WTFRC Project #AH-02-204
Molecular biology-based assay for improved fruit thinning (Final report)
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Objectives
The natural tendency of commercial apple varieties to overcrop leads to small and poor quality fruit. In order to optimize fruit size and quality, growers must remove excess fruit early in the season. Currently this is accomplished through the application of various chemical thinners (growth regulators). However, the outcome of this strategy is inconsistent, cultivar-specific and highly dependent on environmental conditions. In addition, the use of the most popular and effective chemical thinners is being restricted. In order to improve on current methods and to find replacements, it is crucial to identify the modes of action of these thinning agents. The goal of this three-year project is to develop a molecular biology-based assay to study the action of chemical fruit thinners and to evaluate the potential of new thinners. The information generated from this work will allow initial screening of new potential thinning chemicals without the need for initial field trials. This research also provides insight into the mechanism of abscission induction, which benefits both fruit and flower thinning technologies. This research has applications to prevent preharvest fruit drop, and to optimize mechanical harvesting techniques. In addition, although not a goal of this project, the genes that regulate abscission identified in this research are appropriate targets for future manipulation through biotechnology.

The specific aims of this project are to:
1) Develop a research model to study the molecular biology of fruit abscission
2) Identify major genes involved in fruit abscission
3) Develop an assay using such genes to determine the genetic mechanism of thinning of NAA, BA, and carbaryl
4) Use gene promoter analysis to determine how abscission-related genes are regulated in the apple tree

Significant findings
- We refined a technique to reproducibly induce abscission of immature apples. In this technique, we sever the pedicel after June drop, leaving the remaining pedicel attached to the limb. This initiates a series of events which leads to abscission of the pedicel after approximately five days. We used this technique as a research model to study the early molecular events in abscission.
- We developed procedures to purify RNA (the chemical copy of active genes) from abscission layer tissues removed from the pedicel. We then removed abscission layer tissues and purified RNA from approximately 5,000 individual pedicels sampled before induction and two and four days after induction of abscission.
- We created a cDNA library, or collection of cloned genes, that are active in the abscission layers two and four days following abscission induction. We then analyzed the DNA sequence of approximately 800 genes, and matched the DNA sequences with known gene sequences from other organisms. We found that approximately one-half of these genes were previously unknown and unique to apple.
- We developed web-based bioinformatics modules to organize and view this and other apple genomics data. This resource is freely available to other tree fruit researchers (www.genomics.msu.edu/fruitdb)
- Using a technique called microarray analysis, we examined the activity of all of these genes in the sampled tissues. We used these data to identify abscission-layer-specific genes that were

activated during abscission. Our results show that significant changes in gene activity occurred by four days after the induction of abscission.

- Several abscission-responsive genes are especially interesting given their potential function. These include pollen allergens, aquaporin, beta-galactosidase, pectate lyase, lipoxygenase, and several genes that have been found to be also activated during pathogen attack (Table 1, below).
- We isolated the regulatory, promoter regions of early abscission genes. As further evidence that the genes we discovered are involved in abscission, we identified the counterpart of the pectate lyase gene in the research plant *Arabidopsis thaliana* and found that this gene also becomes activated in the fruit-pedicel abscission zone (below).
- We are initiating the use of this gene promoter marker technology in apple trees to quickly visualize the activity of these genes in response to growth regulators and environmental factors.
- We identified a transcription factor (regulatory protein) that may control the formation of the abscission zone in apple. This protein is the apple counterpart of a tomato gene called *JOINTLESS*. Tomato cultivars that have mutations in the *JOINTLESS* gene have been utilized extensively for mechanical harvesting and processing, because these fruit lack an abscission zone entirely and cannot abscise. In a planned future project, we hope to study the function of this gene in apple. If we are able to disrupt the activity of this gene in apple, we expect that the apple flower/fruit will lack an abscission zone.
- We are now taking advantage of new and better microarray technologies that will allow us to screen up to 25,000 genes at one time. The availability of these new technologies, which was not envisioned in the original proposal, greatly expands the scope of this research.
- We expect to finish work on this project this winter, and intend to use these tools to screen potential thinning compounds in Spring '05 and '06.

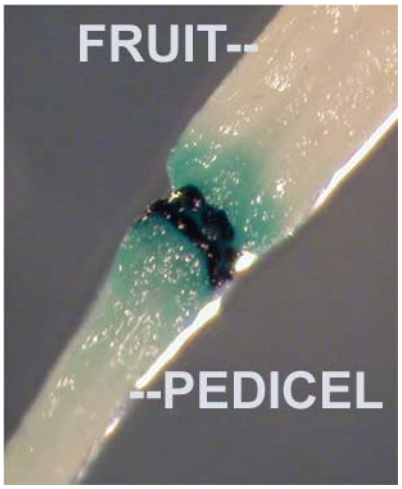
Methods

Field experiments were carried out on cv. Delicious, Golden Delicious, McIntosh, and Gala at the MSU Horticultural Research Stations in E. Lansing, MI and Clarksville, MI. Field experiments done in 2002 were replicated in 2003 and 2004. We analyzed a time-course of abscission activation using an abscission model that we developed (below). We dissected abscission layer tissues daily and used these for microarray analysis to identify abscission-activated genes. We also generated and dissected matched sets of abscission layer tissues from high-abscission-potential and low-abscission-potential fruitlets. These sets were generated by two approaches: (1) Manipulating leaf-to-fruit ratio to generate high crop load (abscission of some fruitlets is expected) and low crop load (retention of nearly all fruitlets is expected). Abscission layer tissues were collected at 5-d intervals, beginning 5 d after full bloom and continuing until June drop. (2) Fruit abscission was promoted on entire trees by treatment with Terbacil, a photosynthesis inhibitor. Treatments were initiated when average size of fruit was 10 mm, and samples were taken every 5 d until June drop. We also evaluated three thinning strategies: 100 ppm BA, 1000 ppm carbaryl, and 30 ppm NAA, applied at 10 mm fruit size, for their effects on gene activity. Abscission zone tissues were collected at 1-d intervals after treatment, and throughout the inductive stage. Tissues were collected for analysis by microarray technology, which is ongoing.

Results and Discussion

In **Year 1** of this project, we initiated and refined field experiments to affect fruitlet abscission; these were replicated in Year 2 and Year 3 (see *Methods*). We developed a technique to reproducibly induce abscission of immature apples. In this technique, we sever the pedicel after June drop, mark the

remaining pedicel with tape, and leave the pedicel attached to the limb; the pedicel then abscises after four or five days. We also refined procedures to purify RNA (the chemical copy of active genes) from abscission layer tissues dissected from the pedicel. We then dissected abscission layer tissues and purified RNA from approximately 5,000 individual pedicels sampled at two days and four days after the induction of abscission.



In **Year 2** we created a cDNA library, or collection of cloned genes, that are active in the abscission layers two and four days following abscission induction. We then analyzed the DNA sequence of approximately 800 ESTs (expressed sequence tags, which correspond to active gene fragments) from our cDNA library. We found that approximately one-half of the respective genes were clearly homologous to genes from other organisms, whereas one-half were apparently unique to apple. Using a technique called microarray analysis, we examined the activity of all of these genes in the sampled tissues. We used this activity data to identify abscission-layer-specific genes that were activated during abscission. Our results show that significant changes in gene activity occurred by four days after the initiation of abscission. To date, we have identified several genes that we believe are of particular significance (Table 1). As further evidence that these genes play important roles in abscission, we identified the counterpart of the abscission-activated pectate lyase gene in the research reference plant Arabidopsis and found that this gene also becomes activated in the fruit-pedicel abscission zone. In the photograph, the region of activity of the gene is seen as a dark blue color in the abscission zone.

Table 1. A partial list of abscission-layer-specific genes that we found to be activated by four days after induction of abscission.

Gene	Potential function in abscission
MalD1, MalD4	These genes have been previously studied in apple because the MalD proteins are responsible for allergies to apple products. Like most allergens, these proteins have also been found in pollen. We speculate they assist in the migration of pollen through the style, a process that requires cell separation. Likewise in the abscission layer, these proteins may foster cell separation through previously unknown mechanisms.
Aquaporin	Aquaporins are proteins found in the cell membrane, the predominant barrier for molecular traffic into and out of the cell. These proteins permit entry of water into the cell. As such, aquaporins are required for cell expansion. We speculate that these proteins regulate the swelling of abscission layer cells seen late in abscission.
Beta-galactosidase, Pectate lyase	Both of these proteins are enzymes that degrade pectins. Pectins are a major component of plant cell walls. Thus, it seems likely that these genes play a key role in degrading the cell walls of the abscission zone tissues.
Lipoxygenase	Lipoxygenases produce free radicals, which cause peroxidative damage to membranes and may cause degradation of carbohydrates in the cell wall. These also may help to disintegrate the abscission layer cells.
Previously unknown genes	Although the function of these genes is unknown, at least two have previously been found to be activated in response to pathogen infection. Pathogen infection involves at least two chemical messengers: ethylene, and small oligosaccharides that originate from breakdown of the cell wall. Although ethylene has been hypothesized to play a signaling role in abscission, a potential role for oligosaccharides has not been studied.

In **Year 3** we began the process of isolating the regulatory, promoter region of some of the genes that are activated during abscission. These segments of DNA will be used for gene promoter analysis.

The genes identified so far are activated relatively late in abscission. That we did not yet identify genes that are activated earlier in abscission, is possibly due to the fact that such genes are rare and that we screened only 800 genes. We are now taking advantage of new and better microarray technologies at MSU that will allow us to screen up to 25,000 genes at one time. These technologies are more sensitive and require much less starting material. The availability of these new technologies, which was not envisioned in the original proposal, will greatly expand the scope of this research. This work is ongoing and is expected to be finished this spring.

We also developed web-based bioinformatics modules to organize and view this and other apple genomics data. This resource is freely available to other tree fruit researchers (www.genomics.msu.edu/fruitdb).

Our results were presented at the 2002 International Conference on Abscission (Orangeville, Ont.) and at the 2002 and 2003 Great Lakes Fruit, Vegetable, and Farm Market Expo. New results will be presented at the 2004 Great Lakes Fruit, Vegetable, and Farm Market Expo. We trained one Ph.D. graduate student, one undergraduate student, and three high school students in field and lab techniques.

Budget

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Project duration: 2002-2004
Current year: 2004
Project total (3 years): $63,596

Year-by-year breakdown:

Item	***Year 1 (2002)***	***Year 2 (2003)***	***Year 3 (2004)***
Salaries[1]	*15,612*	*16,237*	*16,887*
Benefits[2]	*420*	*420*	*420*
Wages	*0*	*0*	*0*
Benefits	*0*	*0*	*0*
Equipment	*0*	*0*	*0*
Supplies[3]	*5,200*	*4,200*	*3,900*
Travel	*0*	*0*	*0*
Miscellaneous[4]	*700*	*0*	*0*
Total[5]	*21,532*	*20,857*	*21,207*

[1]Stipend for one graduate student (Ph.D. level). This did not include tuition, fees, or benefits, which are paid by the P.I. from other funds.
[2]Health benefits
[3]This amount was adequate given the existing infrastructure at MSU-Horticulture.
[4]Charges for DNA sequencing, which was done at the Michigan State University Biotechnology Support Facility

Leverage of funding

Funding by the Washington Tree Fruit Research Commission was instrumental in obtaining funding from the MSU Agricultural Experiment Station (MAES) for related work on alternate bearing in apple (three-year project, total expected funding: $96,000) and from the Michigan Apple Research Committee ($52,000). The MAES also awarded $5,000 to help develop online software to manage

the sequence information generated during this and related projects (www.genomics.msu.edu/fruitdb). Preliminary results from this project were used in an application to the National Sciences Foundation Plant Genomics Program submitted in October '04, and will be used for an application for funding from the USDA Developmental Processes of Crop Plants Program to be submitted in January '05. The NSF or USDA funding sources would provide approximately $120,000 per year for continuation of this project without additional expense to WTFRC.