2
2009

This is to certify that the
dissertation entitled

MOLECULAR ASPECTS OF FRUIT ABSCISSION IN *MALUS DOMESTICA* AND FLORAL ORGAN ABSCISSION IN *ARABIDOPSIS THALIANA*

presented by

Lingxia Sun

has been accepted towards fulfillment
of the requirements for the

Ph.D degree in Horticulture

Major Professor's Signature

7/9/09

Date

MOLECULAR ASPECTS OF FRUIT ABSCISSION IN *MALUS DOMESTICA* AND FLORAL ORGAN ABSCISSION IN *ARABIDOPSIS THALIANA*

By

Lingxia Sun

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirement
for the degree of

DOCTOR OF PHILOSOPHY

Horticulture

2009

ABSTRACT

MOLECULAR ASPECTS OF FRUIT ABSCISSION IN *MALUS* AND FLORAL ORGAN ABSCISSION IN *ARABIDOPSIS THALIANA*

By

Lingxia Sun

Organ abscission is a developmentally and environmentally regulated cell separation process initiated in specialized tissues, the abscission zones (AZs). Understanding the basis of organ abscission is of fundamental interest to elucidate underlying mechanism of organ abscission and is of applied interest as abscission regulation is critical to achieve optimized market value. A model developed from physiological studies has established that the balance between auxin and ethylene within the AZs regulates leaf abscission. Genetic analyses in model plant species have identified both ethylene-dependent and ethylene-independent pathways to regulate floral organ abscission. In this dissertation, I sought to: investigate natural variation in fruit abscission-related traits in *Malus* species, identify gene expression changes within the pedicel abscission zone during apple fruit abscission, analyze promoter activity of members of the *PECTATE LYASE-LIKE* gene family in cell separation and wall loosening in Arabidopsis. In the first study, I evaluated 144 *Malus* accessions representing wild species, domestic cultivars, and hybrids for abscission-related traits. I found that seasonal timing of fruit abscission in wild species and hybrids showed a broad distribution similar to that seen for domestic cultivars, and that internal ethylene concentration at the time of abscission varied by over three orders of magnitude. Wild species, domestic cultivars, and hybrids all included representatives that showed abscission of fruit prior to substantial production of ethylene, as well as accessions that

retained fruit for a significant period of time following ethylene production. For all accessions that retained fruit, fruit removal resulted in abscission of the pedicel, and exogenous ethylene promoted abscission, suggesting that the abscission zone was functional. Our results suggest important roles for mechanisms independent of fruit ethylene production in abscission. In the second study, we identified transcriptional changes accompanying the transition from competent-quiescent to activated AZs in the apple fruit pedicel. The abscission-associated genes identified in this work contribute to our understanding of fruit abscission, while suggesting a common molecular mechanism of fruit abscission induced under various conditions. In the third study, we documented the spatial and temporal promoter activity of 23 of the 26 Arabidopsis *PLL* genes throughout development. Numerous gene family members showed activity in localized domains programmed for abscission, such as the abscission zones (AZs) of the sepal, petal, and stamen, and seed, as well as the fruit dehiscence zone. Several other members showed activity in cell types expected to facilitate separation, including the endosperm layers during seed germination, and root endodermal and cortical layers during lateral root emergence. Other *PLL* promoters were active in domains not obviously programmed for separation, including the apparent vestigial AZs of the branch and pedicel. These results suggest potential for unique and overlapping activity of *PLL* genes, and provide guidance for analysis of individual gene function through reverse genetics.

DEDICATION

To my family, for their support and love, and to my husband, Yiyong, for his understanding, encouragement, and endless love

ACKNOWLEDGEMENTS

I would like to thank my committee members; Drs Steve van Nocker, Randy Beaudry, Martin J Bukovac, Jianping Hu, and Rebecca Grumet, for their guidance throughout my graduate studies. I would like to give special thanks to my advisor, Dr. Steve van Nocker, for providing me the support and encouragement I needed during my study in his lab.

I would like to thank Philip Forsline and Bill Srmack from USDA-ARS Plant Genetic Resources Unit for their help during the time I stay there for my research. I also would like to thank Dr. Chris Watkins from Cornell University for allowing me using his instrument.

I would like to express my gratitude to my fellow labmates, past and present, Hua Zhang, Philip Ludwig, Jullisa Ek-Ramos, YingYan, Sookyung Oh, Sunchung Park, for their technical and moral support. Also, I would like to thank Kaori Ando from Rebecca Grumet's lab, Ann Armenia and Veronica Vallejo from Ning Jiang's lab for their friendship and moral support.

I also would like to thank College of Agricultural Natural Resources for the Dissertation Completion Fellowship, and Office for International Students & Scholar for tuition fee assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

Literature Review

1. Introduction

Many types of plant organs, such as leaves, flowers, fruits, and seeds, undergo separation from the main plant body during plant development. Plants have evolved successful strategies of growth, development, and survival by utilizing organ shedding. Shedding of ripened fruit and dispersal of seeds facilitate reproduction, and dropping of senescent leaves and abscising flowers after fertilization allows for nutrient recycling. In addition, dropping of infected organs protects plants from pathogen invasion, and shedding of old branches enables remodeling of plant structure. Furthermore, shedding of excess flower buds and fruits ensures optimal growth of remaining organs.

Abscission is a cell separation process that occurs at a pre-determined position, called the abscission zone (AZ). This is generally arranged transversely to the axis of the distal organs (Addicott, 1982) (Figure 1.1). Abscission is a highly co-ordinated and active process including changes in cell structure, metabolism and gene expression within the AZ in response to either developmental signals or various environmental conditions. Early work mainly focused on morphological and biochemical events occurring in abscission and manipulation of abscission through the use of the plant hormones, ethylene and auxin. Recent technological advances in plant genomics allows for identification of genes regulating abscission of floral organs, mostly in Arabidopsis. These studies have led to a general model of the organ abscission process across plant species (Figure 1.1) (Sexton and Roberts, 1982; Osborne, 1989; Patterson, 2001; Robert et al., 2002). In this model, cells at the base of abscising organs undergo patterning and

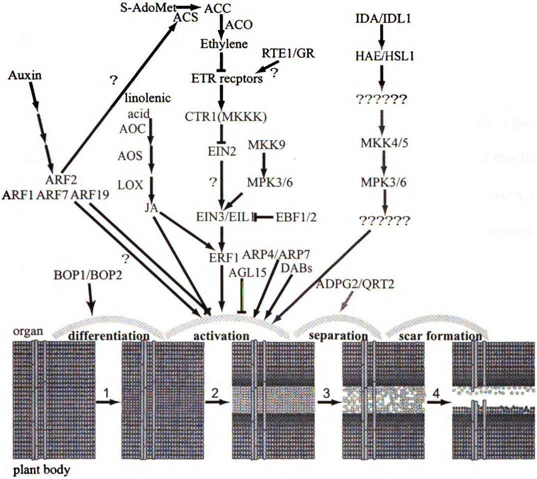


Figure 1.1 Model of floral organ abscission and genes acting in each step in *Arabidopsis*. **Step 1:** Initially, cells at the base of floral organs undergo patterning and differentiation to form morphological distinct abscission zone (AZ) (shown in yellow). *BOP* genes regulate AZ formation indirectly through influencing proximal identity of floral organs. **Step 2:** Next, multiple components and pathways are involved in AZ activation. *ETR1* and EIN2 in the ethylene signaling pathway promote abscission. *ARF2*, along with *ARF1*, *ARF7* and *ARF19*, also stimulate abscission, and *ARF2* promotes abscission in part by stimulating ethylene biosynthesis. *AOS* also promotes abscission presumably through interacting with the ethylene signaling pathway. An ethylene-independent pathway from ligand *IDA/IDL1* to receptors *HAE/HSL1* to downstream effectors has been shown to be crucial for AZ activation. *ARP4/ARP7* also promotes abscission in an ethylene-independent way presumably through chromatin modification. *DABs* specifically influence the timing of floral organ abscission through an unknown mechanism. *AGL15* acts as an inhibitor of floral organ abscission probably through maintaining cell in an embryonic state. **Step 3:** Following AZ activation, many cell wall modifying enzymes are associated with the cell separation process. *ADPG* and *QRT2* promote abscission through loosening the middle lamella between adjacent cells. **Step 4:** Following cell separation, protective layers form at the site of separation to prevent plant from pathogen attack. Many defense related genes are associated with this step.

differentiation to form morphologically distinct layers of small, cytoplasmically dense AZ cells. The AZ is then activated by developmental, hormonal, or environmental cues. This is followed by the activation of cell wall modifying enzymes within the AZ triggering the cell separation process. Lastly, protective layers are formed at the site of organ detachment to protect plants from pathogen attack. In the first part of this literature review, I will address molecular events occurring in each step of organ abscission and hormonal factors regulating abscission. In the second part, I will briefly review chemical fruit thinning and preharvest fruit drop in apple.

2. Processes of Organ Abscission

2.1 Differentiation of the Abscission Zone

During plant development, the AZ is precisely differentiated at the base of many types of organs to be shed, and currently the AZs of leaf, flower, and fruit are well-studied at the morphological level. The AZs in these organs are morphologically distinct from neighboring tissues and characterized by layers of small, densely cytoplasmic cells prior to the onset of abscission (Addicott, 1982, Sexton and Roberts, 1982; Osborne, 1989). The size of the AZ varies significantly among organs and species, for example, 1-2 rows in the AZs of sepals, petals, and stamens in *Arabidopsis*, and 20-30 rows in the fruit pedicel AZs in oil palm (Patterson, 2001; Henderson et al., 2001). The timing of AZ cell differentiation also exhibits a wide range among different organs in the various plant species. For example, differentiation of bean leaf AZ cells may occur in the primordial leaf, while AZ cells of fruit pedicel in pome fruits are differentiated after flower bud opening (Osborne, 1989).

Recent genetic approaches in model plant species, Arabidopsis and tomato, have identified several genes regulating AZ differentiation. *JOINTLESS (J)* and *J-2* are best known for their roles in promoting pedicel AZ formation in tomato (Mao et al., 2000; Yang, et al., 2005). Tomato sheds flowers and fruit at a swollen 'joint' on the pedicel, a site called the pedicel AZ. The *j* mutant fails to form the pedicel AZ, and this leads to a complete blockage of abscission of flower and fruit (Butler, 1936). *JOINTLESS* encodes a MADS-domain transcriptional factor and functions in directing differentiation of the pedicel AZ (Mao et al., 2000). Besides the lack of pedicel AZ formation, the inflorescence meristems in the *j* mutants converts to the vegetative growth after forming only a few flowers and remain determinate (Rick and Sawant, 1955). *J* is preferentially expressed in the main shoot and floral meristems and weakly expressed in the axillary and sympodial meristems (Szymkowiak et al., 2006). However, there was no detectable expression of *J* in any stage of the developing flower pedicels (Szymkowiak and Irish, 2006). These results implied that lack of AZ formation in the *j* pedicels presumably results from an indirect effect of the suppression of sympodial meristem identity in the inflorescence (Szymkowiak and Irish, 2006). The *j-2* mutant also lacks the formation of the pedicel AZ (Rick, 1956). In addition, the *j-2* mutant also has pleiotropic defects in shoot architectures including the production of many flowers and the conversion of sepals to leafy structures (Reynard, 1961). Currently, the *J-2* gene has not been identified and is only known being localized at centromeric region of tomato chromosome 12 (Budiman et al., 2004).

Arabidopsis shed petals, stamens, and sepals after pollination at the AZ located at the base of these organs (Bleecker and Patterson, 1997). *BLADE-ON-PETIOLE 1(BOP1)* and *BOP2* are redundant transcriptional regulators of leaf and floral patterning in Arabidopsis and required for the formation of the AZs (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005; Ha et al., 2007; Mckim et al., 2008). The *bop1 bop2* double mutants fail to abscise their floral organs (Hepworth et al., 2005; Norberg et al., 2005). Histological analysis revealed that the AZs of floral organs and cauline leaves are not properly differentiated in the *bop1 bop2* double mutants, suggesting that *BOP1* and *BOP2* are essential for differentiation of the AZs in the floral organs and leaves (McKim et al., 2008). In addition, the *bop1 bop2* double mutants are abnormal in organ patterning including formation of two sepal/petal mosaic organs and leafy projections from petioles and floral bracts (Hepworth et al., 2005; Norberg et al., 2005; Ha et al., 2007). Furthermore, nectary development is deficient in the *bop1 bop2* double mutants (Mckim et al., 2008). Consistent with its role in the AZ development, *BOP1* is expressed at the base of sepals, petals, and stamens (McKim et al., 2008). These results suggest that BOP 1 and 2 are not specifically committed to AZ differentiation, but appearing have roles in suppressing cell differentiation in the multiple developmental events.

Arabidopsis produces dry dehiscent fruit that is composed of the replum with its attached seeds, the carpel valves, and the valve margins. Seed dispersal requires two sequential cell separation events: fruit dehiscence and seed detachment. Fruit dehiscence takes place along the valve margins at the dehiscence zone (DZ); this process is controlled by a group of transcriptional factors (Liljegren et al., 2004). Mature seeds are released from the funiculus, a stalk-like structure connecting seeds to the replum, at a site referred to as the seed AZ (Pinyopich et al., 2003). The *seedstick* (*stk)* mutants do not properly differentiate the seed AZs and fail to release seeds from mature fruit (Pinyopich et al., 2003). In addition, the *stk* mutants exhibit shorter fruit and enlarged funiculus associated with increased cell expansion and cell division (Pinyopich et al., 2003). These results suggest that *STK*, a MADS domain transcriptional factor, may participate in differentiation of the seed AZ through directing development of the funiculi (Pinyopich et al., 2003).

2.2 Activation of the Abscission Zone

Early studies have shown that the plant hormones ethylene and auxin are the main regulators of organ abscission. Those studies mainly addressed the physiological actions of hormonal regulation and applications in agricultural practice (Addicott, 1982). Forward and reverse genetics approaches have identified many mutants with defects in abscission activation in model plant species (Figure 1.1). Many components in ethylene biosynthesis and perception are known to be involved in organ abscission. A few components in auxin and jasmonate signaling in Arabidopsis presumably regulate organ abscission through an ethylene-dependent pathway. A recently identified ethylene-independent pathway has been show to be crucial for abscission. A few additional genes regulate abscission in an ethylene-independent manner through unknown mechanisms. In this section, I will address the major findings of studies of activation of abscission.

2.2.1 The balance model between ethylene and auxin in abscission

Various developmental and environmental factors affecting organ abscission have been extensively investigated in many plant species (Addicott, 1982; Taylor and Whitelaw 2001). Aging, fruit ripening, and pollination are three important developmental factors promoting abscission (Addicott, 1982). In addition, reduced photoperiod, nutrition deficiency, and water stress from drought, salt, cold, and heat conditions also promote organ abscission as a result of the decline in the growth and vigor of the plant (Addicott, 1982). Furthermore, wounding stress from insect and pathogen attack leads to organ abscission as a function to prevent spread of disease (Addicott, 1982). All these developmental and environmental signals influencing organ abscission have been linked to affect the levels of the plant hormones, ethylene and auxin.

Ethylene is an unsaturated hydrocarbon that binds to copper through a covalent bond (Abeles, 1992). Ethylene has profound effects on many plant developmental processes including stimulation of seed germination, inhibition of triple response in dark-grown seedlings, stimulation of cell expansion, induction of leaf epinasty, organ senescence and abscission, and flowering in some plant species (Abeles et al., 1992). Synthetic ethylene-releasing compounds and inhibitors of ethylene biosynthesis and perception have been used commercially in agricultural practices to manipulate abscission. It has been shown that ethylene production in fertilized flowers, senescent leaves and ripening fruit is positively correlated with abscission in many plant species (Brown, 1997). Fruit abscission in some species occurs without dramatic increase of ethylene production, suggesting that ethylene sensitivity within the AZs controls abscission. However, young leaves typically produce more ethylene than older leaves, but abscise less (Morgan et al., 1992). It has been shown that auxin levels in the distal organs affect ethylene sensitivity within the AZ (Brown, 1997).

Natural auxins in plants are indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-Cl-IAA), and indole-3-butyric a cid (IBA); IAA, however, is the most abundant and physiologically relevant (Taiz and Zeiger, 2003). Auxins affect many developmental events including promoting formation of lateral roots, regulating floral bud development, promoting fruit development, and inducing vascular differentiation (Taiz and Zeiger, 2003). Synthetic auxins, such as 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 1-naphthaleneacetic acid (NAA), have been used to thin fruit and prevent pre-harvest fruit drop (Greene, 2003). Early leaf-explants studies have shown that the removal of leaf blades promoted petiole abscission, whereas application of auxin to the cut surface inhibited abscission (Addicott, 1970; Addicott, 1982). Auxin-transport inhibitors accelerated leaf abscission in the absence of exogenous ethylene (Morgan and Durham, 1972). IAA content within the AZs of cotton boll was positively correlated with boll retention in field-grown cotton (Guinn and Brummett, 1987). All of these results support that auxin prevents organ abscission. Many studies also revealed that ethylene inhibited basipetal auxin transport, and auxin itself can stimulate ethylene production (Abeles & Rubinstein, 1964; Beyer and Morgan, 1971). Thus the interaction between ethylene and auxin appears to regulate activation of organ abscission.

This ethylene-auxin interaction with regard to leaf abscission is outlined in a model proposed in early studies (Rubinstein and Leopold, 1963; Abeles and Rubinstein, 1964; Sexton and Roberts, 1982). This model revealed that there is no single key abscission regulator, and that abscission induction is dependent on the complex interplay between ethylene and auxin signaling. It is generally hypothesized that auxin flow across the AZ controls the sensitivity to ethylene. According to the model, if basipetal auxin flux to the AZ is maintained, abscission is inhibited. Loss of auxin flow promotes abscission by increasing the AZ sensitivity to ethylene. Any factor affecting the supply of auxin and ethylene to the distal organs will influence the sensitivity of the AZ to ethylene. In addition, ethylene can inhibit auxin transport, and auxin itself can stimulate ethylene production (Beyer and Morgan, 1971; Beyer, 1973).

This leaf-explants abscission model can be applied to flower abscission induced either by development cues or by exogenous ethylene (van Doorn and Stead, 1997; Taylor, 2001). Abscission of senescent leaves and flowers after fertilization is usually accompanied with gradual auxin decline that coincides with gradual increase of ethylene (Brown, 1997). The extent to which this model can be applied to other organs, such as fruit, is still not clear. However, it is known that young developing fruit are a strong source of auxin, which is transported basipetally across the AZ of the fruit pedicel, and that loss of auxin transport is associated with fruit abscission (Drazeta et al., 2004; Else et al., 2004).

2.2.2 Involvement of ethylene biosynthesis and perception in abscission

Early work has recognized that ethylene can promote organ abscission (Rubinstein, 1965). Studies of mutants with deficiency in ethylene response have shaped our current understanding of ethylene biosynthesis and perception on regulation of abscission. Ethylene is synthesized from methionine through three enzymes: S-adenosyl-methionine (AdoMet) synthase, 1-aminocyclopropane -1-carboxylic acid synthase (ACS), and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (Yang and Hoffmann, 1984). Ethylene biosynthesis is stimulated by various stresses (Abeles, 1992), flower senescence (Bulfler et al., 1980), fruit ripening (Yang and Hoffmann, 1980), and auxin (Jone and Kende, 1979; Yu et al., 1979), while ethylene biosynthesis is inhibited by aminoethoxy-vinylglycine (AVG) and aminooxyacetic acid (AOA) through blocking activity of ACS (Yang and Hoffmann, 1984). Both *ACSs* and *ACOs* are encoded by a multigene family in plants (Vandenbussche et al., 2006). Increased expression of *ACSs* or *ACOs* has been associated with the organ abscission among many plant species (Mishra et al., 2008; dal Cin et al., 2005, Yuan et al., 2005; Murayama et al., 2006). Transgenic melons with reduced expression of a fruit ripening-related *ACO* revealed decreased fruit abscission (Ayub et al. 1996). Apple cultivars with *ACS1-2*, a dysfunctional allele of *ACS1*, exhibited lower pre-harvest fruit drop than genotypes carrying *ACS1-1*, a functional allele of *ACS1* (Sato et al., 2004).

Many components of the ethylene signaling pathway have been identified in various plant species, and a general model has been established based on studies in *Arabidopsis thaliana* (Chen et al., 2005; Hall et al., 2007; Kendrick and Chang, 2008). Ethylene is perceived by a family of membrane-bound receptors sharing homology with prokaryotic histidine kinase receptors (Chang et al., 1993). Newly identified components, *REVERSION-TO-ETHYLENE SENSITIVITY1 (RTE1)/GREEN-RIPE (GR),* repress ethylene response through *ETHYLNE TRIPLE RESPONSE* (*ETR*) receptors in the membrane through an unknown mechanism. These receptors negatively regulate the downstream ethylene response; in the absence of ethylene, receptors suppress the downstream response through *CONSTITUTIVE TRIPE RESPONSE 1* (*CTR1*). Receptor binding of ethylene inactivates CTR1, a negative regulator of the ethylene response, which leads to activation of *ETHYLENE INSENSITIVE 2* (*EIN2*). In the nucleus, *EIN2* activates *EIN3* and *EIN3-like 1* (*EIL1*) that are controlled by the 26S proteasome-dependent protein degradation pathway. *EIN3* then activates ethylene responses through binding to the EIN3-binding site (EBS) in the promoter of *ETHYLENE RESPONSE FACTOR 1* (*ERF1*).

Although many genes are known to be involved in the ethylene signaling pathway, only a small subset of these genes in Arabidopsis and tomato has been investigated for their role in the timing of organ abscission. Gain-of-function mutation in *ETR1* in Arabidopsis led to ethylene insensitivity and resulted in delayed senescence and abscission of floral organs (Bleecker, et al., 1988; Bleecker and Patterson, 1997). The ethylene receptor family in Arabidopsis is composed of five gene members and classified into two subfamilies (Hua and Meyerowita, 1998). Loss-of-function receptor mutants from any single member within the family did not show any phenotypic defects, while loss-of-function of either triple *etr1 etr2 ein4* or quadruple *etr1 etr2 ein4 ers2* mutants led to a constitutive ethylene response in the absence of ethylene (Hua and Meyerowita, 1998). However, the effects of these mutations on floral organ abscission have not been investigated.

NEVER-RIPE (*NR*) encodes an ethylene receptor in tomato (Lanahan et al., 1994). Semi-dominant *nr* mutants have defects in fruit ripening, flower senescence, and abscission of flowers and fruit (Rick and Butler, 1956). In unfertilized tomato flowers, frequency of flower abscission at pedicel was significantly lower in the *nr* mutants than that in wild type plants (Lanahan et al., 1994). In addition, exogenous ethylene failed to promote abscission of flower explants in *nr* mutants (Lanahan et al., 1994). In tomato, the ethylene receptor family consists of six members and negatively regulates the ethylene signaling pathway (Lashbrook et al., 1998). Transgenic plants with reduced expression of *LeETR1* showed decreased plant size and delayed abscission of leaves and flowers (Whitelaw et al., 2002). These results suggest that levels of ethylene receptors in tomato possibly modulate the timing of organ abscission and fruit ripening.

Loss-of-function *ein2* mutants in Arabidopsis completely blocked the ethylene response and showed delayed senescence and abscission of floral organs (Ecker, 1995). *LeEIL1*, *LeEIL2*, and *LeEIL3* in tomato are functionally redundant transcription factors that act as positive regulators of ethylene signaling (Tieman et al., 2001). Reduced expression of multiple *LeEILs* led to decreased leaf epinasty, delayed fruit ripening, and delayed floral organ abscission (Tieman et al., 2001).

AZ differentiation among all the mutants mentioned above is indistinguishable from that in wild type plants, suggesting that these genes do not play roles in AZ formation. Presumably, other signaling components are also involved in activation of abscission. Interestingly, none of them showed complete blockage of floral organ abscission, suggesting that these components in ethylene signaling are not essential for activation of abscission.

2.2.3 Regulators in abscission through interaction with ethylene biosynthesis

Auxin

As mentioned above, the balance between ethylene and auxin is the predominant effector of organ abscission (Addicott. 1982). Recent studies have shown that either an auxin maxima or an auxin gradient within the given cells is capable of determining developmental reprogramming (Tanaka et al., 2006). Auxin is synthesized in both tryptophan-dependent and tryptophan-independent pathways (Vanneste and Friml, 2009;

Woodward and Bartel, 2005). The unique feature of auxin as a signaling molecule is its ability to move between cells in a directional manner. In plants, auxin transport is achieved through both a long-distance source-to-sink pathway and short-distance cell-to-cell polar transport (Merchant et al., 2002; Vanneste and Friml, 2009). In Arabidopsis, the direction of polar auxin transport is mediated by PIN proteins (Billou et al., 2005; Tanaka et al., 2006). Despite the diversity of cellular responses controlled by auxin, a simple pathway from perception to transcriptional response has been established for auxin signaling (Vanneste and Friml, 2009). Briefly, the presence of auxin stabilizes interactions between auxin receptors *TRANSPORT INHIBITOR RESPONSE 1 (TIR1) /AUXIN-BINDING F-BOX (AFB)* and *Aux/IAA* transcriptional regulators, which trigger ubiquitinylation of *Aux/IAA*. The proteolysis of Aux/IAA results in derepression of *AUXIN RESPONSE FACTORs (ARFs)*, which activates downstream transcriptional responses.

Recent transcriptome profiling analysis revealed that transcription levels of a few *Aux/IAA* in *Mirabilis jalapa* were reduced during petiole and stem abscission induced by auxin depletion (Meir et al., 2006). A few members of the *ARF* gene family were found to regulate the timing of floral organs abscission in Arabidopsis (Ellis et al., 2005; Okushima et al., 2005). *ARFs* encode proteins containing an amino-terminal domain that binds to auxin response elements, an activation/repression domain. Single loss-of-function *arf2* mutants exhibited a slight delay in floral organ senescence and abscission, whereas both *arf1 arf2* double mutants and *arf1 arf7 arf19* triple mutants showed a significant delay in abscission of floral organs. The expression of *ACS2*, *ACS6*, and *ACS8* was lower in flowers of *arf2* mutants than that in wild type plants (Okushima et al., 2005). These results point out the possibility that *ARF2* regulates abscission through promoting ethylene biosynthesis.

Jasmonates

Jasmonates (JAs) are a group of plant hormones including jasmonic acid and its metabolites, such as methyl JA (MeJA). Application of exogenous MeJA promotes organ abscission in bean, citrus, and cherry tomato (Beno-Moualem et al., 2004; Hartmond et al., 2000; Ueda et al., 1996). Acceleration of fruit abscission in citrus induced by MeJA or a synthetic analog, coronatine, appears to result from increased production of ethylene (Burns et al., 2003; Hartmond et al., 2000). Recent biochemical, genetic, and molecular analyses have illustrated JA biosynthetic and signaling pathways (Katsir et al., 2008). Briefly, JA is initially synthesized in chloroplasts from linolenic acid and converted to 2-oxo-phytodienoic acid (OPDA) through lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). In peroxisomes, OPDA is converted to various forms of JA by a few β-oxidation steps. Perception of JA is through a simple signaling pathway. In essence, the presence of bioactive JA signals initiated formation of coronatine-insensitive 1 (COI1)-JA-Jasmonate ZIM domain (JAZ) in which JAZ proteins are degraded through the ubiquitin-26S proteasome pathway. Degradation of JAZ proteins leads to the derepression of transcription of JA-responsive genes. Recent genetic analyses regarding of cell separation in Arabidopsis revealed that abscission of floral organs is delayed in JA-deficient mutant, *aos* (Ogawa et al., 2009), suggesting that *AOS* promotes floral organ abscission. The *aos ein2* double mutants showed more severe delayed floral organ abscission, implying *AOS* presumably promote abscission through interaction with ethylene signaling (Ogawa et al., 2009).

2.2.4 Ethylene-independent regulators in activation of abscission

An ethylene-independent signaling pathway

Genetic screens in Arabidopsis identified a mutant called *inflorescence deficient in abscission (ida)* that fails to abscise sepals, petals, and stamens (Butenko et al., 2003). *IDA* encodes a 77 amino acids protein with an amino-terminal signal peptide and a carboxyl-terminal motif (EPIP) (Butenko et al., 2003). Scanning electron microscopy (SEM) observation revealed that AZ differentiation in *ida* mutants is indistinguishable from that in wild type plants (Butenko et al., 2003). Under exposure to exogenous ethylene, the *ida* mutants showed ethylene responses similar as those in wild type with the exception of floral organ abscission, suggesting that IDA regulates abscission in an ethylene-independent manner or downstream of ethylene (Butenko et al., 2003). In Arabidopsis, there are five additional IDA-LIKE (IDL) proteins that contain amino-terminal peptides and conserved carboxyl-terminal EPIP (Butenko et al., 2003). Overexpression of *IDA* and *IDLs* in Arabidopsis led to similar phenotypes including premature floral organ abscission and silique dehiscence in addition to ectopic abscission of inflorescence branches, cauline leaves, and pedicels (Stenvik et al., 2006; 2008). In addition, expression of *IDLs* partially rescues *ida* phenotypes, suggesting functional redundancy among these genes (Stenvik et al., 2008).

Stenvik and co-workers also revealed that EPIP domain of IDA and IDL1 can substitute the function of IDA (Stenvik et al., 2008). Application of synthetic EPIP peptides to the MS medium promoted premature abscission of flower explants from wild type and induced abscission of flower explants from *ida* mutants (Stenvik et al., 2008). These results suggest that IDA and IDL1 can be recognized as signaling molecules to regulate floral organ abscission.

In the search for potential targets of IDA, two groups have identified two leucine-rich repeat (LRR) receptor-like protein kinases, HAESA (HAE) and HAESA-LIKE1 (HSL1), that presumably serve as receptors for IDA/IDL1 proteins (Cho et al., 2008; Stenvik et al., 2008). *HAE* belongs to a gene family with two additional paralogues, *HSL1* and *HSL2* (Jin et al., 2000; Cho et al., 2008). The *hae hsl2* double mutants fail to shed their floral organs, and SEM revealed that AZ differentiation in *hae hsl2* is indistinguishable from that in wild type plants (Cho et al., 2008). The *hae hsl2* double mutants exhibit ethylene responses same as those in wild types with exception of floral organ abscission (Cho et al., 2008). These evidences indicate that *HAE* and *HSL2* are required for floral organ abscission and redundantly regulate abscission in an ethylene-independent manner. Genetic interactions among *ida* and *hae hsl2* revealed that *IDA* acts upstream of *HAE HSL2* or downstream of ethylene (Cho et al., 2008; Stenvik et al., 2008).

In addition, Cho et al (2008) also found that a few components in the Mitogen-Activated Protein (MAP) kinase cascade were involved in floral organ abscission in Arabidopsis (Cho et al., 2008). In the canonical MAP Kinase (MAPK) signaling cascade, a MAP kinase kinase kinase (MKKK) activates a MAP kinase kinase (MKK), which in turn activates a MAP kinase (MPK). Majority of transgenic lines in Arabidopsis with reduced expression of MKK4 and MKK5 (*MKK4-MKK5RNAi*) were arrested in the cotyledon stages resulting from excessively clustered stomata (Wang et al., 2007). The surviving *MKK4-MKK5RNAi* transgenic lines fail to abscise their floral organs (Cho et al., 2008). SEM and petal break strength studies showed that AZ differentiation in *MKK4-MKK5RNAi* transgenic lines was indistinguishable from that in wild type (Cho et al., 2008). Exogenous ethylene can not induce abscission of floral organs in the *MKK4-MKK5RNAi* transgenic lines, suggesting MKK4 MKK5 regulate abscission in an ethylene-independent way or downstream of ethylene. Consistent with its roles in abscission, GUS reporter gene driven under *MKK4* and *MKK5* promoters was expressed in the floral organs and the AZs of sepals, petals, and stamens (Cho et al., 2008).

MKK4 and *MKK5* have been shown to activate *MPK3* and *MPK6* in plant defenses and function in stomata patterning (Wang et al., 2007). Single *mpk3* or *mpk6* loss-of-function did not show any phenotypic defect, while *mpk3 mpk6* double mutants were embryo lethal. To investigate functions of *MPK3* and *MPK6* on abscission, mutated form of *MPK3* ($MPK3^{KR}$), converting a lysine to arginine, was transformed into *mpk6* mutant (*mpk6* $MPK3^{KR}$) (Cho et al., 2008). 10% of *mpk6* $MPK3^{KR}$ transgenic lines fail to abscise their floral organs (Cho et al., 2008). Consistent with their role on abscission, GUS reporter gene driven under *MPK3* and *MPK6* promoters was expressed in the floral organs and the AZs of sepals, petals, and stamens (Cho et al., 2008). These results suggest that *MPK3* and *MPK6* are positive regulators of floral organ abscission in Arabidopsis

Genetic analyses revealed that *MKK4 MKK5* act downstream of *IDA*, *HAE HSL2* to regulate abscission. These studies have established a putative ethylene-independent signaling pathway on regulating activation of abscission from peptides (*IDA* and *IDLs*), to receptors (*HAE HSL2*), to downstream cytoplasmic effectors (*MKK4*, *MKK5*, *MPK3*, *MPK6*).

Chromatin modification

ACTIN RELATD PROTEINS (ARPs) are components of chromatin-remodeling complexes (Olave et al., 2002). Arabidopsis contains nine *ARP* genes (McKinney et al., 2002), and *ARP4* and ARP7 have been shown to promote floral organ abscission in Arabidopsis (Kandasamy et al., 2005a; 2005b). Although an *arp7* null mutant is embryo-lethal, transgenic plants with reduced expression of *ARP7* revealed a severe delay in floral organ abscission and other pleiotropic growth defects including dwarfed plants, retarded root growth, altered flower development, and reduced fertility (Kandasamy et al., 2005a) The *ARP7*-suppressed transgenic plants exhibited similar triple ethylene responses as those in wild type, whereas exogenous ethylene did not promote floral organs abscission in *ARP7*-suppressed transgenic plants (Kandasamy et al., 2005a). These results suggest that *ARP7* acts on organ abscission in an ethylene-independent manner or downstream of ethylene perception. *ARP4* is the closest paralog of *ARP7*, and *ARP4*-suppressed transgenic plants also revealed pleiotropic defects including delayed abscission of floral organs (Kandasamy et al., 2005b). These results suggest that *ARP4* and *ARP7* act similarly in regulating the timing of floral organ abscission. Considering the pleiotropic effects of reduced expression of either *ARP4* or *ARP7*, these genes appear to regulate gene transcription on a global level and control various developmental events including abscission.

MADS-box domain regulation

AGAMOUS-LIKE 15 (*AGL15*) encodes a MADS-domain transcriptional factor (Fernandez et al., 2000). *AGL15* was preferentially expressed in developing embryos and also expressed in the vegetative shoot apical meristems and at the base of floral organs and cauline leaves (Fernandez et al., 2000). Loss-of-function *agl15* mutants did not show any visible phenotypic defects (Lehti-Shiu et al., 2005), however, overexpression of *AGL15* led to long-term maintenance of embryonic development, delayed flowering time, fruit maturation, floral organ senescence, and abscission (Fernandez et al., 2000; Harding et al., 2003). The *35S::AGL15* transgenic plants exhibited similar triple responses as those in wild type plants under exposure of exogenous ethylene, and *35S::AGL15 etr1* double mutant showed additive effects including delayed senescence and abscission (Fernandez et al., 2000). These results suggest that *35S::AGL15* inhibits abscission in an ethylene-independent way or downstream of ethylene (Fernandez et al., 2000). Considering the dramatic increase in tissue longevity among *35S::AGL15* transgenic plants, *AGL15* possibly regulate organ abscission indirectly through maintaining or enhancing juvenile state of young tissues (Fernandez et al., 2000).

Regulators with unknown function

Genetic screening identified five *delayed floral organ abscission* (*dab1*, *dab2*, *dab3*, *dab4*, *dab5*) mutants with delayed timing of floral organ abscission. These mutants have been shown to be associated with five loci in Arabidopsis (Patterson and Bleecker, 2004). Microscopy studies revealed that AZ differentiation among these mutants is indistinguishable from that in wild type plants. Rounding of AZ cells during the final cell separation step was delayed in all five mutants, and irregular rounding of AZ cells was observed only in the *dab2-1* mutants. These observations were also associated with the degree of petal break strength in the mutants. All five mutants displayed similar triple responses as those in wild type under exogenous ethylene, suggesting *DABs* regulate abscission either in an ethylene-independent manner or downstream of ethylene (Patterson and Bleecker, 2004)

2.3 Separation of the Abscission Zone

Following activation, the next sequential step of abscission is indicated by the loosening of primary cell walls and the dissolution of the middle lamella within the AZs (Addicott, 1982; Osborne, 1989). Cytological studies have showed that AZ cells are distinguished by increased endoplasmic reticulum and Golgi bodies, enriched starch grains and microbodies during cell separation (Osborne, 1989). Following swelling and dissolution of the middle lamella, cells within the AZ are rounded and separate from each other. In addition, irregular cellulose microfibril arrangement has also been observed in the cells within the AZ (Osborne, 1989). Either high turgor pressure or autolysis, generated by the enlargement of separating cells at the time of separation, may contribute to final rupture of the restraining vascular bundles not undergoing wall breakdown (Sexton and Roberts, 1982).

During cell separation within the AZs, loss of Ca^{2+}, decreased methylated pectins, and a lower wall pH have been observed (Stösser et al., 1969; Poovaiah and Rasmussen, 1973; Addicott, 1982; Osborne, 1989). Increased activities of PG, cellulases, and peroxidases have been observed at time of cell separation in many plant species (Addicott, 1982; Osborne, 1989; Roberts and Sexton, 2002). Immunoanalysis on various components of cell wall polysaccharides has demonstrated the dynamic events that occur in cell wall remodeling during the induced abscission of leaf pedicel in E*uphorbia Pulcherrima* (Lee et al., 2008). High levels of UV-induced autofluorescence were detected at the time of leaf separation, suggesting the accumulation of polyphenolics within AZ cells. A reduction in methylesterification of homogalacturonan (HG) and a dramatic increase of de-esterification of HG were also detected within AZ cells at the time of leaf abscission.

Based on the observation of cell wall modification in cell separation, it is not surprising that many cell wall modifying and hydrolytic enzymes contribute to this process. For example, polygalacturonases (PGs), beta-1, 4-endoglucanases (EGases), and pectate lyases (PL) have been implicated in cell separation within the AZ (Cai and Lashbrook; Laskowski et al. 2006; Swarup et al., 2008; Leslie et al., 2007). In addition, expansins and pathogenesis-related (PR) chitinases have also been associated with the cell separation process (Belfield et al., 2005; Sampredro and Cosgrove, 2005; Roberts et al., 2002). Most studies on these genes related to organ abscission focused on expression analysis using RT-PCR or GUS reporter gene approaches.

Despite the large number of cell wall modifying enzymes involved in abscission, a very limited number of genes have been characterized in genetic analysis. Recent genetic analysis revealed that three closely related Arabidopsis PGs, *ARABIDOPSIS DEHISCENCE ZONE PG1* (*ADPG1*), *ADPG2* and *QUARTET2* (*QRT2*), contribute to cell separation in anther and silique dehiscence, and in floral organ abscission (Ogawa et al., 2009). Both single mutants of *adpg1* and *adpg2* showed reduced silique dehiscence, whereas double mutants failed to dehiscence siliques (Ogawa et al., 2009). In addition, both single mutant of *adpg2* and *qrt2* showed delayed floral organ abscission, and *adpg2 qrt2* double mutants exhibited slightly greater delay than either single mutant (Ogawa et al., 2009). Furthermore, the *adpg1 adpg2 qrt2* triple mutants exhibited delayed anther dehiscence (Ogawa et al., 2009). Studies on flower explants also showed that T-DNA insertion mutant of *ADPG2* exhibited delayed floral organ abscission (Gonzalez-Carranza, 2007). Taken together, these results suggest partial functional redundancy among these three genes. These results support the notion that *ADPG1* and *ADPG2* are essential for silique dehiscence, *ADPG2* and *QRT2* contribute to floral organ abscission, and all three genes contribute to anther dehiscence. Many previous studies have implied that PGs play very important roles in various cell separation events, and this study confirms the importance of PGs in these events.

3. Hormonal Regulation of Organ Abscission in Agricultural Practices

In order to achieve maximum yield and optimized quality, regulation of organ abscission has been widely used in various agricultural practices (Addicott, 1982). Induced leaf abscission by chemical defoliation facilitates mechanical harvest in cotton production, and chemical defoliation of young nursery trees prevents disease spread during shipment (Addicott, 1982). Synthetic auxin compounds have been widely used in many potted ornamental plants or cut flowers in order to delay abscission of flowers or petals (Addicott, 1982). Many tree fruit species, such as apple, peach and citrus, retain excess young fruits, and fruit thinning has become a necessary agricultural practice to increase fruit size and quality and maintain consistency in annual bearing (Addicott, 1982) Application of ethylene releasing compounds, such as ethephon, can accelerate dehiscence of shucks in many nut species during the harvest season (Addicott, 1982) On the other hand, prevention of preharvest drop in many fleshy fruit species, like apple and pear, can avoid yield loss (Addicott, 1982). In the following sections, I will address various aspects of hormonal regulation in apple abscission including fruit thinning and preharvest fruit drop.

3.1 Fruit thinning

Domesticated apple bears abundant flowers, which produce excess fruit that the tree is unable to support. Many trees including apple naturally abscise some of their fruit at an early stage of fruit development, a phenomena called 'June drop'. Unlike mature fruit, young fruit start to senesce only after they are already determined to drop (Bangerth, 2000). Auxin transport to the AZ increases in young fruit shortly after fertilization (Gruber and Bangerth, 1990), and ethylene production in young developing fruit is very low (Blanpied, 1972, Miller et al., 1988). These data indicate that the leaf explant model can not be applied to abscission of young fruit. It has been hypothesized that abscission of young fruit is regulated by a hormonally-controlled dominance among fruit and between fruit and shoot (Bangerth, 2000). Auxin transport from dominant young fruit may repress transport from dominated fruit (Bangerth, 2000). Dominated fruit may initiate abscission apparently in the absence of high levels of ethylene production, perhaps reflecting a predominant role for auxin in mediating abscission of young fruit (Bangerth, 2000).

In order to maximize crop value by optimizing marketable fruit size, yield, and quality, post-harvest storage life, as well as to maintain consistent bearing, fruit thinning is necessary and has been an established commercial practice all around the world (Dennis, 2000). Fruit thinning can be done by hand or chemicals. However, hand thinning is no longer practical in commercial apple production due to the high cost of labor.

The most commonly used commercial thinning chemicals are NAA (Naphthaleneacetic acid), 6-BA (6-Benzyladenine), and Sevin (Carbaryl) (Byers, 2003; Wertheim, 1979;). NAA, a synthetic auxin, is a strong thinner and rate responsive; Sevin is a relatively mild thinner and has a unique advantage as an insecticide; 6-BA, a synthetic cytokinin, is also a mild thinner (Byers, 2003). Combinations among these compounds have been shown very effective on fruit thinning (Byers et al., 2003; Bukovac et al., 2008). Many factors affect the efficiency of chemical fruit thinning, for example, genotypes, temperature, and application time (Byers et al., 2003).

The basis of selectivity in chemical fruit thinning is the presence of distinct vigor among fruitlets. Chemical thinners intensify the natural competition among the fruitlets and maximize the effectiveness. The mechanisms involved in fruit thinning are subject to debate among physiologists. Dennis (2002) reviewed various explanations for the thinning action of commonly used chemicals, especially for NAA, BA, and Sevin. These proposed explanations include seed abortion and inhibition of seed development, blockage of nutrient transport from leaf to fruit, reduction of sink strength of fruit, reduced auxin synthesis by the seed, reduced auxin transport from fruit, elevation of ethylene biosynthesis, and inhibition of photosynthesis (Dennis, 2002).

3.2 Preharvest fruit drop

Fruit abscission in advance of harvest (preharvest fruit drop) is a considerable problem in commercial apple production, especially for McIntosh and its sports. Preharvest fruit drop is also greatly influenced by various environmental factors and cultural practices. Climacteric ethylene production associated with fruit ripening has been implicated in preharvest abscission for some domesticated cultivars (Sun et al., 2009; Walsh, 1977). *MdACS1* in domesticated apple is specifically expressed in the ripening fruit and associated with climacteric ethylene accumulation (Harada et al., 1997; Sunako et al., 1999). The dysfunctional allele, *MdACS1-2,* exhibits low transcriptional activity in ripening fruit associated with a transposon insertion in its promoter region (Sunako et al., 1999). Apple cultivars homozygous for *ACS1-2*, associated with low climacteric ethylene production, showed lower preharvest fruit drop than cultivars carrying *ACS1-1* , the functional allele of *ACS1* (Costa et al. 2005; Sato et al., 2004; Sun et al., 2009). These results imply the importance of ethylene in preharvest fruit drop.

As shown for a leaf explant model, increased production of ethylene in fruit may reduce basipetal transport of auxin to the pedicel AZ, at least in part by decreasing auxin transport capacity (Beyer and Morgan, 1971; Riov and Goren, 1979; Suttle, 1988). In some wild *Malus* species, natural fruit abscission is not correlated with a dramatic increase in endogenous ethylene production, suggesting additional factors regulate fruit abscission in these species (Sun et al., 2009).

Plant growth regulators have been used in commercial practice for many years in order to limit or reduce pre-harvest fruit drop (Edgerton, 1973; Wertheim, 1973). Three classes of compounds including NAA, AVG (aminoethoxyvinylglycine hydrochloride), 1-MCP (1-Methylcyclopropene) are currently available for commercial use (Greene, 1983; 2003; dal Cin et al., 2008; Yuan et al., 2008). NAA, a synthetic auxin, prevents preharvest fruit drop within a short period after application, and its major side effect is to accelerate fruit ripening (Yuan et al., 2005). AVG acts as an ethylene biosynthesis inhibitor and 1-MCP is an ethylene binding competitor. The major side effects of these two compounds are inhibition of fruit ripening. Similar to chemical fruit thinners, factors such as application time and weather influence the efficiency of these chemicals on preventing preharvest drop (Greene, 2003).

3.3 Summary

Studies of fruit abscission in apple have mainly focused on the improved use of synthetic chemicals to either promote or prevent abscission based on production goals, but traits related to fruit abscission have not generally been targeted in breeding efforts. Interestingly, the genus *Malus* includes many wild species that are anecdotally known to retain mature fruit, especially the small-fruited species commonly referred to as crab apples (Fiala, 1994). Publicly available germplasm resources (Hokanson et al., 2001; Kresovich et al., 1995) will allow for efficient evaluation of important traits related to fruit abscission. Genotypes can be developed as contrasting models to understand the biological bases of these traits, and as tools in genetic analyses for mapping of genes that influence these traits. In addition, genomics tools will allow for identification of genes involved in abscission, which provides more insights into the mechanisms of fruit abscission and actions of chemicals used to control fruit abscission.

4. References

Abeles FB, Morgan P, Saltveit M (1992) Ethylene in Plant Biology. Academic Press, Inc., San Diego

Abeles FB, Rubinstein B (1964) Regulation of ethylene evolution and leaf abscission by auxin. Plant Physiol 39: 963-969

Addicott FT (1982) Abscission. University of California Press, Ltd. London.

Addicott FT (1970) Plant hormones in the control of abscission. Bio Rev 45: 485-524

Ayub R, Guis M, Ben Amor M, Gillot L, Roustan J-P, Latché A, Bouzayen M, Pech J-C (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nature Biotechnol 14: 862-866

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

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

Beyer EM, Morgan PW (1971) Abscission: the role of ethylene modification of auxin transport. Plant Physiol 48: 208-212

Blanpied GD (1972) A study of ethylene in apple, red raspberry, and cherry. Plant Physiol 49: 627-630

Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml Jc, Heidstra R, Aida M, Palme K, Scheres B (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433: 39-44

Bleecker A, Patterson SE (1997) Last exit: senescence, abscission, and meristem arrest in Arabidopsis. Plant Cell 9: 1169-1179

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

Beno-Moualem D, Gusev L, Dvir O, Pesis E, Meir S, Lichter A (2004) The effects of ethylene, methyl jasmonate and 1-MCP on abscission of cherry tomatoes from the bunch and expression of endo-1,4-[beta]-glucanases. Plant Science 167: 499-507

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086-1089

Brown K (1997) Ethylene and abscission. Physiol Plant 100: 567-576

Budiman M, Chang S, Lee S, Yang T, Zhang H, de Jong J, Wing R (2004) Localization of jointless-2 gene in the centromeric region of tomato chromosome 12 based on high resolution. Theor Appl Genet 108: 190-196

Bukovac MJ, Sabbatini P, Schwallier PG, Schroeder M (2008) Characterizing the interaction between NAA and BA on apple fruit abscission and development. Hortscience 43: 1794-1801

Burns J, Pozo L, Arias C, Hockema B, Rangaswamy V, Bender C (2003) Coronatine and abscission in citrus. J Am Soc Hort Sci 128: 309-315

Butenko MA, Patterson SE, Grini PE, Stenvik G-E, Amundsen S, Mandal A, Aalen RB (2003) *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. Plant Cell 15: 2296-2307

Bufler G, Mor Y, Reid MS, Yang SF (1980) Changes in 1 -aminocyclopropane- 1 - carboxylic acid content of cut carnation flowers in relation to their senescence. Planta 150: 439-442

Butler L (1936) Inherited characters in the tomato. II. Jointless pedicel. J Hered 37: 25-26

Byers RE (2003) Flower and fruit thinning and vegetative: fruiting balance. Apples: Botany, Production and Uses edited by D.C Ferree and I.J. Warrington pp409-430

Cai S, Lashbrook C (2008) Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: enhanced retention of floral organs in transgenic plants overexpressing Arabidopsis ZINC FINGER PROTEIN2. Plant Physiol 146: 1305-1321

Chen Y-F, Etheridge N, Schaller GE (2005) Ethylene signal transduction. Ann Bot 95: 901-915

Cho SK, Larue CT, Chevalier D, Wang H, Jinn T-L, Zhang S, Walker JC (2008) Regulation of floral organ abscission in Arabidopsis thaliana. Proc Natl Acad Sci U S A 105: 15629–15634

Costa F, Stella S, Van de Weg E, Guerra W, Cecchinel M, Dallavia J, Koller B, Sansavini S (2005) Role of the genes Md-ACO1 and Md-ACS1 in ethylene production and shelf life of apple (*Malus domestica* Borkh). Euphytica 141: 181-190 dal Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A (2005) Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus domestica* L. Borck). Journal of Exp Bot 56: 2295-3005

dal Cin V, Danesin M, Botton A, Boschetti A, Dorigoni A, Ramina A (2008) Ethylene and preharvest drop: the effect of AVG and NAA on fruit abscission in apple (*Malus domestica* L. Borkh). Plant Growth Regul 56: 317-325

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

Dennis FG (2002) Mechanism of action of apple thinning chemicals. Hortscience 37: 471-474

Drazeta L, Lang A, Cappellini C, Hall AJ, Volz RK, Jameson PE (2004) Vessel differentiation in the pedicel of apple and the effects of auxin transport inhibition. Physiol Plant 120: 160-170

Ecker JR (1995) The ethylene signal transduction pathway in plants. Science 268: 667-675

Edgerton LJ (1973) Control of abscission of apples with emphasis on thinning and pre-harvest drop Acta Hort. 34: 333-343

Ellis CM, Nagpal P, Young JC, Hagen G, Guifoyle TJ, Reed HW (2005) *AUXIN RESPONSE FACTOR1* and *AUXIN RESPONSE FACTOR2* regulate senescence and floral organ abscission in *Arabidopsis thaliana*. Development 132: 4563-4574

Else MA, Stankiewica-Davies AP, Crisp CM, Atkinson C (2004) The role of polar auxin transport through pedicels of Prunus avium L. in relation to fruit development and retention. J Exp Bot 55: 2099-2109

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

Fiala J (1994) Flowering Crabapples. Timber Press, Inc, Portland, OR.

Gonzalez-Carranza ZH, Elliott KA, Roberts JA (2007) Expression of polygalacturonases and evidence to support their role during cell separation processes in Arabidopsis thaliana. J Exp Bot

Greene DW (1983) Some effects of AVG on fruit set, fruit characteristics, and vegetative growth of apple trees. . J. Amer. Soc. Hort. Sci. 108: 410-415

Gruber J, Bangerth F (1990) Diffusible IAA and dominance phenomena in fruits of apple and tomato. Physiol Plant 79: 354-358

Greene DW (2003) Endogenous hormones and bioregulator use on apple. Apples: Botany, Production and Uses. edited by D.C Ferree and I.J. Warrington pp437-458. CABI publishing

Guinn G, Brummett DL (1987) Concentrations of abscisic acid and indoleacetic acid in cotton fruits and their abscission zones in relation to fruit retention. Plant Physiol 83: 199-202

Ha CM, Jun JH, Nam HG, Fletcher JC (2004) BLADE-ON-PETIOLE1 encodes a BTB/POZ Domain protein required for leaf morphogenesis in *Arabidopsis thaliana*. Plant Cell Physiol 45: 1361-1370

Ha CM, Jun JH, Nam HG, Fletchera JC (2007) BLADE-ON-PETIOLE1 and 2 control Arabidopsis lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. Plant Cell 19: 1809-1825

Hall B, Shakeel S, Schaller GE (2007) Ethylene receptors: ethylene perception and signal transduction. J Plant Growth Regul 26: 118-130

Harada T, Sunako T, Sakuraba W, Goto S, Senda M, Akada S, Niizeki M (1997) Genomic nucleotide sequence of a ripening-related 1-aminocyclopropane-1-carboxylate synthase gene (Md-ACS-1) in apple (accession no U89156) (PGR97-066). Plant Physiol 113: 1465

Harding EW, Tang W, Nichols KW, Fernandez DE, Perry SE (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of AGAMOUS-Like 15. Plant Physiol 133: 653–663

Hartmond U, Yuan R, Burns JK, Grant A, Kender WJ (2000) Citrus fruit abscission induced by methyl-jasmonate. J. Amer. Soc. Hort. Sci. 125 547-552

Henderson J, Davies H, Heyes SJ, Osborne DJ (2001) The study of a monocotyledon abscission zone using microscopic chemical, enzymatic and solid state ^{13}C CP/MAS NMR analyses. Phytochemistry 56: 131-139

Hokanson S, Lamboy W, Szewc-McFadden A, McFerson J (2001) Microsatellite (SSR) variation in a collection of *Malus* (apple) species and hybrids. Euphytica 118: 281-294

Hua J, Meyerowitz EM (1998) Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell 94: 261-271

Jinn TS, JM, Walker J (2000) *HAESA*, an Arabidopsis leucine-rich receptor kinase, controls floral organ abscission. Genes Develop 14: 108

Jones JF, H K (1979) Auxin induced ethylene biosynthesis in subapical stem sections of etiolated seedlings of *Pisum sativum* L. Planta 146: 649-656

Kandasamy MK, McKinney EC, Deal RB, Meagher RB (2005a) Arabidopsis ARP7 is an essential actin-related protein required for normal embryogenesis, plant architecture, and floral organ abscission. Plant Physiol 138: 2019-2032

Kandasamy MK, Deal RB, McKinney EC, Meagher RB (2005b) Silencing the nuclear actin-related protein AtARP4 in Arabidopsis has multiple effects on plant development, including early flowering and delayed floral senescence. Plant J 41: 845-858

Katsir L, Chung HS, Koo AJ, Howe GA (2008) Jasmonate signaling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol 11: 428-435

Kendrick MD, Chang C (2008) Ethylene signaling: new levels of complexity Curr Opin Plant Biol 11: 479-485

Kresovich S, Lamboy W, McFerson J, Forsline P (1995) Integrating different types of information to develop core collections, with particular reference to *Brassica oleracea* and *Malus domestica*. In: T. Hodgkin T, Brown, AHD, Hintum,TJL and Morales EAV (Eds.), Core Collections of Plant Genetic Resources. John Wiley and Sons, Chichester, UK pp. 147-154.

Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1994) The *never ripe* mutation blocks ethylene perception in tomato. Plant Cell 6: 521-530

Laskowski M, Biller S, Stanley K, Kajstura T, Prusty R (2006) Expression profiling of auxin-treated Arabidopsis roots: toward a molecular analysis of lateral root emergence. Plant Cell Physiol 47: 788-792

Lesile ME, Lewis MW, Liljegren SJ (2007) Organ abscission. Annual Plant Reviews V 25. Plant cell separation and adhesion. edited by Jeremy A. Roberts and Zinnia Gonzalez-Carranza: pp91-106

Lee Y, Derbyshire P, Knox JP, Hvoslef-Eide AK (2008) Sequential cell wall transformations in response to the induction of a pedicel abscission event in Euphorbia pulcherrima (*poinsettia*). Plant J 54: 993–1003

Lehti-Shiu MD, Adamczyk BJ, Fernandez DE (2005) Expression of MADS-box genes during the embryonic phase in Arabidopsis. Plant Mol Bio 58: 89-107

Liljiegren S, Roder A, Kempin S, Gremski K, Ostergaard L, Guimil S, Reyes D, Yanofsky M (2004) Control of fruit patterning in Arabidopsis by INDEHISCNET. Cell 116: 843-853

Mao L, Begum D, Goff SA, Wing RA (2000) Sequence and analysis of the tomato *JOINTLESS* locus. Plant Physiol 126: 1331-1340

Murayama H, Sekine D, Yamauchi Y, Gao M, Mitsuhashi W, Toyomasu T (2006) Effects of girding above the abscission zone of fruit on 'Bartlett' pear ripening on the tree. J Exp Bot 57: 3679-3686

McKim SM, Stenvik G-E, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, Aalen RB, Haughn GW (2008) The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. Development 135: 1537-1546

Marchant A, Bhalerao R, Casimiro I, Eklöf J, Casero PJ, Bennett M, Sandberg G (2002) AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. Plant Cell 14: 589-597

Meir S, Hunter DA, Chen J-C, Halaly V, Reid MS (2006) Molecular changes occurring during acquisition of abscission competence following auxin depletion in *Mirabilis jalapa*. Plant Physiol 141: 1604-1616

McKinney E, Kandasamy M, Meagher R (2002) Arabidopsis contains ancient classes of differentially expressed actin-related protein genes. Plant Physiol 128: 997-1007

Miller AN, Krizek BA, Walsh CS (1988) Whole-fruit ethylene evolution and ACC content of peach pericarp and seeds during development. J Amer Soc Hort Sci 113: 119-124

Mishra A, Khare S, Trivedi PK, Nath P (2008) Ethylene induced cotton leaf abscission is associated with higher expression of cellulase (GhCel1) and increased activities of ethylene biosynthesis enzymes in abscission zone. Plant Physiol Biochem 46: 54-63

Morgan PW, He C-j, Drew MC (1992) Intact leaves exhibit a climacteric-like rise in ethylene production before abscission. Plant Physiol 100: 1587-1590

Norberg M, Holmlund M, Nilsson O (2005) The *BLADE ON PETIOLE* genes act redundantly to control the growth and development of lateral organs. Development 132: 2203-2213

Ogawa M, Kay P, Wilson S, Swain S (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are polygalacturonases required for cell separation during reproductive development in Arabidopsis. Plant Cell 216-33 216-233

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologisa A (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in *Arabidopsis thaliana*: unique and overlapping functions of ARF7 and ARF19. Plant Cell 17: 444-463

Olave IA, Reck-Peterson SL, Crabtree GR (2002) Nuclear actin and actin related proteins in chromatin remodeling. Annu Rev Biochem 71: 755-781

Osborne D (1989) Abscission. Cri Rev Plant Science 8: 103-129

Patterson SE (2001) Cutting loose. Abscission and dehiscence in Arabidopsis. Plant Physiol 126: 494-500

Patterson SE, Bleecker A (2004) Ethylene-dependent and independent processes associated with floral organ abscission in Arabidopsis. Plant Physiol 134: 194-203

Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85-88

Poovaiah B, Rasmussen H (1974) Localization of dehydrogenase and acid phosphatase in the abscission zone of bean leaves. Amer J Bot 61: 68-73

Reynard GB (1961) New source of the j2 gene governing *jointless* pedicel in tomato. Science 134: 2102

Rick CM, Butler L (1956) Cytogenetics of the tomato. Advances 8: 267-382

Rick CM, Sawant AC (1955) Factor interactions affecting the phenotypic expression of the *jointless* character in tomatoes. J Amer Soc Hort Sci 66: 354-360

Riov J, Goren R (1979) Effect of ethylene on auxin transport and metabolism in midrib sections in relation to leaf abscission of woody plants. Plant, Cell Environ 2: 83-89

Roberts JA, Elliott KA, Gonzalez-Carranza ZH (2002) Abscission, dehiscence and other cell separation process. Annu Rev Plant Biol 53: 131-158

Rubinstein B, Leopold AC (1963) Analysis of the auxin control of bean leaf abscission Plant Physiol 38: 262-267

Rubinstein B, Abeles F (1965) Relationship between ethylene evolution and leaf abscission. Botanical Gazette 126: 255-259

Sampredro J, Cosgrove DJ (2005) The expansin superfamily. Genome Bio 6: 242

Sato T, Kudo T, Akada T, Wakasa Y, Niizeki M, Harada T (2004) Allelotype of a ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene defines the rate of fruit drop in apple. J Amer Soc Hort Sci 129: 32-36

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

Stenvik G-E, Butenko MA, Urbanowicz BR, Rose JK, Aalen RB (2006) Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* activates cell separation in vestigial abscission zone in *Arabidopsis*. Plant Cell 18: 1467-1476

Stenvik G-E, Tandstad NM, Guo Y, Shi C-L, Kristiansen W, Holmgren A, Clark SE, Aalen RB, Butenko MA (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in Arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. Plant cell 20: 1805-1817

Stösser R, Rasmussen H, Bukovac M (1969) A histological study of abscisison layer formation in cherry fruits during maturation. J Am Soc Hort Sci 94: 239-243

Sun L, Bukovac MJ, Forsline PL, van Nocker S (2009) Natural variation in fruit-abscission related traits in apple (*Malus*). Euphytica 165: 55-67

Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, Niizeki M, Harada T (1999) An allele of the ripening-specific 1-Aminocyclopropane-1-Carboxylic Acid synthase gene (ACS1) in apple fruit with a long storage life. Plant Physiol 119: 1297-1303

Suttle JC (1988) Effect of ethylene treatment on polar IAA transport, net IAA uptake, and specific binding of N-1-naphtyphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol 88: 795-799

Swarup K, Benková E, Swarup R, Casimiro I, Péret B, Yang Y, Parry G, Nielsen E, Smet ID, Vanneste S, Levesque MP, Carrier D, James N, Calvo V, Ljung K, Kramer E, Roberts R, Graham N, Marillonnet S, Patel K, D.G.Jones J, G.Taylor C, Schachtman DP, Sandberg SMG, Benfey P, Friml J, Kerr I, Beeckman T, Laplaze L, Bennett MJ (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nature cell biology 10: 946-954

Szymkowiak EJ, Irish EE (2006) *JOINTLESS* suppresses sympodial identity in inflorescence meristems of tomato. Planta 223: 646-658

Taiz L, Zeiger E (2003) Auxin: the growth hormone. Plant Physiology, 3rd ed. Sinauer Associates, pp423-461

Tanaka H, Dhonukshe P, Brewer PB, J. Friml (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell Mol Life Sci 63: 2738-2754

Taylor J, Whitelaw C (2001) Signals in abscission. New Phytol 151: 323-339

Tieman DM, Ciardi JA, Taylor MG, Klee HJ (2001) Members of the tomato *LeEIL* (*EIN3-like*) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J. 26: 47-58

Ueda J, Miyamoto K, Hashimoto M (1996) Jasmonates promote abscission in bean petiole explants: its relationship to the metabolism of cell wall polysaccharides and cellulase activity J Plant Growth Regul 15: 189-195

van Doorn W, Stead A (1997) Abscission of flowers and floral parts. J Exp Bot 48: 821-837

Vandenbussche F, Vriezen WH, Van Der Straeten D (2006) Ethylene biosynthesis and signaling: a puzzle yet to be completed. Plant Hormone Signaling. edited by Peter Hedden and Stephen G. Thoams pp125-145 Blackwell Publishing Ltd.

Vanneste S, Friml J (2009) Auxin: A trigger for change in plant development. Cell 136: 1005-1016

Walsh C (1977) The relationship between endogenous ethylene and abscission of mature apple fruits. J Amer Soc Hort Sci 102: 615-619

Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang Z (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell 19: 63-73

Wertheim SJ (1973) Chemical control of flower and fruit abscission in apple and pear. Acta Hort. 34: 321-331

Whitelaw CA, Lyssenko NN, Chen L, Zhou D, Mattoo AK, Tucker ML (2002) Delayed abscission and shorter internodes correlate with a reduction in the ethylene receptor *LeETR1* transcript in transgenic tomato. Plant Physiol 128: 978-987

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot 95: 707-735

Yang SF, Hoffmann NL (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155-189

Yang SF, Hoffmann NL (1984) Ethylene biosynthesis and its regulation in higher plants. Annu Rev Plant Physiol 35: 155-189

Yuan R, Wu Z, Kostenyuk I, Burns J (2005) G-protein-coupled alpha2A-adrenoreceptor agonists differentially alter citrus leaf and fruit abscission by affecting expression of ACC synthase and ACC oxidase. J Exp Bot 56: 1867-1875

Yu YB, Adams DO, Yang SF (1979) Regulation of auxin-induced ethylene production in mung bean hypocotyls. Role of 1 - aminocyclopropane - 1 - carboxylic acid. Plant Physiol 63: 589-590

Yuan R, Li J (2008) Effect of sprayable 1-MCP, AVG, and NAA on ethylene biosynthesis, preharvest fruit drop, fruit maturity, and quality of 'Delicious' Apples. Hortscience 43: 1454-1460

CHAPTER 2

Natural Variation in Fruit Abscission-Related Traits in Apple (*Malus)*

Abstract

Abscission or retention of ripening fruit is a major component of seed dispersal strategies and also has important implications for horticultural production. Abscission-related traits have generally not been targeted in breeding efforts, and their genetic bases remain mostly unknown. We evaluated 144 *Malus* accessions representing wild species, domestic cultivars, and hybrids for abscission-related traits. We found that seasonal timing of fruit abscission in wild species and hybrids showed a broad distribution similar to that seen for domestic cultivars, and that internal ethylene concentration at the time of abscission varied by over three orders of magnitude. Wild species, domestic cultivars, and hybrids all included representatives that showed abscission of fruit prior to substantial production of ethylene, as well as accessions that retained fruit for a significant period of time following ethylene production. For all accessions that retained fruit, fruit removal resulted in abscission of the pedicel, and exogenous ethylene promoted abscission, suggesting that the abscission zone was functional. Our results suggest important roles for mechanisms independent of fruit ethylene production in abscission.

Key words: *Malus*, Abscission, Fruit ripening, Ethylene, Natural diversity

Introduction

During plant development, specific organs may undergo programmed separation from the main plant body, a process called abscission (Osborne, 1989). Abscission plays crucial roles in the health and reproductive success of plants. For example, shedding of senescent leaves facilitates the recycling of mineral nutrients, abscission of floral organs after pollination allows for a focus of energy on reproduction, and dropping of diseased or infected organs reduces the spread of disease (Addicott, 1982). Abscission of ripening fruits and mature seeds is an important process contributing to seed dispersal (Addicott, 1982).

Organ separation typically occurs in a pre-determined position, called the abscission zone. The abscission zone may differentiate very early or relatively late in the development of the organ, and is characterized by a few layers of small, densely cytoplasmic cells, generally arranged transversely to the organ axis (Addicott, 1982; Osborne, 1989; Osborne and Sargent, 1976a,b; Sexton and Roberts, 1982; Stösser et al., 1969a,b; Webster, 1968). During initiation of abscission, these separation layer cells expand and may divide. Subsequently, secretion of hydrolytic enzymes, increased peroxidase activity, and loss of calcium and pectin from the wall of separation layer cells presumably lead to the dissolution of the pectin-rich middle lamella, weakening the cell wall and allowing disintegration of abscission zone tissues (Addicott, 1982; Morre, 1968; Osborne, 1989; Rasmussen and Bukovac, 1969; Stösser et al., 1969b; Wittenbach and Bukovac, 1975). Cells basal to the separation layers may undergo a process of trans-differentiation to form a protective layer continuous with the periderm of the stem (Addicott, 1982). The vasculature, which passes through the separation layers, may not always participate in abscission (Stösser et al., 1969a) thus provides a final connection to the main plant body that can be broken by physical force.

Various environmental and developmental signals have been shown to induce abscission by influencing the ratio between auxins and ethylene within the organ and adjacent abscission zone cells (Addicott, 1982; Brown, 1997; Roberts et al., 2002; Taylor and Whitelaw, 2001). According to a widely accepted model developed with leaf explants, loss of basipetal flow of auxin through the abscission zone, for example during leaf senescence, activates abscission by derepressing sensitivity of separation layer cells to ethylene (Abeles, 1967; Abeles and Rubinstein 1964; Addicott, 1982; Addicott and Lynch, 1951; Osborne, 1989; Rubinstein and Leopold, 1963; Sexton, 1995). Thus a balance between auxin and ethylene signaling, rather than absolute levels of the hormones, seems to be the predominant effector of abscission. The extent to which this leaf model can be applied to other organs, such as fruit, is not clear. However, it is known that developing fruit constitute a strong source of auxin, which is transported basipetally across the separation layers of the fruit pedicel, and that loss of auxin transport is associated with fruit abscission (Drazeta et al., 2004; Else et al., 2004). In plants such as apple that naturally adjust crop load to meet physiological capacity, auxin transport from dominant young fruit may repress transport from dominated fruit (Bangerth, 2000). Dominated fruit may initiate abscission apparently in the absence of high levels of ethylene production, perhaps reflecting a predominant role for auxin in mediating abscission of young fruit (Bangerth, 2000).

The interactive contributions of auxin and ethylene signaling to abscission of mature fruit have not been extensively studied. In many fruits, ripening is accompanied by the production of significant amounts of ethylene (Brady and Speirs, 1991; Reid, 1985). Transgenic melon expected to suppress expression of a fruit ripening-related *ACC OXIDASE* gene, and thus accumulation of fruit ethylene, showed loss of abscission (Ayub et al., 1996), and in domestic apple (*Malus domestica* Borkh.), blocking climacteric ethylene production in the fruit through the use of the ethylene agonist 1-methylcyclopropene was associated with delayed abscission (Sato et al., 2004). Walsh (1977) observed that, for three domestic apple cultivars, abscission was preceded by the accumulation of high levels of ethylene in the fruit. Although such experiments suggest that fruit-produced ethylene can promote abscission, whether this ethylene acts directly or indirectly, and the exact mechanism of this effect, remain unknown. Maturity in many fruits that naturally abscise is not associated with high levels of ethylene production [e.g., sour cherry (Wittenbach and Bukovac, 1974)], suggesting either that low levels are sufficient to promote abscission or that natural abscission can occur independently of ethylene.

It is well known that exogenous ethylene accelerates abscission of ripening fruit in a variety of fruit species, even those that do not produce high levels of endogenous ethylene (Abeles et al., 1992; Brady and Speirs, 1991). As shown for a leaf explant abscission model, increased levels of ethylene in the fruit may reduce basipetal transport of auxin to the abscission zone, at least in part by decreasing auxin transport capacity (Beyer and Morgan, 1971; Riov and Goren, 1979; Suttle, 1988). This mechanism may be superimposed on the endogenous decrease in auxin synthesis in the fruit associated with maturity, a phenomenon that may itself derepress ethylene generation (Abeles and Rubinstein, 1964).

Fruit abscission in advance of harvest (pre-harvest drop) is a considerable production problem for many fruit crops, especially apple and pear (*Pyrus communis* L.). In commercial apple, the timing of natural fruit drop, relative to the optimal commercial harvest date, shows a great degree of variability. Some cultivars, such as McIntosh, are especially prone to preharvest drop. The genetic basis of this variability remains obscure. Sato et al., (2004) found that several apple cultivars homozygous for the dysfunctional *ACS1-2* allele of the *ACC SYNTHASE 1* gene, which is important for climacteric ethylene accumulation in apple (Costa et al., 2005; Harada et al., 2000), showed relatively low degrees of pre-harvest drop, and that homozygosity of *ACS1-2* was associated with that low preharvest drop in a small segregating population. This supports the pharmacological evidence implicating climacteric ethylene in promoting abscission, and suggests that *ACS1* allelotype is an important contributor. However, this study also found that cultivars homozygous for the wild-type *ACS1-1* allele nevertheless can show a range of abscission behavior, ranging from nearly complete retention of fruit to nearly complete pre-harvest drop, revealing the importance of additional factors (Sato et al., 2004). This analysis was confounded by the fact that the optimal timing of commercial harvest is not based solely on maturity indicators, but also on storage potential, which may decline with a delay in harvest. Thus, varieties with greater capacity for storage may be harvested at a more advanced stage of maturity. Accordingly, internal ethylene concentration (IEC) of apple fruit has been found to vary dramatically among cultivars at the optimal time of commercial harvest (Chu, 1988).

Although the relationships between the fruit ethylene production and abscission have been documented for a few domestic apple cultivars (Walsh, 1977), there has been no analysis of this phenomenon at a large scale or including wild apple species. In tomato, variability in timing of abscission relative to climacteric ethylene production has been recognized among species (Grumet et al., 1981). Although the genetic basis of natural variation of this response in tomato has not been extensively studied, it is known that ethylene-independent fruit retention in tomato can be indirectly conferred by loss of function of *JOINTLESS*, a gene required for development of the abscission zone (Butler, 1936). The potential influences of ethylene sensitivity, controlled largely by the availability of ethylene receptors (Klee, 2001), in abscission have not been extensively studied in any fruit.

Retention of ripe fruit would be expected to confer considerable advantages for current production regimes, and would be critical for potential mechanical harvesting of apples. However, this trait has generally not been targeted in breeding efforts. Interestingly, the genus *Malus* includes many wild species that are anecdotally known to retain mature fruit, especially the small-fruited species commonly referred to as crabapples (Fiala, 1994). A representation of *Malus* species and genotypes is maintained at the USDA-ARS Plant Genetic Resources Unit in Geneva, NY. This reference collection includes 28 wild *Malus* species and over 1000 *M. domestica* cultivars originating from throughout the northern hemisphere. A core subset considered to represent the diversity of the entire collection is maintained at the Geneva site, allowing for efficient evaluation of important traits relevant to industrial production (Hokanson et al., 2001; Kresovich et al., 1995). To better characterize variability in endogenous timing of fruit abscission, and help define the influence of fruit-produced ethylene in abscission of mature fruit, we analyzed variation in abscission-related responses among these accessions. Specifically, we assessed (1) seasonal timing of natural fruit abscission, (2) endogenous ethylene production at the time of abscission, (3) pedicel abscission in response to fruit removal, and (4) abscission of fruit in response to exogenous ethylene.

Material and Methods

Plant material

The *Malus* Germplasm Collection is maintained at the United States Department of Agriculture-Agricultural Research Service (ARS) Plant Genetic Resources Unit in Geneva, NY. We targeted for evaluation a subset of accessions previously determined to represent much of the diversity of the entire collection [the apple 'Core Collection' (Hokanson et al., 2001; Kresovich et al., 1995)] as well as accessions previously noted by USDA staff as exhibiting either premature fruit drop or fruit retention into the winter. Plants were five to ten years old, budded on M7 or E7 semi-dwarfing rootstocks, and managed in accordance with commercial practice only for insect or microbial pests. Only healthy and vigorous trees were selected for evaluation. Species, hybrid and cultivar nomenclature exactly followed ARS assignments.

Analysis of fruit abscission

For each accession, we determined the peak timing of fruit abscission by counting the number of naturally abscised fruit at two-week intervals, beginning August 27, 2006 when appreciable fruit abscission in some accessions was first noticed, and ending November 10, 2006, when trees were mostly defoliated. Peak abscission was defined as the observation date when at least 15% of fruit initially recorded for the accession had abscised. In all cases, nearly all remaining fruit abscised by the subsequent observation date (not shown). Accessions that showed less than 15% abscission of initial fruit at the final observation date were defined as non-abscising. Of accessions classified as non-abscising in 2006, all but six also showed less than 15% fruit abscission when evaluated at an equivalent date in 2007, with the remaining six accessions showing 50% or less abscission in 2007. For each abscising accession, on the date defined as peak abscission, 20 fruit were selected that abscised when subjected to gentle force. All collected fruit separated from the branch at the apparent pedicel/branch abscission zone, and showed turgid, undamaged pedicels. On the final observation date, fruit from non-abscising accessions were removed from the tree, leaving the pedicel attached to the fruit. Fruit were maintained under laboratory conditions for 24 h before analyses.

Measurement of internal ethylene concentration (IEC), firmness and starch

All determinations of IEC, firmness, and starch were based on measurements of at least five fruit of each accession. For IEC measurement, 1 ml of internal gas was withdrawn from fruit submerged in water under a vacuum (Beyer and Morgan, 1970), and analyzed by gas chromatography using a Carle Series 400 AGC (Hach Co., Loveland, CO) and certified ethylene standard (Matheson Gas Products, Chicago, IL). Flesh firmness was determined using an Effigy FT-327 penetrometer (Effigy, Alfonsine, Italy) with an 11-mm diameter probe. Starch content was evaluated by rating stain intensity after dipping transverse sections into an iodide solution (5 mM potassium iodide, 17 mM iodine), with a visual scale of 1 (intense staining; highest starch content) to 8 (no staining; lowest starch content) using the Cornell Generic Starch Chart.

***MdACS1* genotyping**

MdACS1 allelotype was evaluated by the PCR using oligonucleotide primers *MdACS1-F* (5'- GGTAATTGGAGTAATGAACTGAGCA-3') and *MdACS1-R* (5'-TCACTATTTGCTTGGACTGGG AAGT-3') that flank the transposon insertion found in *MdACS1-2*, as described by Sunako et al., (1999).

Evaluation of pedicel abscission and ethephon-promoted abscission

This experiment was carried out in early July, 2007 approximately 80 d after full bloom for the standard cv. Gala. For each accession evaluated, 30 fruit were labeled on each of two branches. On one of the two branches, the pedicel was severed midway between the fruit and the branch to induce abscission. Abscission was monitored daily by applying a gentle force on the defruited pedicel. On the remaining branch, marked fruit were evaluated for natural abscission (control). A biological replicate, offset by two days, was carried out using a separate branch, or branch of a separate tree. Abscission zone morphology was evaluated on pedicels of fruit attached to the tree, without the aid of microscopy.

Analysis of promotion of fruit abscission with ethephon was carried out in mid-late September, 2007. A subset of non-abscising accessions identified in 2006 was targeted for analysis. For each accession, branches with similar fruit load were tagged as either experimental or control. For each of the experimental treatments, ethephon [(2-chloroethylphosphonic acid), (600 μl/L active ingredient), Micro Flo, Memphis, TN,] was applied with 0.1 % Silwet L-77 as a foliar spray. The control branch was treated with 0.1 % Silwet L-77 only. The replicate treatment was offset by one day. Fruit IEC was determined as described above. Fruit abscission was quantified by counting the number of abscised fruit at defined intervals following treatment, and expressed as percentage of initial number of fruit recorded for the accession.

Results

Seasonal timing of fruit abscission

To document variation in the seasonal timing of fruit abscission among wild *Malus* species, *Malus domestica* cultivars, and hybrids, we examined 144 diverse accessions at defined intervals during the period of natural fruit abscission. These included 53 accessions representing 28 wild species, 61 *Malus domestica* cultivars, and 30 hybrids (Table 2.1 and not shown). We found that the seasonal timing of fruit abscission was similarly and broadly distributed across observation dates for representatives of wild *Malus* species, *Malus domestica* cultivars, and hybrids, and that all three groups included non-abscising accessions. However, accessions showing abscission at the earliest two observation dates were mainly *Malus domestica* cultivars (25 of 33 accessions), whereas non-abscising accessions were predominantly wild *Malus* species or hybrids (36 of 49 accessions) (Figure 2.1A). We then evaluated the potential relationship between seasonal timing of fruit abscission and fruit size. We categorized fruit into three classes: >100g, 30g-100g, and <30g, and identified representatives of each fruit size class that exhibited abscission at each observation date. Small-fruited accessions, nearly all wild species and hybrids, were overrepresented among the non-abscising class (31 out of 49 accessions) (Figure 2.1B). However, we observed 23 small-fruited accessions that abscised, and 14 large-fruited accessions, nearly all domestic cultivars, that did not abscise (Figure 2.1B). This documents an association between an

Table 2.1 Number of wild species, domestic cultivars, and hybrids used in this study

Species and cultivars	No.
M.angustifolia	1
M.asiatica	3
M.atrosanguinea	1
M.baccata	4
M.bhutanica	1
M.coronaria	5
M.halliana	1
M.hupehensis	1
M.ioensis	3
M.kirghisorum	2
M.mandshurica	1
M.micromalus	1
M.prunifolia	3
M.rockii	1
M.sieboldii	2
M.sieversii	5
M.sylvestris	2
M.turesii	1
M.yunnanensis	2
M. x *arnoldiana*	1
M. x *dawsoniana*	1
M. x *hartwigii*	1
M. x *magdeburgensis*	1
M. x *platycarpa*	1
M. x *robusta*	4
M. x *scheideckeri*	1
M. x *soulardii*	2
M. x *sublobata*	1
M. domestica	61
Hybrids	30

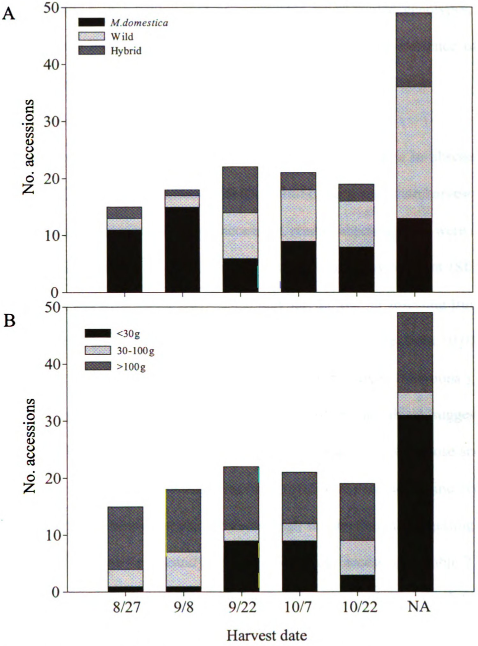


Figure 2.1. Frequency distribution of accessions evaluated for harvest date relative to species (A) and fruit weight (B). NA, non-abscising

association between fruit retention and non-domestic, small-fruited genotypes, lack of abscission among a small number of domestic cultivars, and the existence of alleles specifying early season fruit drop among wild species.

Variation in fruit ethylene concentration at abscission

To help evaluate a potential role for fruit-produced ethylene in abscission, we measured internal ethylene concentration (IEC) of fruit of each accession harvested at the date of peak natural abscission. For each accession, readily abscising fruit were removed from the tree, briefly allowed to equilibrate to the laboratory environment (Sfakiotakis and Dilley, 1973), and assayed for IEC. We found that the IEC in abscising fruit among different accessions varied by greater than three orders of magnitude, from ~0.03 µl/L to 900 µl/L (Figure 2.2 and not shown). Multiple fruit from single accessions generally showed low variability in IEC (standard error ~15% of mean values) suggesting the observed variability reflected true tree-to-tree differences (not shown). Those accessions showing the lowest IEC values in abscising fruit (≤0.5 µl/L) included nine accessions (eight domestic and one wild) that also exhibited high starch content, suggesting that the ripening program had not significantly progressed in these accessions (Table 2.2). We also evaluated IEC in fruit from non-abscising accessions. Unblemished fruit were removed from the tree in early November, allowed to briefly equilibrate to the laboratory environment, and assayed for IEC under the same conditions as for naturally abscising fruit. Surprisingly, the range of IEC from non-abscising fruit was similar to that observed in abscising fruit (~0.07 µl/L to 580 µl/L), although accessions with non-abscising fruit were underrepresented in the group of accessions with highest IEC values (≥10 µl/L) (18 of 78 accessions) (Figure 2.2 and not shown). All non-abscising accessions with high

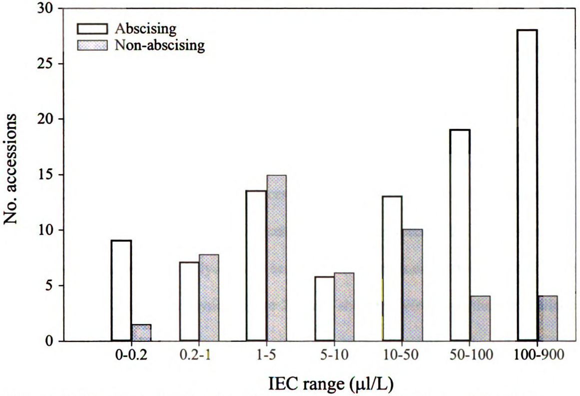


Figure 2.2 Frequency distribution of accessions evaluated for internal ethylene concentration (IEC) at peak harvest date (abscising accessions) or final sampling date (non-abscising accessions).

high IEC values (≥10 μl/L) showed relatively low starch content (starch index ≥6), and at least a subset of these also exhibited low flesh firmness values (≤~90 N) and fully developed skin ground color (Table 2.2)and not shown) suggesting that the ripening program was progressing. This subset included *Malus prunifolia* and *Malus. x asiatica* (= *Malus prunifolia* x *Malus sieversii)* (Luby, 2003), four domestic cultivars, and two hybrids (Table 2.2).

It was previously reported that absence of significant preharvest drop among *M. domestica* cultivars was associated with homozygosity of the dysfunctional *MdACS1-2* allele (Sato et al., 2004; see above). To further understand the variation in seasonal timing of abscission and relationship between abscission and fruit IEC, we determined the *MdACS1* allelotype for the studied accessions. The *ACS1-1* allelotype was identified in >70% of accessions, including 82 accession from wild species, 20 hybrids, and 37 domestic cultivars, whereas only ~9% of accessions (6 hybrids and 6 domestic cultivars) exhibited the *ACS1-2/2* allelotype (Figure 2.3; not shown). We found that each allelic group (*MdACS1-1/-1, -1/2,* or *-2/2)* contained both abscising and non-abscising accessions (Figure 2.3). The *MdACS1-1/-1* allelotype was overrepresented among accessions showing the earliest natural abscission (August 27; 12 of 13 accessions), whereas the *MdACS1-2/-2* allelotype was overrepresented among non-abscising accessions (7 of 49 accessions) (Figure 2.3 and not shown). This suggests that *MdACS1* allelotype is not only a possible determinant of the potential for preharvest drop, but also for the non-abscising character.

To evaluate the relationship between timing of abscission and climacteric associated ethylene production, and to determine if lack of capacity for climacteric

Table 2.2 Selected accessions showing extremes of IEC in abscising and non-abscising fruit

Accession	Species	Cultivar	*ACS1* allelotype	IEC (µl/L) 1d after harvest	IEC (µl/L) 14d after harvest	Firmness (N)	Starch index
Accessions showing low IEC in abscising fruit							
589071	*M.domestica*		*ACS1-1/1-2*	0.03	72.31	86.91	1
588943	*M.domestica*	Liberty	*ACS1-1/1-1*	0.1	593.04	121.04	1
158731	*M.domestica*	Bramtot	*ACS1-1/1-2*	0.24	208.78	107.78	1
199532	*M.domestica*	Toyo	*ACS1-1/1-1*	0.27	212.22	92.83	1
483257	*M.domestica*	Reinette Simirenko	*ACS1-1/1-1*	0.3	286.88	83.44	1
483254	*M.* x *dawsoniana*		*ACS1-1/1-1*	0.04	246.58	117.88	1.2
588848	*M.domestica*	Cortland	*ACS1-1/1-1*	0.45	972.5	90.69	1.6
134808	*M.domestica*		*ACS1-1/1-2*	0.08	635.14	107.69	2
589511	*M.domestica*	Severny Sinap K-21.39	*ACS1-1/1-1*	0.12	510.6	80.23	2
588998	*M.domestica*	Marshall McIntosh	*ACS1-1/1-1*	0.13	1063.7	81.61	5
589780	*hybrid*	PRI 384-1	*ACS1-1/1-1*	0.07	554	73.87	6.2
280401	*M.domestica*	Ein Shemer	*ACS1-1/1-2*	0.37	625.9	79.66	6.6
588866	*hybrid*	Kerr	*ACS1-1/1-1*	0.12	6.23	92.12	8
589391	*M.* x *soulardii*		*ACS1-1/1-1*	0.14	167.31	>133.50	8
Accessions showing high IEC and low starch content in non-abscising fruit							
588747	*M.domestica*	Florina	*ACS1-1/1-2*	20.32		80.37	7.4
589819	*hybrid*	PRI 2050-2	*ACS1-2/1-2*	25.65		76.5	7
589790	*hybrid*	PRI 1484-1	*ACS1-2/1-2*	68.06		68.26	6
162722	*M.domestica*	Damelot	*ACS1-1/1-1*	80.5		72.27	8
199525	*M.domestica*	Amanishiki	*ACS1-1/1-2*	99.9		90.51	6.2
589539	*M.domestica*	Zlatna Resistenta	*ACS1-1/1-2*	157.7		32.49	8
589877	*M.asiatica*		*ACS1-1/1-2*	218		59.1	8
589389	*M.prunifolia*	Macrocarpa	*ACS1-1/1-1*	402.1		52.42	8
589874	*M.asiatica*		*ACS1-1/1-2*	583		111.3	6

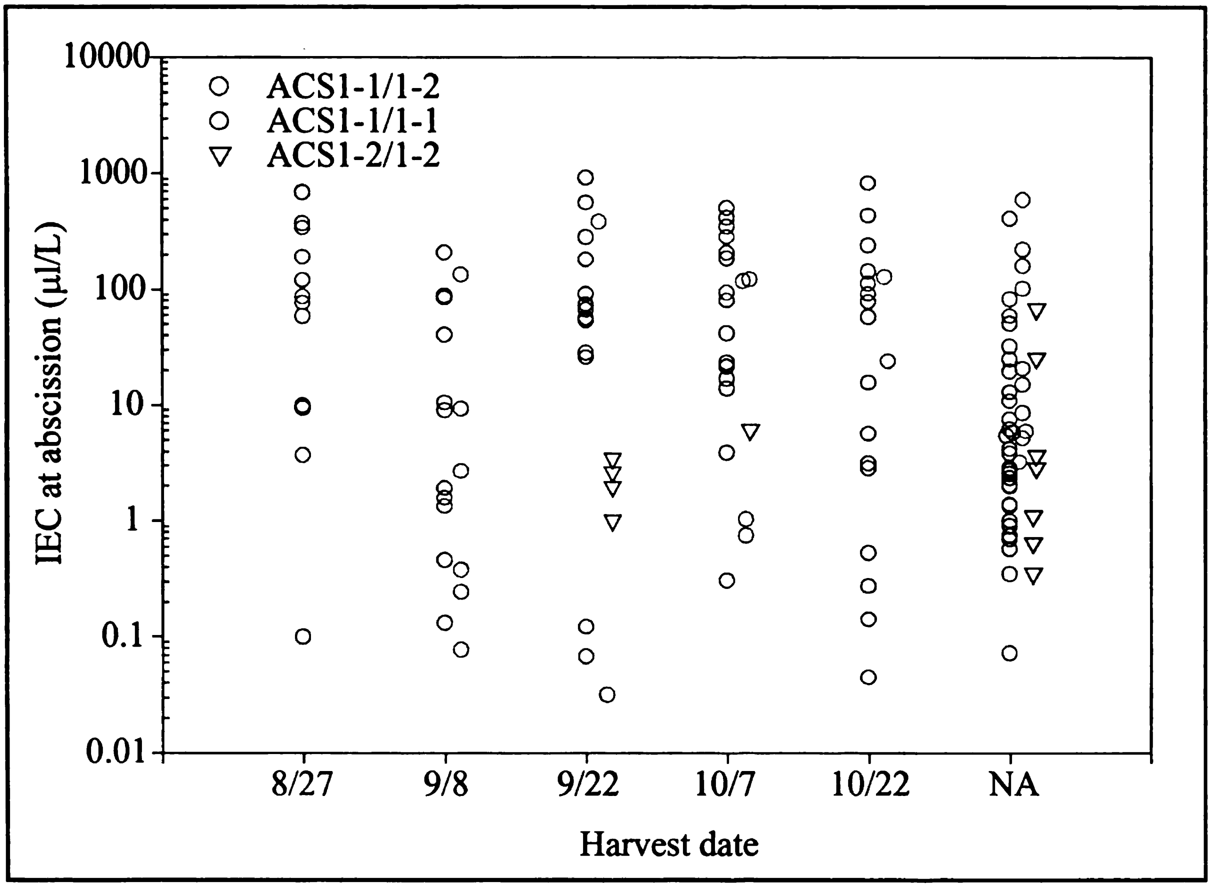


Figure 2.3. Relationship between harvest date, IEC at abscission, and *ACS1* allelotype for studied accessions.

ethylene production may have contributed to the low IEC in some accessions, we measured IEC in harvested fruit after storage in the laboratory environment for 14 days (Gussman et al., 1993; Sfakiotakis and Dilley, 1973). Of 14 abscising accessions evaluated, 13 showed a striking (>500-fold) increase in IEC during this period (Table 2.2). These accessions were naturally abscising, revealing that climacteric ethylene production followed rather than preceded natural fruit drop in these accessions.

Abscission in response to fruit removal and exogenous ethylene

These experiments identified 18 accessions that failed to abscise yet produced high levels of ethylene in the fruit. These were not obviously distinguished from the entire population in terms of *ACS1* allelotype, IEC at harvest, or fruit weight (not shown). All of the non-abscising accessions, including these, exhibited classical abscission zone morphology. To evaluate the functionality of the abscission zone in these accessions, we determined the abscission response of the fruit pedicel when fruit was removed from field-grown plants by cutting the pedicel halfway between the branch and fruit (Barlow, 1950). We previously observed that this treatment invariably resulted in abscission of the remaining pedicel stub at the natural abscission zone within five to eight days, when carried out with the Golden Delicious cultivar approximately 80 days following full bloom (unpublished data). To interpret the results, we applied this analysis to nearly the entire population. For the experiments reported here, a marked subset of fruit on each tree was removed, and the timing of abscission of the remaining pedicel stubs was noted at a daily interval.

Interestingly, all of the 122 accessions used in this experiment showed abscission of pedicel stubs, with the median timing of abscission among accessions ranging from

Table 2.3. Accessions showing extremes of pedicel abscission

Accessions showing relatively rapid and synchronous pedicel abscission

Accession	Species	Cultivar	Days to first abscission	Days to 50% abscission	Days to 95% abscission	Fruit weight (g)	*ACS1* Allelotype
589765	*angustifolia*		3	4	4	12.3	*ACS1-1/1-1*
589877	*asiatica*		4	4	5	48.4	*ACS1-1/1-2*
136488	*atrosanguinea*		3	3	3	7.48	*ACS1-1/1-1*
588907	*baccata*	Himalaica	3	3	3	1.82	*ACS1-1/1-1*
322713	*baccata*	Mandshurica	3	3	4	5.58	*ACS1-1/1-1*
483259	*baccata*	Genvina	3	3	5	9.48	*ACS1-1/1-1*
590062	*bhutanica*		3	4	5	1.48	*ACS1-1/1-1*
589987	*coronaria*		3	3	4	20.9	*ACS1-1/1-1*
589983	*coronaria*		3	3	4	25.56	*ACS1-1/1-1*
588849	*domestica*	Russian	4	5	6	22.05	*ACS1-1/1-1*
589478	*domestica*	Novosibirski Swe	4	4	5	45.55	*ACS1-1/1-1*
589053	*domestica*	Lady	4	5	6	75.92	*ACS1-1/1-1*
588838	*domestica*	Nova Easygro	4	5	6	106.96	*ACS1-1/1-1*
589913	*domestica*	Dorsett Golden	5	6	6	110.28	*ACS1-1/1-1*
589486	*domestica*	Murray	4	4	5	175.46	*ACS1-1/1-1*
588992	*hybrid*	White Angel	3	4	4	2.48	*ACS1-1/1-1*
589819	*hybrid*	PRI 2050-2	5	6	7	198.88	*ACS1-2/1-2*
589820	*hybrid*	Paririe Fire	3	4	5	1.34	*ACS1-1/1-1*
589959	*hybrid*	MA #8	3	4	4	1.96	*ACS1-1/1-1*
589510	*hybrid*	Garry	3	4	4	2.48	*ACS1-1/1-1*
588824	*hybrid*	Almey	3	4	4	3.78	*ACS1-1/1-1*
589250	*hybrid*	Red Jacket	3	3	4	7.96	*ACS1-1/1-1*
588870	*hybrid*	Dolgo	3	3	4	13.86	*ACS1-1/1-1*
589775	*hybrid*	PRI 2382-1	5	6	7	92.82	*ACS1-1/1-1*
588883	*hybrid*	Demir	4	5	6	109.02	*ACS1-1/1-1*
590016	*ioensis*		3	4	5	14.56	*ACS1-1/1-1*
590004	*ionesis*		3	4	5	10.62	*ACS1-1/1-1*
613855	*kirghisorum*		3	4	5	42.96	*ACS1-1/1-1*
589832	*prunifolia*	Xanthocarpa	3	3	4	4.28	*ACS1-1/1-1*
589421	*rockii*		3	4	5	2.8	*ACS1-1/1-1*
613932	*sieboldii*		5	5	6	0.44	*ACS1-1/1-1*
613806	*sieboldii*		3	3	3	0.44	*ACS1-1/1-1*
594104	*sieversii*		4	5	5	26.84	*ACS1-1/1-1*
589008	*turesii*		3	3	4	1.74	*ACS1-1/1-1*
588757	*x hartwigii*	GMAL52	4	4	5	1.92	*ACS1-1/1-1*
588959	*x magdeburgensis*		6	7	8	0.78	*ACS1-1/1-1*
589415	*x platycarpc*	Hoopesii	3	3	3	34.54	*ACS1-1/1-1*
588825	*x robusta*	Robusta 5	3	3	4	3.14	*ACS1-1/1-1*
589383	*x robusta*	Persicifolia	3	4	5	3.94	*ACS1-1/1-1*
589418	*x scheidecke*		3	3	4	3.1	*ACS1-1/1-1*
588922	*x sublobata*	Yellow Autumn C	3	3	4	12.26	*ACS1-1/1-1*
589253	*yunnanensis*	Carmine crab	4	5	6	0.81	*ACS1-1/1-1*

Table 2.3.Continued

Accessions showing relatively rapid and synchronous pedicel abscission							
Accession	Species	Cultivar	Days to first abscission	Days to 50% abscission	Days to 95% abscission	Fruit weight (g)	*ACS1* Allelotype
589765	*angustifolia*		3	4	4	12.3	*ACS1-1/1-1*
589877	*asiatica*		4	4	5	48.4	*ACS1-1/1-2*
136488	*atrosanguinea*		3	3	3	7.48	*ACS1-1/1-1*
588907	*baccata*	Himalaica	3	3	3	1.82	*ACS1-1/1-1*
322713	*baccata*	Mandshurica	3	3	4	5.58	*ACS1-1/1-1*
483259	*baccata*	Genvina	3	3	5	9.48	*ACS1-1/1-1*
590062	*bhutanica*		3	4	5	1.48	*ACS1-1/1-1*
589987	*coronaria*		3	3	4	20.9	*ACS1-1/1-1*
589983	*coronaria*		3	3	4	25.56	*ACS1-1/1-1*
588849	*domestica*	Russian	4	5	6	22.05	*ACS1-1/1-1*
589478	*domestica*	Novosibirski Swe	4	4	5	45.55	*ACS1-1/1-1*
589053	*domestica*	Lady	4	5	6	75.92	*ACS1-1/1-1*
588838	*domestica*	Nova Easygro	4	5	6	106.96	*ACS1-1/1-1*
589913	*domestica*	Dorsett Golden	5	6	6	110.28	*ACS1-1/1-1*
589486	*domestica*	Murray	4	4	5	175.46	*ACS1-1/1-1*
588992	*hybrid*	White Angel	3	4	4	2.48	*ACS1-1/1-1*
589819	*hybrid*	PRI 2050-2	5	6	7	198.88	*ACS1-2/1-2*
589820	*hybrid*	Paririe Fire	3	4	5	1.34	*ACS1-1/1-1*
589959	*hybrid*	MA #8	3	4	4	1.96	*ACS1-1/1-1*
589510	*hybrid*	Garry	3	4	4	2.48	*ACS1-1/1-1*
588824	*hybrid*	Almey	3	4	4	3.78	*ACS1-1/1-1*
589250	*hybrid*	Red Jacket	3	3	4	7.96	*ACS1-1/1-1*
588870	*hybrid*	Dolgo	3	3	4	13.86	*ACS1-1/1-1*
589775	*hybrid*	PRI 2382-1	5	6	7	92.82	*ACS1-1/1-1*

three to 17 days. We identified a subset of 42 accessions that showed relatively rapid and synchronous abscission of all treated pedicels, beginning ~3 days after treatment, and with >50% or >95% of pedicels abscising within 24 or 48 hours, respectively, thereafter (Table 2.3). Abscission of pedicels for the remaining accessions was less rapid and less synchronous, with a subset of 24 accessions showing abscission only after ~5 days, and retention of >50% of pedicels for at least five days thereafter. The synchronous-abscising subset was disproportionally lacking in domestic cultivars, large-fruited accessions, and/or genotypes heterozygous or homozygous for *ACS1-2* ($p<0.05$, Fisher's Exact Test; Table 2.3). Neither the non-abscising accessions producing high levels of ethylene, nor non-abscising accessions considered as a whole, were distinguished from the remainder of accessions in terms of the abscission behavior of the pedicel following cutting.

As an alternative approach to analyze functionality of the abscission zone, we subjected 24 non-abscising accessions to treatment with ethephon, which is metabolized by plant tissues to produce free ethylene. Branches within the same tree were either subject to a foliar spray, or used as mock-treated controls. Of these accessions, 15 showed abscission of >50% of fruit within 12 days of treatment, and all but three showed abscission that was markedly greater than that of the control branches (Table 2.4). The remaining three accessions showed abscission of <2 % of fruit after ethephon treatment; however, fruit from these did not show substantial increases in IEC, suggesting that the ethephon treatment was ineffective (Table 2.4).

Table 2.4. Internal ethylene concentration (IEC) and abscission in response to Ethephon

Accession	Species	Cultivar	treated fruit (µl/L)[1]	IEC of control fruit (µl/L)	Abscission of treated fruit (% initial)	Abscission of control fruit (% initial)
483259	*baccata*	Genvina	11.46	3.64	100	0
589393	*hybrid*		12.26	2.16	100	0
588757	*X hartwigii*	GMAL52	16.07	3.27	100	6.9
589959	*hybrid*	MA #8	21.08	3.85	100	8.4
589820	*hybrid*	Paririe Fire	50.49	3.75	93.1	0
306320	*domestica*	Crittenden	35.96	2.32	91	0
588907	*baccata*	Himalaica	9.91	2.12	87.1	0
588825	*x robusta*	Robusta 5	17.85	1.35	85.5	0
589421	*rockii*		47.14	3.2	84.7	0
590062	*bhutanica*		11.63	3.3	83.3	4.3
613835	*asiatica*		5.96	4.27	80.1	8.9
589008	*turesii*		35.25	31.23	68.8	6.3
588993	*x robusta*		36.75	7.89	67.2	0
589498	*domestica*	Dab 100	5.36	1.03	64.3	2.6
588992	*hybrid*	White	58.57	12.12	57.4	0
594092	*micromalus*		7.83	3.6	34.7	0
589877	*asiatica*		8.5	1.12	31.3	0
589003	*x robusta*	Korea	30.89	27.71	29	5
613932	*sieboldii*		6.76	0.86	24.8	0
613834	*hupehensis*		7.31	0.75	20.9	0
589874	*asiatica*		5.46	1.88	18.5	0
589274	*hybrid*	Masek	4.91	2.14	1.9	0
588856	*hybrid*	Ottawa 8	3.47	1.22	1.4	0
271831	*hybrid*	Vimorin	4.11	1.64	0	0

[1]Mean value of measurements for two replicates 3d, 6d, 9d, and 12d following treatment

Discussion

In this study, variation in abscission-related traits was observed among *Malus* accessions representing the breadth of genetic diversity found in domesticated varieties and in the *Malus* genus. This information is useful both for understanding the underlying biology of these traits and for the more applied goal of developing genotypes with abscission characteristics better suited to modern apple production approaches. We documented the range in seasonal timing of natural abscission for domestic and non-domestic accessions, and the occurrence of accessions lacking fruit abscission. For domestic cultivars, seasonal distribution of abscission is most likely influenced by the broad range in seasonal timing of fruit maturation, a trait perhaps subject to selection during domestication. However, we found a similar range of seasonal timing of abscission among wild species. In this study, replicate observations of seasonal timing of abscission could not be made for most accessions, because only a single specimen was available during the duration of the study. Possibly, some tree-to-tree differences observed were influenced by the physiological status of individual trees, rather than strictly by genotype. However, none of the trees used in this study displayed visible signs of stress. Neither did we observe separation of fruit from the tree independently of the visible abscission zone, a phenomenon that has been connected with abscission related to tissue damage (Walsh, 1977).

We also evaluated the tendency for natural abscission in relation to fruit size. It is well known that many crabapple-type *Malus* genotypes retain fruit into the winter season. Potentially, retention of ripened fruit in small-fruited genotypes is an adaptation that facilitates access and seed dispersal by frugiverous birds (Harris et al., 2002). In contrast, domestication of large-fruited genotypes may have favored those that readily dropped ripe fruit, in order to facilitate collection from the naturally large trees. In this study, we documented the anecdotal observations that lack of abscission is not absolutely coupled to small fruit size. We identified 14 accessions, ten domestic cultivars and four hybrids, that exhibited relatively large (>100 g) fruit and that did not show abscission. Thus, alleles governing this trait may be readily exploited in the development of new commercial varieties.

A trivial explanation for the lack of abscission identified in some accessions in this study is that these genotypes may be adapted to longer growing seasons, and fail to initiate the abscission process at the Geneva site before the onset of dormancy. While this is possibly the case for some of the accessions, we noted that many of the non-abscising accessions showed physiological characteristics that are typically associated with ripening in domestic cultivars, including color development, loss of starch, and loss of firmness, and/or also exhibited relatively high levels of internal ethylene (Table 2.2 and not shown). For these, abscission is apparently unlinked from ripening and/or ethylene production.

Previous studies have shown that internal ethylene concentration can vary strikingly among domestic varieties at the date of optimal commercial harvest, a benchmark based both on fruit maturity and storage potential (e.g., Chu 1988). In this study, we analyzed internal ethylene concentration at the time of abscission, or for non-abscising accessions, at a time late in the season when plants were progressing into dormancy. Blanpied (1972) observed substantial natural abscission in cv. Golden Delicious and McIntosh preceding climacteric ethylene production, suggesting that for these varieties, high levels of fruit ethylene are not required for abscission. There have been no previous large-scale studies to examine genotypic differences in ethylene production in relation to fruit abscission. Here, we found that internal ethylene content of abscising fruit varied substantially among both domestic and non-domestic accessions.

We identified numerous accessions that showed natural abscission even when the IEC was low. These included Marshall McIntosh, a skin color variant of McIntosh. In our study, McIntosh showed only moderate starch content and relatively low flesh firmness at abscission. This suggests that ripening was in progress, and supports both the observation of Blanpied (1972) that this variety abscises in advance of significant ethylene production, and anecdotal observations that this variety is prone to premature abscission. We noted that this accession was distinguished among all of the evaluated accessions by an extremely short pedicel, a trait that may lead to substantial physical force on the abscission zone as the fruit enlarges and becomes constrained by the branch or neighboring fruit. In other accessions that showed abscission at low IEC, ripening had apparently not significantly progressed, as evidenced by the high starch content. Interestingly, eight of the nine accessions that showed abscission in advance of apparent ripening are domesticated cultivars, and the ninth, *M. x dawsoniana*, is believed to have *domestica* parentage, suggesting that abscission in advance of ripening may have been subject to selection during domestication.

In contrast, we also identified accessions that did not show abscission, in spite of high levels of ethylene in the fruit. A potential explanation is that the abscission process was initiated, but was in an early stage, at the time of our measurements. However, this appears unlikely, since our analysis was carried out at a time during the season when trees were mostly defoliated and entering dormancy. In addition, none of the fruit from non-abscising accessions appeared to be nearing abscission, because significant force was required to remove the fruit at the apparent abscission zone when these were harvested (not shown). These accessions, which include four domestic cultivars and two hybrids with Golden Delicious parentage, are an attractive source of alleles conferring this trait for breeding of commercial varieties.

It is interesting to speculate on the determinants that might govern the apparent ethylene-independent retention of fruit seen in these accessions. All of the non-abscising accessions examined in this study showed an adjacent enlargement and constriction at the basal end of the pedicel, a characteristic of the abscission zone in cultivated apple. In addition, our experiments showed that abscission could be induced either through removal of the immature fruit, or, for the subset of the accessions analyzed, by exposure to exogenous ethylene. This suggests that the natural fruit retention seen in these accessions was not due to a homeotic absence of the abscission zone, as seen in tomato mutants for the *JOINTLESS* gene (Mao et al., 2000). However, we cannot rule out the possibility that one or more of these accessions show more subtle defects in abscission zone function that precludes abscission of fruit under natural conditions. For example, incomplete separation layer development has previously been shown as a mechanism associated with loss of grain abscission in domesticated rice (Li et al. 2006). Anatomical comparisons of accessions showing extremes of abscission habits identified here may resolve this question. Another possibility is that the abscission process may be initiated, but remain ultimately ineffective for pedicel breakage. Some genotypes may lack effective production of one or more of the numerous enzymatic activities expected to be required for cell wall disassembly. Here, comparative expression analyses of selected abscission-associated genes among genotypes showing extremes in abscission behavior may be informative. Alternatively, some abscission zone tissues might not fully participate in abscission. For example, vascular tracheids and other cell types may have highly modified secondary walls that likely present a challenge for cell wall disassembly. These may persist after disintegration of other separation layer cell types, and effectively retain the fruit until broken by physical force. Especially for *Malus* genotypes that have small, lightweight fruits, even subtle variation in such cell types or numbers in the abscission zone could predispose the fruit to drop or retention. To explore this, a detailed study of abscission zone development among accessions is needed.

In the model plant *Arabidopsis thaliana*, a gene designated *IDA* is required for floral organ abscission. Interestingly, in *ida* mutants, organ removal force ultimately increases following a sharp decrease at the time of natural organ abscission (Butenko et al. 2003). This identifies a potentially conserved mechanism that may act antagonistically to cell disintegration, and in apple, may preclude efficient abscission in naturally non-abscising accessions.

In conclusion, we documented diversity in fruit-abscission-related traits among *Malus* accessions representing the breadth of genetic diversity seen in *Malus*. Our findings suggest that important mechanism(s) independent of fruit ethylene production act as determinants of natural abscission. Accessions showing phenotypic extremes in abscission-related traits can be developed as contrasting models to understand the biological bases of these traits, and as tools in genetic analyses for the mapping of genes that influence these traits.

References

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in Plant Biology. Academioc Press, Inc., San Diego

Abeles FB, Rubinstein B (1964) Regulation of ethylene evolution and leaf abscission by auxin. Plant Physiol 39: 963-969

Addicott FT (1982) Abscission. University of California Press, Ltd. London.

Ayub R, Guis M, Ben Amor M, Gillot L, Roustan J-P, Latché A, Bouzayen M, Pech J-C (1996) Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nature Biotechnol 14: 862-866

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

Barlow HWB (1950) Studies in abscission I. Factors affecting the inhibition of abscission by synthetic growth-substances J Exp Bot 1: 264-281

Beyer EM, Morgan PW (1970) A method for determining the concentration of ethylene in the gas phase of vegetative plant tissues. Plant Physiol 46: 352-354

Beyer EM, Morgan PW (1971) Abscission: the role of ethylene modification of auxin transport. Plant Physiol 48: 208-212

Blanpied G (1972) A study of ethylene in apple, red raspberry, and cherry. Plant Physiol 49: 627-630

Brandy C, Speirs J (1991) Ethylene in fruit ontogeny and abscission -In The Plant Hormone Ethylene (A.K.Mattoo and J.C Suttle, eds), CRC Press, Boca Raton, FL pp235-258

Brown K (1997) Ethylene and abscission. Physiol Plant 100: 567-576

Butenko MA, Patterson SE, Grini PE, Stenvik G-E, Amundsen S, Mandal A, Aalen RB (2003) *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. Plant Cell 15: 2296-2307

Butler L (1936) Inherited characters in the tomato. II. Jointless pedicel. J Hered 37: 25-26

Chu C (1988) Internal ethylene concentration of McIntosh, Northern spy, Empire, Mutsu, and Idared apples during the harvest season. J Amer Soc Hort Sci 113: 226-229

Costa F, Stella S, Van de Weg E, Guerra W, Cecchinel M, Dallavia J, Koller B, Sansavini S (2005) Role of the genes *Md-ACO1* and *Md-ACS1* in ethylene production and shelf life of apple (*Malus domestica* Borkh). Euphytica 141: 181-190

Drazeta L, Lang A, Cappellini C, Hall AJ, Volz RK, Jameson PE (2004) Vessel differentiation in the pedicel of apple and the effects of auxin transport inhibition. Physiol Plant 120: 160-170

Else MA, Stankiewica-Davies AP, Crisp CM, Atkinson CJ (2004) The role of polar auxin transport through pedicels of *Prunus avium* L. in relation to fruit developement and retention. J Exp Bot 55: 2099-2109

Fiala J (1994) Flowering Crabapples. Timber Press, Inc, Portland, OR

Grumet R, Fobes JF, Herner RC (1981) Ripening behavior of wild tomato species. Plant Physiol 68:1428-1432

Gussman CD, Goffreda JC, Gianfagna TJ (1993) Ethylene production and fruit softening rates in several apple fruit ripening variants. HortScience 28:135-137

Harris SA, Robinson JP, Juniper BE (2002) Genetic clues to the origin of the apple. Trends Genet 18:426-430

Harada T, Sunako T, Wakasa Y, Soejima J, Satoh T, Niizeki M (2000) An allele of the 1-aminocyclopropane-1-carboxylate (*Md-ACS1*) accounts for the low level of ethylene in climacteric fruits of some apple cultivars. Theor Appl Genet 101:742-746

Hokanson SC, Lamboy WF, Szewc-McFadden AK, McFerson JR (2001) Microsatellite (SSR) variation in a collection of *Malus* (apple) species and hybrids. Euphytica 118:281-294

Klee HJ (2001) Control of ethylene-mediated processes in tomato at the level of receptors. J Exp Bot 53:2057-2063

Kresovich S, Lamboy WF, McFerson JR, Forsline PL (1995) Integrating different types of information to develop core collections, with particular reference to *Brassica oleracea* and *Malus x domestica*. In: Hodgkin T, Brown AHD, Hintum TJL and Morales EAV (ed), Core Collections of Plant Genetic Resources John Wiley and Sons, Chichester, UK pp 147-154

Li C, Zhou A, Sang T (2006) Rice domestication by reducing shattering. Science 311:1936-1939

Luby JJ (2003) Taxonomics, classification and brief history. In: Ferree DC, Warrington IJ (ed) Apples: Botany, Production, and Uses. CAB International pp 1-14

Morre JD (1968) Cell wall dissolution and enzyme secretion during leaf abscission. Plant Physiol 43:1545-1559

Osborne D (1989) Abscission. Cri Rev Plant Science 8:103-129

Osborne D, Sargent J (1976a) The positional differentiation of ethylene-responsive cells in rachis abscission zones in leaves of *Sambucus nigra* and their growth and ultrastructural changes in senescence and separation. Planta 130:203-210

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

Rasmussen HP, Bukovac MJ (1969) A histochemical study of abscission layer formation in the bean. Am J Bot 56:69-76

Reid MS (1985) Ethylene and abscission. Hortscience 20:45-50

Riov J, Goren R (1979) Effect of ethylene on auxin transport and metabolism in midrib sections in relation to leaf abscission of woody plants. Plant Cell Environ 2:83-89

Roberts JA, Elliott KA, Gonzalez-Carranza ZH (2002) Abscission, dehiscence and other cell separation process. Annu Rev Plant Biol 53:131-158

Sato T, Kudo T, Akada T, Wakasa Y, Niizeki M, Harada T (2004) Allelotype of a ripening-specific 1-aminocyclopropane-1-carboxylate synthase gene defines the rate of fruit drop in apple. J Amer Soc Hort Sci 129:32-36

Sexton R (1995) Abscission. In: Pessarakli M (ed) Handbook of Plant and Crop Physiology. New York: Dekker, pp 497-525

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

Sfakiotakis EM, Dilley D (1973) Internal ethylene concentrations in apple fruits attached to or detached from the tree. J Amer Soc Hort Sci 98:501-503

Stösser R, Rasmussen HP, Bukovac MJ (1969a) A histological study of abscission layer formation in cherry fruits during maturation. J Am Soc Hort Sci 94:239-243

Stösser R, Rasmussen HP, Bukovac MJ (1969b) Histochemical changes in the developing abscission layer in fruits of *Prunus cerasus* L. Planta 86:151-164

Sunako T, Sakuraba W, Senda M, Akada S, Ishikawa R, Niizeki M, Harada T (1999) An allele of the ripening-specific 1-Aminocyclopropane-1-Carboxylic Acid synthase gene (*ACS1*) in apple fruit with a long storage life. Plant Physiol 119:1297-1303

Suttle J (1988) Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of N-1-naphtyphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol 88:795-799

Taylor JE, Whitelaw CA (2001) Signals in abscission. New Phytol 151:323-339

Walsh CS (1977) The relationship between endogenous ethylene and abscission of mature apple fruits. J Amer Soc Hort Sci 102:615-619

Webster BD (1968) Anatomical aspects of abscission. Plant Physiol 43:1512-1544

Wittenbach VA, Bukovac MJ (1974) Cherry fruit abscission: Evidence of time of initiation and involvement of ethylene. Plant Physiol 54:494-498

Wittenbach VA, Bukovac MJ (1975) Cherry fruit abscission: Peroxidase activity in the abscission zone in relation to separation. J Amer Soc Hort Sci 100:387-391

CHAPTER 3

Molecular Analysis of Abscission Zone Development in Apple Fruit Pedicels

Abstract

Organ abscission is a developmentally and environmentally regulated cell separation process initiated in specialized tissues of the abscission zones (AZs). Physiological studies of leaf abscission have suggested that the interaction between auxin and ethylene signaling within the AZs regulates the initiation of cell separation, whereas genetic analyses of floral organ abscission in *Arabidopsis thaliana* (Arabidopsis) have identified an additional mechanism of abscission apparently unrelated to ethylene. In fruit crops, precise regulation of fruit abscission is crucial to achieve maximum yield and optimized market value. In this study, we identified transcriptional changes accompanying the transition from competent-quiescent to activated AZs in the apple fruit pedicel. The abscission-associated genes identified in this work contribute to our understanding of fruit abscission, while suggesting a common molecular mechanism of fruit abscission induced under various conditions.

Key words: apple, transcriptional profiling, ethylene, auxin

Introduction

Abscission is a process by which plants shed organs, such as leaves, flowers, floral organs, or fruit, at a pre-determined position, called abscission zones (AZs) (Addicott, 1982). The separation layers within the AZ are characterized by small, densely cytoplasmic cells, generally arranged transversely to the organ axis (Addicott, 1982, Osborne, 1989; Sexton and Roberts, 1982). Organ abscission is generally conceptualized as progressing through four steps (Patterson, 2001): (1) differentiation, whereby morphologically distinct cell layers are formed in response to developmental and/or environmental cues; (2) activation, characterized by the acquisition of the ability of the separation layers to respond to abscission-promoting signals; (3) separation of cells within abscission layers, resulting from the production of cell-wall modifying enzymes and accompanied by dissolution of the pectin-rich middle lamella and expansion and rounding of cells; (4) differentiation of protective layer (s) across the newly exposed surface of the plant body.

Pioneering studies of abscission focused on the effects of various plant hormones, especially ethylene and auxins, on differentiation and activation (Addicott, 1982; Sexton and Roberts, 1982). These studies led to a widely accepted model whereby organ abscission is conditioned by the balance between auxin and ethylene signaling, rather than absolute levels of these hormones (Abeles, 1967; Abeles and Rubinstein, 1964; Addicott, 1982; Osborne, 1989; Sexton, 1995). According to this model, auxin flow across the AZ controls the sensitivity to ethylene; when auxin declines, the AZ becomes more responsive to ethylene (Addicott, 1982; Sexton, 1995; Taylor and Whitelaw, 2001). More recent genetic studies in the model plants *Arabidopsis thaliana* (Arabidopsis) and

Solanum lycopersicum (tomato) have identified genes involved in differentiation (Hepworth et al., 2005; Mao et al., 2000; Mckim et al., 2008; Norberg et al., 2005; Pinyopich et al., 2003) and components of auxin or ethylene signaling required for post-differentiation step(s) (Bleecker, et al., 1988; Bleecker and Patterson, 1997; Chen et al., 2002; Ellis et al., 2005; Lanahan et al., 1994; Okushima et al., 2005; Patterson and Bleecker 2004; Tieman et al., 2001). Such studies have also clearly implicated mechanism(s) of abscission acting independently of ethylene (Butenko et al., 2003; Cho et al., 2008; Patterson and Bleecker 2004; Stenvik et al., 2006; 2008).

Outside of the model plants, there has been little work done to identify the genetic components of abscission, especially those that function in early steps. In addition, there have been few studies of genetic mechanisms of fruit abscission. In domestic apple (*Malus X domestica)*, precise control of fruit abscission is critical for production. Similar to many tree fruits, apple naturally bears an abundance of flowers and fruitlets. Although many or most of the fruit abscise early in development, the resulting crop load is typically still too great to allow the remaining fruit to attain marketable size or quality (Byers, 2003). In addition, high crop load can repress floral initiation, leading to the phenomenon of biennial (alternate year) bearing (Byers, 2003). Consequently, flower or fruit thinning by mechanical or chemical methods is generally required to reduce the final crop load (Greene et al., 2003). An additional considerable problem in commercial apple production is the abscission of mature fruit in advance of harvest (pre-harvest drop). Identification of genetic components involved in apple fruit abscission therefore has both fundamental and practical value. In this study, we approached this goal by characterizing temporal changes in transcriptional profiles associated with abscission of the fruit pedicel following fruit removal.

Material and methods

Plant materials and treatments

Experiments were carried out on 15-year-old apple (*Malus domestica* L. Borkh) trees, cv. 'Golden Delicious' and 'Spur McIntosh', grown under natural environmental conditions at the Horticultural Teaching Research Center in Michigan State University (Holt, Michigan). All samples were immediately frozen in liquid nitrogen and stored at -80°C. Two biological replicates were offset by two days for the following three fruit abscission induction experiments.

Analysis of transcriptional responses accompanying fruit removal utilized 'Golden Delicious'. Immature fruit 60 days after full bloom were removed by serving the pedicel with scissors. Abscission of pedicel stubs was monitored daily for six days following fruit removal. For analysis of gene expression, the AZ samples were collected immediately before fruit removal, and 1, 2, 3, and 4 days after fruit removal. Ten trees were used for each replicate in a completely randomized design among the treated and control experiments.

Analysis of transcriptional responses to the application of 6-Benzyl Aminopurine (6-BA) utilized 'Spur McIntosh'. When the average fruit size was 10-12 mm, a commercial 6-BA formulation at 200 ppm [Maxcel, active ingredient 1.9% BA, Valent Biosciences, Libertyville, IL] was applied with 0.1% Silwet L-77 (Helena Chemical, Collierville, TN) as a foliar spray until saturation. Controls received 0.1% Silwet L-77 in water. 50 fruit clusters were labeled on each branch, and four branches were used per replicate. For each fruit cluster, abscission of terminal and lateral fruit was recorded 6, 12, 18, and 24 days after application. For analysis of gene expression, the AZ samples were collected immediately before application, and 2, 4, and 6 days after application.

Pre-harvest fruit drop experiments utilized 'Spur McIntosh'. For the treatment, ethephon [(2-chloroethyl phosphonic acid), active ingredient 21.7%, Micro Flo, Memphis, TN] at 300ppm was applied to the whole apple canopy with 0.1% Silwet L-77 two weeks in advance of the anticipated harvest date. Controls received 0.1% Silwet L-77 in water. Three trees were used per replicate. Fruit abscission was recorded 2, 4, 5, 6, and 8 days after application. Fruit samples used for ethylene measurement were collected immediately before application, and 2, 4, and 5 days after application. For internal ethylene concentration (IEC) measurement, 1 mL of internal gas was withdrawn from fruit through the calyx and analyzed by gas chromatography using a Carle Series 400 AGC (Hach Co., Loveland, CO) and certified ethylene standard (Matheson Gas Products, Chicago, IL). For analysis of gene expression, the AZ samples were collected immediately before application, and 2, 4, and 5 days after application.

Preparation of cDNA arrays, probe preparation, and hybridization

Total RNA was isolated independently from AZ segments collected from 'Golden Delicious' before fruit removal, and 1, 2, 3, and 4 days following fruit removal, following the protocol described by Hu et al (2002). Equivalent amounts of RNA from each sample were pooled, and mRNA was purified using the Clontech mRNA Separator Kit (Clontech, Mountainview, CA). cDNAs were synthesized using the ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA). cDNAs were size-fractionated by gel filtration using Sephadex G-25 medium, cloned into the Uni-ZAP XR vector, and packaged into

ZAP-cDNA GigPackIII Gold cloning kit as specified by the manufacturer (Stratagene, La Jolla, CA). Five thousand cDNA clones were selected from this library, amplified by PCR using T3 and T7 oligonucleotide primers, and applied, in a 32-block format, to UltraGAPS™ coated slides (Corning, Big Flats, NY) using an OminGrid robot (Genemachines, San Carlos, CA) with Chipmaker pins (TeleChem, Sunnydale, CA). After application, cDNAs were crosslinked to slides by exposure to UV radiation as specified by the manufacturer.

For probe preparation, mRNA was purified from total RNA using the PolyATract® mRNA Isolation System (Promega, Madsion, WI). Labeling and hybridization were carried out according to Hedge et al (2002). Briefly, 1 µg of purified mRNA was labeled with aminoallyl-dUTP (Sigma, St. Louis, MO) during reverse transcription with oligo(dT) priming and Superscript II (Invitrogen, Carlsbad, CA). Single-stranded cDNA was labeled with either Cy3- or Cy5-monoreactive dye (Amersham, Piscataway, NJ) and hybridized to cDNA arrays at 42°C overnight in 30µl hybridization buffer [50% formamide, 5x SSC (sodium chloride/sodium citrate buffer), 0.1% SDS (sodium dodecyl sulfate), containing 2 µg yeast tRNA]. Hybridized array slides were scanned using an Affymetrix 428 array scanner (Affymetrix, Santa Clara, CA) and analyzed with GenePix Pro 4.0 software (Axon Instruments, Union city, CA).

Experiment design and data analysis

Probes were prepared from AZ samples collected before fruit removal, day 1, 2, and mixed 3 and 4 days after fruit removal, and pedicels samples mixed at day 3 and 4 after fruit removals. RNA samples represented the pooled collections from 10 trees for each time point. An interwoven loop with side-by-side replication design was used for this experiment (Churchhill, 2002; Yang et al., 2002). Four data sets were derived for each target sample, which included two biological and two technical replications. Microarray data were analyzed using MAANOVA (http://www.jax.org/staff/churchill/labsite/software), an add-on package implemented in the statistical language R (http://www.r-project.org). Raw signal values were first averaged for duplicated spots, then normalized using the 'glowess' on a per-slide basis.

To evaluate the sources of variability using ANOVA in MAANOVA as previously described in Yang et al (2002), normalized signal values were fitted to a mixed model-treating array with biological effects as random factors. For statistical inference on differential expression of genes among treatments, the treatment variances identified from the ANOVA test were further used for the pairwise comparisons between the treatments using Student's t-test with P-value and fold change cutoff at <0.05 and >2.5-fold respectively. K-means clustering was performed on the normalized data using CLUSTER (Eisen et al., 1998). Functional categorization of differentially-expressed genes with E-value less than 1.0E-5 was assigned to the corresponding Arabidopsis annotation in the Gene Ontology (GO) database in The Arabidopsis Information Resources (TAIR) version 7 (www.arabidopsis.org). Otherwise, categories were assigned based on annotation of BLASTx against non-redundant (nr) protein database in NCBI (http://www.ncbi.nlm.nih.gov/). EST assembly was done through EST clustering program developed from Research Technology Support Facility (RTSF) at MSU (http://www.genomics.msu.edu).

Reverse Transcription (RT) - PCR analysis

For semi-quantitative RT-PCR analyses, single-stranded cDNAs were prepared by reverse transcription with oligo(dT) priming and Superscript II. Gene-specific primers were designed using Primer3 (http://primer3.sourceforge.net/webif.php), and cDNA fragments were amplified by PCR using GoTaq DNA polymerase (Promega, Madsion, WI). An apple EST corresponding to ELONGATION FACTOR 1α (Md_EF-1α) was used as an internal control. Gene-specific primers used in these experiments are listed in Table 3.2.

Results and discussion

Abscission kinetics of apple fruit pedicels

In *Phaseolus vulgaris* L. (dry bean), removal of the leaf blade induces formation of the abscission zone in the lower pulvinus followed by abscission of the remaining petiole (Rasmussen and Bukovac, 1969). This method has been widely used to study the kinetics of leaf abscission in many plant species (Meir et al., 2006). In apple, our preliminary experiments showed that independent of fruit development, fruit removal through cutting in the middle of pedicel consistently induced abscission of remaining pedicel stubs within a short period. We utilized this reproducible system of abscission induction to study gene expression changes during pedicel abscission. To avoid 'June drop', a period of natural fruit abscission occurring in June, we induced pedicel abscission 60 days after full bloom (July) in 'Golden Delicious'. Total accumulated pedicel abscission reached 100% within six-days, the experimental period in the treatment, while no fruit abscission was observed in the control experiment (Figure 3.1). This experiment indicated that fruit removal led to activation and subsequent cell separation within pedicel abscission zones.

Analysis of gene expression changes in abscission of fruit pedicels

To document gene expression changes in abscission of fruit pedicels, we performed transcriptional profiling using a cDNA-based microarray representing genes expressed both in the quiescent and activated AZs. A total of 146 ESTs representing 118 genes were found to be either up- or down- regulated during abscission of fruit pedicels (Table 3.1). Among these 146 ESTs, the highest transcript frequency was seen in 14 ESTs, and expression patterns of these 14 ESTs during fruit pedicel abscission were highly consistent (Figure 3.2). In addition, expression patterns of all other redundant ESTs encoding the same gene were also consistent (data not shown). These results support the reliability of this microarray experiment.

Functional classification of these 118 genes using GO Consortium biological process revealed that 28% of total genes were classified as 'unknown biological process' and 21% of total genes had potential roles in response to stress (Figure 3.3). It is not surprising, since the treatment resulted in wounding, that a high percent of differentially expressed genes are grouped as stress response genes. Additionally, it is possible that abscission process itself induces many stress-related genes. Support for the latter is that percentage of genes in response to stress within differentially expressed genes exceeds average of whole genome transcripts by two-fold in stamen AZ transcriptional profiling experiment in Arabidopsis (Cai and Lashbrook, 2008). These 118 differentially expressed ESTs were clustered into five groups based on the similarity of their temporal responses during abscission of fruit pedicels (Figure 3.4). Up-regulated clusters G1, G2, and G3 contain 22% (26 genes), 16% (19 genes), and 9% (11 genes) of total respectively, and down-regulated G4 and G5 contain 29% (34 genes) and 23 % (28 genes) respectively (Figure 3.4). Gene identities are annotated in Table 3.1.

To examine the reliability of temporal responses of these differentially expressed genes identified in this microarray experiment, we chose six genes from four clusters for semi-quantitative RT-PCR analysis. Orthologous of these genes from other species have been implicated in organ abscission (Cai and Lashbrook, 2008). Genes encoding purple acid phosphatase27 (PAP27) and proton-dependent oligopeptide transport (POT) from cluster G1 revealed elevated expression in the pedicel AZs at day 1 after fruit removal, and genes sharing homology with peroxidase and transcriptional factor NAC21, from cluster G2, showed increased expression at day 1, 2 and 3/4 (mixed AZ sample of day 3 and day 4 after treatment) after fruit removal (Figure 3.5). Also, pectate lyase-like from cluster G3 is up-regulated at day 2 and 3/4, and Aux/IAA from cluster G5 is down-regulated during fruit pedicel abscission (Figure 3.5). Expression patterns of these six genes in the pedicel AZs through semi-quantitative RT-PCR analysis were largely consistent with those observed in the microarray experiment (Figure 3.5). In addition, we performed RT-PCR analysis on the adjacent pedicel samples harvested at the same time as those in the AZs for these six genes. Surprisingly, these genes showed transcriptional responses in the adjacent pedicel tissues similar to those in the AZs (Figure 3.5). This implies that these genes are not specifically associated with activation and separation of the fruit pedicel abscission zone.

Chemical fruit thinning

In domesticated apple cultivars, each flower cluster contains one king fruit and 3-5 lateral fruit. Preliminary experiments showed that 6-BA promotes fruit thinning more

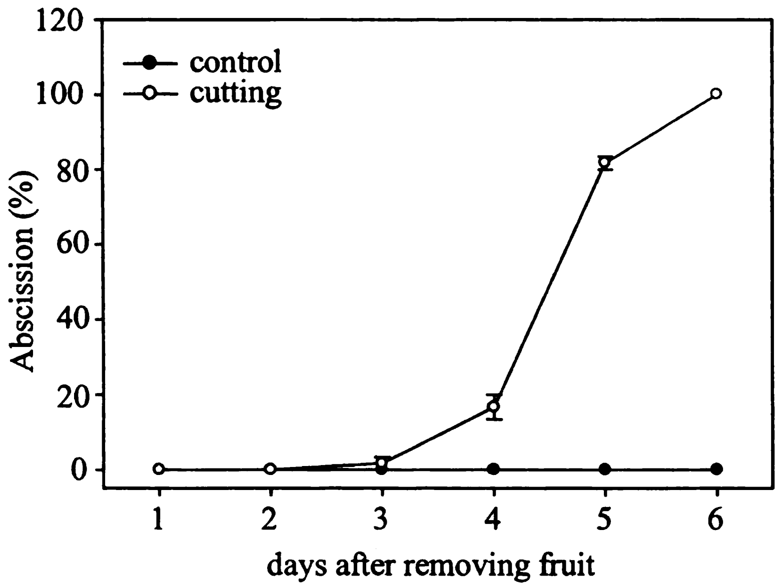


Figure 3.1 Effect of fruit removal on fruit pedicel abscission in apple. Fruit was labeled individually at the midway of the pedicel in the control and cutting treatments. Abscission of remaining pedicel or fruit was recorded at daily intervals and expressed as a percentage of abscission. Values represent means from two replicates with 30 fruit per treatment. Error bars indicate the standard error. The open circles represent the induced pedicel abscission, and circles filled black indicate fruit abscission in control.

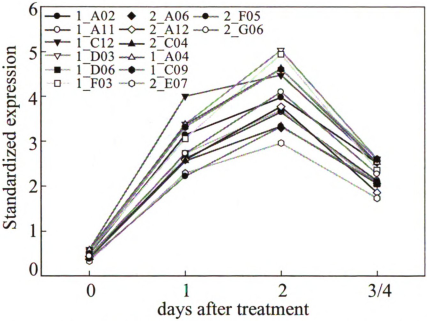


Figure 3.2 Expression profiles of 14 redundant ESTs assembled in the same contig. Standardized expression values are means of normalized absolute values divided by 10000. X-axis represents time courses after fruit removal. Annotation of represented EST (Md1_A04) was shown in Table 3.1

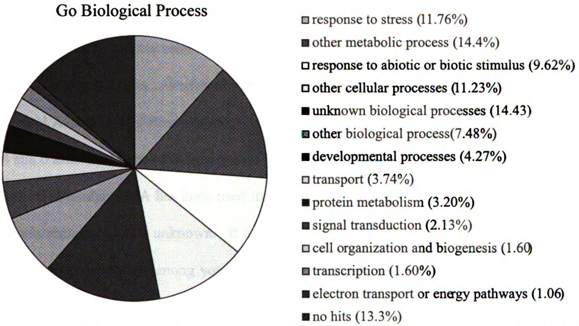


Figure 3.3 Functional categorization of 118 differentially expressed genes based on GO Biological Process. Functional categorization for genes sharing close homology with proteins in Arabidopsis was performed using AGI (Arabidopsis Genome Initiative) IDs in the TAIR website. Functional categorization of genes sharing close homology with non-Arabidopsis proteins was manually classified.

efficiently in lateral fruit than in king fruit. In this experiment, total abscission of lateral fruit induced by 6-BA reached approximately 95% 24 days after treatment, and that of king fruit is 40% (Figure 3.6A). Total accumulated abscission of lateral fruit in control and that of king fruit was only 8% (Figure 3.6A). The reason for the high percentage of abscission in lateral fruit compared to the control is that the fruit thinning experiment extended from late May through 'June drop'. In order to know the temporal responses of those differentially expressed genes during the fruit thinning process, we performed RT-PCR analyses for some selected genes using the AZ samples collected from the lateral fruit treated with 6-BA. We found that expression of *Aux/IAA* gradually decreased in the pedicel AZs two days after treatment, whereas *NAC* showed significant expression at day 4 and 6 after treatment within the pedicel AZs (Figure 3.6B).

Although 6-BA has been used in apple fruit thinning for many years, its mode of action remains largely unknown. It has been hypothesized that a correlative abscission relationship is present among young fruitlets growing within the same cluster, in which the dominant king fruit inhibits auxin export of dominated lateral fruit (Bangerth et al., 2000). This hypothesis, however, remains untested. The similar temporal responses of these selected genes during abscission induced by cutting and chemical the dominant king fruit inhibits auxin export to dominated lateral fruit (Bangerth et al., 2000). This hypothesis, however, remains untested. The similar temporal responses of these selected genes during abscission induced by cutting and chemical thinners suggest a common molecular pathway for fruit abscission induced by these two conditions.

Preharvest fruit drop

It has been shown that preharvest fruit drop is associated with ethylene production

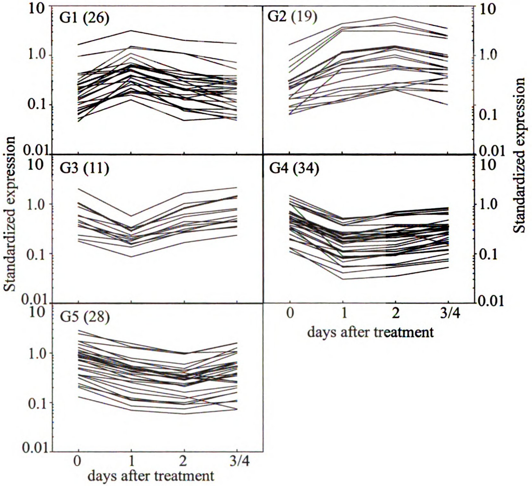


Figure 3.4 Classification of 118 AZ differentially expressed transcripts into five clusters with similar temporal responses in pedicel abscission. Standardized expression values are means of normalized absolute expression values divided by 10000. X-axis represents time courses after treatment. Number of genes in each cluster is shown in the upper left corner of plots. Red bold lines represent average value of genes within each cluster.

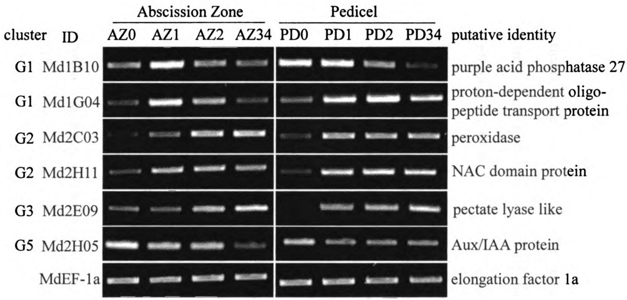


Figure 3.5 Expression profiles of selected differentially expressed genes within the AZs and pedicles in pedicel abscission induced by fruit removal. Expression analysis of these genes was performed through RT-PCR using two biological replicates. AZ0, AZ1, AZ2, and AZ34 represent the AZ samples collected at day 0, 1, 2, 3 and 4 after treatment. AZ34 represents the mixed AZ samples from day 3 and 4. PD0, PD1, PD2, and PD34 are pedicel samples collected at the same time points with those AZs. Detailed annotation for each gene can be found in Table 3.1. MdEF-1α was used an internal control for the RT-PCR analysis.

in fruit (Sato et al., 2004). We induced premature fruit abscission in preharvest season through application of the ethylene releasing compound, ethephon. Total accumulated fruit abscission in ethephon treatment reached 100% with a peak at day 6 after application, while fruit abscission in control experiment was only 5% (Figure 3.7A). Internal ethylene concentration (IEC) in fruit harvested from the ethephon treatment dramatically increased two days after application, while that in fruit from the control treatment remained in the basal level for the duration of the experiment (Figure 3.7B). We performed RT-PCR analysis for selected genes using RNAs from the fruit pedicel AZs collected from ethephon treatment. Genes sharing homology with *pectate lyase-like* (*PLL*) in Arabidopsis showed increased expression at day 2, 4, and 5 after ethephon treatment, and expression of *Aux/IAA* decreased dramatically during fruit abscission induced by ethephon (Figure 3.7C). The parallel expression patterns of these two genes observed in pedicel abscission induced by cutting and ethephon treatments implicate at least partially shared molecular mechanism in fruit abscission under these conditions.

Increased expression of *PLL* and decreased expression of auxin response factor (*ARF*) in citrus was observed during the abscission of leaf explants induced by the application of ethylene (Agusti et al., 2008). These results suggest that genes involved either in fruit or leaf abscission and are induced by exogenous ethylene might be conserved across organs and species. It has been assumed that preharvest fruit abscission resulted from the increased production of ethylene in the fruit (Sato et al., 2004). However, there are still no experiments to show if ethylene level within the pedicels AZs will increase upon application of exogenous ethylene, or if this increase is from diffusion from fruit or other organs. Application of a high concentration of ethephon did not lead

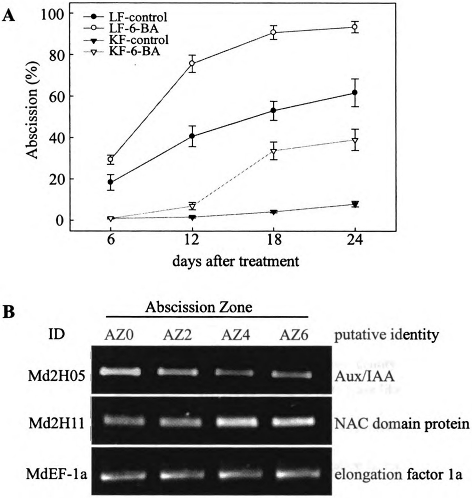


Figure 3.6 Effects of 6-BA application on fruit thinning (A) and expression profiles of selected differentially expressed genes within the AZs in fruit abscission induced by 6-BA (B). **(A)** Percentage of fruit abscission was plotted against time after treatment. Lateral fruit and king fruit were labeled on each cluster separately, and fruit abscission was recorded at day 6, 12, 18, and 24 after application in the both control and treatment. Fruit abscission is presented as percentage of total abscission. LF (lateral fruit), KF (king fruit) **(B)** Expression profiles of selected genes were performed through RT-PCR using two biological replicates. AZ0, AZ2, AZ4, and AZ6 represent AZ samples collected at day 0, 2, 4, 6 from lateral fruit after 6-BA treatment. Detailed annotation for each gene can be found in Table 3.1. MdEF-1α was used as internal control for the RT-PCR analysis.

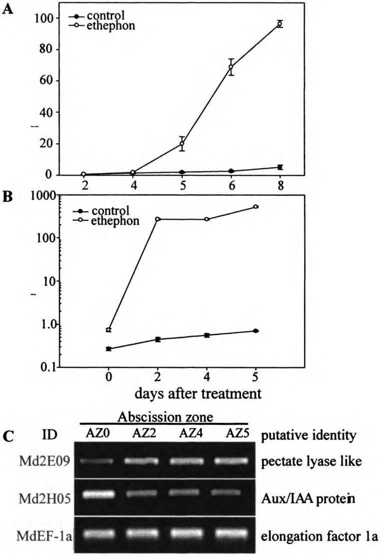


Figure 3.7 Effects of ethephon application on fruit abscission prior to fruit maturation(A), ethylene production in fruit (B), and expression profiles of selected differential expressed genes within the AZs (C). (**A**) Percentage of fruit abscission was plotted against time after ethephon treatment. Fruit abscission was recorded at day 2, 4, 5, 6, and 8 after application in the both control and treatment. Fruit abscission was presented as the percentage of total abscission. Black circles represent the control and the open circle represent the treatment. Error bars represent the standard error. (**B**) Ethylene production in fruit was plotted against time after application. Internal ethylene concentration (IEC) was measured on 10 fruit samples per replicate collected from the control and treatments at day 0, 2, 4, and 5 after application. Error bars represent standard error. (**C**) Expression profiles of these selected genes were analyzed through RT-PCR using two biological replicates. AZ0, AZ2, AZ4, and AZ5 represent AZ samples collected at day 0, 2, 4, and 5 after ethephon treatment. Detailed annotation for each clone can be seen in Table 3.1. MdEF-1α was used as internal control for the RT-PCR analysis.

to abscission of leaves, but leaves are the main organs to absorb the compound and release ethylene. Our previous study on natural variation in fruit abscission in *Malus* species showed there is non linear relationship between ethylene production in fruit and fruit abscission (Sun et al., 2009). Taken together, there are presumably other factors that regulate preharvest fruit drop.

Implications of differentially expressed genes in abscission

Genetic studies in *Arabidopsis thaliana* have identified both ethylene-dependent and -independent pathways that regulate floral organ abscission (Binder and Patterson, 2009). In addition, Cai and Lashbrook (2008) used laser-capture microdisection (LCM) system to collect enriched population of AZ cells, and they revealed potential new abscission regulators involved in the natural abscission of stamens. In this study, we identified a small subset of genes differentially expressed in the abscission of fruit pedicels induced by fruit removal (Table 3.1). Some of these differentially expressed genes showed similar temporal expression patterns as those homologous counterparts in stamen AZ transcriptome profiling, suggesting a general molecular mechanism for organ abscission across species.

Several genes involved in cell wall remodeling including peroxidase, extensin precursor, and pectate lyase-like, were up-regulated during pedicel abscission induced by fruit removal (Table 3.1). The repeating unit, Ser-Pro_4 in extensin, a cell wall hydroxyproline-rich glycoprotein, is highly conserved across dicots (Merkouropoulous and Shirsat, 2003). For those 14 highly expressed ESTs shown in Figure 3.2, the contig assembled from these 14 ESTs shared close homology with one of the extensin precursors in *Prunus Dulcis* (Table 3.1; Garcia-Mas et al., 1992). The $\underline{\text{SPPPP}}X_{5\text{-}15}\underline{\text{YK}}$

repeat in the extensin identified in this study is conserved when compared with that of AtEXT4 in Arabidopsis (Table 3.3). GUS expression driven by the promoter of *AtEXT4* was observed in the AZs of sepals, petals, and stamens (Merkouropoulos et al., 2003). In addition, *AtEXT4* showed increased expression during stamen abscission in Arabidopsis (Cai and Lashbrook, 2008). Furthermore, this gene was also found to be induced by various abiotic and biotic stresses in Arabidopsis ((Merkouropoulos et al., 1999). These results suggest that extensin is not only associated with organ abscission but also stress responses.

Many early studies showed that peroxidase activity is increased during abscission of bean leaf and cherry fruit (Hall and Sexton, 1974; Poovaiah et al., 1973, 1974). Peroxidases are encoded by a large gene family in Arabidopsis and involved in multiple plant developmental and defensive events (Passardi et al., 2005; Cosio and Dunand, 2009). Stamen AZ transcriptome profiling showed that 14 peroxidases were up-regulated during stamen abscission in Arabidopsis (Cai and Lashbrook, 2008). Leaf AZ transcriptome analysis in *Capsicum* revealed that the level of hydrogen peroxide (H_2O_2) was increased in leaf abscission (Sakamoto et al., 2008). These results suggest that peroxidases presumably contribute to cell wall loosening in abscission through controlling level of H_2O_2 in the abscission zones. Pectate lyase-like (PLL) genes are also encoded by a large gene family in plants (Palusa et al., 2007). The apple gene (Md_2E09) encoding *PLL* identified in this study shares close similarity with *PLL19* in Arabidopsis, and regulatory region of this gene in Arabidopsis drove GUS expression in the AZs of floral organs in abscission (Sun PhD thesis, Chapter 4). In addition, *PLL18*, phylogenetic closely related to *PLL19*, was up- regulated during stamen abscission in

Arabidopsis (Cai and Lashbrook, 2008). Our study also showed that *PLL* has elevated expression in the pedicels after fruit removal. These results indicate that this *PLL* in apple is not specifically committed to fruit abscission.

Among up-regulated genes, some share close similarity with plant specific transcriptional factors, NAC superfamily proteins (Table 3.1), with a consensus sequence known as the NAC domain (petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2) (Aida et al., 1997). Members of this gene family are expressed in various tissues and organs along plant development, and some of them have functions on organ fusion and 2005). Stamen AZ transcriptome profiling also revealed that *ANAC021* is up-regulated in natural abscission of stamens (Cai and Lashbrook, 2008). Our study showed that increased expression of an apple *NAC* gene (*Md_2H11*) was also observed in the pedicels during pedicel abscission induced by fruit removal (Figure 3.5). These findings suggest the potential involvement of apple *NAC* gene in organ abscission, but not specially committed to activation of abscission zone separation (Ooka et al., 2003). One differential expressed ESTs (*Md_2H11*) shares close similarity with ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 21 (Table 3.1). *ANAC021* (originally named as *NAC1*) is induced by auxin and is a target of microRNA 164 during lateral root development (Xie et al., 2000, Guo et al., 2005). Stamen AZ transcriptome profiling also revealed that *ANAC021* is up-regulated in natural abscission of stamens (Cai and Lashbrook, 2008). Our study showed that increased expression of an apple *NAC* gene (*Md_2H11*) was also observed in the pedicels during pedicel abscission induced by fruit removal (Figure 3.5). These findings suggest the potential involvement of apple *NAC* gene in organ abscission, but not specially committed to activation of abscission zone.

We observed that two *Aux/IAA* members showed reduced transcription in fruit pedicel abscission (Table 3.1). The one, *Md_2H05,* homologous to *IAA4* in Arabidopsis, also decreased its expression during fruit abscission induced by 6-BA (fruit thinning) and ethephon (preharvest drop) (Figure 3.6B and 3.7C). Aux/IAAs are encoded by a large gene family in plants and are early auxin-responsive genes (Liscum and Reed, 2002). Meir and co-authors showed that two *Mj-Aux/IAA* gene family members in *Mirabilis jalapa* were down-regulated in the AZs during the abscission of petioles induced by auxin depletion through deblading (Meir et al., 2006). A recent transcriptome profiling analysis in citrus revealed that several auxin- response factors (ARF) were down-regulated in abscission of citrus leaves 24 hours after ethylene application (Agusti et al., 2008). Consistent with earlier mentioned leaf explant model, down-regulation of auxin signaling may promote organ abscission (Addicott, 1982).

Conclusion

In this study, we presented a transcriptional profile analysis of pedicel AZ and identified a small population of differentially expressed genes within the pedicel AZs during fruit abscission induced by fruit removal in 'Golden Delicious'. The differentially expressed genes identified in this work provide a resource for further functional characterization of genes associated with fruit abscission. This work also suggests a potential common molecular mechanism for pedicel abscission induced by fruit removal, chemical thinners in early fruit developmental stages, and ethephon in preharvest season.

Table 3.1. Gene transcripts differentially up- or down-regulated in the fruit pedicel AZ in fruit absicssion
Expression pattern of different groups was shown in Figure 3.
The clone IDs highlight in bold were the genes used in RT-PCR analysis

Clone ID	Homology	Score	E-value	Identity	Cluster
Md2_C11	XP_002331862.1| predicted protein [Populus trichocarpa]	131	5.00E-06	39%	G1
Md1_H07	XP_002320378.1| predicted protein [Populus trichocarpa]	133	2.00E-06	35%	G1
Md2_H01	XP_002304653.1 predicted protein [Populus trichocarpa] 50 4e-06	118	1.00E-04	64%	G1
Md2_H03	NP_568287.1 JAS1/JAZ10/TIFY9 (Jasmonate-zim-domain protein 10) [At]	104	3.00E-05	81%	G1
Md2_H07	NP_565103.1 unknown protein [At]	363	1.10E-31	53%	G1
Md1_B10	NP_199851.2 ATPAP27/PAP27 (purple acid phosphatase 27) [At]	349	1.40E-29	65%	G1
Md1_G05	NP_198460.1 CYP716A1 (cytochrome P450, family 716, subfamily A) [At]	477	9.40E-44	58%	G1
Md1_D08	NP_197797.1 acidic endochitinase (CHIB1) [At]	207	3.80E-15	38%	G1
Md1_A01	NP_197784.2 terpene synthase/cyclase family protein [At]	196	4.70E-13	33%	G1
Md1_E03	NP_197784.2 terpene synthase/cyclase family protein [At]	187	4.40E-12	38%	G1
Md1_H06	NP_196290.1 peroxidase [At]	233	6.70E-18	67%	G1
Md1_H05	NP_196179.1 oxidoreductase, 2OG-Fe(II) oxygenase family protein [At]	648	7.10E-62	42%	G1
Md2_C01	NP_196177.1 FAD8 (fatty acid desaturase 8)	238	6.70E-18	76%	G1
Md2_E08	NP_195780.1 ATFER1 (FERRETIN 1); ferric iron binding [At]	649	5.60E-62	78%	G1
Md1_D04	NP_193037.1 oxidoreductase, zinc-binding dehydrogenase family protein [At]	613	3.60E-58	71%	G1
Md1_H03	NP_193037.1 oxidoreductase, zinc-binding dehydrogenase family protein [At]	276	1.90E-22	62%	G1
Md2_H04	NP_193037.1 oxidoreductase, zinc-binding dehydrogenase family protein [At]	584	4.30E-55	68%	G1
Md2_A04	NP_188851.2 CWLP (cell wall-plasma membrane linker protein) [At]	440	7.80E-40	58%	G1
Md1_E05	NP_186799.2 CA1 (carbonic anhydrase 1) [At]	325	1.20E-27	46%	G1
Md1_G04	NP_177024.1 proton-dependent oligopeptide transport (POT) protein [At]	343	5.80E-29	53%	G1
Md2_D04	NP_173813.1 MLP423 (mlp-like protein 423) [At]	141	6.2E-08	33%	G1
Md2_C12	NP_001030865.1 unknown protein [At]	252	6.50E-20	47%	G1
Md2_B11	EEF29405.1conserved hypothetical protein [Ricinus communis]	175	2.00E-42	56%	G1
Md1_B11	CAN82925.1hypothetical protein [Vitis vinifera]	143	2.00E-07	38%	G1
Md1_B07	AAR99061.1 (-)-germacrene D synthase [Populus trichocarpa]	210	3.00E-15	37%	G1
Md1_G07	No hits found				G1
Md1_B08	AAD38144.1 homeobox leucine zipper protein [Prunus armeniaca]	240	6.00E-19	50%	G2
Md1_E12	ACI15346.1 NAC domain protein NAC5 [Gossypium hirsutum]	112	1.00E-03	40%	G2
Md1_F10	EEF39717.1conserved hypothetical protein [Ricinus communis]	106	5.00E-03	65%	G2
Md2_B05	XP_002325400.1| NAC domain protein [Populus trichocarpa]	189	7.00E-13	44%	G2
Md2_D09	XP_002320889.1 predicted protein [Populus trichocarpa]	128	9.00E-06	37%	G2
Md2_B01	NP_567626.1 ATM4/ATMYB102 (arabidopsis MYB-like 102) [At]	140	5.80E-06	33%	G2
Md1_A12	NP_565498.1 translocon-associated protein alpha [At]	443	3.80E-40	60%	G2

Table 3.1. Continued

Md2_E06	NP_200648.1 peroxidase [At]	156	3.00E-09	39%	G2
Md1_D01	NP_200208.1 aminotransferase [At]	467	1.10E-42	57%	G2
Md2_C03	NP_196153.1 peroxidase [At]	656	1.00E-62	67%	G2
Md2_F11	NP_189264.3 CYP71B37 (cytochrome P450, family 71) [At]	335	1.40E-28	48%	G2
Md2_H11	NP_175997.1 NAC1 (Arabidopsis NAC domain containing protein [At]	353	1.30E-30	43%	G2
Md2_F08	NP_174223.1 unknown protein [At]	139	6.20E-08	38%	G2
Md1_A03	No hits found				G2
Md1_A04	emb_X65718.1 extensin [Prunus dulcis]	363	1.00E-96	78%	G2
Md1_D10	No hits found				G2
Md2_A02	No hits found				G2
Md2_B10	No hits found				G2
Md2_D01	No hits found				
Md1_F06	NP_850563.1 universal stress protein (USP) family protein [At]	112	1.60E-10	43%	G3
Md2_E09	NP_567707.1 pectate lyase family protein [At]	680	2.90E-65	86%	G3
Md1_G11	[illegible]_567673.1 Atnye1/nye1 (non-yellowing 1) [At]	535	1.70E-56	75%	G3
Md1_B05	NP_566623.1 oxidoreductase, 2OG-Fe(II) oxygenase family protein [At]	464	2.20E-42	56%	G3
Md1_F07	NP_566585.1 CNBT1 (cyclic nucelotide-binding transporter) [At]	362	1.20E-30	53%	G3
Md1_F09	NP_177601.1 pentatricopeptide (PPR) repeat-containing protein [At]	160	5.80E-17	46%	G3
Md1_E07	No hits found				G3
Md1_E08	No hits found				G3
Md1_E09	No hits found				G3
Md1_F05	No hits found				G3
Md1_H01	No hits found				G3
Md1_G06	XP_002336373.1 predicted protein [Populus trichocarpa]	156	7.00E-09	36%	G4
Md2_C08	NP_973646.1 heat shock protein-related [At]	239	2.70E-17	34%	G4
Md1_C03	NP_850562.1 universal stress protein (USP) family protein [At]	174	1.20E-11	63%	G4
Md1_C07	NP_566533.1 unknown protein [At]	140	8.90E-08	78%	G4
Md2_A10	NP_565351.1 CRCK3 (calmodulin-binding receptor-like cytoplasmic kinase 3) [A	339	5.90E-29	40%	G4
Md1_H04	NP_565203.1 ST1 (mercaptoppyruvate sulfurtransferase1) [At]	447	1.40E-40	65%	G4
Md1_H08	NP_565116.1 GASA1 (gast1 protein-homolog 1) [At]	346	7.20E-30	51%	G4
Md2_D02	NP_564276.1 AHK3 (Arabidopsis histidine kinase 3) [At]	231	2.40E-16	51%	G4
Md2_F01	NP_563922.1 zinc finger (C3HC4-type RING finger) family protein [At]	160	4.70E-10	45%	G4
Md1_B06	NP_200125.1 CNGC1 (cyclic nucleotide gated channel 1) [At]	245	4.20E-18	42%	G4
Md1_D05	NP_199251.1 FAD-binding domain-containing protein [At]	255	1.80E-19	73%	G4
Md1_F02	NP_198644.1 PR5K (PR5-like receptor kinase) [At]	391	3.70E-39	47%	G4
Md1_B03	NP_193719.1 ATPP2-A1 (Arabidopsis thaliana phloem protein [At]	530	2.30E-49	48%	G4

Table 3.1. Continued

Md2_C06	NP_190756.1 HMGB1 (high mobility gropu B1 [At]	376	4.00E-33	55%	G4
Md2_D08	NP_190505.1 glucosamine/galactosamine-6-phosphate isomerase [At]	495	1.20E-45	62%	G4
Md2_C09	NP_190446.1 ATP binding / DNA binding [At]	180	1.40E-10	32%	G4
Md1_G10	NP_190150.1 ATMPK3 (mitogen-activated protein kinase 3 [At]	400	4.00E-35	72%	G4
Md1_A06	NP_189304.2 protein kinase family protein [At]	376	4.70E-33	45%	G4
Md1_E02	NP_187716.1 ATFER2 (FERRITIN 2); ferric iron binding [At]	462	3.60E-42	82%	G4
Md2_G11	NP_187041.1 glutamate dehydrogenase [At]	573	6.30E-54	63%	G4
Md2_A03	NP_176958.1 unknown protein [At]	122	5.00E-06	38%	G4
Md1_C02	NP_173786.1 oxidoreductase, zinc-binding dehydrogenase family protein [At]	550	1.70E-51	68%	G4
Md1_B09	NP_173259.1 calcium-binding protein [At]	198	3.50E-14	44%	G4
Md1_D09	NP_030235.1 ACT domain-containing protein [At]	464	2.20E-42	69%	G4
Md1_C01	hits found				G4
Md1_C08	No hits found				G4
Md1_F11	No hits found				G4
Md1_F12	No hits found				G4
Md2_A01	No hits found				G4
Md2_B09	No hits found				G4
Md2_C10	No hits found				G4
Md2_D11	No hits found				G4
Md2_F10	No hits found				G4
Md2_H08	No hits found				G4
Md1_D12	NP_849820.1 dormancy/auxin associated family protein [At]	251	4.10E-23	50%	G5
Md1_B01	NP_683303.1 DVL4/RTFL17 (rotundifolia like 17) [At]	149	5.40E-09	73%	G5
Md2_E11	NP_567441.1 ATARD2; acireductone dioxygenase [At]	397	2.80E-35	58%	G5
Md2_E12	NP_565289.1 ATUBC2 (ubiquiting-conjugating enzyme 2) [At]	503	1.70E-46	69%	G5
Md1_A09	NP_565052.1 phosphoric monoester hydrolase [At]	558	2.50E-52	58%	G5
Md2_E01	NP_563648.1 cathepsin B-like cysteine protease [At]	411	9.30E-37	44%	G5
Md1_C10	NP_201491.1 disease resistance protein (CC-NBS-LRR class) [At]	198	5.60E-13	32%	G5
Md1_D07	NP_201491.1 disease resistance protein (CC-NBS-LRR class) [At]	505	3.80E-46	49%	G5
Md2_B03	NP_201491.1 disease resistance protein (CC-NBS-LRR class) [At]	285	2.60E-22	36%	G5
Md2_H05	NP_199183.1 IAA4 (indoleacetic acid-induced protein 4) [At]	479	5.80E-44	63%	G5
Md1_C05	NP_199147.1 malate dehydrogenase [At]	748	1.80E-72	83%	G5
Md1_F08	NP_198735.1 GLP2A (germin-like protein 2A) [At]	328	5.80E-28	45%	G5
Md2_G02	NP_197943.1 RD22 (RESPONSIVE TO DESSICATION 22) [At]	275	2.40E-22	46%	G5
Md1_A08	NP_196720.1 TINY2 (TINY2); DNA binding / transcription factor [At]	162	2.30E-10	51%	G5
Md1_G01	NP_195190.1 SQS1 (sualene synthase 1) [At]	541	6.00E-50	66%	G5

Table 3.1. Continued

Md2_F09	NP_195190.1 SQS1 (sualene synthase 1) [At]	260	1.60E-20	49%	G5
Md2_G04	NP_194094.1 BTI1 (virb2-interacting protein 1) [At]	349	3.40E-30	52%	G5
Md1_A05	NP_192853.1 TUF (vacuolar ATP synthase subunit E1) [At]	686	6.70E-66	76%	G5
Md1_C11	NP_192179.1 SULTR3 (sulfate transporter 3) [At]	267	1.40E-20	58%	G5
Md1_H09	NP_188271.1 IAA26/PAP1 (phytochome-associated protein 1) [At]	352	1.70E-30	48%	G5
Md1_H10	NP_187246.1 germin-like protein [At]	448	1.00E-40	58%	G5
Md1_G03	NP_179837.1 hydroxyproline-rich glycoprotein family protein	190	2.40E-13	37%	G5
Md1_E11	NP_179219.1 glycosyl hydrolase family 17 protein [At]	372	3.00E-32	50%	G5
Md2_B06	NP_177592.1 ATHVA22A (Arabidopsis thaliana HVA22 homologue) [At]	555	5.10E-52	65%	G5
Md2_G01	NP_176208.1 29 kDa ribonucleoprotein / RNA-binding protein cp29	576	3.00E-54	67%	G5
Md1_A07	No hits found				G5
Md1_H11	No hits found				G5
Md2_D07	No hits found				G5

Table 3.2. Primers used in RT-PCR analysis

1B10-For	5' - GGTACACGAAACTCCAGC - 3'
1B10_Rev	5' - CCAGAAATCTCACTGGCTTGC - 3'
1G04_For	5'- GGTGGCTAGAAAGTTCACCGG -3'
1G04_Rev	5'- GCCAGCACTAAACTTGTGCACC -3'
2C03_For	5'- CTCAAAGGGACTTGGTAGC -3'
2C03_Rev	5'- CAAAGTCAGCATTGAAGGT -3'
2E09_For	5'- GCCATTGGTGGGAGTGCAG -3'
2E09_Rev	5'- GGCCACAAGCTCGATATGC -3'
2H05_For	5' - GATAAGCATGGATGGAGCTCC - 3'
2H05_Rev	5' - CCGAGCTGGATGTCTTCATG - 3'
2H11_For	5' - ATTGTTGGGCATACCGACATC - 3'
2H11_Rev	5'- GATAGCCGCCAAACCCAGC -3'
MdEF_For	5'- AGGACGGACAGACCCGTGAG -3'
MdEF_Rev	5'- GAGGAAGACGGAGGGGCTTG -3'

Table 3.3 Amino acid sequences of extensins in apple, almond, Arabidopsis.
Highlighted regions represent peptide repeat units

Contig2180 extensin precursor [Malus domestica]
MGKMVPSQVTSLVVTLLVAIVSLTLPSETSANYPYT
SPPPPASPPYHYK SPPPPSPSPPVHYSPPKHPYHPK SPPPPVYAPPKHPYHYK
SPPPPKKPYHYK SPPPPYPTPSKYSPPKHPYHYK SPPPPPKKPYHYK
SPPPPSPTPPKYSPPKHPYHYK SPPPPPKKPYHYK SPPPPPPPVPTPSPPKKPYHYK
SPPPPPPVYK SPPPPVYSPPPPPKKPYKPPTPPAVHPPHGYIYSSPPPPHHY

Emb|X.65718 | extensin precursor [Prunus Dulcis]
MGKMGSSSVTSLVVTLLVAIVSLSLPSETSANYPYS
SPPPPVSPPYHYK SPPPPSPTPPVHYSPPKHPYHYK SPPPPVHYSPPKHPYHYK
SPPPPVYSPPKHPYHYK SPPPPSPSPPKHPYHYK SPPPPSPSPPKHPYHYK
SPPPPSPSPPKHPYHYK SPPPPSPSPPKHPYHYK SPPPPSPPKKPYHYK
SPPPPPSPTPPVYSPPKHPYHYK SPPPPSPPKKPYHYK SPPPPTPVYKPPVY
SPPPPPKKPYKPPTPPVHTAPPHPYIYSSPPPPHHY

ref|NP_849895.1|Extensin 4 [Arabidopsis thalina]
MGAPMASFLVLAFSLAFVSQTTANYFYS
SPPPPVKHYSPPPVYK SPPPPVKHYSPPPVYK SPPPPVKHYSPPPVYK SPPPPVKYYSPPPVYK
SPPPPVYK SPPPPVKHYSPPPVYK SPPPPVKHYSPPPVYK SPPPPVKHYSPPPVYK
SPPPPVKHYSPPPSYTTLHHHRFTTHLLQSYTTLHHHRFTTHLLQLYTTPHHHPRSTTNTNLLLLRT
PLLTSTPTLPLQITTSSSLLDYLIVSNGVDDDTQENKESFGDIIKDTRSLTGELKRIH
MSKEKVSHFNVFSLFINTLL

References

Abeles FB, Rubinstein B (1964) Regulation of ethylene evolution and leaf abscission by auxin. Plant Physiol 39: 963-969

Addicott FT (1982) Abscission. University of California Press, Ltd. London.

Abeles FB (1967) Mechanism of action of abscission accelerators. Physiol Plant 20: 442-454

Agusti J, Merelo P, Cercos M, Tadeo FR, Talon M (2008) Ethylene-induced differential gene expression during abscission of citrus leaves. J Exp Bot 59: 2717-2733

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: an Analysis of the cup-shaped cotyledon mutant. Plant Cell 9: 841-857

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

Binder BM, Patterson SE (2009) Ethylene-dependent and -independent regulation of abscission. Stewart Postharvest Review 5: 1-10

Bleecker AB, Patterson SE (1997) Last exit: senescence, abscission, and meristem arrest in Arabidopsis. Plant Cell 9: 1169-1179

Bleecker AB, Estelle MA, Somerville C, Kende H (1988) Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science 241: 1086-1089

Butenko MA, Patterson SE, Grini PE, Stenvik G-E, Amundsen S, Mandal A, Aalen RB (2003) *INFLORESCENCE DEFICIENT IN ABSCISSION* controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. Plant Cell 15: 2296-2307

Byers RE (2003) Flower and fruit thinning and vegetative: fruiting balance. Apples: Botany, Production and Uses edited by D.C Ferree and I.J. Warrington pp409-430

Cai S, Lashbrook C (2008) Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: enhanced retention of floral organs in transgenic plants overexpressing Arabidopsis ZINC FINGER PROTEIN2. Plant Physiol 146: 1305-1321

Chen G, Alexander L, Grierson D (2002) Constitutive expression of EIL-like transcription factor partially restores ripening in the ethylene-insensitive Nr tomato mutant. J Exp Bot 55: 1491-1497

Cho SK, Larue CT, Chevalier D, Wang H, Jinn T-L, Zhang S, Walker JC (2008) Regulation of floral organ abscission in Arabidopsis thaliana. Proc Natl Acad Sci U S A105: 15629–15634

Churchill GA (2002) Fundamentals of experimental design for cDNA microarrays. Nature Genetics Supplement 32: 490-495

Cosio C, Dunand C (2009) Specific functions of individual class III peroxidase genes. J Exp Bot 60: 391-408

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95: 14863-14868

Ellis CM, Nagpal P, Young JC, Hagen G, Guifoyle TJ, Reed HW (2005) *AUXIN RESPONSE FACTOR1* and *AUXIN RESPONSE FACTOR2* regulate senescence and floral organ abscission in *Arabidopsis Thaliana.* Development 132: 4563-4574

Garcia-Mas J, Messeguer R, Arus P, Puigdomenech P (1992) The Extensin from *Prunus amygdalus*. Plant Physio 100: 1603-1604

Greene DW (2003) Endogenous hormones and bioregulator use on apple. Apples: Botany, Production and Uses. Edited by D.C Ferree and I.J. Warrington pp437-458. CABI publishing

Guo H-S, Xie Q, Fei J-F, Chuad N-H (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for Arabidopsis lateral root development. The Plant Cell 17: 1376-1386

Hall J, Sexton R (1974) Fine structure and cytochemistry of the abscission zone cells of Phaseolus leaves II. Localization of peroxidase and acid phosphatase in the separation zone cells. Ann Bot 38: 855-858

Hedge P, Qi R, Albernathy K, Gay C, Dharap S, Gaspard R, Earle-Hughes J, Snerud E, Lee N, Quackenbush J (2000) A concise guide to cDNA microarray analysis - II. Biotechniques 29: 548-562

Hepworth SR, Zhang Y, McKim S, Li X, Haughna GW (2005) BLADE-ON-PETIOLE–dependent signaling controls leaf and floral patterning in *Arabidopsis*. The Plant Cell 17: 1434-1448

Hu C, Honda C, Kita M, Zhang Z, Tsuda T, Moriguchi T (2002) A simple protocol for RNA isolation from fruit trees containing high levels of polysaccharides and polyphenol compounds. Plant Mol Bio Rep 20: 69a-69g

Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1994) The *never ripe* mutation blocks ethylene perception in tomato. Plant Cell 6: 521-530

Laskowski M, Biller S, Stanley K, Kajstura T, Prusty R (2006) Expression profiling of auxin-treated Arabidopsis roots: toward a molecular analysis of lateral root emergence. Plant Cell Physiol 47: 788-792

Lesile ME, Lewis MW, Liljegren SJ (2007) Organ abscission. Annual Plant Reviews V 25. Plant Cell Separation and Adhesion. Edited by Jeremy A. Roberts and Zinnia Gonzalez-Carranza: pp91-106

Liscum E, Reed JW (2002) Genetics of Aux/IAA and ARF action in plant growth and development. Plant Mol Bio 49: 387-400

McKim SM, Stenvik G-E, Butenko MA, Kristiansen W, Cho SK, Hepworth SR, Aalen RB, Haughn GW (2008) The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis. Development 135: 1537-1546

Mao L, Begum D, Goff SA, Wing RA (2000) Sequence and analysis of the tomato *JOINTLESS* locus. Plant Physiol 126: 1331-1340

Merkouropoulos G, Barnett DC, Shirsat AH (1999) The Arabidopsis extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic and salicylic acid, and codes for a protein with unusual motifs. Planta 208: 212-219

Merkouropoulos G, Shirsat AH (2003) The unusual Arabidopsis extensin gene At*Ext1* is expressed throughout plant development and is induced by a variety of biotic and abiotic stresses. Planta 217: 356-366

Meir S, Hunter DA, Chen J-C, Halaly V, Reid MS (2006) Molecular changes occurring during acquisition of abscission competence following auxin depletion in *Mirabilis jalapa*. Plant Physiol 141: 1604-1616

Norberg M, Holmlund M, Nilsson O (2005) The *BLADE ON PETIOLE* genes act redundantly to control the growth and development of lateral organs. Development 132: 2203-2213

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologisa A (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR genegamily members in *Arabidopsis thaliana*: unique and overlapping functions of ARF7 and ARF19. Plant Cell 17: 444-463

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K, Kikuchi S (2003) Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Research 10: 239-247

Osborne D (1989) Abscission. Cri Rev Plant Science 8: 103-129

Palusa SG, Golovkin M, Shin S-B, Richardson DN, Reddy ASN (2007) Organ-specific, developmental, hormonal and stress regulation of expression of putativepectate lyase genes in Arabidopsis. New Phytol. 174: 537-550

Patterson SE (2001) Cutting loose. Abscission and dehiscence in Arabidopsis. Plant Physiol 126: 494-500

Patterson SE, Bleecker AB (2004) Ethylene-dependent and independent processes associated with floral organ abscission in Arabidopsis Plant Physiol 134: 194-203

Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. Plant Cell Rep. 24: 255-265

Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85-88

Poovaiah BW, Rasmusse.Hp, Bukovac MJ (1973) Histochemical localization of enzymes in abscission zones of maturing sour and sweet cherry fruit. J Am Soc Hort Sci 98: 16-18

Poovaiah B, Rasmussen H (1974) Localization of dehydrogenase and acid phosphatase in the abscission zone of bean leaves. Amer J Bot 61: 68-73

Rasmussen HP, Bukovac MJ (1969) A histochemical study of abscission layer formation in the bean. Am J Bot 56: 69-76

Sakamoto M, Munemura I, Tomita R, Kobayashi K (2008) Involvement of hydrogen peroxide in leaf abscission signaling, revealed by analysis with an in vitro abscission system in *Capsicum* plants. Plant J 56: 13-27

Sexton R (1995) Abscission. In: Pessarakli M (ed) Handbook of Plant and Crop Physiology. New York: Dekker, pp497-525

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

Stenvik G-E, Butenko MA, Urbanowicz BR, Rose JK, Aalen RB (2006) Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* activates cell separation in vestigial abscission zone in *Arabidopsis*. Plant Cell 18: 1467-1476

Stenvik G-E, Tandstad NM, Guo Y, Shi C-L, Kristiansen W, Holmgren A, Clark SE, Aalen RB, Butenkoa MA (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION Is sufficient to induce abscission in Arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. Plant cell 20: 1805-1817

Taylor J, Whitelaw C (2001) Signals in abscission. New Phytol 151: 323-339

Tieman DM, Ciardi JA, Taylor MG, Klee HJ (2001) Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J 26: 47-58

Sun L, Bukovac MJ, Forsline PL, van Nocker S (2009) Natural variation in fruit-abscission related traits in apple (*Malus*). Euphytica 165: 55-67

Xie Q, Frugis G, Colgan D, Chua N-H (2000) *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Gene Dev 14: 3024-3036

Yang YH, Speed T (2002) Design issues for cDNA microarray experiments. Nature Rev Genet 3: 579-588

CHAPTER 4

Analysis of promoter activity of members of the *PECTATE LYASE-LIKE (PLL)* gene family in cell separation and wall loosening in Arabidopsis

Abstract

Pectate lyases depolymerize pectins by catalyzing the eliminative cleavage of (1->4)-alpha-D-galacturonan. Pectate lyase-like (*PLL*) genes exist as large and complex families in plants, but their cellular and organismal roles have not been well characterized. As a first step to understand the functional diversity of *PLL* genes in plants, we documented the spatial and temporal promoter activity of 23 of the 26 Arabidopsis *PLL* genes throughout development. Numerous gene family members showed activity in localized domains programmed for abscission, such as the abscission zones (AZs) of the sepal, petal, and stamen, and seed, as well as the fruit dehiscence zone. Several other members showed activity in cell types expected to facilitate separation, including the endosperm layers during seed germination, and root endodermal and cortical layers during lateral root emergence. Other *PLL* promoters were active in domains not obviously programmed for separation, including the apparent vestigial AZs of the branch and pedicel. These results suggest potential for unique and overlapping activity of *PLL* genes, and provide guidance for analysis of individual gene function through reverse genetics.

Key words: pectate lyase, GUS expression, cell separation, cell wall loosening, Arabidopsis

Introduction

Pectins, a class of polysaccharides containing predominately 1, 4-linked α-D-galacturonic acid (GalA) residues, are a major component of primary plant cell walls, and within the wall form a matrix in which a network of cellulose and hemicellulose is embedDED (Ridley et al., 2001; Schols and Vorgen, 2002; Willats et al., 2001). Pectins not only contribute to the mechanical strength and physical properties of primary cell walls (Jarvis, 1984; O'Neill et al., 2004; Wilson et al., 2000), but also function in intercellular adhesion (Iwai et al., 2002; Jarvis et al., 2003; Knox, 1992), and can act as signaling molecules in morphogenesis and pathogen defense (Ridley et al., 2001).

Plant growth and development is accompanied by dynamic remodeling of the cell wall, which in turn requires modifications of the various cell wall components including pectin. In accordance with the often complex structure of some pectins, an assortment of pectinase activities modify or degrade these polymers. Pectinesterases target methyl-esterified homogalacturonan (HG) yielding substrates for polygalacturonase (PG) and pectate lyase (PL), which cleave the GalA backbone (Tucker and Seymour, 2002). Rhamnogalacturonase (RGase) and rhamnogalacturonan lyase depolymerize branched regions of rhamnogalacturonan (RG), whereas β-galactosidases and α-arabinosidases can degrade the galactan/arabinan or arabinogalactan side chains (Tucker and Seymour, 2002). Pectate lyases (PLs, EC 4.2.2.2) have been most extensively studied in *Erwinia chrysanthemi*, a major causal agent of soft-rot diseases that affect a wide range of plant species (Barras et al., 1994; Collmer and Keen, 1986; Kotoujansky, 1987). Their action not only results in maceration of plant tissues but also can activate plant defense systems (De Lorenzo et al., 1991; Fagard et al., 2007; Norman-Setterblad et al., 2000). Plant

PECTATE LYASE–LIKE (*PLL*) genes encode proteins with strong amino acid sequence homology with the PelC isoform of bacterial PLs (Marín-Rodríguez, et al., 2002) and exist as large families in plants where studied. The abundance of *PLL* genes in plants, including 26 in Arabidopsis, 12 in rice, and 22 in poplar, has arisen from multiple gene duplication events (Palusa et al., 2007; Zhang, 2003), a process that may enhance plasticity in adaptation to changing environments (Lynch and Force, 2000). Theoretical population genetics models predict that gene redundancy is evolutionarily stable only when duplicated genes differ in some aspect of their function, suggesting that individual members of large families such as *PLL* may have some unique function (Force, et al., 1999).

Various analyses of *PLL* genes from plants revealed expression in a broad range of organs including root, leaf, flower, pollen, filament, style, pistil, and ripening fruit (Benítez-Burraco et al., 2003; Chourasia et al., 2006; Futamura et al., 2002; Marín-Rodríguez et al., 2002; 2003; Mita et al., 2006; Palusa et al., 2007; Pua et al., 2001). *PLL* genes in several studies also showed elevated expression in response to auxin, wounding, and/or pathogen infection (Domingo et al., 1998; Laskowski et al., 2006; Milioni et al., 2001; Palusa et al., 2007; Vogel et al., 2002). Transcriptional analysis in Arabidopsis revealed that a small subset of *PLL* genes were up-regulated during stamen abscission and in cortical cell separation during the emergence of the lateral root (Cai and Lashbrook, 2008; Laskowski et al., 2006; Swarup et al., 2008). Reduced expression of *PL1* in transgenic strawberry suggested a natural role in tissue softening during fruit ripening (Jiménez-Bermúdez et al., 2002). All of these data implicate *PLL* genes in various plant growth and development events including cell separation and wall loosening.

During plant growth and development, there are many events in which adjacent cells separate in a coordinated manner (Roberts et al., 2002). Cell separation resulting in organ abscission, or anther or fruit dehiscence, occurs in predetermined positions, called abscission zones (AZs) and dehiscence zones (DZs), respectively (Laskowski et al., 2006; Leslie et al., 2007; Ostergaard et al., 2007). Intercellular space formed in leaves and stems can result from restricted separation of cells at the tricellular regions (Jarvis, 1998). Fruit ripening also involves limited cell separation, in which only middle lamella is degraded, with tricellular junctions and plasmodesmata often remaining intact (Hallett et al., 1992; Roy et al., 1994;). Targeted cell separation is also involved in the processes of seed germination, lateral root emergence, and shedding of columella root cap cells (Kuriyama et al., 2007; Mollet et al., 2007; Wen et al., 2007). In this study, we examined spatial-temporal expression patterns of promoters of *PLL* family members in the various cell separation and wall loosening events that occur in Arabidopsis growth and development.

Material and Methods

Plant growth conditions

Wild-type *Arabidopsis thaliana* (ecotype Col-0) was used for all experiments. Standard growth conditions were 16-h-cool white fluorescent light/8-h-dark photoperiods at 22°C. For growth in sterile culture, seeds were surface-sterilized and germinated on one-half-strength Murashige and Skoog (MS) medium supplemented with 10mM MES [2-(N-morpholino) ethanesulfonic acid] (Sigma, St. Louis, MO), pH adjusted to 5.7, and solidified with 0.8% phytoagar (Invitrogen, Carlsbad, CA). For auxin treatment, 5-d-old seedlings growing vertically on the above medium were transferred to medium containing 1 μM indole-3-acetic acid (IAA) (Sigma, St. Louis, MO) for 24 h.

Phylogenetic analysis and protein domain identification

The peptide sequence of an enzymatically confirmed pectate lyase from banana, *Musa acuminata PL1* (GenBank accession AF206319; Marin-Rodriguez, et al., 2003), was utilized as a query in BLASTP (Basic Local Alignment Search Tool Protein) analysis of Arabidopsis translated open reading frames [The Arabidopsis Information Resource (TAIR) Version 7; http://www.arabidopsis.org], and closely related proteins [Expect (E) value < 8E-36] were used for phylogenetic tree construction. Predicted amino acid sequences were aligned using ClustalW2 (http://www.clustal.org) (Larkin et al 2007), and MEGA4 (Tamura et al. 2007) was used to construct the phylogenetic tree. Annotated PLL protein domains were identified in the Pfam database (http://pfam.sanger.ac.uk; Finn et al., 2008) as follows: PF03211, Pectate_lyase; PF00544, Pec_lyase_C; PF09492, Pec_lyase; PF04431, Pec_lyase_N. Amino-terminal signal peptides were defined by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) using a maximum S-score larger than 0.95 and signal peptide probability greater than 0.80 (Emanuelsson et al., 2007). The number of ESTs for each gene in the NCBI Unigene database was found at NCBI (http://www.ncbi.nlm.nih.gov/).

Transgenic plant construction

To engineer beta-glucuronidase (GUS) as a reporter for *PLL* promoter activity, approximately 2 kb of genomic DNA, upstream from the start codon, was amplified by PCR using gene-specific primers (Table 4.1) containing unique restriction sites, and

Table 4.1 Primers used for amplification of *PLL* promoters

At4g24780	5' -AACCATGGTTCTCTCTCTCTCTCACTTGG- 3' 5' -ATAAGCTTCCACCTATCATTACCAACACC- 3'
At5g63180	5' -TTCCATGGATGAGTGAAGAGAGA AAGACA-3' 5' -CGAAGCTTTAACACTGATAAATGAGTAGA- 3'
At1g67750	5' -AACCATGGTCTTGTCTCTCGAGAGGATT- 3' 5' -CGAAGCTTTATCAATGTGTATCATGAAT- 3'
At3g27400	5' -ACCATGGTCTTCTAAACATAGATTGAGA- 3' 5' -CGGAATTCGCAAATGGCACTATAAACCAC- 3'
At4g13710	5' -AACCATGGTGAAGCTTTCTTCTTCTTCT- 3' 5' -AAGAATTCTGCACAAGAGACATAAAAGT- 3'
At3g24230	5' -AACCATGGTGTGCAAACAAAGGGAAAATG- 3' 5' -ATGAATTCACGAAAAATATAGCGTGACGG- 3'
At1g04680	5' -AACCATGGTGGAGAGGCAGAAGCTGAGCC- 3' 5' -AAGGTACCCACTTCAAGTCTTCGAAAGTA- 3'
At4g13210	5' -AACCATGGTGTTGGTTGTTGTTAGAGTT- 3' 5' -TTGGTACCGCCGAAACAATAACCTCTTT- 3'
At3g24670	5' -AACCATGGTGTTGGATATATCAAAGCTCT- 3' 5' -AAGGTACCCAGGGTGCTTGTAAATTATGT- 3'
At5g48900	5' -AACCATGGTGTTCTTGCTCTGTTCTGTT- 3' 5' -AAGGTACCAAATCATGTTTTCCCGCCAA- 3'
At3g07010	5' -AACCATGGTGTGACAGCCATTGTTATGGC-3' 5' -AAGGTACCCTCGTAAGTTCCTTACCTATG3'
At3g53190	5' -AACCATGGTGCTGAAGAAACTTGTGATT- 3' 5' -GAAAGCTTAATCAGTAACTTTATTGACA- 3'
At5g09280	5' -AACCATGGTTTTCCGGCAAATCCGACTGA- 3' 5' -AAGGTACCAGTTTATTCAGGTCATGTGT- 3'
At4g22090	5'- AACCATGGTAGTAATGTTGCATTTACTT-3' 5'- AAGGTACCATGAGGCAGCTGCCACCCTT-3'
At1g30350	5' -CGCCATGGTTTCTTGAAAATGTGATGCT- 3' 5' -AAGGATCCCCAAAGCCTTTTGCTGATA- 3'
AT4g22080	5' -AACCATGGGGTTGGGTGGGTTTATGGTT- 3' 5'-ATAAGCTTTATTCAATACTCTTTTCACG- 3'
At2g02720	5' -AACCATGGTTGAATCAGCAGTGGTGAGA- 3' 5' -ATAAGCTTGATTGCGATCATCAAAAGTT- 3'
At3g54920	5' -AACCATGGCGTTAGTGGCGGATTTTGAC- 3' 5' -ACAAGCTTTCAGAAGATACCACAATCGC- 3'
At1g14420	5' -TACCATGGTTTAAATATATTGCAAATGC- 3' 5' -ATAAGCTTTTAATTACTTGTATGATAAT- 3'
At5g15110	5' -GCCCATGGCTTTCTTTTTTTGTTTTCAA- 3' 5' -CAGGATCCAAGATATATATAATAGTCTC -3'
At3g01270	5' -AACCATGGTTTATTTGATTACCCCTTTC- 3' 5' -TTGGATCCTGTTGCTTATCTGAGAAAGT- 3'
At3g55140	5' -AACCATGGCGTTCGTTGTTATGCGACGT- 3' 5' -ACAAGCTTTCTTGTACATACAGCAGAGA- 3'
At3g09540	5' -GGCCATGGATCTAATTTATATAACAAATG- 3' 5' -ATAAGCTTCCATGTTTAACATACTTCTT- 3'

confirmed as lacking mutation by sequencing. These PCR products were cloned into the pCAMBIA1305 vector (Cambia, Black Mountain, Australia) modified to contain an *Nco*I site at the start codon of the *GUS* gene and multiple cloning sites replacing the pCAMBIA Lac Z alpha and CaMV35S regions. This resulted in *PLL:GUS* fusions that preserve the authentic 5' UTR and start codon position. Plasmid DNAs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into wild-type plants using the floral dipping method (Clough and Bent, 1998). Transgenic plants were subjected to selection with the herbicide glufosinate (Basta; Beyer CropScience, Barmen, Germany).

Histochemical GUS assay

Populations derived from at least four independently transformed transgenic lines were analyzed for each *PLL:GUS* construction. Analysis utilized T2 plants from transgenic lines segregating approximately 3:1 for herbicide resistance. For histochemical GUS assays, plants or plant parts at various developmental stages were immersed in GUS staining solution [0.5 mM X-gluc, 0.5% (v/v) Triton X-100, 50 mM sodium phosphate buffer (pH adjusted to 7.2)] under vacuum infiltration for 5 min, and incubated at 37°C for various lengths of time. After staining, tissues were incubated in 70% ethanol for several hours to remove chlorophyll. Staining was visualized using a Nikon dissecting microscope equipped with a digital camera. Reproductive organs used in this analysis were at stages 11 to 20 as described by Smyth et al (1990).

Expression analysis of AtGenExpress data

Microarray data were collected from the AtGenExpress Development data set (Schmid et al., 2005; http://www.weigelworld.org/resources). Only results from wild-type plants were included in the analysis. The log_2-transformed absolute signal values were used in hierarchical clustering based on average linkage (Cluster version 3; Eisen et al., 1998), and results were visualized in TreeView (Eisen et al., 1998).

Results

Phylogenetic Analysis

Based on peptide sequence homology with a known pectate lyase from banana and annotated protein domains, the Arabidopsis genome encodes for 26 pectate-lyase-like proteins. Neighbor-joining analysis partitioned these protein sequences into five subfamilies (Figure 4.1). This genomic content and phylogenetic organization is consistent with that previously reported for *PLLs* from multiple plant species including Arabidopsis (Futamura et al., 2002; Palusa et al., 2007). All Arabidopsis PLLs exhibited a recognizable Pec_lyase_C (Pfam00544) domain (Yoder et al., 1993; Figure 4.1) and 23 of the proteins contain a probable amino-terminal signal peptide. The carboxyl-terminal glycosyl-phosphatidylinositol (GPI) anchor previously identified in PMR6 (POWDERY MILDEW RESISTANCE 6, also called PLL13) was not obviously present in other PLLs, suggesting a specialized function of PMR6 associated with disease resistance (Vogel et al, 2002) (Figure 4.1).

Estimation of *PLL* gene expression through analysis of public transcriptome data

As a first step to assess expression pattern of *PLL* genes in Arabidopsis, we analyzed publicly available transcriptome data. Expressed sequence tags (ESTs) found in public databanks corresponding to individual *PLL* gene family members were found to be sourced from various tissues and stages across Arabidopsis growth and development, as

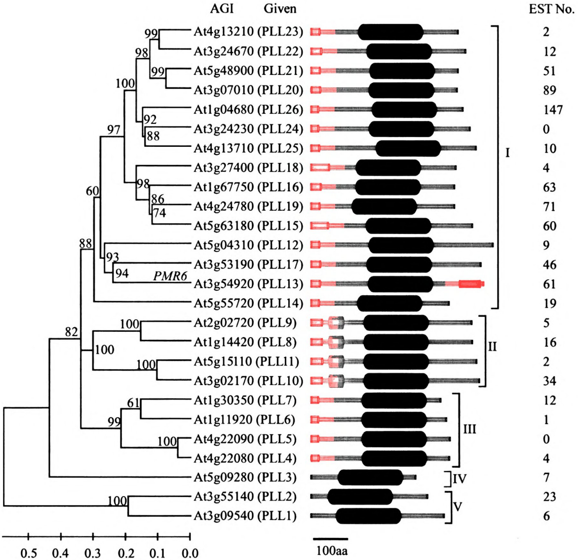


Figure 4.1. Phylogenetic tree of Arabidopsis PLL amino acid sequences. Bootstrap values are shown above nodes and indicate how consistently the data support the given taxon bipartition. EST No. gives the numbers of ESTs for that gene currently found in the NCBI database. The Pec_lyase_C domain is indicated as long green rectangles. The amino-terminal signal peptide is represented by short yellow rectangles. The Pec_lyase_N domain is indicated as blue rectangles. The GPI anchor in PMR6 is indicated by a red rectangle. The scale bar at lower left shows the relative distance between tree branches. **"Images in this thesis/dissertation are printed in color"**

well as various environmental conditions (not shown). Representational frequency of ESTs for individual *PLL* genes was highly variable both across the gene family and within subfamilies (Figure 4.1). Analysis of publicly available microarray data also suggested that there was at least some unique developmental pattern of expression for most *PLL* genes (Figure 4.2). A striking exception is the four members of subfamily II (*PLL8-PLL11*), which showed a similar expression pattern localized predominately to pollen and stamens (Figure 4.2). Several genes (*PLL3*, *4*, *6*, *7*, *12*, and *24*) were transcriptionally silenced across most sampled tissues, whereas *PLL2* was apparently expressed ubiquitously, suggesting a very general function (Figure 4.2). These data are generally consistent with the results of recent RT-PCR analysis of selected *PLL* genes (Palusa et al., 2007) and support a collectively ubiquitous function for *PLL*s in growth and development and the potential for functional specialization by many of these genes.

Analysis of *PLL* promoter activity

Approaches for analysis of gene expression can be difficult when applied to large gene families, due to sequence homology among family members and/or inability to resolve expression at the cellular level. Here, we utilized a histological reporter gene (*GUS*) driven by individual *PLL* gene promoters. We engineered the *GUS* coding sequence adjacent to ~2 kb of 5' UTR/promoter sequence, preserving the authentic start codon of the *PLL* genes, and expressed the *PLL:GUS* fusions in transgenic Arabidopsis. For each *PLL* gene, we analyzed at least four independent transgenic lines. GUS activity patterns were generally consistent between independent lines, with the exception of three genes (*PLL6*, *PLL12*, and *PLL14*) that showed weak and variable activity and were not analyzed further. No GUS activity was observed in transgenic plants transformed with a

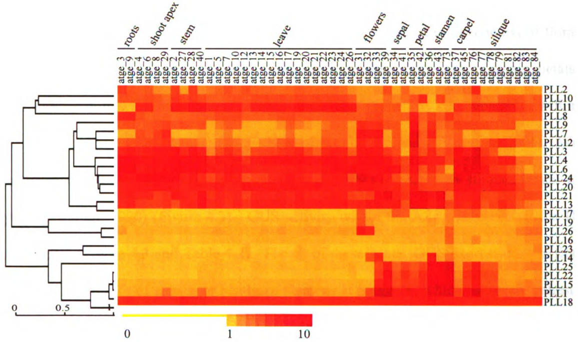


Figure 4.2. Hierarchical clustering of RNA accumulation for 25 *PLLs* along developmental stages based on public microarray data (AtGenExpress; Schmid et al., 2005). Growth stages and plant parts were labeled according to the AtGenExpress. Microarray signal value is indicated by color from yellow (lowest) to red (highest). *PLL5* was not represented on the microarray used in this analysis. **"Images in this thesis/dissertation are printed in color"**

promoterless *GUS* construction, or in non-transgenic plants. Based on the known roles for pectins in cell adhesion and cell wall architecture, we focused our analysis on developmental events associated with cell separation and cell wall remodeling.

***PLL:GUS* expression associated with cell separation**

Floral organ abscission zones.

Cell separation in Arabidopsis has been best characterized in the context of floral organ abscission (Leslie et al., 2007). Arabidopsis exhibits abscission of sepals, petals, and stamens following pollination. Abscission is conditioned by cell separation within the AZ, a tightly localized region at the base of the floral organs (Patterson, 2001). We analyzed developing flowers at various stages until Stage 18, when siliques began to yellow (Smyth, 1990) (Figure 4.3). GUS activity within the AZs was observed for 18 *PLL* genes (Figure 4.3 and Table 4.2). For all of these genes, GUS expression was first detected in the AZs of sepals, or petals, or stamens at Stage 16, which followed anthesis by about two days and was marked by the withering of sepals and petals (Smyth et al., 1990) (Figure 4.3). At Stage 17, which was marked by the abscission of sepals, petals and stamens, stronger GUS activity was detected within the AZs of all three organ types. Five *PLL* genes (*PLL4*, *5*, *7*, *20*, and *23*) showed markedly weaker GUS staining relative to the remaining 13 genes (Figure 4.3 and not shown). For all genes, *GUS* expression in the AZs was detectable but very weak at Stage 18 (not shown).

Fruit dehiscence zone and seed abscission zone

The Arabidopsis fruit consists of two valves separated by a replum. Mature fruits dehisce due to cell separation in the so-called separation layer of cells that is distributed

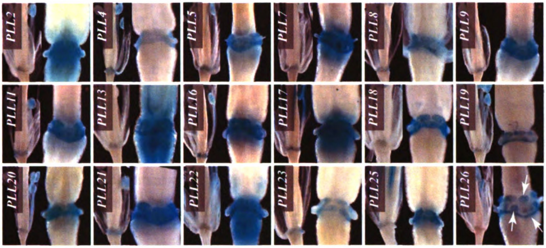


Figure 4.3. Spatial and temporal *PLL:GUS* expression in the abscission zones of sepal, petal, and stamen. Picture labels are given number of genes indicated in Figure 4.1. Each frame shows flowers at Stage 16 (left) and 17 (right). Arrowheads indicate location of abscission zone of sepals, petals, and stamens. **"Images in this thesis/dissertation are printed in color"**

along the valve margins (dehiscence zone; DZ) (Liljegren et al., 2004). Mature seeds are released from the funiculus, a stalk-like structure connecting seeds to the replum, at a site referred to as the seed AZ (Pinyopich et al., 2003). We analyzed GUS activity during development of flowers and fruit from Stage 16 through Stage 19, marked by valve separation, and Stage 20, marked by seed abscission of seeds (Smyth, 1990) (Figure 4.4). We found that 16 of the *PLL* promoters drove *GUS* expression within the apparent DZ of developing siliques at the onset of stage 18, which proceeded separation of the valves by approximately 24 h (Figure 4.4A and Table 4.2). GUS activity was first seen at the basal and apical ends of siliques, where valve separation was initiated, and then became established along the entire length of the fruit as valve separation progressed (Figure 4.4A and not shown). We also found that 16 *PLL* promoters drove GUS activity within the apparent seed abscission zone (Figure 4.4B and Table 4.2). Activity was not seen until Stage 20, concomitant with seed abscission.

<u>Cell separation events associated with radicle emergence and lateral root initiation</u>

During seed germination in Arabidopsis, the radicle penetrates a single endosperm cell layer (Liu et al., 2005). GUS activity was observed in the endosperm cell layer of 1-d-old seedlings for *PLL16:GUS* and *PLL22:GUS* transgenic plants (Figure 4.5 '*PLL22a, PLL16a*'). The stronger GUS activity was observed in the endosperm layers of *PLL22:GUS* transgenic plants (Figure 4.5).

Arabidopsis lateral roots initiate from the pericycle cell layer and need to penetrate the overlaying cortical and epidermal layers during emergence, a process that requires separation of these cells (Laskowski et al., 1995). We analyzed GUS activity for

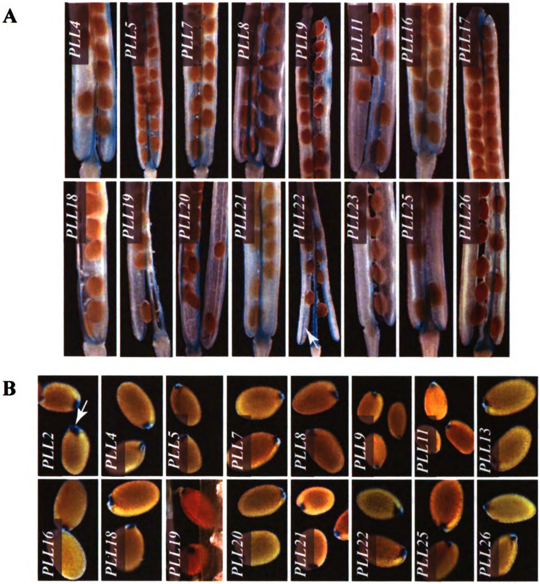


Figure 4.4. *PLLs:GUS* expression in the dehiscence zone of siliques (A) and abscission zone of mature seeds (B). Picture labels are given number of genes indicated in Figure 4.1. Arrowheads indicate location of dehiscence zone of siliques and abscission zone of seeds. **"Images in this thesis/dissertation are printed in color"**

all 23 *PLL* promoters in roots of seedlings growing on media supplemented with auxin, which promotes lateral root initiation (Casimiro et al., 2003). Under these conditions, we observed GUS activity for six *PLL* promoters in the apparent cortical and endodermal layers of primary roots (Figure 4.5, Table 4.2). Activity for *PLL16* and *PLL21* was restricted to cell layers directly overlaying new lateral root primordia, whereas activity for the remaining four, *PLL19*, *22*, *23*, and *26* was distributed evenly along the length of root, including the regions of initiation (Figure 4.5).

<u>Vestigial abscission zone of pedicels and inflorescence branches, and base of trichomes</u>

Unlike many plants, Arabidopsis does not shed leaves, branches, or entire flowers or fruit. Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*) led to abscission of inflorescence branches and pedicels, which suggested that Arabidopsis presumably has dormant and vestigial AZs at the base of these organs (Stenvik et al., 2006). GUS activity was detected at the base of pedicels for two transgenic lines (Figure 4.5). Intensity of GUS staining at the base of pedicels from flowers at stage 17 (Figure 4.5, *PLL15c, PLL24c*) was stronger than that of flowers at stage 15 (Figure 4.5, *PLL15b*, *PLL24b*). GUS activity was also observed at the base of trichomes from rosette and cauline leaves for four *PLL* genes (Figure 4.5, Table 4.2)

***PLL:GUS* expression associated with cell wall loosening events**

Various analyses of *PLLs* in plants have shown that they were expressed in a broad range of tissues (Marín-Rodríguez et al., 2002; Palusa et al., 2007). We further carried out analysis of GUS expression driven under *PLL* promoters with seedlings at different developmental stages and flowers from stage 11 (stigmatic papillae appear) to

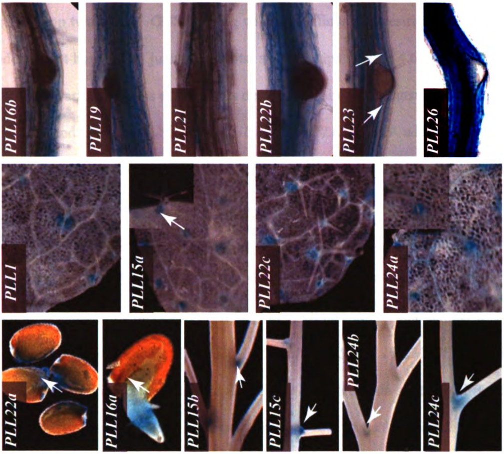


Figure 4.5. *PLL:GUS* expression in the tissues involved in various cell separation processes. Picture labels are as indicated for Figure 4.1. For transgenic line with more than one picture being displayed, lower case letters were used to indicate different organs at different developmental stages. Top row: *PLL::GUS* expression at the endodermis and cortical cell layers during lateral root emergence. Middle row: *PLL:GUS* expression at the base of trichome. Arrowheads indicate location of base of trichomes. Bottom row: *PLL22a* and *PLL16a* are *GUS* expression in the endosperm cell layers during seed germination. Arrows indicate location of endosperm cell layer. *PLL15b* and *PLL24b* are GUS expression at the base of pedicels of young flowers; *PLL15c* and *PLL24c* are GUS expression at the base of mature siliques. **"Images in this thesis/dissertation are printed in color"**

stage 15 (stigma extends above long anthers) (Smyth et al., 1990).

Seedlings

GUS activity was detected in the 1-d-old seedlings for 22 *PLL* transgenic lines (Figure 4.6, top two rows). GUS staining within seedlings was restricted to hypocotyls, cotyledons, root-hypococtyl junctions, roots, and root tips (Figure 4.6, Table 4.2). GUS activity in the various regions of roots was detected within 12 transgenic lines (Figure 4.6, Table 4.2), and *PLL13:GUS*, *PLL17:GUS*, and *PLL22:GUS* additionally showed expression in the root tips (Figure 4.6, Table 4.2). *PLL25::GUS* was uniquely distinguished by a specific localization within the root tip (Figure 4.6, '*PLL25*').

With the seedling differentiation, strong GUS activity was detected in the roots in the 5-d-old seedlings for 12 *PLL* transgenic lines (Figure 4.6, bottom row). GUS expression in various regions of roots in 5-d-old seedlings was similar as that in 1-d-old seedlings. Robust expression of *PLL13:GUS*, *PLL17:GUS*, and *PLL22:GUS* was limited to the root apex region, including *PLL22:GUS* in the columella root cap (Figure 4.6). *PLL25:GUS* was specifically expressed in the root apical meristems and root differentiation zones (Figure 4.6 '*PLL25a*').

Hydathodes

Hydathodes are highly specialized structures evolutionarily related to stomata, and they are permanently open pores to release water and solutes from xylem (Esau, 1977). Hydathodes in Arabidopsis are positioned at the leaf margins, and close to the ending of xylem vessels. Evident GUS expression was observed in fully-open cotyledons and rosette leaves for seven *PLL* transgenic lines (Figure 4.7A, Table 4.2). GUS activity

Figure 4.6 ***PLL:GUS*** **expression in the seedlings.** Picture labels are as in Figure 4.5. <u>Top two rows</u>: GUS expression in the 1-d-old seedlings; <u>bottom row</u>: GUS expression in the root at 5-d-old seedlings. Arrows in the bottom row indicate the enlarged picture of root apical meristem region. **"Images in this thesis/dissertation are printed in color"**

was only restricted to the hydathodes within cotyledons and rosette leaves (Figure 4.7).

Stipules

In Arabidopsis, stipules are present on the newly formed leaves and degenerate with leaf expansion. GUS activity within shoot apex was observed for 19 *PLL* transgenic lines (Figure 4.7B). Within the shoot apex, we observed strong GUS activity only in the stipules of primordia and newly formed leaves (Figure 4.7B, Table 4.2). GUS expression was evident in the first pair of true leaves in 6-d-old seedlings and in successive leaves including cauline leaves in the older seedlings (Figure 4.7B, example *PLL19*, *PLL19a*, *PLL19b*, *PLL19c*, *PLL19d*). For all leaves, expression was the strongest in newly formed leaves and decreased with leaf expansion concomitant with degeneration of stipules (Figure 4.7B).

Flowers

GUS activity was detected in developing flowers at stage 15 for 23 *PLL* transgenic lines (Figure 4.8A). GUS expression within flowers was restricted to stigma, style, junction of anther and stamen filament, and stamen filament, pollen, and developing seeds at stages from 11 to 15 (Figure 4.8 A and B, Table 4.2). GUS activity in styles was observed for 14 *PLL:GUS* transgenic lines, whereas GUS activity in stigma was detected in other eight transgenic lines (Figure 4.8, Table 4.2). We observed evident GUS staining in mature pollen for 13 *PLL* transgenic lines and at junction of stamen and filament for 18 transgenic lines (Figure 4.8A, Table 4.2). We also observed that *PLL1:GUS* was only expressed in the styles, and *PLL10:GUS* was only detected in the mature pollen (Figure 4.8A). *PLL13:GUS* was distinguished by its expression in the developing seeds at stage 11 to 15 (Figure 4.8 A and B).

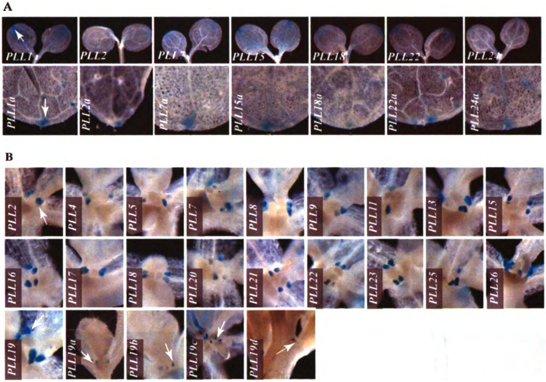


Figure 4.7. ***PLL:GUS*** **expression in the hydathodes (A) and stipules within shoot** apex (B). Picture labels were described as in the Figure 4.5. **A.** Top row: GUS expression in the hydathodes of cotyledons; bottom row: GUS expression in the hydathodes of rosette leaves. Arrowheads indicate the location of hydathodes (hy). **B.** Top two rows: GUS expression in the stipules of 6-d-old seedlings. Bottom row, *PLL19* is used as an example to show expression in the stipules at different stages of leaf development. *PLL19a*: stipules visible at the base of new formed leaf; *PLL19*: enlarged image of *PLL19a* within shoot apex; *PLL19b*: weak stipule expression at the base of expanding leaf; *PLL19c*: stipules at the base of rosette leaves at different stages of development, *PLL19d*, single stipule detected at the base of expanded cauline leaves. Arrowheads indicate the location of stipules. **"Images in this thesis/dissertation are printed in color"**

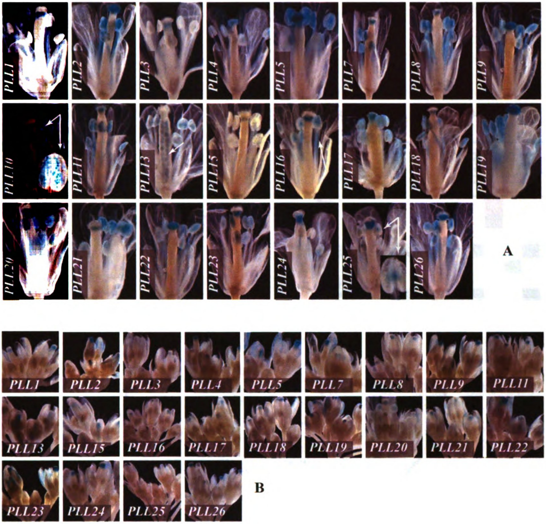
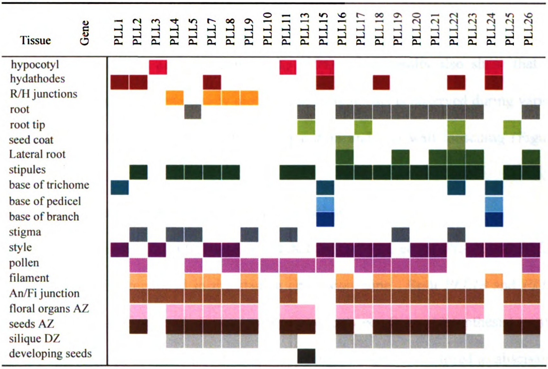


Figure 4.8. ***PLL:GUS*** **expression in the different parts of flower at stage 15 (A) and stage (11-14) (B).** Picture labels were described as in the Figure 4.5. *PLL10* as an example showing expression in the pollen (enlarged picture), *PLL25* as an example showing expression in the junction between anther and filament (enlarged picture). Arrowheads indicate stigma, style, pollen, junction between anther and filament, seeds, and filament. **"Images in this thesis/dissertation are printed in color"**

Table 4.2. Summary result of Figure 4.3 to 4.8. "Images in this thesis/dissertation are printed in color"



Discussion

PLLs are abundantly and ubiquitously expressed throughout plant development

Pectate lyase is the primary virulence agent in the soft rot disease caused by *Erwinia sp.* and has been extensively studied for its activity and function (Collmer and Keen, 1986; Barras et al., 1994). Whole genome sequencing of plant species has revealed that *PLLs* are encoded by a large gene family, such as 26 in Arabidopsis, 12 in rice, 22 in poplar (Palusa et al., 2007). Publicly available microarray data reveals that *PLLs* are expressed in various tissues and organs along plant development (Schmidt et al., 2005; Ma et al., 2005; Zimmermann et al., 2004). Our results also show that the promoter activity of *PLL* gene family members in Arabidopsis is observed during various developmental events associated with cell separation and cell wall loosening (Figures 4.3-4.8).

PLL:GUS expression associated with cell separation

Cai and Lashbrook (2008) determined that the two *PLL* genes, *PLL18* and *PLL23*, are developmentally regulated in the stamen abscission zone. Both *PLL18* and *PLL23* belong to the class of genes that were up-regulated between Stage 13 (anthesis) until at least the end of Stage 15. The fact that the remaining *PLL* genes identified as abscission-associated in our study were not identified by Cai and Lashbrook could be due to the potential greater sensitivity of our approach or to the possibility that posttranscriptional mechanisms play a predominant role in the regulation of their expression. The highly overlapping promoter activity patterns within floral abscission zones suggest that these genes could act in a highly redundant manner.

Overlapping GUS expression patterns observed with in the AZs of floral organs, and seeds, and the DZs of siliques among multiple gene members suggest that the function specialization related to cell separation presumably results from co-expression of many *PLL* genes. Three closely related polygalacturonases, QUATER2 (QRT2), ARABIDOPSIS DEHISCENCE ZONE POLGACTURONASE1 (ADPG1) and ADPG2, were partially redundantly expressed in the AZs of floral organs and seeds, the DZs of siliques and anthers (Ogawa et al., 2009). In addition, these members also exhibited expression in other cell separation events, such as lateral root initiation and radicle emergence. These results imply that the possibility of *PLLs* participate in other cell separation events.

Detection of GUS activity in the endosperm layer during radicle emergence for two *PLLs* suggests that they are actively transcribed during the seed germination process. Other cell wall modifying enzymes, such as PG, are reported to be expressed in the endosperm region adjacent to the emerging radicles (González-Carranza et al., 2007). These results implied that these cell wall modifying enzymes presumably contribute to the targeted cell separation occurring within the endosperm layer during radicle emergence.

Increased transcriptional accumulation of *PLL16* and *PLL26* has been observed during lateral root emergence induced by auxin (Laskowski et al., 2006, Swarup et al., 2008). These results support that these six *PLLs* were transcriptionally expressed in the cortical and endodermal layers overlaying new lateral root primordia during lateral root emergence induced by auxin, implying their contribution to this targeted cell separation event.

Overexpression of *IDA* in Arabidopsis led to the abscission of pedicels and inflorescence branches, suggesting the potential presence of these dormant AZs and the capability of responding to some specific signals (Stenvik et al., 2006). Interestingly, transgenic lines showing expression at the base (vestigial AZs) of pedicels and inflorescence branches were not overlapping with those showing expression in the AZs of floral organs (Table 4.2). These results support the hypothesis that there is evolutionary function specialization regarding organ abscission among different members of *PLL* gene family.

We observed that several *PLL* promoters drove GUS expression at the base of trichomes (Figure 4.5). Other cell wall modifying enzymes, such as PGs and expansins, have also shown expression at the base of trichome through a GUS reporter approach (Gonzalez-Carranza et al., 2007; Cho and Cosgrove 2000). One possible explanation for expression of these genes at the base of trichomes is that they may contribute to the cell wall remodeling during initiation and emergence of trichomes from the leaf epidermis.

***PLL:GUS* expression associated with cell wall loosening events**

We observed almost half of gene family members drove evident GUS expression in various regions of roots (Figure 4.6). This result is largely supported by publicly available microarray data and a comprehensive RT-PCR analysis which also show abundant transcriptional expression in roots these genes (Palusa et al., 2007; Schmidt et al., 2005). The majority of members with expression in roots also showed a robust level of expression in the root elongation zones, additionally, three genes showed expression in the root tips (Figure 4.6). *PLL25:GUS* was distinguished by its expression in root tips and the root differentiation zone (Figure 4.6). These results suggest that *PLL* genes are differentially expressed in various regions of roots and may contribute to cell wall loosening events occurring in root elongation, differentiation, and radicle emergence.

We detected evident GUS expression in the hydathodes among several *PLL:GUS* transgenic lines (Figure 4.7A). Genes associated with cell separation, for example, *IDA-like4* (*IDL4*) showed expression in the stomata (Stenvik et al., 2008). The structural features of hydathodes suggest their involvement in cell wall loosening or targeted cell separation during formation of hydathodes (Roberts et al., 2002).

GUS activity patterns observed in the stipules suggested that these *PLLs* were temporally and spatially expressed in the stipules in a developmentally-dependent manner. Gene members that drove GUS expression in the stipules are largely consistent with members expressed in the shoot apex from the public microarray data analysis (Figure 4.7B and Figure 4.2). The function of stipules in Arabidopsis development remains largely unknown. It has been shown that stipules are primary sites for high accumulation of free-auxin, which is associated with vascular differentiation and leaf morphogenesis in seedlings (Aloni et al., 2003; Cheng et al., 2007, Barkoulas et al., 2008). This information implies that these *PLLs* may be auxin responsive and presumably contribute to the degradation of stipules along leaf development in Arabidopsis.

Gene members that drove GUS expression in pollens are largely agreed with those in transcriptiome analysis (Palusa et al., 2007). The first *PLL* gene reported in higher plants is from pollen based on sequence homology with PelC in bacteria (Wing et al., 1990). Multiple *PLLs* have been shown to be expressed in pollen in a wide range of plants (Marín-Rodríguez et al., 2002). These data imply that *PLLs* presumably contribute to the initial loosening of pollen cell wall to facilitate pollen tube emergence. Some studies also showed that *PLLs* from tomato and tobacco were expressed in the styles and pistils (Budelier et al., 1990; Wu et al., 1996). In our study, the majority of *PLL* gene family members in Arabidopsis drove GUS expression either in stigma or styles (Table 4.2), suggesting that *PLLs* may participate in the degradation of stigmatic papillae or softening of stylar tissue to facilitate pollen tube growth.

***PLL:GUS* expression in an overlapping and specializing manner**

It is not known whether phylogenetically closely related paralogs in a large gene family share similar expression patterns. Four phylogenetic closely-related paralogs in subfamily II, *PLL8-11*, drove GUS expression in mature pollen (Figure 4.8, Table 4.2), and transcriptome analysis showed that they are highly expressed in the stamens and mature pollen (Figure 4.2). In addition, GUS expression patterns driven by these four *PLL* promoters in other tissues were divergent (Table 4.2). We also observed that the *PLLs* used in this study drove GUS expression in a partially redundant and distinct manner in various tissues and organs (Table 4.2). These results imply that each *PLL* gene member has partially overlapping and specialized biological function during plant development.

Implications

This analysis is the first report of comprehensive GUS expression analysis driven by *PLL* promoters, and it is also the first step in determining the functions of *PLLs* in plant development. Comprehensive expression analysis is useful for providing guidance to reverse genetic screening in order to identify the phenotypic abnormality of gene-specific mutants. For example, a loss-of-function mutant of *PLL25* may have defects in root differentiation, while mutants of *PLL13* may reveal the abnormality in seed development. The overlapping expression observed in multiple *PLLs* will facilitate the silencing of these genes simultaneously.

References

Aloni R, Schwalm K, Markus L, Ullrich CI (2003) Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in Arabidopsis. Planta 216: 841-853

Barras F, van Gijsegem F, Chatterjee AK (1994) Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu Rev Phyto 32: 201-234

Benitéz-Burraco A, Blanco-Portales R, Redondo-Nevado J, Bellido ML, Moyano E, Caballero J-L, Muñoz-Blanco J (2003) Cloning and characterization of two ripening-related strawberry (*Fragaria x ananassa* cv. Chandler) pectate lyase genes. J Exp Bot 54: 633-645

Budelier K, Smith A, Gasser C (1990) Regulation of a stylar transmitting tissue-specific gene in wild type and transgenic tomato and tobacco. Mol Gen Genet 224: 183-192

Barkoulas M, Hay A, Kougioumoutzi E, Tsiantis M (2008) A developmental framework for dissected leaf formation in the Arabidopsis relative *Cardamine hirsuta*. Nature Genet 40: 1136-1141

Cai S, Lashbrook C (2008) Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: enhanced retention of floral organs in transgenic plants overexpressing Arabidopsis ZINC FINGER PROTEIN2. Plant Physiol 146: 1305-1321

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting Arabidopsis lateral root development. Trends Plant Sci 6: 1350-1385

Cho H-T, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicle abscission in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 97: 9783-9788

Cheng Y, Dai X, Zhao Y (2007) Auxin synthesized by the YUCCA flavin monooxygenases Is essential for embryogenesis and leaf formation in Arabidopsis. Plant Cell 19: 2430-2439

Chourasia A, Sane V, Nath P (2006) Differential expression of pectate lyase during ethylene-induced postharvest softening of mango (*Mangifera indica* var. Dashehari). Physiol Plant 128: 546-555

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735-743

Collmer A, Keen N (1986) The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 24: 383-409

De Lorenzo G, Cervone F, Hahn M, Darvill A, Albersheim P (1991) Bacterial endopectate lyase: evidence that plant cell wall pH prevents tissue maceration and increases the half-life of elicitor-active oligogalacturonides. Physiol Mol Plant Pathol 39: 335-344

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95: 14863-14868

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP, and related tools. Nature Protocols 2: 953-971

Esau K. (1977) Hydathodes. In Anatomy of Seed Plants. pp. 205–206. John Wiley & Sons, New York.

Fagard M, Dellagi A, Roux C, Périno C, Rigault M, Boucher V, Shevchik V, Expert D (2007) *Arabidopsis thaliana* expresses multiple lines of defense to counterattack *Erwinia chrysanthemi*. Mol Plant Micro Interact 20: 794-805

Force A, Lynch M, Pickett FB, Amores A, Yan Y-l, Postlethwait J (1999) Preservation of duplicate genes by complementary, degenerative mutations. Genetics 151: 1531-1545

Finn RD, Tate J, Mistry J, Coggill PC, Sammut JS, Hotz HR, Ceric G, Forslund K, Eddy SR, Sonnhammer EL, Bateman A (2008) The Pfam protein families database Nucleic Acids Research 36: D281-D288

Futamura N, Kouchi H, Shinohara K (2002) A gene for pectate lyase expressed in elongating and differentiating tissues of a Japanese willow (*Salix gilgiana*). J Plant Physiol 159: 1123-1130

González-Carranza ZH, Elliott KA, Roberts JA (2007) Expression of polygalacturonases and evidence to support their role during cell separation processes in Arabidopsis thaliana. J Exp Bot 58: 3719-3730

Iwai H, Masaoka N, Ishii T, Satoh S (2002) A pectin glucuronyltransferase gene is essential intercellular attachment in the plant meristem. Proc Natl Acad Sci USA 99: 16319-16324

Jarvis MC (1984) Structure and properties of pectin gels in plant cell walls. Plant Cell Environ 7:153–64

Jarvis M, Briggs S, Knox J (2003) Intercellular adhesion and cell separation in plants. Plant Cell Enviro 26: 977-989

Knox J (1992) Cell adhesion, cell separation and plant morphogenesis. Plant J 2: 137

Knox J (2002) Cell and developmental biology of pectins In: Seymour GB and Knox JB (ed) Pectins and their manipulation CRC press LLC pp131-146.

Kotoujansky A (1987) Molecular genetics of pathogenesis by soft rot *Erwinias*. Annu Rev Phytopathol 25: 405-430

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948

Laskowski MJ, Williams ME, Nusbaum HC, Sussex IM (1995) Formation of lateral root meristems is a two-stage process. Development 121: 3303-3310

Laskowski M, Biller S, Stanley K, Kajstura T, Prusty R (2006) Expression profiling of auxin-treated Arabidopsis roots: toward a molecular analysis of lateral root emergence. Plant Cell Physiol 47: 788-792

Lesile ME, Lewis MW, Liljegren SJ (2007) Organ abscission. Annual Plant Reviews V 25. Plant Cell Separation and Adhension. edited by Jeremy A. Roberts and Zinnia Gonzalez-Carranza pp91-106

Liljiegren S, Roder A, Kempin S, Gremski K, Ostergaard L, Guimil S, Reyes D, Yanofsky M (2004) Control of fruit patterning in Arabidopsis by INDEHISCNET. Cell 116: 843-853

Liu P-P, Koizuka N, Homrichhausen TM, Hewitt JR, Martin RC, Nonogaki H (2005) Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes. Plant J 41: 936-944

Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. Genetics 154: 459-473

Ma L, Sun N, Liu X, Jiao Y, Zhao H, Deng XW (2005). Organ-specific expression of Arabidopsis genome during development. Plant Physiol 138: 80–91

Marín-Rodríguez M, Orchard J, Seymour GB (2002) Pectate lyases, cell wall degradation and fruit softening. J Exp Bot: 2115-2119

Marin-Rodriguez M, Smith D, Manning K, Orchard J, Seymour GB (2003) Pectate lyase gene expression and enzyme activity in ripening banana fruit. Plant Mol Biol 51: 851-857

Ogawa M, Kay P, Wilson S, Swain S (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are polygalacturonases required for cell separation during reproductive development in Arabidopsis. Plant Cell 216-33 216-233

O'Neill MA, Ishii T, Albersheim P, Darvill AG (2004) Rhamnogalacturonan II: structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu Rev Plant Biol **55:** 109-139

Palusa SG, Golovkin M, Shin S-B, Richardson DN, Reddy ASN (2007) Organ-specific, developmental, hormonal and stress regulation of expression of putativepectate lyase genes in Arabidopsis. New Phytol 174: 537-550

Patterson SE (2001) Cutting loose. Abscission and dehiscence in Arabidopsis. Plant Physiol 126: 494-500

Pinyopich A, Ditta GS, Savidge B, Liljegren SJ, Baumann E, Wisman E, Yanofsky MF (2003) Assessing the redundancy of MADS-box genes during carpel and ovule development. Nature 424: 85-88

Ridley B, O'Neill M, Mohnen D (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling Phytochemistry 57: 929-967

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Scholkopf B, Weigel D, Lohmann JU (2005) A gene expression map of *Arabidopsis thaliana* development. Nature Genetics 37: 501-506

Schols HA, Voragen AGJ (2002) The chmeical structure of pectins. In: Seymour and Knox (ed) Pectins and their manipulation. pp1-25 CRC press LLC

Seymour G, Tucker G (2002) Modification and degradation of pectins In: Seymour and Knox (ed) Pectins and their manipulation CRC presss LLC pp150-173.

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in Arabidopsis. Plant Cell 2: 755-767

Stenvik G-E, Butenko MA, Urbanowicz BR, Rose JK, Aalen RB (2006) Overexpression of *INFLORESCENCE DEFICIENT IN ABSCISSION* activates cell separation in vestigial abscission zone in *Arabidopsis*. Plant Cell **18:** 1467-1476

Stenvik G-E, Tandstad NM, Guo Y, Shi C-L, Kristiansen W, Holmgren A, Clark SE, Aalen RB, Butenkoa MA (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION Is sufficient to induce abscission in Arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2. Plant cell 20: 1805-1817

Swarup K, Benková E, Swarup R, Casimiro I, Péret B, Yang Y, Parry G, Nielsen E, Smet ID, Vanneste S, Levesque MP, Carrier D, James N, Calvo V, Ljung K, Kramer E, Roberts R, Graham N, Marillonnet S, Patel K, Jones JDG, Taylor CG, Schachtman DP, Sandberg SMG, Benfey P, Friml J, Kerr I, Beeckman T, Laplaze L, Bennett MJ (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nature Cell Bio **10:** 946-954

Roberts JA, Elliott KA, Gonzalez-Carranza ZH (2002) Abscission, dehiscence and other cell separation process. Annu Rev Plant Biol 53: 131-158

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599

Tucker GA, Seymour GB (2002) Modification and degradation of pectins. In Pectins and their manipulation Blackwell Publishing, CRC Press Edited by Graham B. Seymour and J. Paul Knox: 150-168

Vogel JP, Raab T, Somerville C (2002) PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in Arabidopsis. Plant Cell 14: 2095-2106

Valkama E, Salminen J-p, Koricheva J, Pihlaja K (2004) Changes in leaf trichomes and epicuticular flavonoids during leaf development in three *Birch Taxa*. Ann Bot 94: 233-242

Wilson RH, Smith AC, Kacurakova M, Saunders PK, Wellner N, Waldron KW (2000) The mechanical properties and molecular dynamics of plant cell wall polysaccharides studies by Fourier transform infrared spectroscopy. Plant Physiol 124: 397–406

Willats WG, McCarney L, Mackie W, Knox PJ (2001) Pectin: cell biology and prospects for functional analysis. Plant Mol Biol 47: 9-27

Wing R, Yamaguchi J, Larabell S, Ursin V, McCormic S (1990) Molecular and genetic characterization of two pollen – lexpressed genes that have sequence similarity to pectate lyases of the plant pathogen *Erwinia*. Plant Mol Biol 14: 17-28

Wu Y, Qiu X, Du S, Erickson L (1996) PO149, a new member of pollen pectate lyase-like gene family from alfalfa. Plant Mol Biol 32: 1037-1042

Yoder M, Keen N, Jurnak F (1993) New domain motif: the structure of pectate lyase C, a secreted plant virulence factor. Science 260: 1503-1507

Zhang J (2003) Evolution by gene duplication: an update. Trends Ecol Evolu 18: 292-298

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L. and Gruissem, W. 2004.

GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136: 2621–2632.

CONCLUSION

Abscission or retention of ripening fruit is a major strategy of seed dispersal and also has important implications for horticultural production. Abscission-related traits have generally not been targeted in breeding efforts, and their genetic bases remain mostly unknown. As a first step to elucidate the genetic bases of abscission related traits, we documented diversity in fruit-abscission-related traits among *Malus* accessions representing the breadth of genetic diversity seen in *Malus*. Our findings suggest that important mechanism(s) independent of fruit ethylene production act as determinants of natural abscission. Accessions showing phenotypic extremes in abscission-related traits can be developed as contrasting models to understand the biological bases of these traits, and as tools in genetic analyses for mapping genes that influence these traits.

In fruit crops, precise regulation of fruit abscission is crucial to achieve maximum yield and optimized market value. We presented a transcriptional profile analysis and identified a small population of differentially expressed genes within the pedicel AZs during pedicel abscission induced by fruit removal in apple cv. 'Golden Delicious'. These differentially expressed genes identified in this work provide a valuable resource for further functional characterization of genes associate with fruit abscission. This work also suggested a potential common molecular mechanism on pedicel abscission induced by fruit removal, chemical thinners in early fruit developmental stages, and ethephon in preharvest season.

As a first step to understand the functional diversity of *PLL* genes in plants, we documented the spatial and temporal promoter activity of 23 of the 26 Arabidopsis *PLL* genes throughout development. Our results suggest potential for unique and overlapping activity of *PLL* genes, and provide guidance for analysis of individual gene function through reverse genetics..