the regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism *via* selective interaction with DNA. The EST database search results indicated that similarity of this up-regulated gene fragment (EST0467) have been extracted from developing orange fruit peel (CK935049) and from orange shoot meristems (CF837836).

Photosynthesis-Related Gene Fragments:

EST0119 ribulose 1, 5-bisphosphate carboxylase small subunit; transiently expressed

Ribulose-1, 5-bisphosphate carboxylase (Rubisco) is a critical enzyme that comprises approximately half the amount of the soluble protein in leaves. It is present in much lower levels in other photosynthetic organs of the plant. Rubisco is composed of eight identical large sub-units (LSU) and eight identical small subunits (SSU). The LSU and the SSU proteins are products of the chloroplast *rbcL* gene and the nuclear *rbcS* genes, respectively. The subunits are found in chloroplasts in the requisite stoichiometric amounts (Wanner and Guissem, 1991). The biosynthesis of Rubisco is believed to be regulated by the control of *rbcS* gene expression (Rodermel *et al*., 1988). Many *rbcS* genes have been shown to have a different in expression in different organs during development (Wanner and Guissem, 1991). In

photosynthetically active young tomato fruit, only *rbcS*1 and 2 mRNA were found. Both mRNA decreased to undetectable levels in ripe tomato fruit (reviewed by Wanner and Guissem, 1991). The transcription and stability of individual *rbcS* mRNAs are altered by organ, organ development and by light. The differential expression pattern of EST0119 was detected in both years of this study. Transient expression was found in fruits harvested at stages I, III, III+30, IV in the first year, and higher in stage I+30 than in stage I and IV in the second year. The member of similar ESTs isolated during various developing stages of fruit growth supports the concept that the synthesis of the Rubisco small subunits is related to development. Our inconsistent expression pattern may suggest that this putative protein may have been affected by the orchard environment, as shown previously by Wanner and Guissem (1991).

Energy/ Respiration-Related Gene Fragments:

EST0357 NADH-ubiquinone oxidoreductase; up-regulated

The mitochondrial NADH-ubiquinone oxidoreductase is the first of the electron transfer complexes in the respiratory chain, and is termed complex I. In eukaryotes, the genes coding for complex I proteins are distributed unevenly between the mitochondrial and nuclear genomes. Two nuclear genes isolated from potato were expressed at elevated levels in flowers, and declined with leaf aging (Schmidt-Bleek *et al*., 1997). Differential analysis of floral tissues has yielded significant transcript differences between individual cell layers for several genes specifying mitochondrial proteins (Smart *et al.*, 1994). Significant differences occurred with fruit ripening in this study, where an increase in transcript level was found.

General Metabolism-Related Gene Fragments:

EST0375 Leucoanthocyanidin dioxygenase; up-regulated

The translated product of EST0375 matched a leucoanthocyanidin dioxygenase (LDOX) gene in *Arabidopsis*. It functions to convert leucoanthocyanidins to monomeric anthocyanidin *via* the anthocyanins and polymeric proanthocyanidin biosynthetic pathway. Abrahams *et al.* (2003) concluded that LDOX consistently participated in proanthocyanidin biosynthesis during early ripening, and in anthocyanin synthesis at grape "veraison." The transcript level of LDOX was also enhanced by exogenous treatment with ethephon in non-climacteric grape berries (El-Kereamy *et al*, 2003). LDOX has been hypothesized to be a key enzyme in flavonoid biosynthesis, and its expression level appears to depend on fruit development and/ or ethylene level. The increasing transcript level of EST0375 in this study, and its EST similarity from *Medicago truncatula* during flower development (BQ147734) supports the idea that the transcript level of this TDF is ripening related.

EST0103 Putative 3-beta hydroxysteroid dehydrogenase/isomerase protein; up-regulated

The enzyme 3-beta-hydroxysteroid dehydrogenase regulates progestin and androgen levels in humans. It also shares a common ancestor with enzymes involved in the synthesis of anthocyanins in plants. This evolutionary connection, and the structural similarities between flavonoids, licorice-derived compounds, and

mammalian steroid hormones, provides an interesting perspective on the hormone-like activity of flavonoids and other plant-derived compounds in humans. Some of the hormone-like activity of plant-derived compounds appears to be due to their binding to steroid and prostaglandin dehydrogenases (see Baker, 1995). The 3-beta-HSD gene family may have evolved to facilitate differential patterns of tissue- and cell-specific expression and regulation involving multiple signal transduction pathways. These could be activated by several growth factors, steroids, and cytokines (Simard *et al*, 2002). Therefore, EST0103 is an interesting up-regulated gene candidate that plays a role in fruit ripening, and may also affect the health of fruit-consuming humans.

EST0327 Leucine-rich repeat (LRR) protein/ nodulation receptor kinase; up-regulated

LRR has been suggested as a possible surface protein which is responsible for cell interaction. It contains cell adhesion domain and ChW-repeats. Nodulation receptor kinase (NORK) is proposed to function in the Nod-factor perception/transduction system that initiates a signal cascade leading to nodulation (Endre *et al.*, 2002). EST similarity including developing peach fruit (BU040104) and developing *Medicago truncatula* stem (AW688582) closely matched this up-regulated TDF.

Lipid Metabolism-Related Gene Fragments:

EST0079 (up-regulated), EST0401 (transiently expressed in attached fruits): 1-acyl-sn-glycerołЗ- phosphate acyltransferase (plsC, LPAAT)

Different expression patterns of two identical gene fragments in the region of massive part of their sequence (149 /176 or 190 bp) were found in the cDNA-AFLP displaying gel. No expression in stage I (attached fruit) of EST0079 occurred, but a strong expression of EST0401 in all attached fruits from stage I to VI was found. The microarray and the Northern analysis verified the pattern found in EST0401. No significant difference was observed between stage I and stage VI probes. In addition, the hybridization intensity of EST0401 to the probe of stage VI was significantly higher than the I+30 detached apple fruit in another comparison microarray. In that no difference was found in EST0079. The different hybridization patterns between these two identical gene fragments can not be explained based on their size, as EST0079 is only 14 base pairs shorter than EST0401. The best match between the deduced sequences of EST0079 and EST0401 to database sequences is to an acyl-sn-glycero-3-phosphate (lysophosphatidic acid, LPA) acyltransferase (LPAAT) from almond. Lysophosphatidyl acyltransferase (LPAAT) is a pivotal enzyme controlling the metabolic flow of lysophosphatidic acid into different phosphatidic acids which has been reported to be a key intermediate for chloroplast membrane lipid biosynthesis (Kim and Huang, 2004).

EST0019 Phytochelatin synthetase-like protein

The closest match for the cDNA sequence EST0019 was to a phytochelatin synthetase-like protein in strawberry. This was investigated because of potential role in strawberry fruit ripening (AY642687). It has been well documented that plants, yeast and algae allsynthesize phytochelatins to detoxify intracellular metal ions. The similarity to EST0019 in the database from other plant species may suggestthat this gene fragment is associated with development (peach, BU047431; grape, CF213680), abiotic stress responses (grape, CD718171) and also fragrance production (rose, BQ104401) (Guterman *et al*., 2002). Its expression decreased in attached apple fruit during ripening using cDNA-AFLP. However, no significant difference was found in the subsequent microarray study comparisons of stage I and stage VI apple fruits (ratio=0.99).

EST0020 C2H2 zinc-finger protein; down-regulated in detached fruits

Our BLAST search results for EST0020 revealed that it encodes a protein with a high similarity to the SERRATE (SE) gene of corn. This gene encodes a single, C2H2-type, zinc finger protein (AF311223). The gene has been implicated as to be essential in normal development of shoot and flower meristems, developing leaves, and embryos. Mutational analyses including over-expression and co-suppression studies in Arabidopsis have been used to study this gene (Prigge and Wagner, 2001). They proposed that the SE gene regulates changes in gene expression *via* chromatin modification, due to its putative zinc finger and nuclear localization motifs. In our apple research, a decreasing expression of EST0020 occurred in detached fruits by cDNA-AFLP, but no significant difference was observed in our microarrays. This is

not, however, the first report of this EST in fruits. It has also been reported previously in developing peach mesocarp (BU047431).

EST0026 U-box domain-containing protein; up-regulated

PUB proteins have been shown to play a regulatory role in plants *via* the ubiquitin-dependent protein degradation process. The up-regulated TDF, EST0026, encodes a U-box domain-containing protein. Plant U-box (PUB) proteins have been found to exist in five distinct subclasses. It has been suggested that they play diverse roles. One has been shown to be essential for self-incompatibility in Brassica species (Azevedo *et al*, 2001). PUB proteins function as an E3 ubiquitin ligase with specific E2 ubiquitin-conjugating enzymes in *Arabidopsis* (Andersen *et al*, 2004), or as an E4 ubiquitination factor in yeast (Azevedo *et al*, 2001).

EST0085 Endo-1,4-beta-D-glucanase

EST0085 was expressed only in early stages in 2002 but not significant in 2003, encoded for a protein, endo-1,4-beta-D-glucanase (EGase), with probable influence on the cell wall. EGases which form a large family of hydrolytic enzymes in prokaryotes and eukaryotes have been known to act on the hemicellulose or cellulose fraction of the cell wall. In higher plants, potential substrates in vivo are xyloglucan and non-crystalline cellulose in the cell wall. It has been suggested a role for EGases in various developmental processes such as leaf abscission, fruit ripening and cell expansion on the basis of differential gene expression (Harpester *et al*., 2002). Salentijin *et al.* (2003) indicated that higher expression was found in firmer strawberry cultivar (Holiday) than softer one (Gorella). Thus, Salentijin *et al.* (2003)

suggested that this phenomenon gives the reason why transgenic strawberry with antisense of endo-1,4-beta-D-glucanase did not show firmer texture in Woolley and colleague's study (2001). However, Harpster *et al*. (2002) indicated a different prospect that higher activity was found during ripening but not during development in pepper fruit. Further studies should be focused on the expression and activity of this enzyme before any advanced conclusion to be addressed.

EST0086/0088 Hydrolase, Alpha/Beta-hydrolase fold enzymes

"The alpha/beta-hydrolase fold family of enzymes is rapidly becoming one of the largest groups of structurally related enzymes with diverse catalytic functions. Members in this family include acetylcholinesterase, dienelactone hydrolase, lipase, thioesterase, serine carboxypeptidase, proline iminopeptidase, proline oligopeptidase, haloalkane dehalogenase, haloperoxidase, epoxide hydrolase, hydroxynitrile lyase and others. The enzymes all have a Nucleophile-His-Acid catalytic triad evolved to efficiently operate on substrates with different chemical composition or physicochemical properties and in various biological contexts. For example, acetylcholine esterase catalyzes the cleavage of the neurotransmitter acetylcholine, at a rate close to the limits of diffusion of substrate to the active site of the enzyme. Haloalkane dehalogenase is a detoxifying enzyme that converts halogenated aliphatics to the corresponding alcohols, while haloperoxidase catalyzes the halogenation of organic compounds. Hydroxynitrile lyase cleaves carbon-carbon bonds in cyanohydrins with concomitant hydrogen cyanide formation as a defense mechanism in plants" (refer to Holmquist, 2000).

EST0128 Putative copper/zinc superoxide dismutase copper chaperone precursor; up-regulated

An up-regulated stress-associated enzyme, a putative copper/zinc superoxide dismutase copper chaperone precursor, was encoded by the similarity of EST0128 in soybean. It is an oxidoreductase that catalyzes the reaction between superoxide anions and hydrogen to yield molecular oxygen and hydrogen peroxide. The enzyme protects the plant cell against toxic levels of superoxide.

EST0132 Small GTP-binding (SMG protein)

Up-regulated EST0132, a similarity of small GTP-binding (SMG) protein from lotus (Z73937, rab2A), showed elevated levels of cDNA-AFLP during fruit ripening process but non-significant difference of expression intensity found in microarray. SMGs have been suggested to play an important role in organ development including nodule in the root on the basis of differential expression pattern (Borg *et al.*, 1997; O'Mahony and Oliver, 1999). The large catalogue of SMGs with diverse patterns of transcript accumulation levels have been reported previously. Borg and colleagues (1997) suggested that most of them have household functions, and a few may be required for differentiation and formation that are important for specialized cells. Many EST homoloues isolated from various developing flowers, fruits, and seeds suggested the connection of small GTP-binding protein to fruit development.

EST0161 Zinc metalloproteinase-like

"Zinc metalloproteinase that functions as part of a regulatory loop controlling local concentrations of peptide substrates and associated peptide-mediated signal transduction processes. Metalloproteinases or metalloendopeptidases (EC subclass 3.4.24) are present across all kingdoms of living organisms; they are ubiquitous and widely involved in metabolism regulation through their ability either to extensively degrade proteins or to selectively hydrolyze specific peptide bonds. They must be subjected to exquisite spatial and temporal control to prevent this vast potential from becoming destructive. They are mostly zincdependent hydrolytic enzymes. Members target inner peptide bonds of proteins or oligopeptides and catalyze extensive processing events like digestion or degradation of intake proteins and tissue development, maintenance, and remodeling" (see Gomis-Ruth, 2003). This unverified TDF had an EST similarity from developing peach mesocarp.

EST0222 and EST0390 Calmodulin-binding family protein; transiently expressed at stage I

Two clones encoding for members of proteins of calmodulin-binding family were transiently expressed in the early stage of apple maturation when analyzed with cDNA-AFLP. Gene expression analysis using a different set of samples also indicated that both similarities had a similar content of transcripts during maturation and ripening. Calmodulin is a ubiquitous multifunctional calcium receptor, and is one of the best-characterized calcium sensors in eukaryotes. There are some similarities in Ca^{2+}/calmodulin-mediated signaling in plants and animals. In animals, calmodulin-binding proteins are found in many tissues. They have a variety of functions

including F-actin cross-linking properties and the inhibition of cyclic nucleotide phosphodiesterase and calcium and magnesium ATPases (Yang and Poovaiah, 2003). Plants possess multiple calmodulin genes, and many calmodulin target proteins, including unique protein kinases and transcription factors. Some of these proteins are likely to act as 'hubs' during calcium signal transduction. Although these two differentially-expressed ESTs changed with maturation, they might not be directly related to ripening as they have not been reported in developing fruits.

EST0233 putative valyl-tRNA synthetase; transiently expressed at stage I and III

EST0233 encoded for a valyl tRNA synthase. It is identified as an enzyme that activates valine with its specific transfer RNA, or act with another branched-chain essential amino acid that has stimulatory activity. It also appears to be needed to synthesize a precursor in the penicillin biosynthetic pathway. The role of this enzyme in plants is not well documented, although a similar EST was reported in an abiotic grape database (CB008322).

EST0245 ABC transporter; EST0332 probably ABC transporter

These two homologs of ATP-binding cassette (ABC) transporters which are members of a large family of active transport proteins energized directly by ATP hydrolysis did not show differential expression pattern in microarray among stage I, I+30 and VI. ABC transporters are able to use the energy of ATP hydrolysis directly to pump organic molecules (especially large anionic molecules) across a membrane. An ABC transporter has been found to secrete anti-fungal terpenes across the plasma membrane of tobacco cells. More commonly, ABC transporters are found at the

tonoplast, where they are sometimes referred to as glutathione conjugate pumps, or GS-X pumps, since they often transport molecules that have been covalently attached to glutathione.

EST0261 Putative ripening-responsive protein

The protein deduced from the EST0261 sequence is a putative ripening-responsive protein from Arabidopsis with similarity to ripening regulated protein DDTFR18 of tomato. This TDF which was cloned due to transiently suppressed in fruit harvested in stage IV and V exclusively. Therefore, our result suggested that this putative ripening-responsive protein may be also regulated by other factors such as environmental or developmental stages because this transiently suppression occurred in the fruit harvested in late commercial harvest period. Since it has been characterized as ripening related, more studies should be addressed on the expression pattern in various fruit developing stages.

EST0314 Dehydroquinate dehydratase; up-regulated

Most aromatic rings found in natural products are derived from chorismate, a product of the shikimate (prechorismate) pathway. Chorismate is synthesized in seven steps from erythrose 4-phosphate and phospho*enol* pyruvate. In plants the bifunctional enzyme 3-dehydroquinate dehydratase- (DHQase, EC 4.2.1.10)/ shikimate: NADP dehydrogenase catalyzes the third and fourth steps of that pathway (Bischoff *et al*., 2001). Aromatic amino acids such as phenylalanine, tyrosine, and tryptophane are derived from chorismate.

EST0321 Glycosyltransferases

The fragment of glycosyltransferase was isolated due to decreasing expression pattern in 2002, however, its pattern reversed in 2003. Since the individual glycosyltransferases in a polymorphic multigene family play multiple roles in plants, the different hybridization pattern found might indicate that other physiological reactions were taken place as well. "These transfer reactions of glycosyltransferases generally use UDP-glucose or galactose as receptors that include hormones such as auxins, cytokinins, gibberellins and abscisic acid, secondary metabolites such as flavonoids, and xenobiotics such as herbicides and pesticides. Because of the wild range of substrates, the functions of this enzyme are broadly from the regulation of developmental and metabolic homeostasis to detoxification pathways. Moreover, the transgenic plants overexpressing the respective glycosyltransferases revealed a useful candidate for phytoremediation" (reviewed by Lim and Bowles, 2004).

EST0336 Acidic ribosomal protein; up-regulated

The verified up-regulated EST0336 had similarity extracted from developing fruits of apricot (CV822736) and peach (BU040164) in the EST database. Its translated product matched an acidic ribosomal protein from *Arabidopsis*. Acidic ribosomal protein has been studied in mammalian systems, and suggested to be the most-suitable housekeeping gene for normalizing mRNA levels in human pulmonary tuberculosis studies (Dheda *et al*., 2004). The role of acidic ribosomal protein in plants is poorly documented.

EST0355 Pyridoxal-5'-phosphate-dependent enzyme, beta family protein

The inconsistent expressed TDF encodes to a pyridoxal-5'-phosphate (PLP; vitamin B6 derivative) -dependent enzyme which catalyzes manifold reactions in the metabolism of amino acids, neurotransmitters (serotonin, norepinephrine), sphingolipids, and aminolevulinic acid (Alexander *et al*., 1994). Most of these PLP-dependent enzymes can be assigned to one of three different families of similar proteins, the alpha, beta and gamma families. The beta family includes L- and D-serine dehydratase, threonine dehydratase, the beta subunit of tryptophan synthase, threonine synthase and cysteine synthase. These enzymes catalyze beta-replacement or beta-elimination reactions. Because these enzymes have intricate evolutionary relationships, Percudani and Peracchi (2003) suggested that assigning the function of PLP-dependent enzymes simply on the basis of sequence criteria is not straightforward on the basis of the consequence of their common mechanistic features. Thus, many genes for PLP-dependent enzymes remain functionally unclassified, and several of them might encode undescribed catalytic activities (promiscuity).

EST0362 Putative pyruvate kinase; transiently expressed at stage III

Pyruvate kinase is a key enzyme in glycolysis. It catalyzes the transfer of a phosphoryl group from phosphoenol pyruvate (PEP) to ADP yielding pyruvate and ATP. The product pyruvate can be converted to acetyl CoA and can enter the TCA cycle. There are many isoenzymes of pyruvate kinase found in various tissues and in a range of different species.

EST0371, EST0372 Eukaryotic release factor 1 family protein / eRF1 family protein

Eukaryotic release factor 1 (eRF1) abolishes readthrough and competes with suppressor tRNAs at all three termination codons, UAG, UGA, UAA, in messenger RNA. It is known from experiments with bacteria and eukaryotic viruses that readthrough of termination codons located within the open reading frame (ORF) of mRNAs depends on the availability of suppressor tRNA(s) and the efficiency of termination in cells. Consequently, the yield of readthrough products can be used as a measure of the activity of polypeptide chain release factor(s) (RF), key components of the translation termination machinery. eRF1 is functional towards all three termination codons located in a natural mRNA and efficiently competes *in vitro* with endogenous and exogenous suppressor tRNA(s) at the ribosomal A site (reviewed by Janzen and Geballe, 2004). This Developing peach mesocarp EST similarity was found.

EST0412 Chloroplast phosphoglycerate kinase; transiently expressed at stage III+20

Phosphoglycerate kinase is as an enzyme catalyzing the transfer of a phosphate group from 3-phospho-D-glycerate in the presence of ATP to yield 3-phospho-D-glyceroyl phosphate and ADP. Genes encoding both isoenzymes of tobacco phosphoglycerate kinase (PGK, EC 2.7.2.3) have been shown to be differentially expressed in a developmental and tissue-specific manner in different plant organs (Bringloe *et al*, 1996).

EST0413 Clathrin heavy chain

The differential expression pattern of EST0413 was not assessed in microarray. Its homolog encodes to clathrin heavy chain, which is the main structural coat protein of coated vesicles. Clathrin heavy chains play a key role in the intracellular transport between membranous organelles as well as the interaction with cytoskeletal proteins.

EST0427 putative purple acid phosphatase precursor (or ACP5)

"Purple acid phosphatases (PAPs) known as the largest group of plant phosphatases are tartrate-resistant and contain a binuclear metal site which results of pink/purple color of its concentrated water solution. The role of PAPs is ascribed to iron transport, bonde resorption but remains unknown in plant" (for review, Olczak *et al*., 2003). PAPs are thought to be in charge of phosphate acquisition in root and/or in vacuoles, whereas taking part in utilization of extracellular environment and/or vacuolar phosphate metabolite. Some PAPs play an auxiliary role in the utilization of phytate, one of the most important phosphate storage compounds in plants. In addition, the elevated expression of genes encoding to PAPs in stressed or senescent plant organs demonstrates the defensive and antioxidant functions in plant (Pozo, *et al*., 1999). However, our irregular expression patterns could not match these concepts as reported.

EST0433 Glyoxysomal beta-ketoacyl-thiolase precursor

"Glyoxysome is microbodies which occur in plant cells, and in some eukaryotic microorganisms, and which contain enzymes of the glyoxylate cycle. Beta-ketoacyl-thiolase catalyzes the final step of fatty acid oxidation in which acetyl-CoA is released and the CoA ester of a fatty acid two carbons shorter is formed. Thiolases are ubiquitous and form a large family of dimeric or tetrameric enzymes with a conserved, five-layered ababą catalytic domain. Thiolases can function either degradatively, in the β-oxidation pathway of fatty acids, or biosynthetically. Biosynthetic thiolases catalyze the biological Claisen condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. This is one of the fundamental categories of carbon skeletal assembly patterns in biological systems and is the first step in a wide range of biosynthetic pathways, including those that generate cholesterol, steroid hormones, and various energy-storage molecules" (reviewed by Modis and Wierenga, 1999). This TDF was transiently expressed in stage III in cDNA-AFLP with increasing pattern in microarray. It appeared to be differential expressed in developing almond seeds (BU574305) due to their nucleotides sequence similarity.

EST0441 Pentatricopeptide repeat-containing protein (PRP)

"The pentatricopeptide repeat (PPR) protein is named after the characteristic tandem array of a 35 amino acid motifs that make up the major part of each of these proteins. The motif is found in a few animal and fungal proteins but the family has greatly expanded in higher plants and the *Arabidopsis* genome contains more than

450 members of the PPR family (1-2% of all *Arabidopsis* proteins). PPR proteins make up a considerable proportion (about 6%) of the unknown function proteins in *Arabidopsis* based on sequence similarity. The vast majority of these proteins are predicted to play constitutive, often essential roles in mitochondria and chloroplasts. The PPR proteins are considered to have a certain common role in the modification of specific RNA in organelles and in RNA processing or translation" (for review, see Lurin *et al*., 2004). The up-regulated pattern of this TDF in cDNA-AFLP was not verified in microarray.

EST0447 Transducin family protein / WD-40 repeat family protein/ putative stress protein; up-regulated

This up-regulated TDF expression was found in using cDNA AFLP but was not verified by microarray. WD-40 repeats (also known as WD or beta-transducin repeats) are short ~40 amino acid motifs, often terminating in a Trp-Asp (W-D) dipeptide. WD-containing proteins have 4 to 16 repeating units. These are thought to form circularized beta-propeller structures. WD-repeat proteins are from a large family found in eukaryotes that are implicated in functions ranging from signal transduction and transcription regulation to controlling the cell cycle and apoptosis. The common function of WD-repeat proteins is their coordination of multi protein complex assemblies. In these assemblies the repeating units serve as a rigid scaffold for protein interactions. Proteins specificity is determined by the sequences outside the repeats. Examples of such complexes are the G proteins (where the beta subunit

is a beta-propeller), TAFII transcription factor, and the E3 ubiquitin ligase (see the EMBL-EBI webpage).

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