2009 Apple Research Review
January 21-23, 2009
Hoilday Inn at TRAC
Pasco, WA
21 January

Time	Page	PI	Project Title	Funding period
8:00		T. Schmidt	Welcome and introduction	
8:15		McFerson	Technology Roadmap Update/SCRI report	
			Final Reports	
8:30	1	Dhingra	Optimization of Rosaceae rootstock micropropagation	08
8:45	6	Dhingra	Apple genome sequencing	08
9:00	10	Dhingra	Cultivar improvement via trait-targeted sport induction	07-08
9:15	15	Rudell	Metabolomics: characterizing fruit with chemistry	08
9:30	23	Dhingra	Establishing trait–gene relationships and gene discovery in apples	07-08
9:45	28	Aldwinckle	Functional genomics of flowering in apple	07-08
10:00	37	van Nocker	Auxin and ethylene dynamics in the abscission zone	06-08
	46	Cheng	High temperature stress on apple fruit peel (written report only)	06-07
	56	Elfving	Sprayable 1-MCP for managing apple postharvest quality (written report only)	07
	60	Peace	Adapting available genomics tools to enhance WA apple breeding (written report only)	07
10:15			**Break**	
Group #			**Continuing Projects Poster Session 10:30-12:00**	
1	74	T. Schmidt	Chemical thinning of apple	internal
1	81	Elfving	Management of vegetative growth in apple trees with bioregulators	08-09
1		McFerson	WTFRC technology projects (see technology reports in appendix)	
2	85	Zhu	Functional genomics and marker development for apple sensory qualities (extension)	07-09
2	92	Ross	Sensory profiles and consumer acceptance of apple breeding selections	08-09
2	100	Shetty	Washington apple varieties for management of type 2 diabetes	08-09
3	108	Auvil	Apple rootstock and scion evaluation	internal
3	114	Wisniewski	Mapping M. sieversii: A valuable genetic resource for apple breeding	08-09
3	120	Mattheis	Integration of storage technologies for fruit quality management	08-10
3	125	Hanrahan	Programs to increase packouts of apples	internal

FINAL PROJECT REPORT

Project Title: Optimization of rosaceae rootstock micropropagation

PI:	Amit Dhingra	**Co-PI(2):**	Tom Auvil
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Total Project Request: Year 1: $13,000

Other funding Sources - None

Total Project Funding: $13,000
Budget History

Budget History

Item	2008	2009	
Salaries	10,000		
Supplies	3,000		
Travel			
Miscellaneous			
Total	**13,000**		

NOTE: THIS IS A TWO-YEAR PROJECT WITH FUNDING REQUESTED ONLY FOR THE FIRST YEAR. SOME OF THE SUB-OBJECTIVES WILL BE ACCOMPLISHED IN THE COMING MONTHS.

OBJECTIVES

In order to rapidly multiply G 41 and G 935 rootstock material several parameters were tested and optimized. We had three main objectives to accomplish our goal of accelerating rootstock multiplication.

1. Media Optimization: This objective focuses on identifying optimal conditions of the growth media from the stage of multiplication to transfer of the rooted plantlets to soil in a green house. It has three sub-objectives:

A. Identify optimal growth media formulation for accelerated G 41 multiplication
B. Optimize multiple root formation
C. Standardize transfer of rooted plants to soil in a green house condition

2. Photobiological Regulation of Rootstock Multiplication: Light has the capacity to regulate plant growth.
A. Under this objective, we aim to optimize G 41 multiplication and/or multiple root formation using different light regimes

3. Temporary Immersion System (TIS): This is a novel micropropagation technology that simulates plant growth in nature, only in an abbreviated timeline. The objective is to compare plant growth rates in TIS vs traditional media.

SIGNIFICANT FINDINGS

1. None of the reported media or the recipes provided by commercial lab was found to be suitable for G 41 rootstock micropropagation.

2. It takes over 10 weeks for two-fold multiplication of a given G 41 explant.

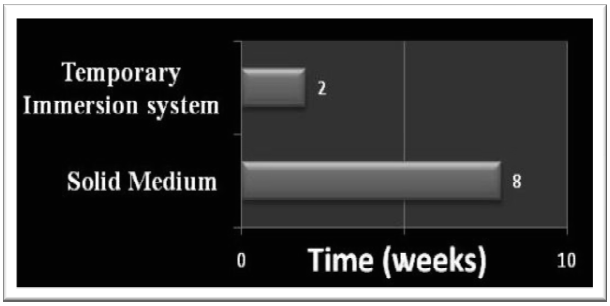
3. With the temporary immersion system the multiplication time can be reduced to two weeks.

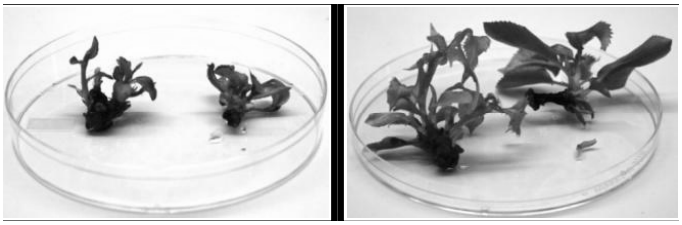
RESULTS AND DISCUSSION

1. Media Optimization: The media commonly used in the commercial labs was based on the formulation described by Murashige and Skoog (1964). This media called MS Media was formulated for tobacco. We were quickly able to optimize media for healthy growth of G-41 by changing the nitrogen source in the media (Figure 1). Box on the right represents the media formulation provided by commercial labs. Healthy plant growth is routinely obtained on the media optimized in the laboratory. Currently we are standardizing rooting. Our goal is to obtain multiple roots that enable better survival of the explant in the green house. There have been some explants that root and have been successfully transferred to green house. However, copious rooting remains elusive in this genotype.

2. Photobiological regulation of rootstock multiplication: Once the media was formulated we tested the impact of different light wavelengths on G 41 multiplication. It was found that any light intensity higher than 30 micromoles per meter square per second was detrimental for shoot multiplication. Our next goal is to test different light regimes for rootstock micropropagation. The construction of specialized growth chambers has concluded successfully. The impact of light regimes will now be tested. The growth chambers use LEDs that offer a "green" solution to electricity use. LEDs last longer and do not produce much heat.

3. Temporary Immersion system.
Standardized media formulation without agar was utilized for temporary immersion system. Figures below illustrate the speed with which the G-41 rootstocks can be multiplied. The graph shows the gain of time in multiplication of the rootstock. A savings of 6 weeks during multiplication is being utilized to develop an efficient rooting system. Second figure shows relative growth of an explant in two weeks in solid media (left panel) and TIS produced explant (right panel). Last figure shows the magnitude of multiplication if an explant is left in the TIS for four weeks.





With these encouraging results the stage is set to incorporate other troublesome rootstocks or scions in the program. Over the next few months we will test the impact of different wavelengths of light on G-41 multiplication.

ADDITIONAL DEVELOPMENTS

Several undergraduate students have had the opportunity to contribute to this project. Two of them, Danielle Druffel (civil engineering major) and Maureen McFerson (Food Science

major) have been awarded a fellowship from CAHNRS at WSU to carry out the research embodied in the objectives of this proposal.

PRESENTATIONS AND PUBLICATIONS

A. Invited Presentations:
1. A Dhingra: Rosaceae Micropropagation and Biotechnology. 4th Acclimatization and Establishment of Micropropagated Plants 2008. Bangalore, India December 2008
2. A Dhingra: Woody Plant Micropropagation. NNII presentation. Yakima, WA December 2008

B. Poster Presentations:
1. S Tariq, M McFerson, S Schaeffer, N Tarly, G Fazio and A Dhingra: Go Forth and Multiply! Establishing an efficient system for G-41 rootstock micropropagation. Annual WSHA meeting, December 2008, Yakima, WA.
2. J Able, E Shay, F Ali Khan, D Druffel, J Cruz, D Kramer and A Dhingra: Building controlled electronic systems to direct plant growth. Annual WSHA meeting, December 2008, Yakima, WA.
3. S Schaeffer, T Koepke, D Jiwan, D Druffel, D DeMars, F Ali Khan, N Tarlyn, T Yang and A Dhingra: Improving tissue culture, micropropagation and biotechnological approaches in Rosaceae Crops. 4th International Rosaceae Genomics Conference, Pucon Chile March 2008.

EXECUTIVE SUMMARY AND FUTURE DIRECTIONS

Rapid, reliable, cost-effective micropropagation is essential to accelerate progress in genomics, genetic, and breeding (ggb) projects and, fully integrate the emerging biotechnological tools in horticultural crop improvement. Of immediate significance to the Pacific Northwest industry is the scaling up of superior genotypes for rapid commercialization. Several years of progress towards developing a superior genotype is often impeded at the initial step of building liner beds. The infrastructure established with support from this project aims to cater to this very issue.

Future Directions: Encouraged by the current micriprpagation results we have requested for consolidated funding from WTFRC to continue tissue culture based projects in all three major US Crops. The next steps in the project are to test the impact oF LEDs in root growth optimization.

FINAL PROJECT REPORT

Project Title: Apple genome sequencing

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Total Project Request: **Year 1:** 32,000

Other funding Sources

Agency Name: Agriculture Research Center and Dept of Horticulture and LA
Amount awarded: $150,000
Notes: Seed funding to initiate the Apple Genome Sequencing Initiative at WSU

Agency Name: USDA-NRI
Amount awarded: $150,000
Notes: Development of BAC library for the double haploid apple

Total Project Funding: **$32,000**

Budget History

Item	**2008**		
Salaries			
Benefits			
Wages			
Benefits			
Equipment			
Supplies	30,000		
Travel	2,000		
Miscellaneous			
Total	32,000		

OBJECTIVES

Apple scion and rootstock improvement by utilizing *genomics, genetics and breeding* has emerged as one of the highest research priorities. The ever-changing global and environmental scenarios challenge us to employ creative approaches to accelerate apple improvement. The basic foundation of genetics, genomics breeding activities is grounded in a *genome sequence*. Availability of the apple genome sequence will provide ultimate utility to all the massive body of previous research performed in apples.

This project had only one objective

1. The specific objective was to augment the Apple Genome Sequencing Initiative (AGSI) with acquisition of approximately 300 Mega bases of apple genome sequence

SIGNIFICANT FINDINGS

The apple genome project is ongoing and the 300 million bases of DNA sequence generated with support from this project are expected to help in assembling of the genome information.

RESULTS AND DISCUSSION

Genetically unique doubled haploid material P21 R1 A50 n°13 = clone X9273, derived from Golden Delicious was used to isolate nuclear DNA. The material was obtained from the group at INRA, Angers, France. Utilization of double haploid genotype in conjunction with next generation technologies has generated a lot of interest in several genomics research groups.

As per the proposed timeline, we have achieved 2X coverage of the double haploid genome. The data is being constantly augmented with sequence data from other sources and assembly of this information is undergoing.

ADDITIONAL DEVELOPMENTS

This project has enabled establishment of several important collaborations with international groups. Some of the major developments are summarized below.

1. Formation of the international consortium of apple genome sequencing. The formal partners include researchers from INRA, France; IASMA, Italy; University of Western Cape, South Africa; HortResearch NZ.

2. Next-Generation Sequencer: The apple genome project enabled us to leverage a demo unit from Roche Inc in June 2008. Finally the University procured this equipment and it is valued at $650,000.

3. Roche Inc: In addition to loaning the equipment, Roche Inc as part of the collaboration has agreed to provide us additional sequence information data worth over $100,000. This will increase the coverage of the genome by an additional 2X and make the genome assembly more feasible.

4. USDA-NRI Funding: The preliminary data generated from this project further enabled to attract USDA-NRI funding worth $150,000.

5. Collaboration with DOE-Joint Genome Institute: DOE JGI director, Dr. Dan Rokhsar has agreed to generate additional sequence information to augment the ongoing work. In addition, they will also

assist in genome assembly work. DOE-JGI has sequenced several large plant genomes like maize, poplar, and medicago to name a few.

6. Collaboration with University of Guelph: U of G has invested $1.5 million in establishing an very well equipped post-harvest facility. The sequence information generated with this project will be linked with post-harvest biochemistry studies to devise appropriate storage conditions for apple cultivars.

PRESENTATIONS AND PUBLICATIONS

Peer-reviewed publication
1. A Dhingra, A Kalyanaraman and R Bumgarner: Plant Genomes Revealed: Unraveling the structure and function of large plant genomes with next-generation genomics. Current Genomics in press 2009 *Invited Review*

Invited Presentation
1. A Dhingra: International Consortium for Apple Genome Sequencing. 4th International Rosaceae Genomics Conference. Pucon, Chile March 2008.
2. A Dhingra: Apple Genome Project: Laying the foundation for a better apple. University of Guelph, Ontario November 2008.
3. A Dhingra: Apple Genome Project. IASMA Italy February 2009
3. A Dhingra: Rosaceae research meets next generation genomics. Department of Plant Molecular Biology, Univ of Delhi South Campus, India December 2008
4. A Dhingra: Next-generation genomics for horticultural crop improvement. National Botanical Research Institute, Lucknow, India December 2008

Poster Presentation
1. A Dhingra, A Kalyanraman and R Bumgarner: Laing the foundation for a better apple. Annual WSHA meeting, Yakima, December 2008
2. A Dhingra et al. Apple Genome Project: Annual PAG conference, San Diego, CA January 2009

EXECUTIVE SUMMARY AND FUTURE DIRECTIONS

The blueprint of apple genome is coming together rapidly as technological advances continue in the area of next-generation genomics. The funding from this project was to seed a larger initiative. In total we have attracted over $ 1 million in funding with this project just for the apple genome project. What this means is rapid progress towards the finish line. Importantly, this project has furthered WSU and WTFRC's mission of enhancing PNW's profile in horticultural genomics research.

Data generation is to be followed by data distillation and assimilation in a biologist's programs. While the USDA-NRI project will enable establishment of basic genomics resources, we have requested for additional funding from WTFRC to support data distillation and assimilation. The data will also serve as preliminary information for a NSF grant ready to be submitted in January and July 2008.

FINAL PROJECT REPORT

Project Title: Cultivar improvement via controlled sport induction (CSI)

PI:	Amit Dhingra	**Co-PI(2):**	Bruce Barritt
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City:	Pullman	**City:**	Wenatchee
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Total project funding request: **Year 1:** 26,150 **Year 2:** 36,725 **Year 3:** $0

Other Funding Sources - none

Total Project Funding: 62,875

Budget History

Item	2007	2008	
Salaries			
Benefits			
Wages	10,000	15,000	
Benefits	1150	1,725	
Equipment			
Supplies	13000	18,000	
Travel	2000	2,000	
Miscellaneous			
Total	26,150	36,725	

Footnotes:

OBJECTIVES

Naturally occurring sports have been a source of improvement to apple cultivars in the past but it is a chance process that is long-term and unpredictable. We had proposed a procedure to accelerate this natural process of sport induction and regulate it by targeting economically important traits. As cause and effect or gene-trait relationships continue to be established both in our program and by other programs, the scope of this platform will expand to encompass numerous important traits.

Proposed objectives of the project were:

1. Using tissue culture, establish cultures from selected apple cultivars

2. Standardize the technique for efficient sport induction

3. Perform a pilot experiment with tissue culture material for identifying allelic diversity for the genes that regulate fruit firmness (ACC-synthase and ACC-oxidase)

SIGNIFICANT FINDINGS

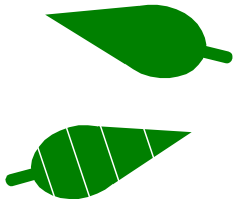
1. Leaf material from all apple cultivars tested is better for rapid regeneration of new plants. Regeneration from fruit derived cell lines were extremely slow in regeneration that can result in large number of somaclonal variants.

2. Leaf size and light intensity are highly critical for shoot regeneration from leaf material in M26, Gala, Pinova apple cultivars.

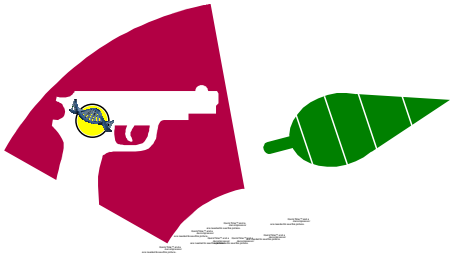
RESULTS AND DISCUSSION

Leaf regeneration has been optimized for Royal Gala, Pinova, and M26 leaves as seen in the figure. M26 leaves produced large amounts of regenerants on N6MS media supplemented with BAP and NAA. Royal Gala and Pinova, however, were unable to produce many regenerants on this media. Instead, regenerants were copiously produced on N6MS media containing TDZ, IBA and BAP.

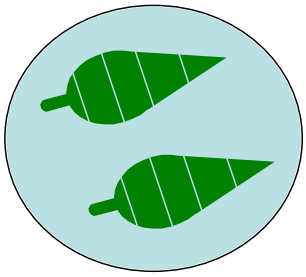
Apple leaves ranging from 2 to 5 cm were selected from healthy looking plants. These leaves were cut into 1cm strips and placed upon N6MS media. Plates were placed in a dark room temperature cabinet for a week. After dark incubation, plates were transferred to a 75 deg F light chamber with light intensities of 15 to 100 µmol per meter square per second. The leaves were kept under these conditions for three to four weeks and the numbers of generated shoots were compared.



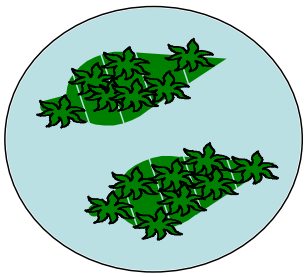
Cut leaves into 1 cm strips



Shoot gold particle with GRON into leaf



Place leaves on N6MS media and into dark cabinet 10 days



The initial N6MS media contained BAP and NAA. If plants did not generate from leaves on this media, new leaves were tested under the same condition with varying concentrations of IBA, TDZ, and BAP.

Controlled Sports Induction or CSI (general schematic above) is being performed using the regeneration system developed in the lab. Leaf segments are bombarded using a gene gun. This instrument introduces thousands of small gold particles covered in RNA/DNA hybrid oligonucleotides called GRONs (Gene Repair Oligonucleotides) into the leaf cells. The GRONs target specific genes for mutation and produce a protrusion in the leaf's genomic DNA. This protrusion tricks the plant into "repairing" its DNA, thus mutating it's own DNA in a specific manner. In the end no extraneous DNA is added into the plant's genome. Thus this is not a transgenic method and does not produce GMOs.

We have targeted the ACS genes. In addition, PPO gene responsible for flesh browning, ALS gene, a mutation in which causes herbicide resistance, TFL gene that reduces juvenility in apple have been targeted. The plants are being regenerated to identify mutants at the present stage. Several regenerants are in the process of being screened.

ADDITIONAL DEVELOPMENTS

1. Graduate Student Support: This project is being carried out by Scott Schaeffer who was a lab manager for a year and has recently enrolled into the graduate program in the Dhingra Lab. Scott is pursuing his graduate studies under the Molecular Plant Sciences Program that has been ranked 2nd in the nation recently. This proposal has been accepted for NIH Protein Biotechnology Graduate Training Program that provides Scott with 2 years of complete support for his Ph.D. work. That amounts to $ 70,000 for two years.

2. Equipment Grants: Research proposed in this project has been supported further by the procurement of a gene gun that obviates the need for radiation-based mutagenesis. The gene gun is worth $25,000.

3. Undergraduate training: Four undergraduate students have been trained in this project so far providing them with a unique opportunity to get involved in horticultural plant research.

PRESENTATION AND PUBLICATIONS

A. Invited Presentations:

1. A Dhingra: Rosaceae Micropropagation and Biotechnology. 4th Acclimatization and Establishment of Micropropagated Plants 2008. Bangalore, India December 2008

2. A Dhingra: Woody Plant Micropropagation. NNII presentation. December 2008

3. A Dhingra: Novel Approaches for improving tissue culture, micropropagation and biotechnological applications in horticultural crops. AEMP 2007 Faro, Portugal. September 2007

B. Poster Presentations:

1. S Schaeffer and A Dhingra: Perfecting the apple. Annual Washington State Horticulture Association meeting, Yakima, WA December 2008.

2. S Schaeffer, T Koepke, D Jiwan, D Druffel, D DeMars, F Ali Khan, N Tarlyn, T Yang and A Dhingra: Improving tissue culture, micropropagation and biotechnological approaches in Rosaceae Crops. 4th International Rosaceae Genomics Conference, Pucon Chile March 2008.

3. D Druffel, J Poff, C Tong, et al.: Novel approaches for improving tissue culture, micropropagation and biotechnological applications in horticultural crops. Annual Washington State Horticulture Association meeting, Wenatchee, WA December 2007.

EXECUTIVE SUMMARY AND FUTURE DIRECTIONS

The aim of the proposal was to establish a rapid and efficient method of creating controlled sports in a given variety. Several successful varieties suffer from major production or storage issues. As we establish cause and effect relationship between trait and genes with gene discovery and apple genome project, this platform will be increasingly utilized to remove the shortcoming in a variety. Mutations in ethylene genes for fruit firmness, PPO gene for reducing flesh browning, ALS gene for herbicide resistance and TFL gene for reducing juvenility are being generated. Some of these regenerants can be utilized as source of novel traits in the breeding program as well. The outcome of this exercise will be mutated, non-transgenic plants. The improved clonal cultivars can be tested commercially and used directly as this approach involves no transgenic modification. During mutagenesis (sport induction) other mutations, some deleterious, may also be generated but those can be eliminated in the segregating population. The clonal variants will also serve as defined donors or parents of desirable traits for Marker Assisted Breeding. Materials developed using this technology may offer opportunities for new intellectual property in the form of novel clonal variants. The data generated from the activities mentioned above will be leveraged to attract long-term federal funding for continued apple improvement.

Future Directions: This work is not being submitted for renewal this year. Our future aim is to utilize this platform to develop targeted mutations on a contract basis from interested groups. This project will be sustained from such a revenue source. In addition, the projects being submitted to NSF and USDA in the coming year will benefit from the infrastructure develop with support from this project.

FINAL PROJECT REPORT

Project Title: Metabolomics: characterizing fruit with chemistry

PI:	David Rudell	**Co-PI:**	Jim Mattheis
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City:	Wenatchee	**City:**	Wenatchee
State/Province/Zip	WA 98801	**State/Province/Zip:**	WA 98801

Cooperators: Dr. Maarten Hertog, Dr. Yanmin Zhu, Dr. B. Markus Lange, Dr. Bruce Whitaker

Other funding Sources

Agency Name: USDA-ARS
Amount requested or awarded:
Notes: ARS provides a permanent, full-time GS-9 Biological Sciences Technician and analytical instrumentation.

Total Project Funding: $47,520

Budget History:

Item	**2008**		
Salaries	0		
Benefits	0		
Wages[1]	23,000		
Benefits	10,000		
Equipment	0		
Supplies[2]	5000		
Travel	0		
Miscellaneous[3]	9520		
Total	**47,520**		

[1] Time-slip employees
[2]Consumables, analytical standards
[3]Intsrument maintenance and repair

Objectives:

1. Characterize metabolites (phytochemicals) (500+ expected) that are detectable in apple fruit.
2. Generate an analytical library to rapidly identify apple metabolites (phytochemicals).
3. Apply the analytical library to characterize how storage duration, 1-MCP, and diphenylamine impact apple fruit metabolites (phytochemicals).

Significant Findings:

Objectives 1 and 2.

1. 600+ apple peel phytochemicals were characterized including many that contribute to quality aspects including aroma, sweetness, sourness, nutritive value, color, and physiological disorders.
2. Chemical identifiers for peel phytochemicals characterized in the study were compiled in an analytical library to reference in future research.

Objective 3.

1. Changes in apple peel chemistry resulted from storage initiation, storage duration, and 1-MCP or DPA treatment.
2. 1-MCP treatment slowed or prevented changes in peel chemistry.
3. Peel from DPA treated apples contained more typical flavor phytochemicals, ethylene precursors, and novel putative antioxidants compared to untreated controls.
4. Differences in peel chemistry between controls and DPA-treated fruit were detectable 8 weeks and differences in some individual phytochemicals detectable 10 weeks prior to the onset of visible scald suggesting these phytochemicals may be useful for early scald diagnostic tests.
5. Changes in peel chemistry resulting from biochemical stress that leads to scald were far more extensive than previously expected.

Results and Discussion:

Biochemical characterization and library construction

Chemical profiling of apple peel uncovered a very broad collection (600+) of known, newly discovered (in apples), and unknown metabolites. To evaluate a more complete set of phytochemicals, three different analysis procedures were employed (Fig. 1). Phytochemicals found and routinely measured included volatiles (apple aroma), sugars, acids, amino acids, pigments, vitamins, antioxidants, and compounds involved in energy storage, signal transduction, and building other phytochemicals. The results provided a more complete picture of changes in peel chemistry occurring during storage compared to previous work focusing on a few phytochemicals or groups of phytochemicals.

An objective of the current project was generation of apple phytochemical libraries using specialized software (AMDIS, NIST05, Chemstation) that "cleans up" analytical data for each metabolite and stores it in a format that can used for future research. These libraries contain reference information from all of the phytochemicals characterized in this study. Progress was made laying some of the groundwork for compiling an on-line metabolic library using the data generated in this study for apples and other Rosaceous crops in Genomic Database for Rosaceae in cooperation with Dr. Dorrie Main. This information may aid biochemical and genetic comparisons to develop breeding strategies as well as fruit quality and storability research.

Broad or "comprehensive" phytochemical evaluation of fruit provides a means for understanding some of the changes occurring in response to production and postharvest practices that lead to desirable or undesirable characteristics in our fruit. Using these extensive analytical techniques leads to a better understanding of storage-related apple fruit biochemistry that is expected to be useful for developing new postharvest tools, or suggesting new areas of fruit chemistry that are related to edible quality or disorders. Developing comprehensive phytochemical evaluation

techniques and libraries, something that has not been previously accomplished in apple, pear, or cherry, is crucial to the work performed in this and later studies that generate new information related to apple storage and superficial scald.

DPA, 1-MCP, and storage duration alter apple peel chemistry

Comprehensive phytochemical evaluation techniques proved useful for distinguishing different treatments and effects of storage duration in 'Granny Smith' apples treated with 2000 ppm DPA, 1 ppm 1-MCP, or washed with water (untreated) and stored for up to 6 months in air at 33 ºF. Peel was sampled at 0, 1, 2, and 4 weeks and 2, 3, 4, and 6 months. The apple peel chemistry of 1-MCP treated peel was immediately different from those of the control and DPA treated fruit (Fig. 2). 1-MCP treated peel was most similar to that of pre-storage fruit, although changes were still considerable. The peel chemistry of the control and DPA treated fruit were different around 4 weeks and increasingly so at longer storage durations.

1-MCP, but not DPA, reduced ripening. Phytochemicals associated with the ethylene pathway and non-ethylene volatiles were strongly associated with longer storage durations and less so in the 1-MCP treated fruit. Conversely, less ripe fruit had more malic acid (responsible for sourness), certain amino acids, volatiles associated with "green" fruit and many other phytochemicals. Unlike levels of other amino acids, isoleucine, a building block of an important class of volatiles associated with ripe apples, increased with volatile production and ripening demonstrating the utility of our method for looking at many aspects of phytochemical production simultaneously instead of just the end products.

Comprehensive phytochemical profiling demonstrated its worth as a tool for providing a more complete picture of chemical changes occurring during apple storage than would be gained using more directed techniques. While control and DPA treated fruit may have not physically looked different in any way at 1-2 months, phytochemical differences were present well before visible symptoms of scald were observed. Following a broad array of phytochemicals rather than just volatiles or sugars and acids provides new areas to explore that may potentially lead to new tools for monitoring apple condition and storability much like chemical profiling tools commonly employed for monitoring human health.

Superficial scald and the apple peel chemistry

Clear differences in peel chemistry between control and DPA treated fruit were observed following 1 month of storage and the patterns continued to diverge until 6 months (Fig. 2). Scald incidence (rated 1-4; 1 = 0%, 2 = less than 25%, 3 = less than 50%, and 4 = greater than 50%) was also rated at all sampling periods (Fig. 3). Slight scald symptoms appeared at 3 months storage, approximately 2 months following detection of differences in apple peel chemistry. In a parallel experiment, 'Granny Smith' apples were treated with 2000 ppm DPA at harvest or following 1, 2, or 4 weeks or 2 months cold storage to determine when DPA treatment became ineffective. Delayed treatments after 1 or 2 weeks were partially effective, while treatment after 4 weeks, the point where peel chemistry began to differ, was not (Fig. 2). This suggests that a significant amount of the biochemical events that lead to scald development occurred prior to 4 weeks storage. In effect, 2-4 weeks was the "point of no return" for scald development.

Statistical data mining techniques identified phytochemicals most associated with the different phases of scald inception and development. Major groups of phytochemicals were associated with untreated (scalded peel or peel that would develop scald) or DPA treated (unscalded) fruit. The presence of many phytochemicals that increased in peel from untreated fruit appeared to result from biochemical stress that changes chemical structure and, potentially, function. Likewise, compounds associated with DPA treated fruit decreased in untreated peel during storage. While typical volatiles increased in DPA treated apples, different volatile phytochemicals, that may affect flavor, were produced in scalded tissue. Other compounds with antioxidant activity were more

prevalent in DPA treated fruit as storage duration increased indicating their depletion in the control fruit may be related to oxidative biochemical stress.

Individual phytochemicals that changed prior to actual symptom development were uncovered. These groups included more than 51 phytochemicals whose fluctuations could potentially be used to diagnose peel that will develop scald up to 10 weeks prior to appearance of the symptoms. Differences between individual scald-associated phytochemicals in control and DPA treated peel increased or decreased and other phytochemicals continued to appear up to 6 months storage.

In a parallel experiment, the wax layer, cuticle layer, and live epidermal and hypodermal cells were analyzed separately to localize phytochemicals within the entire peel structure. Many phytochemicals associated with scald-induction are in live epidermal and hypodermal cells, where changes would more likely lead directly to tissue death and browning. Given the nature of these phytochemicals, associations with crucial tissue functions such as structural integrity and cellular communications are possible.

Conclusions and future directions

Comprehensive profiling of 600+ apple peel phytochemicals proved a valuable tool for discovering key changes in apples affecting quality and storability. By understanding these changes, it may be possible to develop tools that predict quality changes before they occur and reduce losses during storage or marketing.

Further research examining factors influencing scald development (harvest maturity, cultivars, storage environments) are needed to validate which phytochemicals are effective markers. Similarly, identification of changes in apple peel chemistry that cause scald will identify phytochemicals and genes that could also be used as benchmarks for breeding new cultivars that are not susceptible to scald.

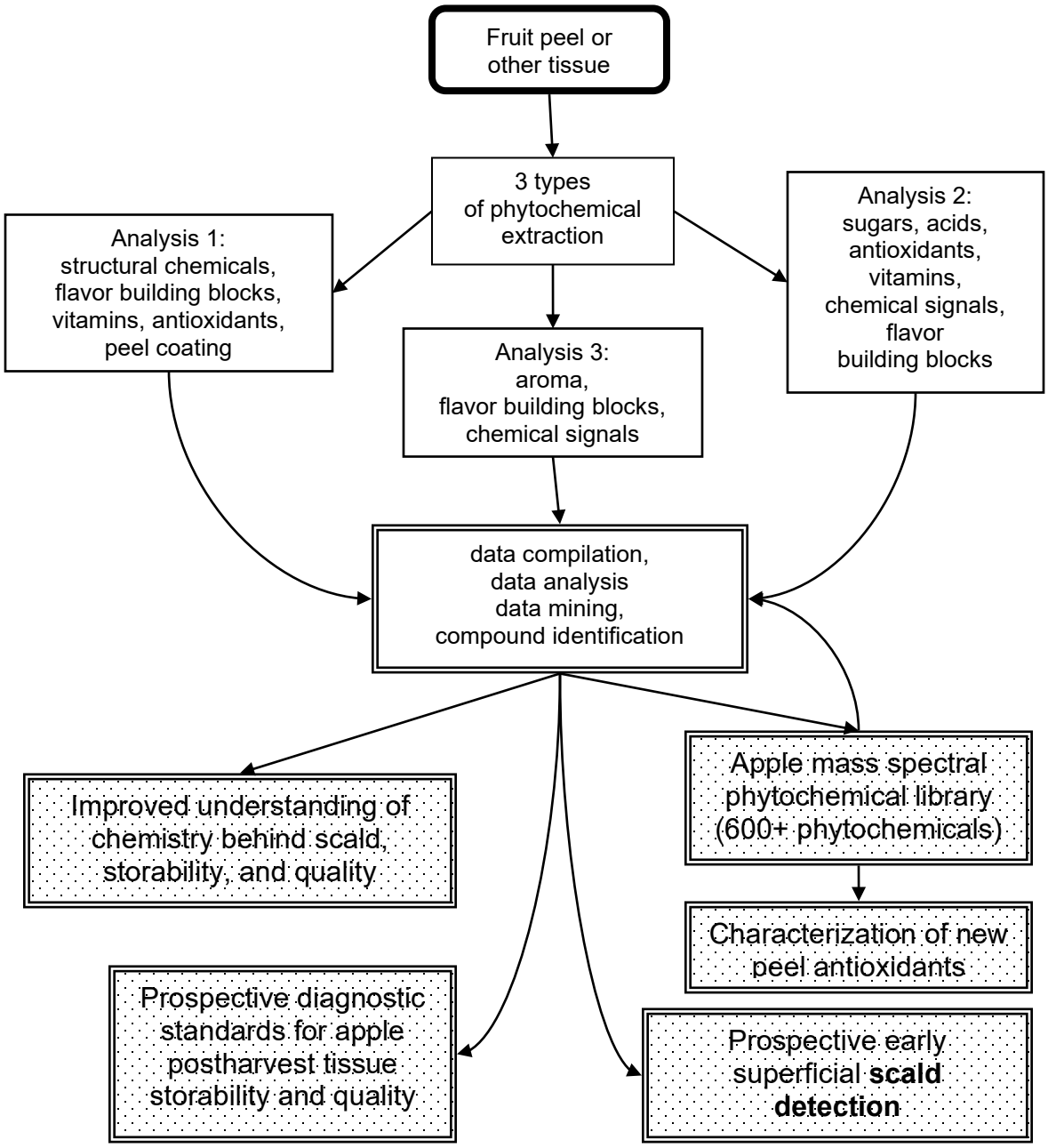

Fig. 1. Peel sample processing, sample analysis, data compilation/analysis, and experimental outcomes flow chart.

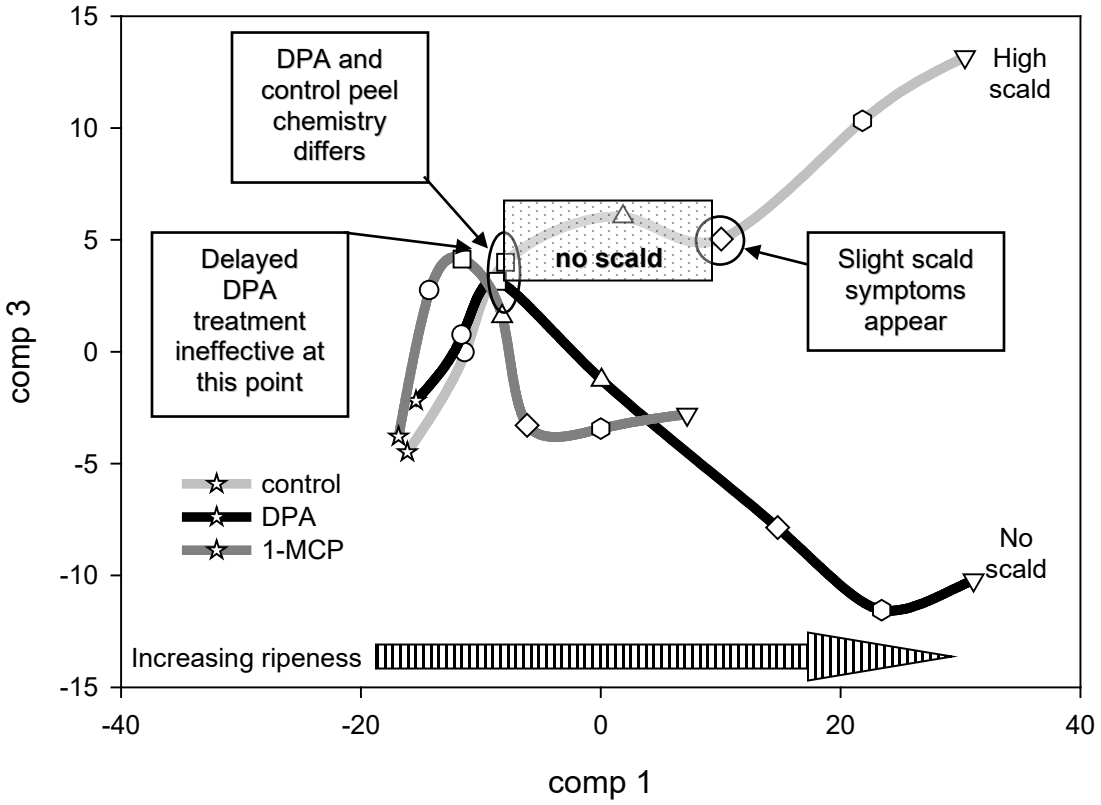

Fig. 2. Graph (PCA scores plot) reflecting total peel chemistry changes with respect to relative ripeness and scald development. After harvest apples were treated with 1 ppm 1-MCP, 2000 ppm DPA, or untreated. 600+ phytochemicals were evaluated in peel sampled from each treatment at 1 week (**star**), 2 weeks (**circle**), 4 weeks (**square**), 2 months (**triangle**), 3 months (**diamond**), 4 months (**hexagon**), and 6 months (**inverted diamond**) of 33°F air storage.

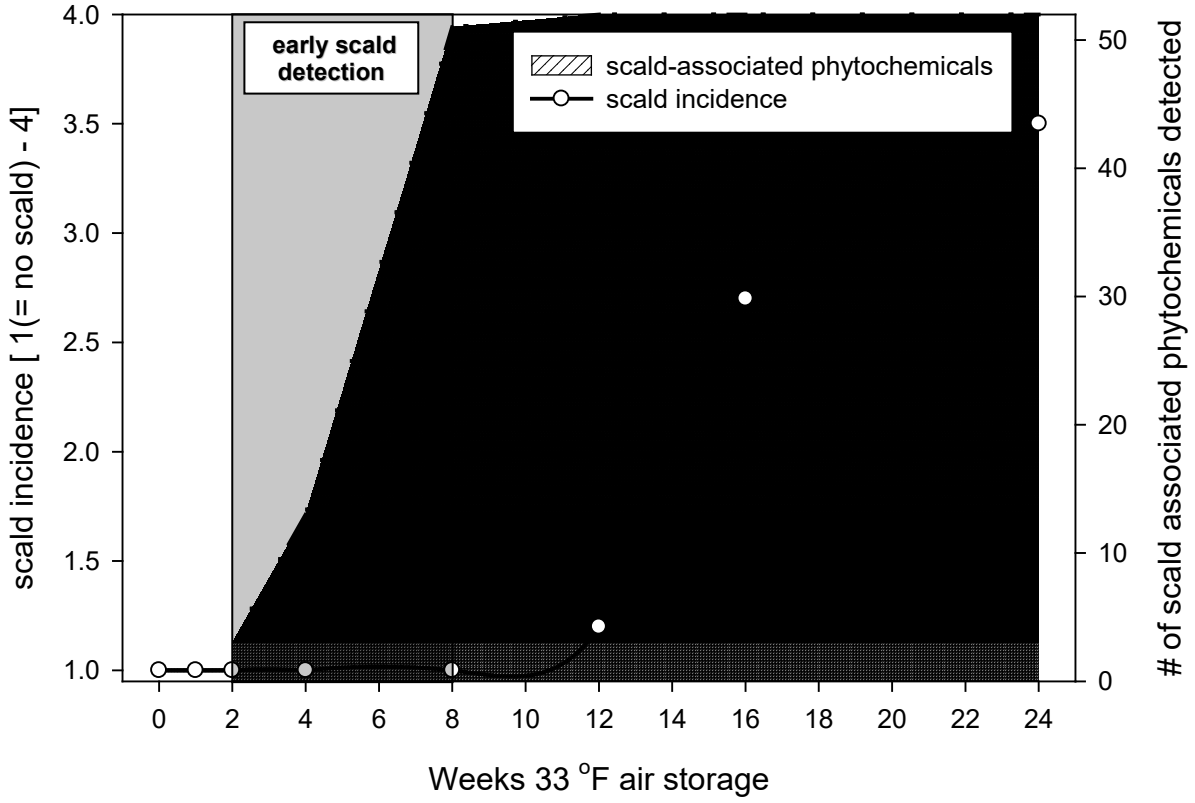

Fig. 3. Number of prospective early scald diagnostic markers compared to actual scald incidence. Differences in some prospective scald markers were found as early as 2 weeks after harvest increasing to 51 at 8 weeks of storage. Scald symptoms were slight at 12 weeks storage.

Metabolomics: Characterizing Fruit with Chemistry (*Rudell*)

Executive Summary

Project outcomes:

1. Prospective diagnostic markers for early superficial scald detection.
2. An apple fruit mass spectral phytochemical library containing descriptions of 600+ individual phytochemicals.
3. Partial characterization or identification of novel putative antioxidants and various flavor and quality related phytochemicals.
4. Improved understanding of the peel chemistry behind superficial scald, storability, and quality to direct and expedite future research.

Significant Findings:

1. 600+ apple peel phytochemicals were characterized including many that contribute to quality including aroma, sweetness, sourness, nutritive value, color, and physiological disorders
2. Chemical identifiers for peel phytochemicals characterized in the study were compiled in an analytical library to reference in future research.
3. Changes in apple peel chemistry resulted from storage initiation, storage duration, and 1-MCP or DPA treatment.
4. 1-MCP treatment slowed or prevented changes in peel chemistry.
5. Peel from DPA treated apples contained more typical flavor phytochemicals, ethylene precursors, and novel putative antioxidants compared to untreated controls.
6. Differences in peel chemistry between controls and DPA-treated fruit were detectable 8 weeks and differences in some individual phytochemicals detectable 10 weeks prior to the onset of visible scald suggesting these phytochemicals may be useful for early scald diagnostic tests.
7. Changes in peel chemistry resulting from biochemical stress that leads to scald were far more extensive than previously expected.

Future directions:

1. Continue to find peel phytochemicals that link scald to cultivar, harvest maturity, CA storage, and other factors.
2. Continue to identify phytochemicals that are important to fruit maturation, ripening, and scald.
3. Identify phytochemical/gene associations that trigger or control scald for use as harvest/storage management and breeding selection tools.
4. Evaluate prospective early superficial scald diagnostic markers for use as storage management tools.
5. Use apple peel chemistry to increase the understanding of scald and related disorders to find pre-harvest predictive tests.

FINAL PROJECT REPORT

Project Title: Establishing trait – gene relationships and gene discovery in apples

PI:	Amit Dhingra	**Co-PI(2):**	Bruce Barritt
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Cooperators: None

Total project funding request: **Year 1:** 21,690 **Year 2:** 21,690 **Year 3:** $0

Other Funding Sources - none

Total Project Funding: 43,380

Budget History

Item	2007	2008	2009
Salaries			
Benefits			
Wages	6,000	6,000	
Benefits	690	690	
Equipment			
Supplies	11,000	11,000	
Travel	2,000	2,000	
Sequencing	2,000	2,000	
Miscellaneous			
Total	21,690	21,690	

Footnotes:

OBJECTIVES

Empirical knowledge of desirable trait-gene relationships is vital for effective marker assisted apple breeding and as a target for rapid crop improvement via controlled sport induction. With this basic guiding principle, the specific objectives as outlined in the proposal were:

1. *Prioritization of a subset of apple traits of greatest economic and immediate importance*.

2. *Identification and grouping of contrasting genotypes to be used for the study based on available knowledge in the breeding program*.

3. Perform side-by-side expressed genomic comparisons using a method termed Differential Display (DD).

SIGNIFICANT FINDINGS

1. **Trait prioritization**: Three traits critical to fruit quality, crispiness, juiciness and firmness were selected by the "think-tank" that comprises of industry group, Bruce Barritt (current apple breeder) genomics researchers and Fred Bliss (Consultant to WTFRC). All subsequent objectives and activities are based on improving these traits.

2. **Genotype identification**: HoneyCrisp has been designated as the reference genotype for the WA apple-breeding program recently. However, we had selected this genotype two years ago to identify important genes for desirable traits. In order to perform gene discovery experiments for the traits listed in objective 1 samples were collected from the following genotypes: HoneyCrisp (crisp not firm) and Golden Delicious (mealy, no texture, no firmness, disintegration of character, soft). The rationale behind selecting these genotypes is that this group represents the two extremes in fruit characters and presents the phenotypic differences needed for our investigations. Two sets of tissues have been harvested for each genotype. The peel and the cortex represent contrasting sites of action physiologically. Thus we have taken very thin peel and cork bored cortex samples for our experiments.

3. **Identification of differentially expressed genes**: So far we have identified **114** differentially expressed genes in comparative experiments performed with Honeycrisp and Golden Delicious varieties using Differential Display.

RESULTS AND DISCUSSION

Identification of traits or phenotype (crispiness, juiciness and firmness) that define two contrasting genotypes (HoneyCrisp and Golden Delicious) set the stage for precise identification of genes involved in imparting these traits. Instead of utilizing breeding populations (genetics), developing fruit from these two genotypes was selected for our work that directly looks into the tissues for important genes. It is one of the many approaches for gene identification and relies on gene function rather than gene inheritance. The method used here is called differential display and it utilizes RNA (Ribonucleic acid) that is the functional output of the DNA in the genes. RNA by nature is very labile and standard RNA isolation methods are very unsuitable to extract best quality RNA. In our program we have now established a streamlined and efficient method that yields high quality RNA vital for the success of these experiments.

Sterilized peel and core samples were obtained from HoneyCrisp and Golden Delicious apples that were either collected biweekly at the Tukey Orchard in Pullman, WA (figure 1).

Samples were immediately frozen in liquid nitrogen after collection and stored at -80 C to limit degradation of RNA or any major changes in gene expression. Peel and core samples were ground in the Spex SamplePrep 6870 freezer mill by using three, four-minute grinding cycles at 15 impacts per second. This mill has enhanced RNA recovery by over 30 times compared to conventional methods. High quality RNA was isolated from the ground tissue using a Qiagen RNeasy Plant Mini Kit following the kits protocol with a few minor alterations. RNA was eluted from the column with DEPC treated water and stored at -20 C. RNA quantity was measured on a highly accurate Nanodrop spectrophotometer and the quality was determined by testing a small amount of RNA on a agarose gel.

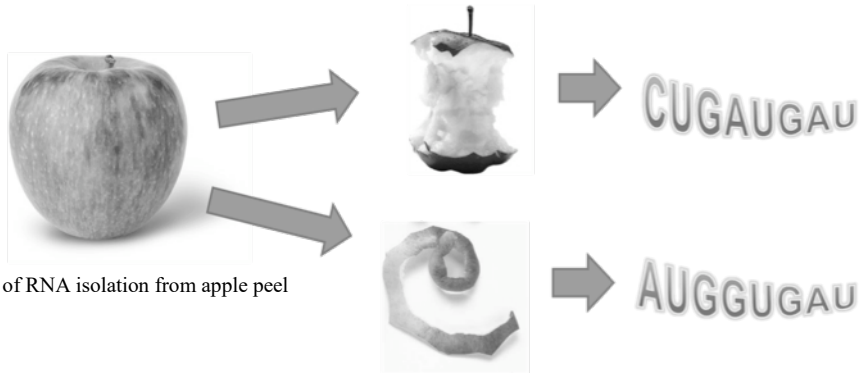

Figure 1. Schematic of RNA isolation from apple peel and core samples.

Out of 240 different possible combinations, differential display was performed using 8 primer combinations. Processed RNA samples were visualized for differential gene expression as labeled in Figure 2. These differentially expressed genes were directly picked out of the gel using a razorblade and stored at -20 C. Samples were boiled in 40 µl of water for 10 minutes to remove DNA from the gel and bring into solution.

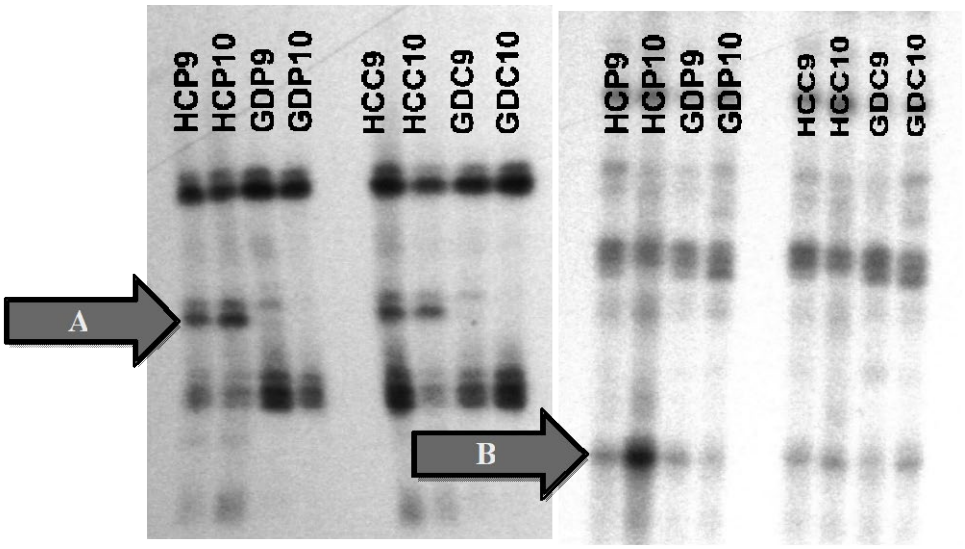

Figure 2: Differential display gel of Honeycrisp and Golden Delicious peel and core RNA samples. HC-Honeycrisp; GD-Golden Delicious; P-Peel; C-Core; 9-September; 10-October. "A" displays a gene expressed in Honeycrisp peel and core but not in Golden Delicious peel and core. "B" displays a gene upregulated in Honeycrisp Peel, but not in other tissues.

From the results reported here it is clear that we can quickly identify important genes that are related to any given phenotype. We have identified **114** differentially expressed genes in comparative experiments performed with HoneyCrisp and Golden Delicious varieties using Differential Display with only 8 combinations of primers (tools to fish out genes) out of 240 possible ones. There will of course be redundancy of up to 30% if all 240-primer combinations are used. However, such an experiment can potentially take up to 3 years of constant gel running and enormous amount of resource input. Fortunately, we have been able to leverage $650,000 equipment that can process multiple samples in its entirety in a matter of a week and at the cost of about $1500 per sample. We have analyzed some samples using the next-generations sequencing method and have some useful datasets that are being analyzed currently.

Here is a brief breakdown of the 114 differentially expressed genes:

- o These genes represent either genes unique to HoneyCrisp or Golden Delicious along with genes that vary in expression in one variety and not the other.
- o 34 potentially interesting genes have been directly taken out of the gel and amplified from the experiment.
- o 14 genes have been captured and are being sequenced to analyze the DNA sequence to relate them with the observed phenotypic differences between HoneyCrisp and Golden Delicious.

ADDITIONAL DEVELOPMENTS

Leveraged Funding:

1. Graduate Student Support: This project is being carried out by Scott Schaeffer who was a lab manager for a year and has recently enrolled into the graduate program in the Dhingra Lab. Scott is pursuing his graduate studies under the Molecular Plant Sciences Program that has been ranked 2nd in the nation recently. This proposal has been accepted for NIH Protein Biotechnology Graduate Training Program that provides Scott with 2 years of complete support for his Ph.D. work. That amounts to $ 70,000 for two years.

2. Travel Support for Scott Schaeffer: This research was featured at the International conference organized by ASPB in Merida Mexico. Scott Schaeffer received a travel grant from the society to attend the conference.

2. Equipment Grants: We have been able to leverage over $ 700,000 in equipment funds from the college and the department to enable genomics-related experiments that will directly benefit this project and increase the impact of investments made by WTFRC. Equipment includes next-generation Genome Sequencer, a high sensitivity spectrophotometer to accurately measure RNA and DNA, a freezer mill to grind hard tissue like the bud spurs and Bioanalyzer for RNA quality control.

PRESENTATIONS AND PUBLICATIONS

A. Invited Presentations

1. A. Dhingra: Apple Genome Project and transcriptomics for a better apple. Inauguration ceremony of the University of Guelph post-harvest facility. Guelph, Ontario, Canada November 2008.
2. A. Dhingra: Apple Varieties and the Role of Genomics Research, USApple Outlook Conference, Chicago, August 2007

B. Poster Presentations

1. S Schaeffer and A Dhingra: Perfecting the apple. Annual Washington State Horticulture Association meeting, Yakima, WA December 2008.
2. S Schaeffer, S Tariq, T Magrath and A Dhingra: Unraveling the fruit development program in apple. American Society of Plant Biologists Annual Conference, Merida, Mexico July 2008 – Travel grant for Scott Schaeffer.

EXECUTIVE SUMMARY AND FUTURE DIRECTIONS

Directed and efficient apple improvement is possible if we understand the relationship between desirable traits and the underlying gene or genes. There are two major ways of establishing this very important relationship. One is based on studying inheritance of the gene in breeding populations and the other relatively newer but precise method is to study the function of individual genes in already identified genotypes that possess the desirable traits. Both approaches are equally important to make rapid progress. In other crop improvement programs rapid progress has been made with the infusion of functional genomics as in case of rice, wheat, corn and soybean and even horticultural crops like apple as is being done at HortResearch in NZ. Our program focuses on functional genomics a field of genomics that deals with gene function. We can rapidly delve into the pertinent tissues (like peel and core of apple) of contrasting genotypes to identify important genes and catch them in action. Our advantage is having access to world-class market-ready genotypic material grown right here in WA and technological infrastructure that is unparalleled compared to other genomics programs around the globe. Several of the differentially expressed genes in a developing fruit are expected to be involved in any number of biological processes that not only impart crispiness, juiciness and firmness but also involved in production of amino acids, vitamins and other volatile compounds. The latter compounds have multiple functions in determining fruit aroma, resistance to pests and pathogens, shelf life and many such traits. Thus, the data generated with support from this project has far-reaching implications in terms of developing a unique knowledge infrastructure.

Two apples may have the same genes but the difference lies in their regulatory regions. In that sense a gene is similar to any car. The sitting area may be the same but the cars differ in type of engine and brakes. The engine and brakes in a gene are called its regulatory regions. As we are completing the apple genome, we will have direct access to the regulatory regions to not only look at the gene but also have a holistic view of why and how a variety is different from one another. Importantly, this knowledge is expected to increase the precision of how we improve apple in WA. These genes are also going to be the targets for controlled sports induction that is also being carried out in our program. In addition the transgenic systems for apple in the program will enable functional testing of the genes rapidly. This activity is expected to generate intellectual property and our programs will be the first ones to utilize this knowledge for generating apple varieties via the breeding program. Note: Transgenic methods are not for variety development but only for research purposes. Overall, the identification of genes responsible for Pacific Northwest apple traits is necessary for the local economy to remain competitive in the world market place. As a juggernaut in the apple industry, we can use this information to improve the quality and attractiveness of our products. New apple varieties could be developed tailored to the Northwest needs by selecting plants expressing the genes necessary for a given trait.

Future Directions: The data generated from this proposal is being included as preliminary information for a proposal that is being resubmitted to National Science Foundation. The project deals with peel-specific genes that participate in aromatic amino acid and vitamin biosynthesis in apple. Since we have been successful at utilizing this platform for gene identification, we have submitted a one-year proposal to extend these investigations to post-harvest conditions. Apple peel and core samples will be collected from WA and University of Guelph post-harvest stored-apples and the information is expected to provide an insight into the continuum of post-harvest storage of apple varieties. Importantly, it will provide insight into how we can connect post-harvest fit with genes thus including this information in designing apple for the market.

FINAL PROJECT REPORT

Project Title: Functional genomics of flowering in apple

PI:	Herb Aldwinckle	**Co-PI(2):**	Steve VanNocker
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Address:	630 W. North Street	**Address:**	A390C Plant & Soil Sciences
City:	Geneva	**City:**	East Lansing
State/Province/Zip	NY 14456	**State/Province/Zip:**	MI 48824
Cooperators:	M John Bukovac, Michigan State University		

Other funding Sources

Agency Name: MSU Agricultural Experiment Station /Project GREEEN
Amount requested or awarded: 30,000

Total project funding: $96,319

Budget History:
Organization Name: Cornell University

Item	2007	2008	
Salaries	14,500	15,370	
Benefits	7,434	8,034	
Wages			
Benefits			
Equipment			
Supplies	3,000	1,000	
Travel			
Miscellaneous			
Total	24,934	24,404	

Footnotes: The salary and benefits were for a technician to work 50% time on transferring silencing constructs into apple. Supplies are for tissue culture, chemicals, enzymes, plastic ware, and potting supplies.

Budget 2:
Organization Name: Michigan State University

Item	2007	2008	
Salaries	12,854	13,240	
Benefits	885	912	
Wages			
Benefits			
Equipment			
Supplies	5,000	3,390	
Travel	600	600	
Miscellaneous	9,500		
Total	28,839	18,142	

The salary and benefits requested were for 1/2 grad student for first two years. Note that for the grad student, this amount includes tuition/fees ($4215) and stipend ($8640). Supplies were for molecular studies, and include enzymes, primers, and reagents. Expenses for travel were for one trip/year in years 1 and 2 from Michigan to New York.
Miscellaneous costs were for DNA sequencing of genes related to flowering.

Objectives:

1. Genomic census of *FT/TFL* gene family members in apple. We will identify all possible *FT/TFL* gene family members in apple, and determine their DNA sequence. As mentioned above, from previous studies we know that at least five exist in apple. This step is important, because additional genes may exist with even more important functions.

2. Gene expression atlas of the apple *FT/TFL* genes. We will extensively analyze the activity pattern of all *FT/TFL* genes identified through Objective 1, concentrating on flowering. This analysis will include expression in various parts and organs of the plant, changes in expression in response to phytohormones, effects of biotic and abiotic stresses on expression, and temporal control of expression during development (e.g. flower induction). The goal of this approach is to identify those members of the *FT/TFL* family that have the most important role in flowering.

3. Functional analysis. The best way to unambiguously determine the function of a gene is to examine the phenotypic consequences of loss of that gene's activity. In other words, how does flowering occur without that gene? In apple, genes can be repressed through a technique called RNA interference (RNAi), which PI Aldwinckle has several years' experience with other apple genes. We will examine the effect on flowering of suppression of the most important genes identified in Objective 1 and 2.

Summary of Significant Findings:

1. Genomic census of *FT/TFL* gene family members in apple.

- We used a novel molecular technique to isolate and sequence genomic DNAs corresponding to developmentally regulated genes from flower bud and leaf tissues (S Park, S Oh, S Mookerjee and S van Nocker, manuscript in preparation). This resulted in the acquisition of ~108,000 (flower bud) and ~226,000 (leaf) new apple genomic sequences representing ~28 million nucleotides of the apple genome and corresponding to nearly all apple genes previously identified by expressed sequence tag (EST) analysis (accession: http://vannocke.hrt.msu.edu/public/0822.fna; http://vannocke.hrt.msu.edu/public/1120.fna). Additional funding for this objective (one month postdoc salary equivalent) was leveraged through Michigan State University. This effort provided the genomic sequence for *FT/TFL* family members that was required to design gene-specific primers used in Objective 2 and construct the plant transformation vectors utilized in Objective 3. Based on this sequence we constructed a revised phylogenetic tree representing the apple *TFL/FT* gene family (Fig. 1).

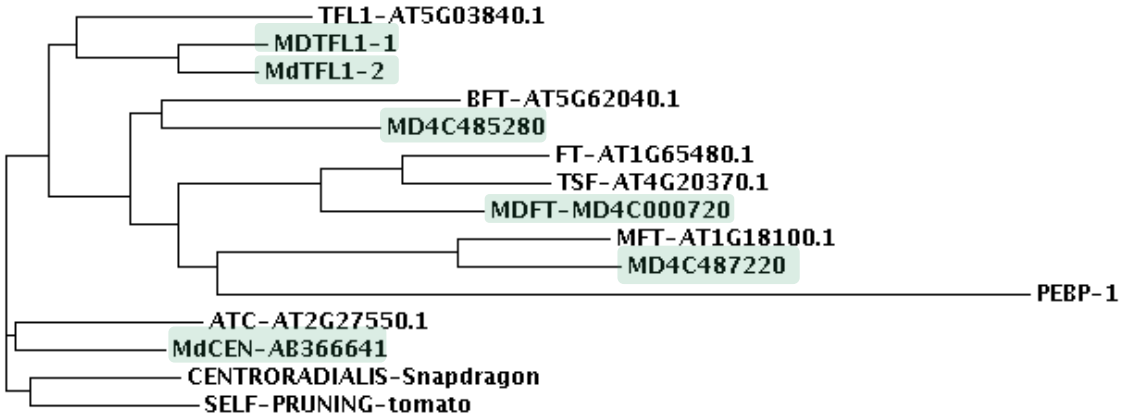

Fig 1. Phylogenetic tree representing the *TFL1*/*FT* gene family from Arabidopsis, along with the homologs from apple, snapdragon, tomato, and human.

2. Gene expression atlas of the apple *FT/TFL* genes.

- *MdTFL1-1* and *MdTFL1-2* expression was detected only in the root, stem and apex of seedlings. *MdFT* expression was detected in the root, stem, apex and leaf of seedlings, and also in the flower bud and fruit. *MdBFT* was expressed in the root and stem of the seedling, and also in developing fruit. *MdMFT* was expressed most strongly in the seed (Fig. 2A). *MdCEN* had a strong level of expression in the root but was also detected in the vegetative tissues (stem, apex, and leaf) (Fig. 2B) (See Appendix A for sample information).

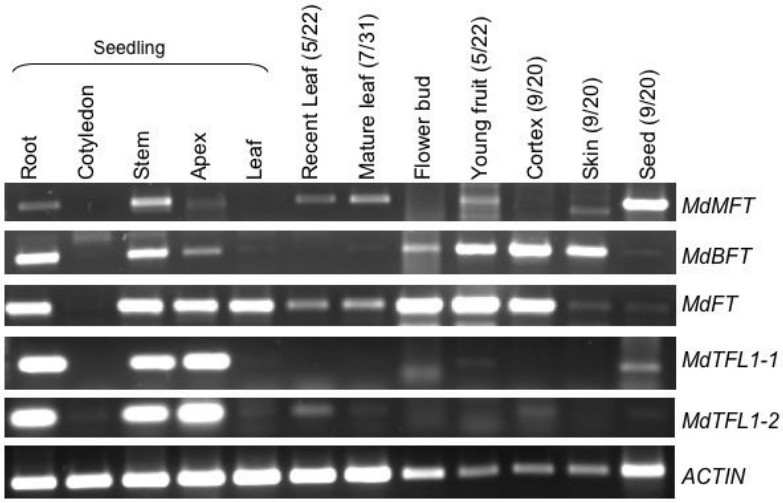

Fig 2A. Expression patterns for *MdTFL1-1*, *MdTFL1-2*, *MdFT*, *MdMFT*, and *MdBFT* in various parts of apple.

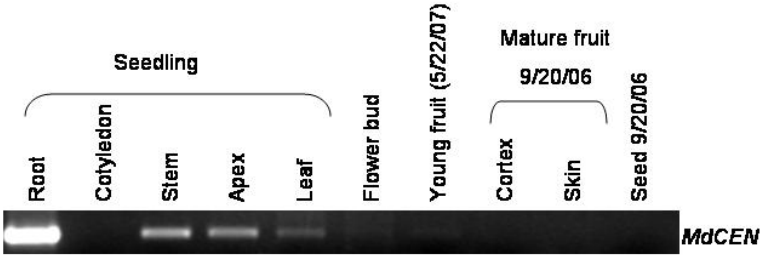

Fig 2B. Expression pattern of *MdCEN* in various apple tissues and parts.

- We then analyzed expression of *FT/TFL1* gene family members in various parts and organs of the mature flower (Fig 3). Although *MdMFT* expression was not detected in immature flowers (Fig. 2A) it was found in the style at the time of anthesis. *MdBFT* expression was mainly localized to the flower pedicel. *MdFT* was detected in all parts of the flower analyzed (Fig. 3).

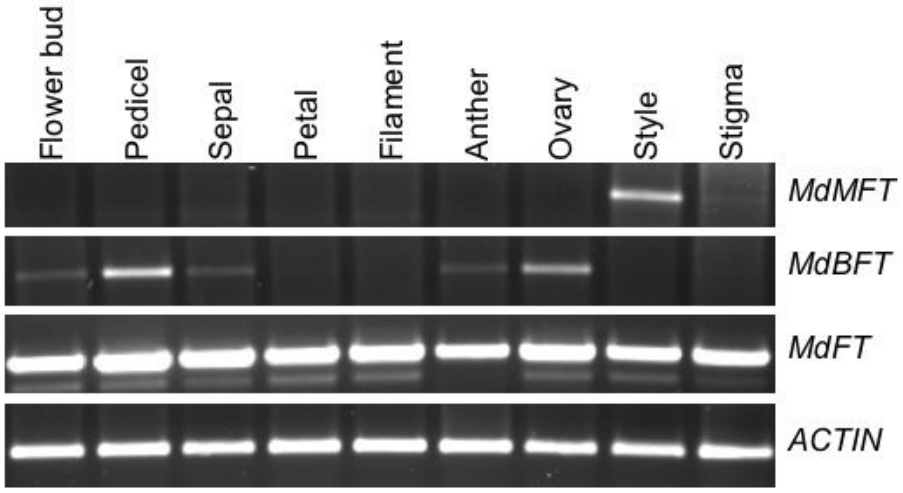

Fig 3. Expression pattern of *MdMFT*, *MdBFT*, and *MdFT* in different parts of the mature flower.

- We also analyzed expression of those genes expressed in the fruit (*MdFT* and *MdBFT*; Fig. 2A) at various stages of fruit development and in various parts of the fruit (Fig 4). *MdBFT* was expressed in all parts of the fruit and in all developmental stages tested, with relatively higher expression in the cortex. *MdFT* was detected in both the fruit skin and cortex in the early stages and only in the cortex in the later stages of development.

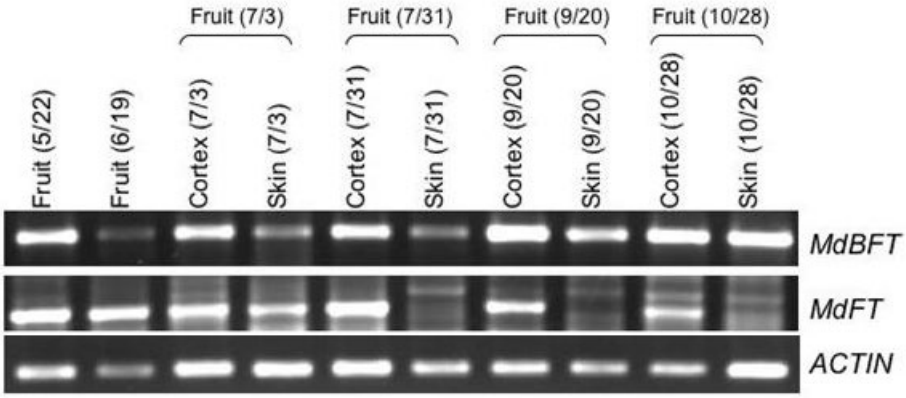

Fig 4. Expression of *MdBFT* and *MdFT* in apple fruit tissue at different stages of development.

- Because *MdMFT* and *MdTFL1-1* were detected in seed tissues (Fig 2A), we analyzed expression of these genes at different stages of seed development (Fig 5). *MdMFT* expression was strongest in seeds in green fruit, but was detected at very early stage and in ripe fruit. *MdTFL1-1* was expressed almost exclusively at an early developmental stage (Fig. 5).

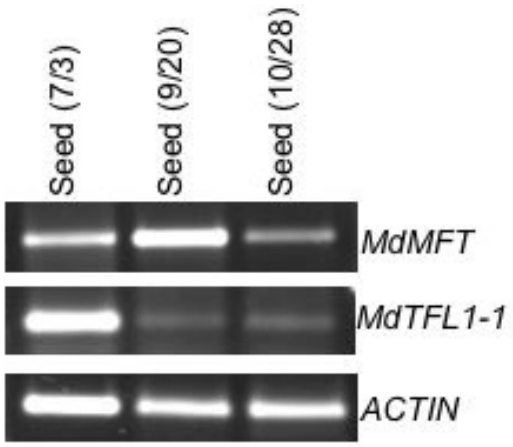

Fig 5. Expression of *MdMFT* and *MdTFL1-1* in different stages of seed development.

- Finally, we analyzed the expression of the two apple *TFL1* homologs (*MdTFL1-1* and *MdTFL1-2*) in flowering-committed shoot apices during floral initiation and development (Fig. 6). Interestingly, *MdTFL1-1* was expressed at increasingly levels during the period of floral initiation, with strongest expression at weeks 12 to 14 AFB concomitant with strong expression of the flowering initiator gene *AFL1* (the apple homology of *LFY)*. After this point, it was silenced, with expression reestablished at week 20 and beyond. *MdTFL1-2* was expressed constitutively throughout the season (Fig. 6).

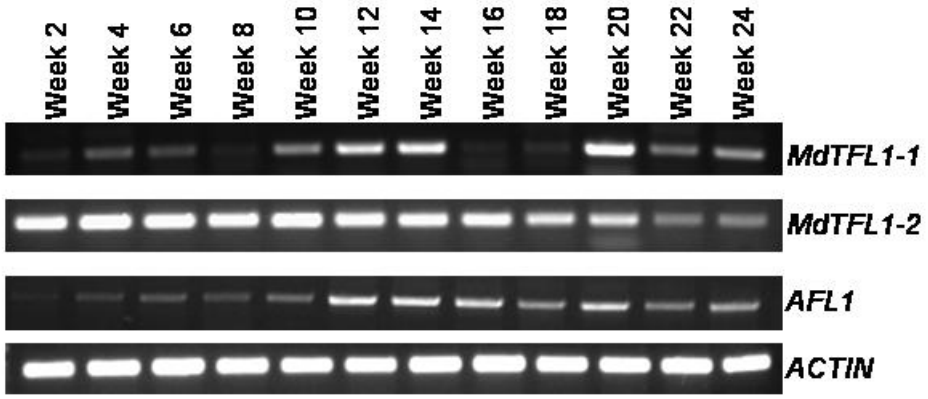

Fig 6. Expression patterns of *MdTFL1-1*, *MdTFL1-2*, and *AFL1* in 'Gala' buds collected from week 2-24 after full bloom.

3. Functional analysis

- **Artificial miRNA (amiRNA) technology was used to design amiRNAs targeting *MdTFL1-1*, *MdTFL1-2*, or both *MdTFL1-1* and *1-2*, while minimizing the risk of suppressing off-targets. This means that these genes can be silenced individually and their distict roles dissected. Initial transformation experiments with the amiRNA silencing constructs for TFL1-2 were done in Aldwinckle's lab, and transformed plants are now being selected.** Transformation experiments with 6 amiRNA silencing constructs for *MdTFL1-1*, *MdTFL1-2*, or both *MdTFL1-1* and *1-2*, were done in Aldwinckle's lab, and multiple transformed plants of Gala variety were produced (Table 2).

Table 2. Transformed lines of Gala apple obtained with 6 amiRNA silencing constructs for *MdTFL1* genes

Silencing Construct	# transformed lines of Gala
TFLa::2	35
TFLa::10	22
TFLa::21	10
TFLa::51	25
TFLa::55	10

The transformed lines have been confirmed as transformed by PCR. They have been propagated in tissue culture and observed for morphological differences from non-transformed Gala. This far no differences have been observed. Selected lines will be further propagated and grown as own-rooted or grafted plants to determine whether flowering time is affected by silencing the two genes.

Recent results from other labs have shown limited response by Gala to silencing of TFL1, but earlier resonse in other varieties. Therefore we have now done transformation experiments with the amiRNA silencing constructs on M.26 apple rootstock, which is also readily transformed (Table 3).

Table 3. Putatively transformed lines of M.26 apple rootstock obtained with 3 amiRNA silencing constructs for *MdTFL1* genes

Silencing Construct	# regenerated lines of M.26
TFLa::2	49
TFLa::10	20
TFLa::21	regenerants now appearing

Since we get very few escapes among the regenerants on the highy selective regeneration medium, most of the regenerated lines are very probably transformed with the silencing constructs. The regenerated shoots are small (2-10 mm) at this time, and too young to exhibit possible morphological differences. They will be grown and propagated in tissue culture and subsequently grown as plants for observation of any alteration in flowering onset. Experiments will also be done with the other 3 amiRNA silencing constructs on M.26.

Discussion:

Genomic census of *FT/TFL* gene family members in apple.

We used a novel gene identification technique that appears to be very efficient in identifying gene regions within stretches of genomic DNA. This effort characterized the majority of transcribed gene space in the apple genome, and more than tripled the amount of publicly available sequence data for apple. Based on this we probably have identified all of the *FT/TFL* related genes in this apple. Our phylogenetic analysis provides clues for function (based on relationship with genes of known function) and suggest that *TFL1-1* and *TFL1-2* are the only authentic homologs of *TFL1*. Apple apparently has only one homolog of the *FT* gene, which is duplicated in Arabidopsis.

Gene expression atlas of the apple *FT/TFL* genes..

Results of Objective 2 are exciting for a number of reasons:

1. This is the first comprehensive analysis of this family of genes in apple. Based on widespread expression in non-reproductive tissues, the activity of these genes is probably not limited to flowering regulation, but probably extends to other developmental events.

2. The *MdBFT* gene likely has a function in the developing fruit, and should be further analyzed as a potential determinant of fruit quality.
3. Interestingly, both *MdMFT* and *MdTFL1-1* are strongly expressed in developing seeds. Potentially, this expression is related to the role of the developing seed in repressing floral initiation, which is already known to involve the phytohormon GA.
4. *MdFT*, which by homology with the *FT* gene of Arabidopsis is expected to promote flowering, is strongly expressed in seeding tissues and in the fruit. It is well known that flowering is repressed in seedlings and young plants through a phenomenon known as juvenility; if *MdFT* has a role as expected in promoting flowering in apple, its expression in seedlings suggests juvenility must involve a control point 'downstream' from *MdFT* activity.
5. We detected two seasonal peaks of expression of *MdTFL1-1,* first during the period of floral initiation, and second during the period of inflorescence development (Fig. 6). This pattern was reproducible in at least two biological replicates. *MdTFL1-2* expression, in contrast, was ubiquitous. We interpret this data as showing a specialized role for *MdTFL1-1* in flowering. Based on its known activity to antagonize *LFY* activity and repress inflorescence determinacy in Arabidopsis, we suggest that the early peak of expression (weeks 12-14) limits *AFL1* activity to the initiation of lateral flowers, and that its subsequent downregulation derepresses *AFL1* activity in the center of the inflorescence apex, allowing for terminal flower formation.

Based on these results, we now hypothesize that *MdTFL1-1* and *MdTFL1-2* have become functionally specialized in apple, with *MdTFL1-1* devoted to regulating inflorescence architecture, and *MdTFL1-2* devoted to maintenance of juvenility. This suggests that juvenility and inflorescence architecture can be regulated independently in apple – a tremendous opportunity for improving production.

3. Functional analysis.
Confirmation of the role of the *MdTFL1-1* and *MdTFL1-2* genes depends on determining what occurs when they are silenced (turned off). The powerful amiRNA technique should allow us to do this. We have obtained multiple lines of Gala with each of 6 silencing constructs. However thus far none of these lines have shown the phenotype (visible effect) of silencing either or both of the genes, although we will continue to observe them as they develop for any effects. Recent data from another lab in Germany produced similar inconclusive results with Gala. Therefore we are now exploring the effects of silencing the *MdTFL1-1* and *MdTFL1-2* genes in other varieties. We have started with the M.26 rootstock which is the best apple variety to transform. Although it is a rootstock, the effect of the *MdTFL1-1* and *MdTFL1-2* genes on flowering of M.26 should be applicable to fruiting varieties. We have just recovered multiple lines with three of the silencing constructs. These are still too young to show any early flowering effect, but we expect to be able to assess them meaningfully in the next two months. Experiments with the other silencing constructs are also planned. Results will be confirmed in a fruit variety, such as Fuji, which we can now also transform quite well.

Appendix A. **Material used for gene expression atlas:** Gala vegetative, floral, and fruit samples collected during years 2006-07.

Tissue		**Collection date**
Seedling	Root	4 week seedling
	Stem	4 week seedling
	Leaf	4 week seedling
	Cotyledon	4 week seedling
	Apex	4 week seedling
Leaf	Recently expanded leaf	5/22/07
	Leaf 4	5/22/07
	Leaf 4	7/31/07
Apex	Apex	5/22/07
Flower	Bud and parts	04/04/08
Fruit	Fruit	05/22/07
	Cortex	07/03/07
	Cortex	07/31/07
	Cortex	9/20/06
	Skin	07/31/07
	Skin	09/20/06
	Seed	07/03/07

Full bloom dates:
2006: 05/07/06
2007: 05/07/07

Harvest dates:
2006: 09/05/06
2007: 09/04/07- 09/14/07

EXECUTIVE SUMMARY

Functional genomics of flowering in apple

Herb Aldwinckle and Steve Van Nocker

The overall goal of this project was to improve our understanding of the genetic regulation of flowering in apple, so that eventually better varieties without shortcomings like juvenility, biennial bearing and over-cropping can be produced by marker assisted breeding. The research may also yield information that could result in corrective treatments for existing varieties.

We approached the problem first by identifying all possible members of *FT/TFL* gene family, which is known to regulate flowering in other plants, in apple, and determining their DNA sequence. We confirmed that the apple genome contains five genes in this family, with a single gene related to the flowering time regulator *FT* and two duplicate genes related to the juvenility/inflorescence architecture gene *TFL1*. This effort added substantially to the public apple sequence database.

Second, we analyzed the activity pattern of all the identified *FT/TFL* genes. This analysis included expression of the genes in various parts and organs of the plant, and control of expression during development (e.g. flower initiation). The goal of this approach was to identify those members of the *FT/TFL* family that might play the most important role in flowering. We showed that *FT* is expressed in a surprisingly broad pattern, suggesting roles in addition to flowering. Two other members of the family are expressed strongly in fruit and seeds, suggesting unanticipated functions in these plant parts. Our findings on the duplicated apple *MdTFL1* genes suggests that one gene may be responsible for inflorescence architecture, while the other may be involved in repressing juvenility.

Finally we concentrated our functional analysis on these latter two genes. In order to show if these genes are those that are really critical to flowering, we knocked out (or silenced) them, to determine if the pattern of flowering was in fact altered. This work is in progress. We have silenced both genes individually and in combination in Gala apple, but have not yet seen effects on flowering behavior in the silenced plants in tissue culture. We have also silenced the genes in M.26 rootstock, but these experiments are still in their early stages. We will also silence the genes in another fruiting variety, probably Fuji, in case there are strong differences between varieties in the behavior of this family of genes.

It is still too early to say exactly which genes are most critical to onset of flowering in apple. The evidence thus far indicates strongly that one of the *MdTFL1* genes is likely to one of the most important genes involved. The transformation experiments in progress should provide additional important evidence.

Once the genes most critically involved in flowering onset are confirmed, we can proceed to develop markers for the use of those genes in marker-assisted apple breeding. New improved varieties will be of great value to the Washington apple industry, resulting in decreased production costs and higher quality fruit.

FINAL PROJECT REPORT

Project Title: Auxin and ethylene dynamics in the abscission zone

PI: Steve van Nocker
Organization: Michigan State University
Telephone/email: 517-355-5191 / vannocke@msu.edu
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City: East Lansing
State/Province/Zip MI 48824

Cooperators: M John Bukovac, MSU Horticulture

Other funding Sources

Agency Name: Michigan Agricultural Experiment Station
Amount requested or awarded: Matching (Year 1 only).
Notes: This project is included in the PI's MAES 5-year Project. MAES pays partial faculty salary for the PI.

Total Project Funding: $56,236

WTFRC collaborative expenses $500

Budget History:

Item	2006	2007	2008
Salaries	8,676[1]	8,936	9,204
Benefits	819[2]	693	956
Wages	4,200	3,846	4,456
Benefits	0	0	
Equipment	0	0	
Supplies	1,800	1,600	4,200[3]
Travel	150	150	150
Miscellaneous	3,200[4]	3,200	
Total	18,845	18,425	18,966

Footnotes: [1]We have obtained matching funds from the Michigan State Agriculture Experiment Station. [2]Supported $^1/_2$ effort by a graduate student (stipend). [3]Costs include production and screening of microarrays. [4]Costs for DNA sequencing in Year 1 and Year 2.

OBJECTIVES

Interactions between two endogenous plant growth regulators, ethylene and auxins, play a crucial role in programming abscission of flowers and fruit. We proposed a model for the initiation of flower and fruit abscission, where loss of directional (polar) auxin flow through the abscission layers triggers enhanced ethylene signaling in abscission layer cells, culminating with activation of genes that promote cell separation. The objective of this project was to test this model through a methodical characterization of auxin and ethylene signaling components in the flower and fruit abscission layers, and to analyze the effects of cultural practices (including application of bloom and postbloom thinners) and environment on the interactions between auxin and ethylene signaling components.

Our specific objectives were:

1) Identification of auxin and ethylene signaling components active in the flower and fruit abscission layers. We sought to identify apple counterparts of known components of auxin and ethylene signaling (enzymes involved in biosynthesis, degradation, receptors, transporters, signaling intermediates, and regulatory proteins).

2) Design and construction of a microarray tool for gene expression profiling in apple

3) Studies of gene activity through microarray analysis. We sought to study these components in abscission-promoting circumstances, such as:

a) Flower abscission or retention associated with pollination/fruit set.
b) Fruitlet abscission associated with competition within a cluster.
c) Fruit abscission promoted by wounding.
d) Natural fruit abscission associated with maturity and ripening.
e) Flower abscission associated with bloom thinners.
f) Postbloom thinning by PGRs.
g) Fruit abscission promoted by reduced photosynthate
h) Fruit removal
i) Effects of PGRs on mature fruit retention or abscission

4) Construction of a map of regulatory pathways involving auxin and ethylene. The gene activity profiles reveal activity of each gene with respect to the individual abscission-promoting circumstances, and thus create a blueprint for the roles of auxin and ethylene in abscission. Hypothetically this can be used as a predictive tool for the design of more effecting thinning strategies.

SIGNIFICANT RESULTS (by objective)

1) Identification of auxin and ethylene signaling components active in the flower and fruit abscission layers.

- We analyzed the *expressed sequence tag (EST)*[1] information currently available in public sequence databanks
- We performed extensive literature searches to identify and catalog all suspected auxin-related and ethylene-related genes characterized in other plants.
- We used bioinformatics techniques to identify apple counterparts of known components of auxin and ethylene signaling, and identified a total of ~414 apple genes potentially involved in auxin signaling, and ~190 apple genes potentially involved in ethylene signaling.

[1] **EST (expressed sequence tag)** is a short DNA sequence of a randomly selected gene active in a given tissue. ESTs are a useful resource for gene discovery and for designing probes for DNA microarrays used to determine patterns of gene expression.

- We supplemented this gene set with 150 genes with roles in cell wall degradation, mostly pectinases, some of which are known to be activated during abscission in other plants.
- We compiled this information in a web-accessible database, Tree Fruit Technology (http://www.genomics.msu.edu/fruitdb). This work was published in the journal *Plant Physiology* (http://www.plantphysiol.org/cgi/content/full/141/3/811).

2) Design and construction of a microarray tool for gene expression profiling in apple

- We designed a microarray containing all DNA sequences (~2,200) of the gene sets that we identified. This microarray is now commercially available (CombiMatrix Corp.) and can be ordered by researchers. This tool is useful in studies of flowering, fruit ripening, color and aroma production, and other developmental processes important for production and storage.

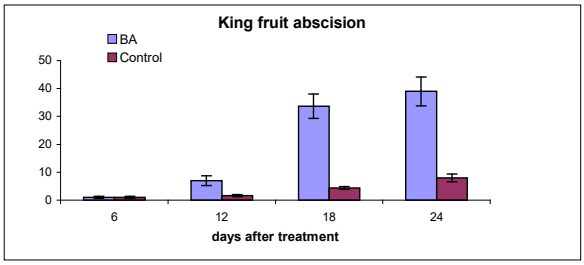
3) Studies of gene activity

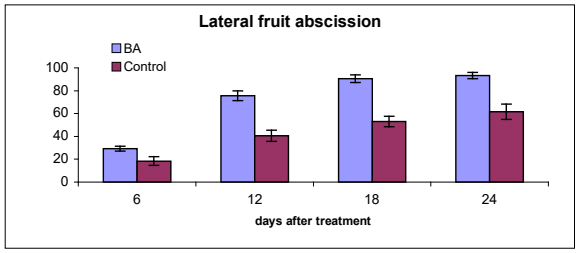
Flower abscission associated with bloom thinners.

- We sampled flower and abscission zone tissues from bloom thinning trials on Gala in the Wenatchee area. This was in collaboration with the more extensive thinning trials done by WTFRC staff. Treatments were ReTain (200 ppm), MCP (Nate Reed, AgroFresh), Ethrel (3 pts/acre), CFO/Lime sulfur, Retain pretreatment/CFO/Lime sulfur, and MCP pretreatment/CFO/Lime sulfur. To dissect the molecular mechanisms of flower abscission promoted by chemical thinners, we dissected abscission zone tissues from flowers from trees treated with lime sulfur. To help evaluate the potential role of ethylene in promoting thinning in response to lime-sulfur, we also analyzed tissues from trees treated with lime sulfur that had also been pretreated with AVG, an inhibitor of ethylene biosynthesis, or MCP, a strong repressor of ethylene sensitivity. When evaluated 2d following application, flowers treated with lime-sulfur were found to generate markedly more ethylene than the control or plants treated with lime sulfur pretreated with AVG. Other treatments did not result in ethylene evolution. We conclude lime sulfur, which WTFRC data showed was by far the most effective, may work through ethylene signaling. Gene expression analysis is in progress.

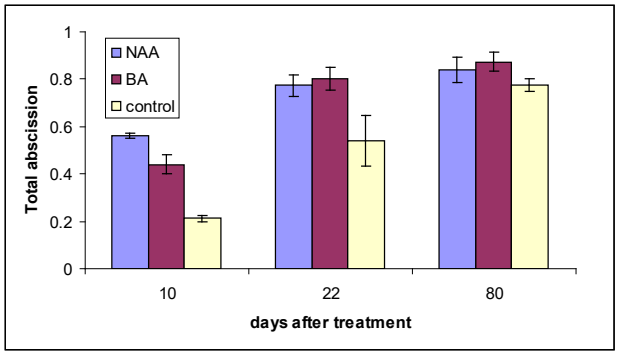
Postbloom thinning by PGRs

Research was conducted on Gala at MSU. Various concentrations of NAA (naphthaleneacetic acid)] and BA (6-Benzylaminopurine, benzyladenine) active ingredient were used in these experiments at 10-12 mm king fruit size. Each compound application and control included two replicates (three trees/replicate). Chemical compounds were dispersed in 0.1% surfactant Silwet-77 immediately before canopy application. All of the control trees were treated with 0.1% Silwet-77.





Abscission was calculated by fruit counts on



representative limbs. Total abscission was expressed at a percentage of total initial 10-12 mm fruit. Abscission zone samples were collected into liquid nitrogen in the above time points.

Both NAA and BA promoted fruit abscission early in the season, but ultimate fruit numbers were not significantly different than control. BA showed no marked preference for promoting drop of king fruit vs. lateral fruit. Still, documenting effects of chemical fruit thinning by NAA and BA early in the season when sampling was done allows interpretation of the gene expression data.

Fruit removal

Work was done in three consecutive years on Gala and Golden Delicious. Fruit were removed from trees after June drop by cutting, leaving the pedicel stub attached to the branch. This was marked and analyzed for abscission. Abscission of the majority of the pedicels occurred five days later. We dissected abscission zone tissues from a separate set of pedicel stubs at 2h, 8h, 1d, 2d and 4d after fruit removal. Two controls were the adjacent segment of the pedicel stub not containing the abscission zone, and abscission zones from non-removed fruit pedicels. Experiments utilized three temporal replicates. These samples were used in gene expression profiling using the microarrays developed in Objective 2.

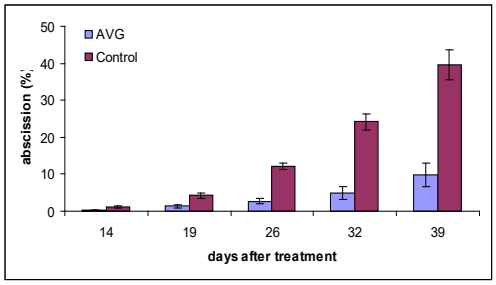
Results from this analysis are available at *http://vannocke.hrt.msu.edu/public/fruitremoval.xls*. This analysis identified many genes that were upregulated or downregulated in the pedicel abscission zone at various times after treatment. However, nearly all of the identified genes were also similarly upregulated or downregulated in the adjacent, non-abscission zone control tissues. Potentially, this suggests that abscission is initiated as a localized *response* to regional changes in gene expression. However, we also identified several genes that did appear to be abscission zone specific, including transcription factors, known components of auxin/ethylene signaling, and cell-wall modifying genes. We are concentrating on a subset of these that we suggest have a particularly important function based on their identities:

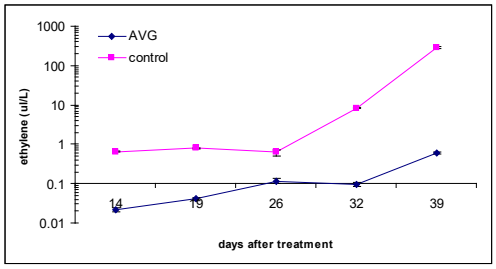
Gene i.d.	**Response**	**Identity**	**Presumed function**
MD4C492040	2h	MdHAE, a HAESA-like gene	Ethylene-independent abscission
MD4C054590	2h	MYB-class transcription factor	Gene regulation
MD4C506930	2h	HB-1 transcription factor	Gene regulation
MD4C464220	2h, 4h	WRKY transcription factor	Gene regulation
MD4C514850	4h	polygalacturonase	Cell wall degradation
MD4C515220	4h	ABA response element binding protein	ABA signaling

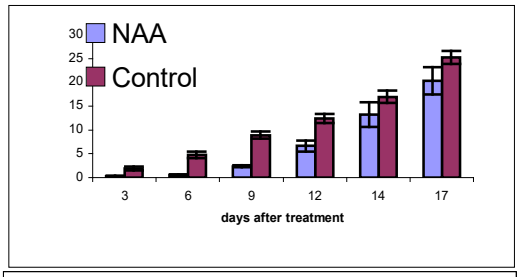
MD4C458550	24h	Pectate lyase	Cell wall degradation
MD4C503670	24h	PHD finger family protein	Gene regulation
MD4C496200	24h	Auxin-responsive SAUR protein	Auxin signaling
MD4C401830	24h	Auxin-responsive SAUR protein	Auxin signaling
MD4C501810	24h	MYB-class transcription factor	Gene regulation
MD4C191660	24h	ACC oxidase	Ethylene signaling
MD4C230030	48h	Auxin-response factor	Auxin signaling
MD4C2400230	48h	AUX1-like protein	Auxin signaling
MD4C268730	48h	Calmodulin-binding protein	Signal transduction
MD4C045340	48h	PIN1-like auxin transporter	Auxin signaling
MD4C188060	96h	MYB-class transcription factor	Gene regulation
MD4C509730	96h	SCL-class transcription factor	Gene regulation
MD4C214670	96h	Auxin-responsive protein	Auxin signaling
MD4C421570	96h	SPL-class transcription factor	Gene regulation

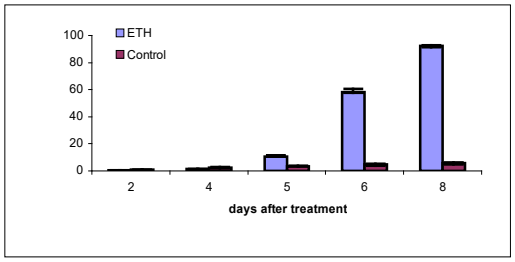
<u>Effects of PGRs on mature fruit retention or abscission</u>

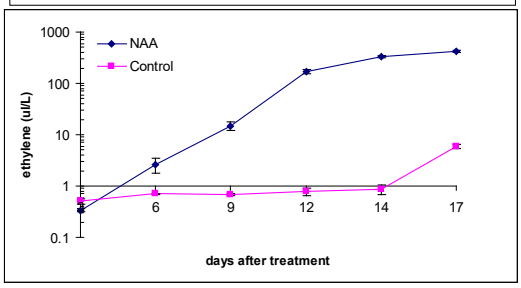
Research was conducted on 20-y-old Spur Macs at MSU. Three different chemical compounds, Retain [active ingredient: aminoethoxyvinylglycine (AVG))], Ethephon [active ingredient: ethephon (2-chloroethyl phosphoric acid)], and Fruitone-N [active ingredient: NAA (naphthaleneacetic acid)] were used in this experiment. Each compound application and control included two replicates (three trees/replicate). All of these three chemical compounds were applied to whole apple tree in this study. Chemical compounds were dispersed in 0.1% surfactant Silwet-77 immediately before canopy application. All of the control trees were sprayed with 0.1% surfactant Silwet-77 at the same time with treatments. Retain was applied one month prior to expected harvest date at 70mg/L active

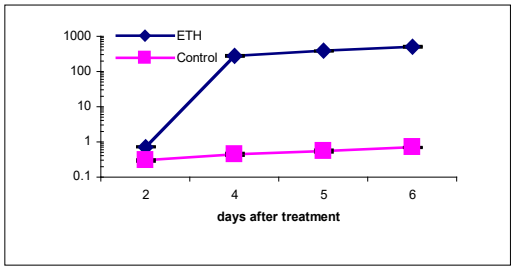












ingredient. Ethephon was applied two weeks before anticipated harvest data at 300 mg/L active ingredient. Fruitone-N was also applied 2 weeks before anticipated harvest date at 20mg/L active ingredient.

Fruit abscission and ethylene measurement were carried out starting from one day up to six weeks after treatment. Thirty fruits from three trees per treatment were collected for ethylene measurement at intervals of 5 to 7 days after retain application, or 2 to 3 days after Ethephon and Fruitone-N application. The number of abscised fruit was counted at each time point as above. Total abscission was expressed at a percentage of total initial fruit load. Abscission zone samples were collected into liquid nitrogen in the above time points.

AVG and NAA significantly influenced both ethylene evolution and fruit abscission. Ethephon promoted almost total fruit drop within 8 d of application in both years. These samples serve as important standards for the interpretation of microarray gene expression data, because they are associated with documented PGR effects.

RESULTS AND DISCUSSION

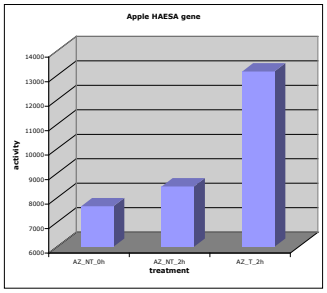
To recap, we developed a model for fruit abscission involving the interaction between two endogenous PGRs, auxin and ethylene. Specifically, at a very early stage in abscission we have found changes in the activity of a number of genes that participate in mediating auxin signal transduction, and this preceded observed changes in the activity of several genes that function in ethylene signaling. Taken together with a variety of studies of the effects of bloom and postbloom thinners, and with very recent findings in basic plant biology, our results allow us to propose a model for the initiation of flower and fruit abscission. In this model, loss of directional auxin transport through the abscission layers triggers enhanced ethylene signaling in abscission layer cells, culminating with activation of genes that promote cell separation.

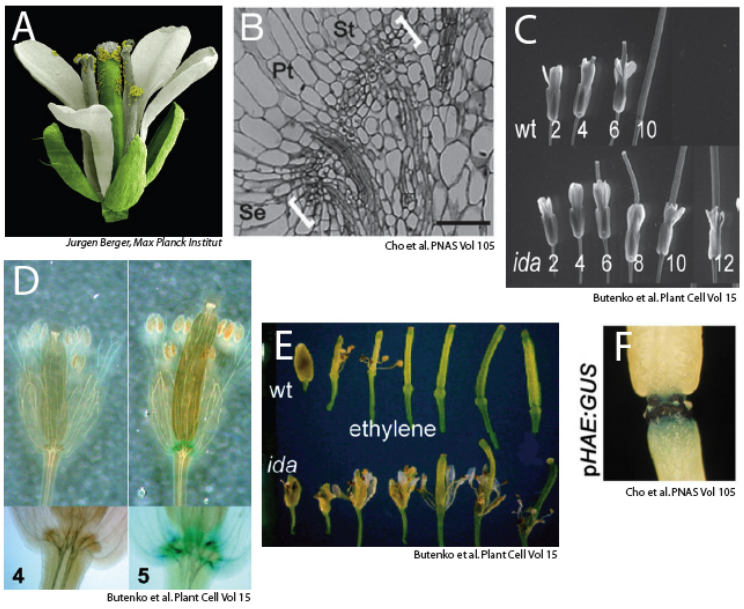
We found that among the first genes to be activated in the abscission zone upon fruit removal are a subset of genes associated with carbohydrate modification. The presumed role of these genes in abscission is to degrade the cell wall, allowing for cell separation. This was initially confusing, because in the system used for this study, separation of abscission layer cells takes place much later (3-4 d after activation), and previous studies demonstrated that a large variety of cell-wall-modifying genes became active only late in abscission. However, a possible scenario is that the early-induced carbohydrate-modifying genes participate in generating a signaling molecule that acts as an initiator of abscission. Hypothetically, degradation of the cell wall contributes to an extracellular pool of small oligosaccharides, some of which are well-known signaling intermediates in other pathways such as defense response. Analogous to the defense pathway(s), this could result in initiation of ethylene production and coordinated advance of the abscission process. Though highly speculative, this idea is supported by two recent findings: 1) Our observation that an *ACO* gene is induced at a later stage of abscission, suggesting activation of ethylene signaling, and 2) Recent findings from Michael McManus' lab (Ann Bot, Oct 2007) showing that abscission depends on a mobile signal, generated in the stele of the pedicel, that works with ethylene to promote abscission. In fact, vasculature is considered to be a main route of polar auxin transport; disruption of auxin flow resulting from a variety of cultural manipulations or environmental trauma could somehow act as a trigger to generate this signal.

In contrast to expectations, we identified numerous abscission-associated genes that, based on identity, are presumed to act as positive regulators of auxin signaling. Our model predicts that initiation of abscission disruption of auxin flower results in decreased auxin in the abscission zone, but the activation of such genes indicates exactly the opposite! One possible explanation is that auxin

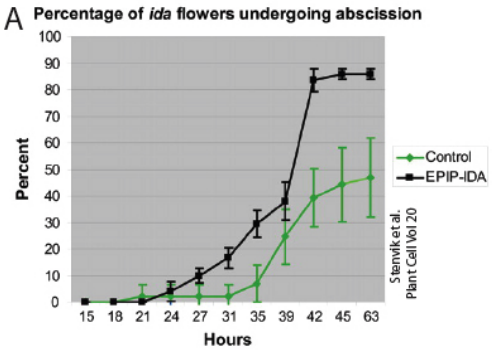
transport is indeed disrupted as expected, and this results in the accumulation of auxin in the disrupted region simply because it can't be exported. We have yet to analyze several experiments that should shed light on this apparent paradox.

Ethylene-independent mechanisms of fruit abscission. We identified a gene that we call *MdHAE* as an abscission early-response gene. This gene was activated within 2 hours of fruit removal in the pedicel abscission zone (below, left). *MdHAE* is the apple counterpart of an abscission-control gene called *HAESA* identified in the reference plant Arabidopsis.

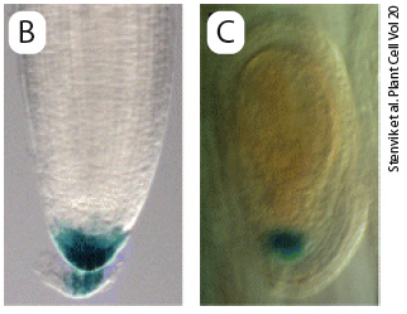




Arabidopsis does not shed fruit but does abscise flower petals. Flower petal shedding closely follows pollination, and takes place through activation of an abscission zone found at the base of the petal (panel B, at right). By looking for mutations that delay petal loss, researchers have identified several genes required for promoting abscission. One of these is called *IDA* (C). This gene becomes activated in the abscission zones just after pollination (D) and produces a small protein, which undergoes proteolysis to generate a small peptide. This peptide is thought to act as the molecular signal to activate other genes required for cell-cell separation. Interestingly, *IDA* seems to act independently of ethylene signaling (E), and so defines a previously unrecognized 'pathway' of abscission. Like apple *MdHAE*, *HAESA* is also activated in the abscission zone (F). *HAESA is though to represent the receptor for the IDA signaling molecule.*



Interestingly, researchers also found that a synthetic peptide corresponding to the active peptide of IDA (EPIP-IDA) could accelerate petal abscission when applied to the flower (Right, panel A). Moreover, they found that *IDA* is one of several related genes in Arabidopsis, that these additional genes, such as *IDL1* or *IDL2*, become activated in other events associated with cell-cell separation, such as in the root cap or in the abscission zone of the seed (B, C), and that *IDA*-like genes are found in other plants as well.



IDL1 gene activity in the root cap

IDL2 gene activity in the seed abscission zone

This data revealed a previously unknown class of gene with regulatory roles in abscission. I suggest that the homologous *MdHAE* gene in apple has a role in promoting flower and fruit abscission, and that derived synthetic peptides from apple counterparts of IDA-like proteins might be useful for thinning.

Executive Summary - Auxin and ethylene dynamics in the abscission zone

The goal of this three-year project is to analyze patterns of gene expression in the flower- and fruit-pedicel abscission zone under various abscission-promoting circumstances, in order to understand the dynamics of auxin and ethylene signaling that trigger abscission, and potentially to gain some information that could be used to design better thinning strategies.

We approached this by designing numerous field experiments that are known to influence flower or fruit abscission. We carried out these experiments in each of the years of the project, and in those cases where the treatments had a substantial effect on abscission, we saved the dissected abscission zone tissues for analysis. Our analysis tool was a DNA microarray, which we designed and constructed in collaboration with a company named Combimatrix. This is now a commercial product that is available from that company.

Among our most exciting findings to date:

- We see rapid induction of an apple gene related to *HAESA*, a gene from Arabidopsis that works in an ethylene-independent pathway of abscission involving a little-studied, abscission-promoting peptide. This leads to the possibility that apple thinning could be promoted by synthetic bioactive peptides!
- A subset of cell-wall modifying genes induced very early in the abscission processes. We hypothesize that these could promote the synthesis of small oligosaccharides that act as a signaling molecules to coordinate abscission.
- Induction of ACO oxidase in the abscission zone. We hypothesize that this gene is the basis for ethylene production that turns on cell-wall-modifying genes.
- Induction of genes thought to be auxin-responsive, suggesting a rise in auxin levels or auxin sensitivity. This is confusing and counter to our expectations that auxin signaling decreases in the abscission zone upon induction.

We encountered three blocks during the course of the project. The first was related to the field work. Some of our treatments, especially the post-bloom thinning trials carried out at MSU, did not affect abscission in the anticipated manner. Thus, many experiments carried out in the first year had to be repeated in the second, and ultimately the third year. Second, we found that the public genomic data for apple that we needed to construct the microarray was disorganized, filled with artifacts and frequently misannotated. Cleaning this up required almost a year of effort on my part. Third, we had some quality control problems with the microarrays. Some of our replicate analyses did not yield high-quality data, and could not be used, forcing us to confirm much of the results by laborious low-throughput techniques.

Genomic technologies have progressed quickly in the past three years, and microarray-based approaches are now mostly obsolete. Current high-throughput sequencing technologies are far more sensitive and comprehensive than microarrays in identifying the type of changes in gene activity patterns that we are interested in, and offer an exciting opportunity to expand the approach. Funds supported half-time effort by a graduate student, Lingxia Sun. The work will be further developed into one of the three research chapters of Lingxia's PhD thesis, and she plans to graduate at the end of the spring '09 semester. Consequently, we have to submit the work for publication before that time to get the reviewer feedback required for the thesis defense. So, we are continuing to work on the project and expect the unfinished aspects of the project to be tied up within the next five months. The work has already resulted in a publication in *Plant Physiology*, one of the two most highly cited plant research journals, and will be the basis for application to the USDA AFRI for major funding to extend the work.

FINAL PROJECT REPORT

Project Title: High temperature stress on apple fruit peel: physiology and detection

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Cooperators:

Other funding Sources: None

Total Project Funding: $82,967

Budget History:

Item	**2007**	**2008**	
Salaries	22,000	23,188	
Benefits	8,249	9,070	
Wages			
Benefits			
Equipment	0	**0**	
Supplies	10,230	**10,230**	
Travel	0	**0**	
Miscellaneous	0	**0**	
Total	40,479	**42,488**	

Objectives

The overall objective is to better understand the underlying physiology of high temperature stress to apple fruit peel with an ultimate goal of detecting and reducing high peel temperature-induced fruit disorders both preharvest and postharvest. The specific objectives are:

1) To determine how high temperature affects the balance of photooxidation and photoprotection of apple fruit peel, leading to sunburn browning;
2) To determine if chlorophyll fluorescence reflects the damage of high temperature on fruit peel and varietal differences during the growing season;

Significant Findings

1) Maximum photosystem II (PSII) quantum efficiency (Fv/Fm) of the sun-exposed peel of well-exposed fruit in the southwest canopy decreased during the day in response to high peel temperatures, and very little recovery was made during overnight dark relaxation, indicating that the high peel temperature has damaged the PSII centers of the peel.

2) After a couple days of high temperature exposure, more fruit in the west side of the canopy had very low Fv/Fm value than those in the east side. This difference corresponds to the different profiles of peel temperatures and sunburn occurrence between the two sides of the canopy. This along with the diurnal Fv/Fm data indicates that Fv/Fm is a very sensitive indicator of high temperature stress in apple peel.

3) Compared with the non-sunburned peel, the sunburned peel had lower chlorophyll content, lower Fv/Fm, lower net oxygen evolution rate, and lower activities of key photosynthetic enzymes, but higher activities of antioxidant enzymes and higher content of antioxidant metabolites and higher xanthophyll cycle activity on a chlorophyll basis, and higher hydrogen peroxide and malondialdehyde content. This indicates that high peel temperature most likely has increased the photooxidation potential, rather than decreased the photoprotective capacity of fruit peel.

4) Controlled temperature treatments of fruit peel samples in the dark showed that high peel temperature led to decreases in Fv/Fm and net O_2 evolution, and appearance of "K" step in chlorophyll a fluorescence transient. This indicates that high temperature has damaged the oxygen evolution complex of the PSII, leading to oxidative stress.

5) Simultaneous high temperature and high light treatment decreased Fv/Fm and O_2 evolution of Gala peel more than high temperature or high light alone. The clear "K" step in chlorophyll fluorescence, which appeared in the high temperature treatment, disappeared under simultaneous high temperature and high light treatment. This indicates that high temperature mainly affects the oxygen evolution complex of the PSII (the donor side) whereas high light mainly affects the acceptor side of the PSII.

6) Apple cultivars differ in their responses to high temperature and high light stress. Of the cultivars tested, Red Delicious is most tolerant of high temperature and high light stress whereas Cameo is the least tolerant. Our data show that chlorophyll fluorescence is an effective tool for testing varietal difference in tolerance to high temperature and high light stress.

Methods

<u>1. Determine diurnal changes of Fv/Fm in relation to peel temperature</u> This experiment was carried out on mature Gala/M.9 trees (spacing:15 X 6.5 feet) at WSU TFREC on July 21. Fifty well-exposed fruit on the southwest part of the canopy were selected the day before and the temperature of the sun-

exposed side of each fruit was monitored with a thermocouple connected to a data logger. In addition, the temperature of the shaded side of 3 fruit was also monitored along with ambient temperature. Every 4 hours starting from pre-dawn (5:00), ten fruit were dark-adapted for 30 min and then measured for Fv/Fm. The pre-dawn values of Fv/Fm were also measured the next day.

2. Determine the distribution of fruit peel Fv/Fm and sunburn occurrence on the east side and west side of the canopy after exposure to high temperatures. Ten well-exposed fruit from each side (east and west) of the canopy were selected and their peel temperatures were monitored as above from July 23 to 25. All the fruit from the east side and west side of the canopy were harvested separately and Fv/Fm of the sun-exposed peel was measured at pre-dawn on July 25. The percentage of fruit with sunburn was counted on separate trees with similar canopy size and structure in the morning on July 26.

3. Compare the sunburned and non-sunburned fruit in terms of photosynthetic capacity, chlorophyll fluorescence, and antioxidant system. The sun-exposed peel of non-sunburned and sunburned fruit (80 fruit each) was taken from the east and west side of the canopy from 9:15 to 10:00AM and from 4:00 to 4:45PM on July 25, respectively. The samples were immediately frozen in liquid nitrogen and stored until analysis.

4. Determine chlorophyll fluorescence and oxygen evolution of the sun-exposed peel of 'Fuji' fruit in response to controlled high temperature treatments. At approximately 100 days after full bloom (mid-August), well-exposed fruit on the west side of the canopy of Fuji/M.9 trees were taken right after sunset and the sun-exposed side of each fruit was marked. All the fruit were dark-adapted overnight at 22C and fruit peel samples (0.5 mm thick, 1 cm^2) were taken from the sun-exposed side. The peel samples were placed between two layers of wet paper towel and the assembly was put onto the bottom of a small aluminum foil vessel with the top covered with aluminum foil. Then, the vessel was directly floated on water in a water bath, the temperature of which was controlled by a refrigerated water bath and the temperature equilibrium between the fruit peel and water was reached within 1 to 2 min. The peel samples were exposed to 25, 35, 40, 42, 44, 46 or 48°C in the dark for 30 min. Chlorophyll a fluorescence transient and photosynthetic O_2 evolution were measured after the peel samples had been kept in the dark at room temperature for 30 min after each temperature treatment.

5. Determine the effect of simultaneous high temperature and high light stress on the sun-exposed peel of Gala fruit. Well-exposed fruit were harvested from the west side of Gala/M.9 trees on August 16, 2007, wrapped in wet paper towel and put into plastic bags immediately. After overnight dark adaptation in the lab, the peel discs with 3 mm thickness were cut from the sun-exposed side and were put onto 4 layers of wet cheesecloth in a stainless steel water jacket, the temperature of which was controlled by a refrigerated water bath. Light was provided by a tungsten lamp. Peel disc were treated with high temperature (45 ºC) in the dark, high light (1600 μmol m^{-2} s^{-1}) at room temperature, or cross stress of high light and high temperature (45 ºC, 1600 μmol m^{-2} s^{-1}) for 0, 15, 30 and 45 min, respectively. After dark-adaptation for one hour, chlorophyll a fluorescence transients and O_2 evolution were measured.

6. Determine varietal difference in response to high temperature and high light stress. In late August, well-exposed fruit of Cameo, Fuji, Gala, Golden Delicious and Red Delicious were harvested from the west side of the canopy, wrapped in wet paper towel and put into plastic bags immediately. After overnight dark adaptation in the lab, the peel discs with 3 mm thickness were cut from the sun-exposed side and were put onto 4 layers of wet cheesecloth on a stainless steel water jacket, the temperature of which was controlled by a refrigerated water bath. The light intensity was controlled by a tungsten lamp. Discs were treated with different temperatures (30, 35, 40, 42, 44, 46 and 48 ºC) in the dark or in the light (1200 μmol m^{-2} s^{-1}) for 30 min. After dark adaptation for 1 h, chlorophyll a

fluorescence transients were measured. For all the experiments, chlorophyll fluorescence was measured with a Handy PEA and oxygen evolution was measured with ChloroLab 2 (Hansatech, UK).

Results and Discussion

1. Diurnal changes of PSII quantum yield (Fv/Fm) of the sun-exposed peel and the shaded peel in relation to peel temperature on a hot day in central WA

On July 21 of 2006, the temperature of the sun-exposed peel of well-exposed fruit in the southwest part of the canopy increased almost linearly from 5:00 to 15:20, reaching 50C at 15:20, and then stayed above 45.9C till 17:30. In contrast, the shaded peel only reached a maximum of 40.7C between 17:00 and 17:30 when the highest ambient temperature was 35.5C.

Fv/Fm of the sun-exposed peel at pre-dawn was around 0.53, and remained essentially unchanged till 9:00. However, the Fv/Fm decreased linearly from 9:00 to 17:00, and then increased slowly from 17:00 to 5:00 the next day. The rapid decrease in Fv/Fm from 9:00 to 17:00 corresponds well with the period of high fruit peel temperature. Even at pre-dawn the next day Fv/Fm has not fully recovered to the previous pre-dawn level, indicating severe oxidative damage has occurred in the peel. In contrast, Fv/Fm of the shaded peel was higher than in the sun-exposed peel and remained unchanged throughout the entire day. The large error bar in the Fv/Fm data for the sun-exposed peel is due to the fact that there were large variations in Fv/Fm value among individual fruits.

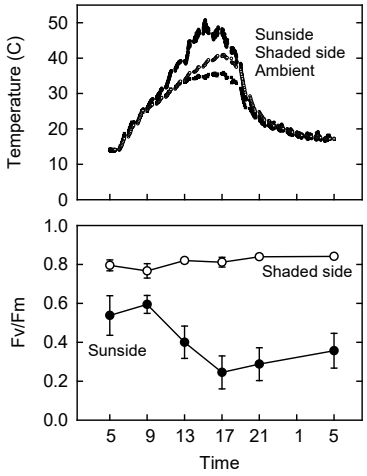


Fig 1

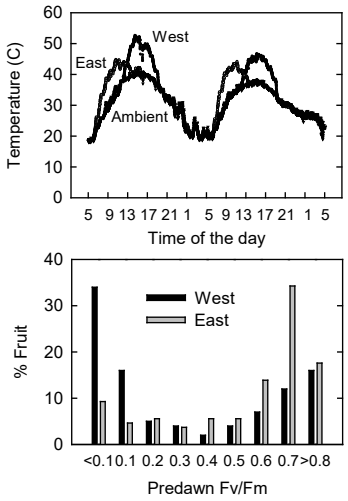


Fig 2

Fig 1. Diurnal changes of peel temperature and Fv/Fm in the sun-exposed peel and the shaded peel of well-exposed fruit in the southwest part of the canopy on July 21, 2006.

Fig 2. Diurnal changes of the temperature of the sun-exposed peel in the east and west sides of the canopy on July 23 and 24 and fruit distribution in terms of Fv/Fm measured at pre-dawn on July 25, 2006.

2. Distribution of fruit peel Fv/Fm and sunburn occurrence in the east side and west side of the canopy after exposure to high temperatures.

We compared the distribution of fruit Fv/Fm between east and west sides of the canopy after a couple days of high temperature exposure (July 23 and 24). As shown in Fig 2, the % fruit with an

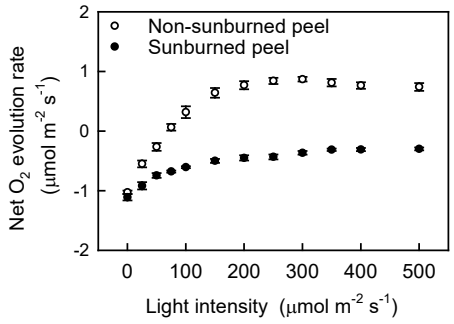
Fv/Fm value less than 0.1 was much higher in the west side (34%) than in the east side (9%) of the tree canopy whereas the % fruit with an Fv/Fm value between 0.7 and 0.8 was much lower in the west side (13%) than in the east side (34%). These numbers correspond well with the difference in temperature profiles between the two sides. Interestingly, both east and west sides had 16 to 18% of the fruit with an Fv/Fm value higher than 0.8, which indicates that both sides have equal number of shaded fruit. Counting the number of sunburned fruit on each side showed that the west side had 21.9% with sunburn whereas the east side had only 6.1%.

3. Comparison of sunburned and non-sunburned fruit peel

Photosynthetic oxygen evolution: As light level increased, net O_2 evolution rates for both non-sunburned and sunburned peels increased almost linearly first, then reached a saturation point, beyond which O_2 evolution showed little response to increasing light level (Fig 3). At each given light level, photosynthetic O_2 evolution rate was significantly lower in the sunburned peel than in the non-sunburned peel (Fig. 3). The quantum yield for O_2 evolution (the initial slope of each curve) was much lower in the sunburned peel than in the non-sunburned peel, whereas the light saturation point was higher in the sunburned peel than in the non-sunburned peel.

Hydrogen peroxide and malondialdehyde: The sunburned peel had higher concentrations of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA, an indicator of oxidative lipid metabolism) compared with the non-sunburned peel (Fig. 4), which clearly indicates that oxidative damage has occurred.

Activities of key photosynthetic enzymes: Compared with the non-sunburned peel, the sunburned peel had lower activities of key photosynthetic enzymes, including ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), NADP-glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, and stromal fructose-1,6-bisphosphatase (Data not shown). However, the activities of these enzymes decreased to a lesser extent than the net O_2 evolution rate.



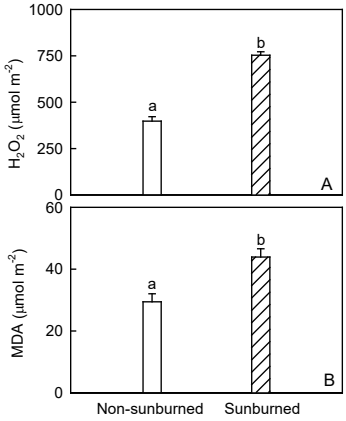


Fig 3 Fig 4

Fig 3. Light response of net oxygen evolution of sunburned and non-sunburned peels.
Fig 4. Hydrogen peroxide and malondialdehyde (MDA) content in sunburned and non-sunburned peels.

Reflectance and pigments: The sunburned peel had higher reflectance averaged over 400 – 700 nm. Reflectance spectra showed that the sunburned peel reflected more light in the range between 420 and 700 nm than the non-sunburned peel (Data not shown).

Chlorophyll, xanthophyll cycle pool size and lutein contents expressed on a peel area basis, and β-carotene and neoxanthin contents expressed on a peel area or Chl basis were lower in the sunburned peel than in the non-sunburned peel, whereas the contents of xanthophyll cycle pool size, zeaxanthin and antheraxanthin, and lutein expressed on a chlorophyll basis were higher in the sunburned peel than in the non-sunburned peel. This indicates that more chlorophylls were degraded relative to xanthophylls and other carotenoids. Almost all the xanthophyll cycle pool was converted to zeaxanthin and antheraxanthin in both sunburned and non-sunburned peels.

Antioxidant enzymes and metabolites: Activities of ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, and glutathione reductase were higher in the sunburned peel than in the non-burned peel, whereas there was no significant difference in superoxide dismutase or catalase activity between the two peel types (Data not shown).

The content of total ascorbate (reduced + oxidized), total glutathione (reduced + oxidized) and reduced glutathione was higher in the sunburned peel than in non-sunburned peel, but the ratio of both reduced ascorbate to total ascorbate and reduced glutathione to total glutathione were lower in the sunburned peel than in the non-sunburned one. No significant difference was observed in reduced ascorbate content between the sunburned and the non-sunburned peels (Data not shown).

Chlorophyll a fluorescence transients: When overnight dark-adapted fruit was exposed to a saturating pulse of light, chlorophyll a fluorescence showed a characteristic rise from minimal fluorescence (Fo) to maximum fluorescence (Fm) in the non-sunburned peel (Fig 5). However, the fluorescence signal of the sunburned peel was much lower and reached Fm at a much earlier stage followed by little change in fluorescence intensities. Chlorophyll fluorescence turns out to be the most sensitive of all the responses we have measured on the sunburned peel.

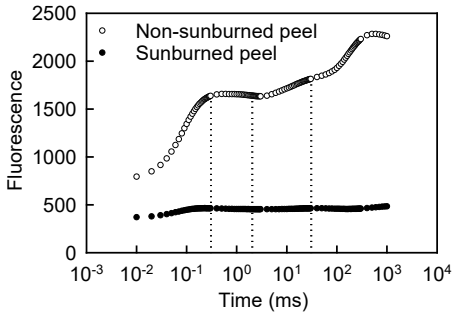


Fig 5. Chlorophyll fluorescence induction curves of sunburned and non-sunburned fruit.

4. Chlorophyll a fluorescence transients, Fv/Fm and photosynthetic oxygen evolution of the sun-exposed peel in response to temperature treatments

When the peel temperature increased from 25 to 35C, neither Fo nor Fm showed any significant change (Fig 6A). However, as peel temperature increased further, Fm decreased whereas Fo increased. In addition, the shape of the chlorophyll fluorescence induction curve changed. When the peel temperature reached 44 - 48°C, a very clear peak (called "K" step) at 300 μs appeared, followed by a pronounced dip. After exposure to 46 and 48°C, maximal fluorescence was already reached at "K" step followed by a rapid decrease to a level close to or below Fo (Fig. 6A). Fv/Fm changed very little from 25 to 40°C, and then dropped rapidly with further increase in temperature (Fig. 6B).

Net photosynthetic O_2 evolution rates remained unchanged as temperature increased from 25°C to 40°C, then dropped rapidly with any further increase in temperature (Fig. 6C). After

exposure to 46 and 48C, the net O_2 evolution rate became negative. However, heat stress showed no effects on dark respiration.

Decreases in Fv/Fm and net O_2 evolution, coupled with appearance of "K" step in chlorophyll a fluorescence transient indicate that high temperature has damaged the oxygen evolution complex of the PSII, leading to oxidative stress. However, the lack of a clear K step in the sunburned peel (Fig 6) suggests that there is interaction between high peel temperature and high light.

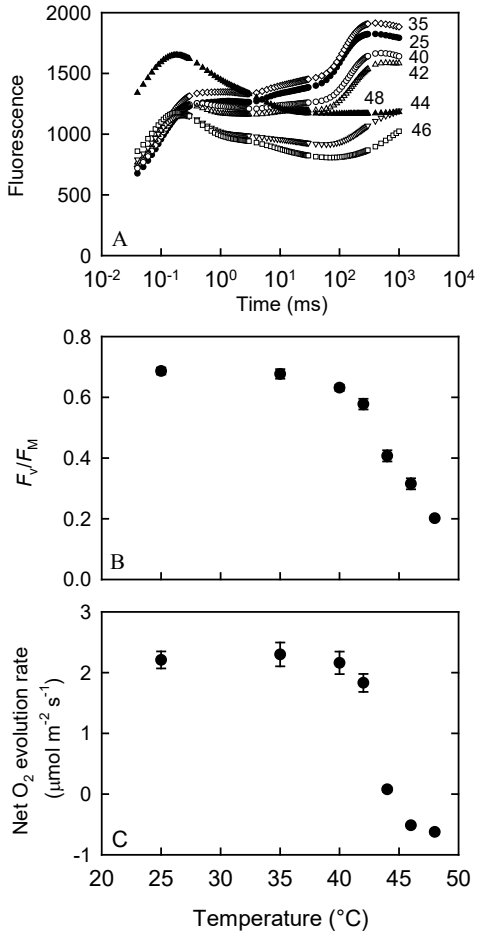


Fig 6. Chlorophyll fluorescence transient (A), maximum PSII efficiency, Fv/Fm (B), and net oxygen evolution of the sun-exposed peel of Fuji fruit in response to temperature treatments.

5. Responses of Fv/Fm, chlorophyll a fluorescence transients and photosynthetic oxygen evolution of the sun-exposed peel in response to high temperature and high light treatments

Simultaneous high temperature and high light treatment decreased peel Fv/Fm more than high temperature or high light treatment alone (Fig 7). Peel oxygen evolution rate was significantly lower in the simultaneous high temperature and high light treatment than in the high temperature or high light treatment alone (Fig 7). Dark respiration was not significantly affected (Data not shown). This clearly indicates that high temperature coupled with high light causes more damage to fruit peel than high temperature or high light alone.

Chlorophyll fluorescence transients at the end of the 45 min treatment showed a clear "K" step in the high temperature treatment (in the dark) alone, but the "K"step disappeared in the simultaneous high temperature and high light treatment (Fig 8A). This suggests that high temperature mainly affects the oxygen evolution complex of the PSII (the donor side) whereas high light mainly affects the acceptor side of the PSII. High light first followed by high temperature treatment (in the dark) induced a clear "K" step in chlorophyll a fluorescence transient (Fig 8B), whereas high temperature (in the dark) first followed by high light treatment did not induce a "K" step, but decreased fluorescence intensity to a very similar level as in the simultaneous high temperature and high light treatment (Fig 8A). This suggests that the photooxidative damage occurring in the sun-exposed peel of apple fruit under high temperature coupled with high light is initiated by high temperature on the oxygen evolution complex of the PSII, and exacerbated by high light.

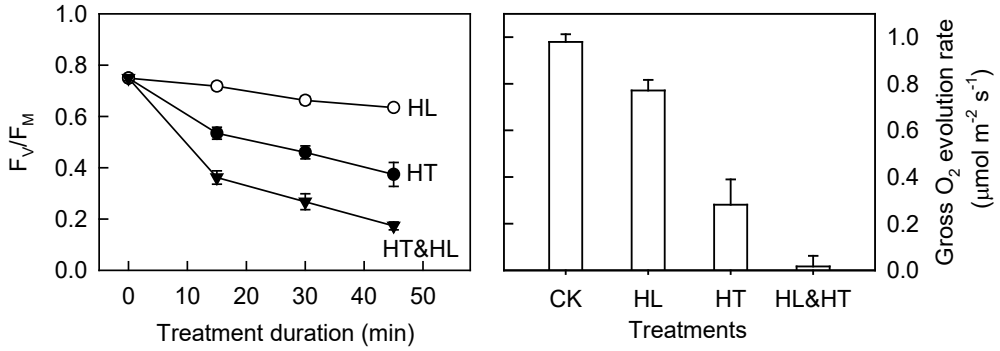


Fig 7. Maximum quantum yield of PSII (Fv/Fm, left) and photosynthetic oxygen evolution (Right) of the sun-exposed side of Gala apple peel in response to high temperature (45 ºC, HT), high light (1600 μmol m^{-2} s^{-1}, HL), and high temperature with high light (HT&HL) treatments.

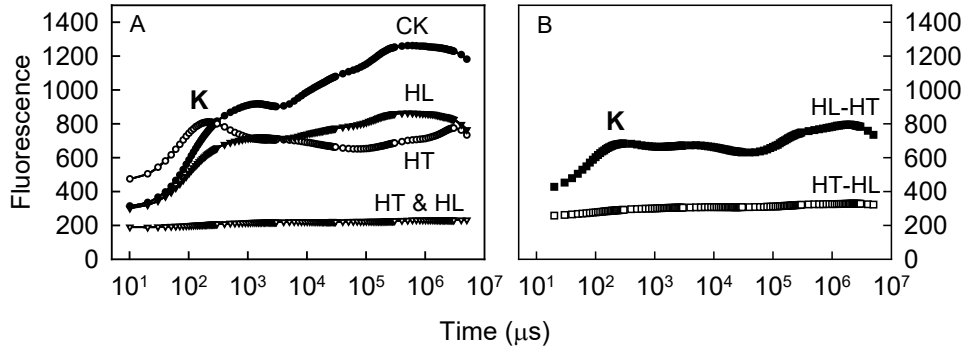


Fig 8. Chlorophyll fluorescence transients of 'Gala" peel in response to high light (HL), high temperature (HT), high light coupled with high temperature (HL&HT), high light followed by high temperature (HL-HT), and high temperature followed by high light (HT-HL) treatments for 45 min.

6. Varietal difference in response to high temperature and high light stress

When treated in the dark, the Fv/Fm value of the sun-exposed peel of Cameo, Fuji, Gala, Golden Delicious and Red Delicious remained unchanged as the treatment temperature increased from 30 to 40 °C (Fig 9A). With further increases in treatment temperature, varietal difference showed up. Fv/Fm of Cameo peel started to decrease at 42°C, whereas that of Red delicious didn't decrease until temperature increased to 46°C. At any given temperature from 42 to 48 °C, Red Delicious had the highest Fv/Fm whereas Cameo had the lowest Fv/Fm.

When treated under high light, Fv/Fm of the sun-exposed peel of all cultivars tested decreased as the treatment temperature increased (Fig 9B). At each given temperature, Red Delicious had the highest Fv/Fm whereas Cameo had the lowest Fv/Fm.

Our data indicate that apple cultivars differ in terms of tolerance to high temperature and high light stress. However, the tolerance mechanism remains to be elucidated.

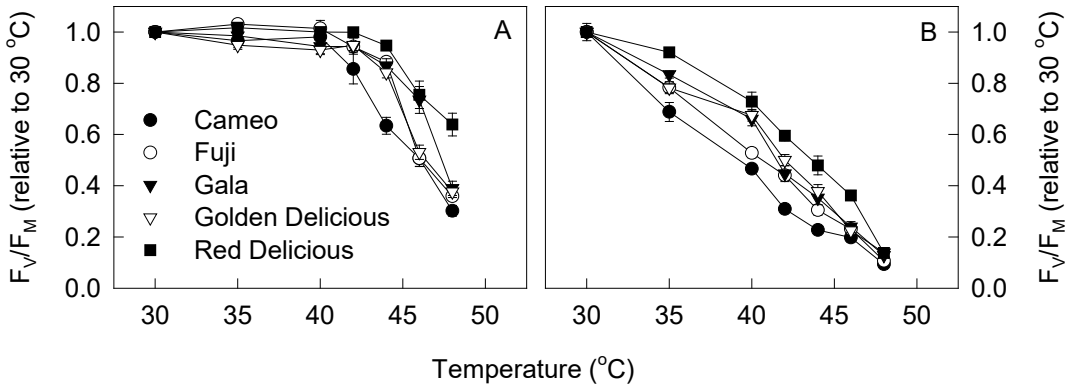


Fig 9. Maximum quantum yield of PSII (Fv/Fm) of the sun-exposed peel of five apple cultivars in response to temperature treatment for 30 min in the dark (A) or under 1200 μmol m^{-2} s^{-1} light (B).

Papers published out of this project

Chen, L. S. and L. Cheng. 2007. The sun-exposed peel of apple fruit has a higher photosynthetic capacity than the shaded peel. *Functional Plant Biology* 34, 1038-1048.

Chen, L. S., P. Li and L. Cheng. 2008. Effects of high temperature coupled with high light on the balance between photooxidation and photoprotection in the sun-exposed peel of apple. *Planta* 228: 745-756.

Li, P. and L. Cheng. 2008. The shaded side of apple fruit becomes more sensitive to photoinhibition with fruit development. *Physiologia Plantarum* 134: 282-292.

Executive Summary

This project was initiated to determine: 1) how high temperature affects the balance of photooxidation and photoprotection of apple fruit peel, leading to sunburn browning; and 2) if chlorophyll fluorescence reflects the damage of high temperature on fruit peel and varietal differences during the growing season.

By combining environmental monitoring under WA conditions, controlled temperature and light treatments, and peel photosynthetic oxygen evolution and chlorophyll fluorescence measurements, we accomplished the objectives of the project. We found 1) Maximum photosystem II (PSII) quantum efficiency (Fv/Fm) of the sun-exposed peel of well-exposed fruit in the southwest canopy decreased during the day in response to high peel temperatures, and very little recovery was made during overnight dark relaxation, indicating that the high peel temperature has damaged the PSII centers of the peel. 2) After a couple days of high temperature exposure, more fruit in the west side of the canopy had very low Fv/Fm value than those in the east side. This difference corresponds to the different profiles of peel temperatures and sunburn occurrence between the two sides of the canopy. This along with the diurnal Fv/Fm data indicates that Fv/Fm is a very sensitive indicator of high temperature stress in apple peel. 3) Compared with the non-sunburned peel, the sunburned peel had lower chlorophyll content, lower Fv/Fm, lower net oxygen evolution rate, and lower activities of key photosynthetic enzymes, but higher activities of antioxidant enzymes and higher content of antioxidant metabolites and higher xanthophyll cycle activity on a chlorophyll basis, and higher hydrogen peroxide and malondialdehyde content. This indicates that high peel temperature most likely has increased the photooxidation potential, rather than decreased the photoprotective capacity of fruit peel. 4) Controlled temperature treatments of fruit peel samples in the dark showed that high peel temperature led to decreases in Fv/Fm and net O_2 evolution, and appearance of "K" step in chlorophyll a fluorescence transient. This indicates that high temperature has damaged the oxygen evolution complex of the PSII, leading to oxidative stress. 5) Simultaneous high temperature and high light treatment decreased Fv/Fm and O_2 evolution of Gala peel more than high temperature or high light alone. The clear "K" step in chlorophyll fluorescence, which appeared in the high temperature treatment, disappeared under simultaneous high temperature and high light treatment. This indicates that high temperature mainly affects the oxygen evolution complex of the PSII (the donor side) whereas high light mainly affects the acceptor side of the PSII. The photooxidative damage occurring in the sun-exposed peel of apple fruit under high temperature coupled with high light is initiated by high temperature on the oxygen evolution complex of the PSII, and exacerbated by high light. 6) Apple cultivars differ in their responses to high temperature and high light stress. Of the cultivars tested, Red Delicious is most tolerant of high temperature and high light stress whereas Cameo is the least tolerant. Our data show that chlorophyll fluorescence is an effective tool for testing varietal difference in tolerance to high temperature and high light stress.

The findings of this project has laid a solid foundation for 1) using chlorophyll fluorescence to monitor and predict damage to apple peel caused by high temperature coupled with high light; and 2) screening for tolerant apple genotypes for breeding tolerant cultivars for high temperature and high light stress; and 3) understanding the molecular mechanism of high temperature/high light stress to apple peel.

FINAL PROJECT REPORT **YEAR: 1 of 1**

Project Title: Sprayable 1-MCP for managing apple postharvest quality

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Cooperators: Eugene M. Kupferman, Horticulturist, WSU-TFREC;
Dwayne Visser, Agricultural Research Technologist III, WSU-TFREC

Other Funding Source: N/A

Total Project Funding: **Year 1: 12,000**

Budget History:

Item	2007
Salaries	5,220
Benefits	1,780
Wages	3,590
Benefits	410
Equipment	0
Supplies	200
Travel	800
Miscellaneous	0
Total	12,000

Objectives:

1. Evaluate effects of ethephon applied 4 weeks or 4 and 3 weeks before normal harvest on fruit quality attributes at harvest 2 or 1 week(s) before normal harvest and at normal harvest.
2. Examine effects of postharvest SmartFresh treatment with or without early ethephon on storability and fruit quality attributes for each harvest date after short-term RA and medium-term CA storage.
3. Assess whether combinations of ethephon applied 4 or 3 weeks before normal harvest and SmartFresh postharvest can enable earlier than normal harvest of fruit with comparable quality and storage characteristics to untreated fruit harvested at the normal timing.

Significant findings:

1. Two treatments of ethephon at 150 mg a.i./liter at a weekly interval had less effect on stimulating drop than a single treatment with 300 mg a.i./liter.
2. Both ethephon treatments induced significant drop; a stop-drop treatment of NAA should be used if this program is repeated (combining ReTain and ethephon treatment on 'Cripps Pink' apple stimulates, rather than retards, fruit drop).
3. Over a two-week interval (three harvests), ethephon stimulated fruit C_2H_4 production in proportion to treatment concentration, not number of applications.
4. Ethephon significantly increased C_2H_4 production for the first two harvests and enhanced starch hydrolysis on the second harvest date.
5. After around 90 days of RA storage or 120 or 240 days of CA storage, 'Cripps Pink' apples treated with ethephon and SmartFresh and harvested one to two weeks early showed quality characteristics as good as untreated apples harvested one or two weeks later.
6. Risk of crop loss due to early fall freezes can be reduced by treating a portion of the 'Cripps Pink' crop with ethephon to permit earlier harvest. Such apples must be treated postharvest with SmartFresh to ensure quality maintenance in storage.
7. Careful attention must be paid to the progress of maturity of ethephon-treated 'Cripps Pink' apples. Fruit drop can be accelerated by this treatment.
8. Do not use preharvest ReTain prior to preharvest ethephon on 'Cripps Pink' apples. Preharvest fruit drop has been significantly increased by this combination.

Methods:

One trial was established in a cropping 'Cripps Pink' apple orchard in Wapato. Multiple-tree plots were chosen to allow sufficient fruit for sequential harvests over 3 weeks. A randomized complete block experimental design was used, with preharvest treatments in a one-way treatment arrangement, subdivided into a 3X2 factorial when harvested fruit samples were separated into replicate non-treated or SmartFresh-treated fruit. Harvest evaluations of fruit characteristics were made, to be followed by subsequent evaluations at 90 days of RA storage, as well as 120 and 240 days of CA storage. Experimental harvest in this trial began 2 weeks before normal commercial harvest, to simulate the avoidance of an early freeze event that otherwise might severely damage a large percentage of the crop.

Results and discussion:

Preharvest ethephon did not result in softer fruit flesh at harvest, a common observation that does not indicate how fruit will behave in storage. Starch hydrolysis and C_2H_4 production were the two fruit characteristics most affected at harvest by preharvest ethephon treatment. The lack of accumulating differences in fruit characteristics at the third harvest suggests that the substantial crop loss to drop may have adjusted the population of remaining fruits such that less mature fruit were the only ones left on ethephon-treated trees at commercial harvest. If so, this observation is not of serious concern, since any program in which ethephon would be used preharvest would also automatically schedule

harvest earlier than the normal commercial harvest time in order to retain a larger proportion of the crop in an early fall freeze event. Follow-up evaluations were carried out after 90 days RA and both 120 and 240 days of CA storage. Fruit treated with ethephon 4 weeks before normal harvest, harvested 1 or 2 weeks earlier than normal, treated with postharvest SmartFresh and then stored were comparable or better in fruit quality characteristics after removal from storage and holding for 7 days at room temperature. Postharvest SmartFresh counteracted the tendency of ethephon to stimulate the climacteric, thus enabling the more mature fruit harvested early to maintain its acceptable quality for long periods. Preharvest ethephon or combinations of preharvest ReTain and ethephon have both increased preharvest fruit drop.

Acknowledgments:
The assistance and support of the following persons and organizations are gratefully acknowledged: Lynnell Brandt, Nancy Buchanan, Clyde Buechler, Dr. Steve Drake, Jeff Henry, Dr. Gene Kupferman, Dr. Nate Reed, Chris Sater, Dwayne Visser, Marcia Walters, Mike Young, AgroFresh, Inc., Bayer CropScience, E.W. Brandt & Sons, Inc., Washington Tree Fruit Research Commission, WSU Agricultural Research Center.

Publications 2008:

Schmidt, T.R., D.C. Elfving, J.R. McFerson and M.D. Whiting. 2008. Gibberellic acid accelerates 'Honeycrisp', but not 'Cameo', apple fruit maturation. **HortTechnology 18:39-44.**

Lenahan, O.M., M.D. Whiting, and D.C. Elfving. 2008. Gibberellic acid is a potential sweet cherry crop load management tool. **Acta Hort. 795:513-516.**

Elfving, D.C. 2008. Bioregulator sprays. p. 75-87. In: T.J. Smith (coord.), **2008 Crop Protection Guide for Tree Fruits in Washington. EB 0419.**

Elfving, D.C. and D.B. Visser. 2008. Bioregulator effects on growth, flowering and cropping in apple trees. **Poster, WA State Horticultural Association Annual Meeting, Yakima, WA.**

Visser, D.B. and D.C. Elfving. 2008. Bioregulators for managing growth, cropping and fruit quality in sweet cherry. **Poster, WA State Horticultural Association Annual Meeting, Yakima, WA.**

Executive Summary:
Preharvest ethephon did not result in softer fruit flesh at harvest, a common observation that does not indicate how fruit will behave in storage. Starch hydrolysis and C_2H_4 production were the two fruit characteristics most affected at harvest by preharvest ethephon treatment. The lack of accumulating differences in fruit characteristics at the third harvest suggests that the substantial crop loss to drop may have adjusted the population of remaining fruits such that less mature fruit were the only ones left on ethephon-treated trees at commercial harvest. If so, this observation is not of serious concern, since any program in which ethephon would be used preharvest would also automatically schedule harvest earlier than the normal commercial harvest time in order to retain a larger proportion of the crop in an early fall freeze event. Follow-up evaluations were carried out after 90 days RA and both 120 and 240 days of CA storage. Fruit treated with ethephon 4 weeks before normal harvest, harvested one or 2 weeks earlier than normal, treated with postharvest SmartFresh and then stored were comparable or better in fruit quality characteristics after removal from storage and holding for 7 days at room temperature. Postharvest SmartFresh counteracted the tendency of ethephon to stimulate the climacteric, thus enabling the more mature fruit harvested early to maintain its acceptable quality for long periods. Preharvest ethephon or combinations of preharvest ReTain and ethephon have both increased preharvest fruit drop.

FINAL PROJECT REPORT

Project Title: Adapting available genomics tools to enhance WA apple breeding

PI:	Cameron Peace	**Co-PI**:	Bruce Barritt
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Other funding sources

Agency Name: WTFRC Apple Review
Amount requested: $158,422 (2008)
Notes: "Apple Scion Breeding" PI: Barritt. Co-PIs: Ross, Peace, Zhu.

Agency Name: USDA-CSREES National Research Initiative
Amount awarded: $400,000 (2009-2010)
Notes: "Functional gene markers for Rosaceae tree fruit texture" PI: Peace. Co-PIs: Costa, van de Weg, Luby, McFerson, Gardiner, Hamblin, and Oraguzie. To be closely coordinated with the current proposed WTFRC Apple project, particularly for activities 2-4 (below).

Agency Name: WTFRC
Amount awarded: $50,000
Notes: "ABI 3730 DNA Analyzer to augment tree fruit breeding and research" PI: Peace. See below also.

Agency Name: Washington Wheat Commission
Amount awarded: $50,000
Notes: PI: See. Matches WTFRC funding (see above) to obtain a refurbished ABI 3730 DNA Analyzer ($100,000) for high-throughput genotyping of tree fruit and cereals, based at Pullman.

Agency Name: WSU Agricultural Research Center
Amount awarded: ~$170,000 (2009)
Notes: Additional support to Dr. Peace and WSU genotyping center(s) for high-throughput DNA extraction and genotyping equipment, complementing the ABI 3730 and removing technical bottlenecks for routine tree fruit genotyping.

Agency Name: WTFRC Technology Review
Amount awarded: $48,235 (2007-2010)
Notes: "Developing flavor gene markers for the Washington tree fruit industry" PI: Peace. Co-PIs: Olmstead, Barritt, Drew

Pending
Agency Name: WTFRC Apple Review
Amount requested: $611,219 (2009-2011)
Notes: "Apple Scion Breeding" PI: Evans. Co-PIs: Peace, Ross, Zhu.

Agency Name: WTFRC Apple Review
Amount requested: $77,616 (2009-2010)
Notes: "Developing an online toolbox for tree fruit breeding" PI: Main. Co-PIs: Evans, Oraguzie, Peace, Jung. Establishment of bioinformatics and databasing support to facilitate the translation of genomics information into application in WSU tree fruit breeding programs.

Agency Name: WTFRC Cherry Review
Amount requested: $45,000 (2009)
Notes: "Establishing the Marker-Assisted Breeding Pipeline for sweet cherry" PI: Peace. Co-PIs: Olmstead, Iezzoni, and Oraguzie. Synergistic project to establish marker-assisted breeding infrastructure for the WSU sweet cherry breeding program.

To be submitted (early 2009)
Agency Name: USDA-CSREES, Specialty Crops Research Initiative
Amount requested: $4,000,000 approximately (plus equal amount matching)
Notes: "RosBREED: Enabling marker-assisted breeding in Rosaceae". PI: Amy Iezzoni. Co-PIs include Peace, Olmstead, and Evans. A synergistic project proposal to establish sustainable marker-assisted breeding infrastructure for U.S. Rosaceae crops, based on the Marker-Assisted Breeding Pipeline concept that involves Pedigree Based Analysis.

Total Project Funding: $40,575

Budget 1 History: WSU

Item	Year 1: 2007	Year 2: 2008
Salaries		
Benefits		
Wages[1]	5000	
Benefits	575	
Equipment		
Supplies[2]	15,000	
Travel[3]	15,000	
Miscellaneous[4]	5,000	
Total	40,575	

Footnotes:
[1] Casual assistance for marker testing (activity 4).
[2] Molecular genetics lab supplies for DNA extraction and marker testing (activity 4).
[3] $3000 in-state (Pullman-Wenatchee), $5000 visit apple germplasm collections and international colleagues, $7000 host international experts (Bliss, van de Weg, Gardiner) for local workshop (activity 3).
[4] Access to software and consulting for Pedigree Based Analysis.

ORIGINAL OBJECTIVES

Our overall goal is to integrate genomics with Washington tree fruit breeding programs, to enhance breeding scope and efficiency. The major objective of the project was to lay the foundation for the larger program by developing the capability and technical infrastructure for applying available genomics information and tools to the Washington apple breeding program (WABP). This will allow, in future years, for more genetically-informed and targeted selection of parents and an efficient means of predicting the genetic value of thousands of seedlings in a short time. Such an enhanced breeding program will also be able to quickly capitalize on future genomic discoveries in apple and other fruit crops.

Specific objectives of this proposal were to:

1) Identify reported genes and linked markers for important traits in apple with the most potential value for the WABP.
2) Adapt the Pedigree Based Analysis approach to the WABP.
3) Establish efficient methods of obtaining DNA samples from thousands of plants at a time.
4) Establish efficient means of routine high-throughput genetic testing of potential parents and seedling populations for the WABP.
5) Coordinate between the WABP and internationally-renowned apple germplasm collections for genetic surveys that identify commercially valuable traits, enabling more rapid exploitation of genes for improved or novel traits that reside in germplasm currently outside of the WABP.
6) Obtain expert advice on important issues by exchange visits with key internationally-eminent scientists and institutions.

SIGNIFICANT FINDINGS

- **A comprehensive review of opportunities and constraints for integrating genomics into the WABP identified numerous specific opportunities for improving the scope and efficiency of this breeding program**. In particular, the approach of marker-assisted breeding (MAB) has the most immediate relevance to breeding, with additional application in understanding genetic predisposition of existing cultivars in Washington orchards.

- **Currently available markers for genes involved in fruit texture and storability (*Md-ACS1* and *Md-ACO1*) are prime candidates for immediate use in MAB**, for providing the first demonstration of the value of MAB to tree fruit breeding in the U.S. Other promising markers are also available, including for other texture attributes, acidity, skin color, and apple flavor (aroma), and are being tested for utility in the WABP.

- The world's most comprehensive **apple germplasm collections**, Geneva (NY, USA) and Brogdale (Kent, UK), contain much genetic diversity that can be channeled into the WABP for future crop improvement – for the development of new cultivars with novel attributes as well as incremental improvements. A replication of the "core collection" of the larger Geneva collection, consisting of 258 diverse accessions, is being propagated for planting in Washington in spring of 2010.

- **Currently available technologies for high-throughput DNA extraction and genotyping can revolutionize the selection of genetically superior seedlings in the WABP**. After comparisons for cost-efficiency, time-efficiency, and practical feasibility for the WABP. Funding for equipment for the optimum high-throughput technologies – the Silica Bead Method for DNA extraction, and ABI DNA Analyzer for genotyping – was obtained from the WSU Agricultural

Research Center (~$170,000), the Washington Tree Fruit Research Commission ($50,000), and the Washington Wheat Commission ($50,000). This involves collaboration with the already established USDA-ARS Western Regional Small Grains Genotyping Laboratory in Pullman, run by Dr. Deven See, leveraging existing equipment and expertise.

- **A successful workshop on the application of MAB for the WABP was held** in May 2007. Strong collaborations with world experts were cemented, and valuable advice obtained to ensure that MAB is efficiently and appropriately integrated into the WABP. An article was published in the July 2007 issue of the Good Fruit Grower by Geraldine Warner, based on an interview conducted during the workshop.

- **Software for implementing Pedigree Based Analysis was obtained** for use in the WABP. The PediMap program was used to visualize the complex pedigree relationships among apple parents used in the WABP. As in most other apple breeding programs worldwide, 'Golden Delicious' is the most prominent founder cultivar for WABP germplasm. Other founders include (Red) Delicious, McIntosh, Rome Beauty, *Malus floribunda* 821 (original source of apple scab resistance for cultivars such as Enterprise and Goldrush), Splendour, and Cox's Orange Pippin.

- **Strategic apple germplasm sets were developed** for a long-term apple germplasm planting to be used for powerful genetic analyses of direct relevance for the WABP. The WABP "Pedigree Set", a set of almost 500 individual trees representing the WABP, was developed with the aid of PediMap. In May 2008, the Pedigree Set was planted together with the Parent Set (38 parent cultivars, five replicate trees each) at the WSU Sunrise Research Orchard. The Geneva core collection replication at Sunrise will provide the bulk of our Diversity Set. A Mapping Set was chosen for future fine-scale genetic mapping of traits of highest priority to the WABP. This set is based on crosses between three contrasting and valuable parents in the WABP: Honeycrisp, Pink Lady, and Crimson Crisp. The Genetic Stock Set will include such material as future marker-assisted introgression lines (to introduce traits from exotic germplasm sources), transgenic lines, and CSI lines. A similar approach will be developed for sweet cherry and pear.

- **A breeders' MAB Decision Support Tool was developed** in the form of an Excel spreadsheet. This tool determines the cost efficiency of marker-assisted seedling selection (MASS) using available markers by calculating potential savings of replacing phenotypic selection with marker selection, and identifying the optimum window for genotyping. A surprising finding of applying this tool for the WABP was that even a single available DNA marker that allows the breeder to cull as little as 10% of the seedlings prior to field planting is cost-efficient to use. This is contrary to the prevailing belief among breeders that half a dozen or more markers for various traits are needed before MASS becomes worthwhile.

- ***Md-ACS1* is of immediate practical use in the WABP**. This marker alone would allow a pre-planting cull of half to three-quarters of many families in the WABP. For example, 75% of Honeycrisp x Pink Lady seedlings, 10,000 seeds of which were produced in 2006, can be identified as inferior by this marker. When applied as a trial test in 2008 for several thousand seedlings, *Md-ACS1* performed to expectations. If used to cull Honeycrisp x Pink Lady seedlings just prior to budding, *Md-ACS1* genotyping would save future field costs averaging more than $12 per seedling genotyped (this calculation includes the small cost of genetic testing).

- **A seven-stage Marker-Assisted Breeding Pipeline was developed** based on our experiences with developing MAB infrastructure for the WABP. Our approach is to dissect, clarify, and perform the practical steps required to translate genomics research (reported marker-trait

associations) into real world application (routine MAB). This MAB Pipeline is of general relevance for tree fruit, Rosaceae, and any perennial crop.

- **This project's PIs/collaborators and concepts have been core ingredients in recent large federal research proposals**. RosCAP was submitted to USDA-NRI in Februray 2008, and RosBREED was submitted to USDA-SCRI in August 2008, but both were unsuccessful. "RosBREED 2" will be submitted to USDA-SCRI in February 2009. The innovative Pedigree Based Analysis approach is a common feature, and has always received positive reviews. The seven-stage MAB Pipeline anchors both RosBREED proposals.

- **We successfully obtained federal funds for a WABP-based project on apple fruit texture genetics**. The project, "Functional gene markers for Rosaceae tree fruit texture", will occur in 2009 and 2010 with $400,000 from USDA-NRI.

- **Routine marker-assisted breeding is now enabled for the Washington apple breeding program**. MAB can begin with the *Md-ACS1* genetic test for reduced fruit softening and long storage capacity, beginning what we expect will be a successful ongoing MAB scheme for the WABP, at the forefront of tree fruit breeding programs worldwide. Routine MAB will reduce costs, improve operational efficiency, and reduce time to release new cultivars in the WABP.

RESULTS AND DISCUSSION

Abbreviations:
HR – HortResearch (New Zealand)
MAB – marker-assisted breeding
MAPS – marker-assisted parent selection
(using markers to "map" out the populations
to be created)
MASS – marker-assisted seedling selection
(using markers for mass genotyping of
many progeny seedlings)
MBM – Metallic Bead Method
PBA – Pedigree Based Analysis (formerly
known as PG – Pedigree Genotyping)
PRI – Plant Research International (Netherlands)
SBM – Silica Bead Method
TAS – Theonyx Automated System
WABP – Washington apple breeding program
WSU – Washington State University

This anticipated one-year project (2007) continued for an additional year (2008) with no further cost to the WTFRC. In addition to the large proportion of time spent on the project by the PI, co-PI, and many collaborators, additional personnel support was provided by WSU in the form of Dr. Peace's Associate In Research, Daniel Edge-Garza. Mr. Edge Garza, also a Masters student at California State University Fresno, worked primarily on this project, conducting various aspects including reviewing, trialing, and troubleshooting DNA extraction and genotyping technologies, performing some of the apple germplasm genotyping, aiding development of the MAB economic analysis spreadsheet (chosen as his Masters research topic), and technical support for the May 2007 workshop.

The past year saw the transition of WSU apple breeding responsibilities from co-PI Barritt to Dr. Kate Evans. PI Peace was on the WSU selection panel that recommended Dr. Evans for the position.

We believe that Washington apple breeding program will be a pioneer of marker-assisted tree fruit breeding on the world stage, particularly for seedling selection. Just a handful of tree fruit breeding programs in Europe use markers (for disease resistance thus far) for seedling selection on thousands of seedlings, although it is unclear whether this is performed routinely each year. HortResearch (New Zealand) is subcontracted to genotype (using a marker that determines gender) tens of thousands of kiwifruit seedlings each year.

Four major uses of markers within a breeding program are marker-assisted parent selection (MAPS), parentage verification, marker-assisted seedling selection (MASS), and cultivar identification or profiling. All four of these applications are components of the more general term of marker-assisted breeding (MAB). MAPS is already widely adopted by many tree fruit breeders, especially for gene markers where specific marker alleles are directly associated with specific phenotypic effects – such as the high and normal ethylene alleles of *Md-ACS1*. Breeders also employ MAPS when they use genetic diversity analyses to choose pairs of unrelated parents for crossing to avoid inbreeding. Parentage verification has to date only been performed in tree fruit breeding programs on advanced selections, where numbers of individuals to check are very low. MASS is the application that is usually meant when the term MAB or MAS (marker-assisted selection) is used, and involves genetic screening of thousands of seedlings followed by rejection of inferior types. This high-throughput marker use poses the greatest logistical challenges. Cultivar profiling is used in the patent process to help distinguish new cultivars from existing ones, and discourage illegal propagation of advanced selections. We hope to address this marker application from 2009, to help safeguard new cultivars arising from the WABP. Cultivar profiling can also be used to identify labeling errors and detect cultivar synonyms.

Activity 1: Germplasm resources

1a: Identify specific opportunities for marker-assisted breeding in the Washington apple breeding program

In late March 2007, PI Peace, with research assistant Daniel Edge-Garza, spent several days in Wenatchee with co-PI Barritt. This trip began the formal process of identifying key points in the WABP where genomics could facilitate breeding efforts, by reviewing the current objectives, activities, and capacity of the WABP, and the technical infrastructure required to implement MAB on a routine, high-throughput basis. Local USDA-ARS collaborators (Drs. Yanmin Zhu, Jim Mattheis, and Dave Rudell) were also consulted. A report was written that gave an overview of the WABP (goals, history, people and institutions involved, overall cost, expected cultivar outputs, and future plans and expectations), described traits of interest, explained the current breeding cycle timeline and selection scheme (overview of breeding scheme, details of the

breeding cycle, and costs for operations), detailed the WABP's physical capacity (crossing, greenhouse, nursery, field space, field phenotyping, lab/storage space, lab phenotyping, data management, and cultivar deployment), and finally, made recommendations on the opportunities and constraints for MAB in this breeding program. Traits of interest to the WABP were listed under the categories of "Highest priority", "Also selected for but of lower priority", and "Don't select for but of interest". For each trait was described their desired level, existing variation and need for new germplasm, and potential for MAB, and this was greatly aided by regular teleconferences with collaborators hosted by Dr. Fred Bliss to prioritize and assess traits for the WABP and their suitability for MAB.

This 16-page report (Appendix 1) on the WABP was the first section of a four-part document printed and distributed to experts joining the workshop held in May 2007 (see activity 3 below). The document was also to be posted on the WSU Horticulture Genomics, Genetics, and Breeding ("hortggb") website; however, this website is not yet operational. The document was provided to candidates for the WSU apple breeding position in early 2008 to familiarize them with the WABP and intended incorporation of MAB. Some recommendations of the WABP review are listed below:

Opportunities and constraints for MAB in the Washington apple breeding program arising from a review of the program in March 2007:

- MAB appears likely to **save money, space, and time** within the Washington apple breeding program, and lead to more and/or better cultivars.
- The simplest use of markers in the breeding program, with considerable benefits, is in **parent genotyping** (i.e. marker-assisted parent selection, or MAPS). Parents and cross choice can be greatly improved by genotyping, such as for firmness with the ACS gene test.
- Enormous **cost savings** are possible with strategic use of MAB in **progeny selection** (i.e. marker-assisted seedling selection, or MASS), particularly by eliminating the costs of establishing and maintaining trees that would not pass field evaluations. If these trees were not planted, an average of $13 per tree ($120,000 for 9,000 seedlings) would be saved, of which approximately two-thirds are costs associated with growing these inferior plants, a quarter is budding costs, and the rest is phenotyping costs.
- There are several **possible ways to use the cost savings of MAB** within the program – one or a combination of the following:
    a. Increase the initial number of seedlings and select the same number of genotypes for 2nd stage onward (should lead to better cultivars)
    b. Increase the initial number of seedlings and increase the number of genotypes for 2nd stage onward (should lead to more cultivars)
    c. Increase the number of locations tested for each selection in the 2nd stage and/or 3rd stage (should lead to reduced time to cultivar release and industry acceptance due to greater confidence in performance across multiple environments)
    d. Increase the number of clones tested at each location in the 2nd stage and/or 3rd stage (should lead to reduced time to cultivar release due to greater confidence in performance variability within sites)
    e. Increase the degree of field and lab assessment, particularly the use of more objective methods
    f. Devote some/more resources to incorporating exotic germplasm for introgressing novel traits
- MAB will be of greatest benefit if it is used to **perform culling** – i.e. individuals with an inferior genotype are eliminated from further assessment as soon as their genotype is known.

- **The more markers used** in MASS, the fewer progeny are likely to be retained, or the more initial seedlings are required to maintain current numbers of selected progeny. If markers reduce the number of progeny too much, then the problem is in the parents used or the original number of seedlings created, not in the value of the markers.
- **If single markers were available** for the following traits, they would be used to cull undesirable progeny (assuming they are independent or synergistic with other traits), and would aid in crossing decisions:
  - Firmness retention: High vs. low ethylene, would cull high ethylene (soft after storage)
  - Crisp vs. not crisp, cull not crisp
  - Juicy vs. not juicy, cull not juicy
  - Acidity: some (TA 0.4+) vs. bland (TA <0.4), cull bland
  - Sweetness: sweet (SSC 15%+) vs. not sweet (<15%), cull not sweet
  - Yellow vs. green ground color, cull green
  - Skin cover vs. no cover, cull no cover
  - Proportion of skin cover: >20% vs. <20%, cull <20%
  - Russet: Low vs. high incidence, cull high incidence
  - Bitter pit or scald susceptibility: Resistant vs. susceptible, cull susceptible
  - Watercore: No or low susceptibility vs. high susceptibility, cull high susceptibility
  - Precocity: precocious or normal vs. slow-bearing, cull slow bearing
  - Disease resistance (powdery mildew and fire blight): No or low vs. high susceptibility, cull high susceptibility
  - Yield: At least commercial standard yield vs. low yield: cull low yield
  - Fruit size: normal (88+ box size) vs. small, cull small
  - Novel traits: Markers for novel traits would facilitate their introgression into an elite background, and therefore their incorporation into new cultivars. However, it is likely that use of exotic (undomesticated) germplasm will require at least one extra generation of breeding to effectively introgress a useful trait.

Available markers for the above traits, which function within the WSU apple breeding program with a strong predictive power, are therefore desired!

1b: Identify specific opportunities in apple germplasm collections

In late March and early April 2007, PI Peace visited the two largest ex situ apple collections in the world: the "Geneva collection" – the USDA-ARS National Clonal Germplasm Repository (Geneva, New York) – and the "Brogdale collection" – the National Fruit Collection (Brogdale, Kent, UK). The objective of these trips was to determine where marker-assisted germplasm characterization would be most efficient and identify specific opportunities inherent in the collections to meet the needs of the WABP. Following these visits, a report on each collection was made that described for each an overview of the collection, the availability of germplasm, phenotypic characterization, molecular characterization, and ended with recommendations for specific opportunities for the WABP. Dr. Peace was hosted by Dr. Chuck Simon while in Geneva, and meetings were also held individually with Drs. Gennaro Fazio, Susan Brown, Angela Baldo, and Herb Aldwinckle (Geneva collection curator Dr. Phil Forsline was not available at the time), each of whom graciously described and showed their apple breeding and/or genomics programs. Dr. Peace was hosted by Dr. Colin Turnbull while visiting the Brogdale collection, and also met Dr. Emma-Jane Allen who was the Scientific Curator of the collection, and Drs. Kate Evans and Ken Tobbutt who conducted apple breeding and genomics research at East Malling Research and recently performed molecular characterization of the Brogdale collection. While in Europe, Dr. Peace also visited Dr. Eric van de Weg, another formal collaborator of this WTFRC project, and other key personnel at Plant Research International in Wageningen, the Netherlands. Opportunities were identified for each collection for obtaining germplasm for breeding, and for conducting genotyping and phenotyping in future research

collaborations. Around the time of the visits, 5-year planning cycles were being hashed out, and so it was an opportune time to coordinate the needs of the WABP with those of the collections. The description of the Geneva collection comprised an 8-page report (Appendix 2), and another 4-page report was completed for the Brogdale collection (Appendix 3). These formed the second and third parts of a document prepared for the expert workshop of activity 3.

Germplasm sets

The breeder/curator for each of the three apple germplasm sources reviewed in early 2008 – WABP, Geneva, and Brogdale – chose a representative subset of 96 individuals, and provided leaf material for each. We extracted DNA from each sample (with each of three methods – activity 4b) for testing with various promising markers (activity 4). For WABP, the subset consisted of 61 cultivars (and advanced selections from other programs) used as breeding parents, and 35 selections. For Brogdale, 96 diverse cultivars were included. The Geneva germplasm comprised 32 diverse cultivars, 49 non-*domestica Malus* species, and 15 interspecific hybrids. The software PediMap, developed at PRI and provided free of charge by Dr. Eric van de Weg, was used to visualize pedigree relationships among the 96 individuals of the WABP set. PediMap analysis revealed more than 50 founders (ancestral cultivars without known ancestors themselves) for the WABP material.

At the project outset, the intent was to choose a "PG-set" – "a set of apple varieties within the WABP (cultivars, advanced selections, etc) that can be used in Pedigree Genotyping [PG, now known as Pedigree Based Analysis, PBA] for future verification of marker-trait associations". The 96 WABP individuals were developed for this purpose. Later, it became apparent that this WABP set was instead a large Parent Set. A true PBA set requires larger numbers of pedigree-linked individuals to effectively validate marker-trait associations, preferably comprising many small populations of 25-50 individuals (M. Bink, personal communication). Pedigree Based Analysis is best served when comprehensive phenotypic data is available for each individual.

To address the needs of future genetic analyses for the WABP, strategic germplasm sets were devised, to be grown at the Sunrise Research Orchard as a long-term germplasm planting. The long-term planting is anticipated to consist of the following five germplasm sets (approximate tree numbers are indicated, all trees to be grafted on M9):

1. Parent Set (50 cultivars x 5 trees each)
2. Pedigree Set (475 seedlings x 1 tree each)
3. Diversity Set (400 accessions x 2-3 trees each)
4. Mapping Set (600-900 seedlings x 1 tree each)
5. Genetic Stock Set (unknown number, variable replication)

The Pedigree Set consists of 16 families derived from crossing among 10 parent cultivars – a subset of the Parent Set and a subset of the WABP 96-set that still well represents the range of germplasm in the WABP (Figure 1). Three of the parents are WSU selections (WSU 3, WSU 5, and WSU 7), thus including the next generation. Crosses among the three "core parents", Honeycrisp, Pink Lady, and Aurora Golden Gala, have 50 seedlings each, while the remaining families have 25 seedlings each. The Pedigree Set parents are ultimately derived from 18 founders, and are joined by complex pedigree relationships.

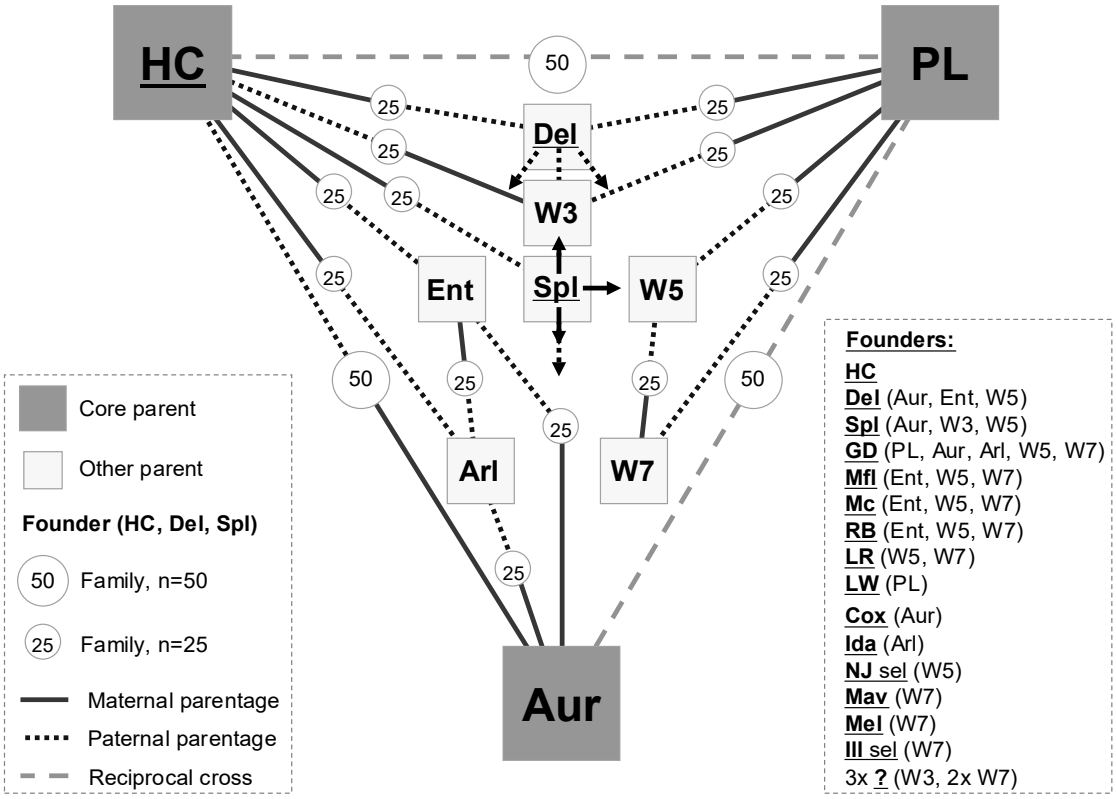

Figure 1: Schematic of the composition of the WABP Pedigree Set. Eighteen founders are represented (15 known and three unknown). Pedigree Set parents derived from each founder are shown in the bottom right section. Three founders (Honeycrisp, Delicious, and Splendour) are immediate parents of the Pedigree Set (in other words, three parents here have no known ancestors). On the main diagram, arrows from the founders Delicious and Splendour point to their descendants.

One advantage of Pedigree Based Analysis is that can use existing breeding germplasm rather than requiring the creation of devoted experimental populations. The 475 seedlings of the Pedigree Set follow this concept, as they are actually part of the breeding program despite being planted separately at Sunrise. The breeder will still be able to collect performance data on these individuals and identify promising selections, although the trees will remain alive for additional genetic analyses. Breeding data will be supplemented with additional phenotypic data where required. The 475 trees, each grafted on M9 rootstock, were planted at Sunrise in May 2008, together with 38 cultivars/selections of the Parent Set.

The bulk of the Diversity Set will be planted in spring of 2010, consisting of the 258 accessions of the Geneva collection's "core collection" (the 96-set Geneva collection for current DNA analysis is a subset of this core), with budwood provided in fall 2008 by USDA-ARS Geneva. The Mapping Set will also be planted at this time. Budded trees for the Mapping Set are from families created for the WABP. As for the Pedigree Set, seedlings of the Mapping Set will remain available for performance testing and selection within the breeding program.

Activity 2: Genomics resources
2a: Review available markers for apple

A table was compiled of marker/gene-trait associations in reported (papers, conference presentations, and personal communications) of potential value for the WABP. New markers and promising genes are continually being reported, and can be added to this list for those traits of priority to the WABP. This table is available to WTFRC members on request.

Activity 2b: Review available high-throughput technologies
Three contrasting methods for high-throughput DNA extraction were identified and considered for their applicability to the WABP. The first is the DNA extraction robot developed in New Zealand and available for testing through HortResearch (Theonyx Automated System, TAS), the second (Silica Bead Method, SBM) is currently used by the USDA-Pullman small grains lab and has the benefit of avoiding several steps of the others by using silica beads for tissue dessication, and the third (Metallic Bead Method, MBM) is run in the wheat genetics lab of Dr. Kulvinder Gill in Pullman and is theoretically the cheapest. These three methods were chosen for comparison in Activity 4b.

High-throughput genotyping technologies were identified and compared (as a desk exercise) for their applicability to the WABP and germplasm collections, including development costs, running costs, and suitability for screening various marker types. This comparison was presented as a 10-page report (Appendix 4), forming the fourth part of the document used in the workshop of activity 3. The ABI (Applied Biosystems) genotyping platform was identified as the single most applicable system. ABI machines are able to genotype several relevant marker types, and do so at the best cost efficiency for a range of sample and marker numbers relevant for the WABP. From our review in early 2007, it was recommended that for the needs of the WABP (and also servicing the stone fruit breeding program), a dedicated ABI machine be obtained. In 2008, Dr. See requested partial funding from the Washington Wheat Commission (WWC) to obtain a refurbished ABI 3730 (48-capillary). Dr. Peace requested equivalent funds ($50,000) from the WTFRC to purchase this machine jointly with Dr. See and the WWC. Currently, this combined proposal has been given the green light. The ABI machine, which will more than cover the genotyping needs of WSU tree fruit breeding programs and regional small grains breeding programs, is expected to be established in Dr. See's lab in Pullman in early 2009, with management of its use to be conducted jointly by Drs. Peace and See. The WSU Agricultural Research Center has also agreed to fund approximately $170,000 in additional equipment chosen by Dr. Peace to remove any remaining physical bottlenecks for DNA extraction and genotyping of tens of thousands of tree fruit seedlings each year.

Activity 3: Prioritization
In May 2007, a week-long workshop was held in Washington (the "Apple Genomics Enabling Team Tour"). External experts hosted were Dr. Eric van de Weg of PRI in the Netherlands, Dr. Sue Gardiner of HortResearch in New Zealand, and Dr. Fred Bliss of Davis, California. The internal participants were PIs Drs. Peace and Barritt, and collaborators Drs. Jim McFerson, Amit Dhingra, Dorrie Main, Matt Whiting, Jim Olmstead, and Yanmin Zhu. Conveniently, Marco Bink (PRI, Netherlands) was also available for the final days. Details of the workshop are in Appendix 5. Action items and recommendations resulting from this productive workshop are reflected in Significant Findings above and activities performed for Infrastructure establishment described below.

Activity 4: Infrastructure establishment
4a: PG-set database
An Excel spreadsheet was developed for housing data on the 96-individual sets of WABP, Geneva, and Brogdale. Columns include cultivar name, female parent, male parent, and genotypes for any markers tested. Additional columns are available for phenotypic data, but have

not yet been used. Pedigree data in this format was used directly in the PediMap program for visualizing pedigree relationships (activity 1). The second, and core, PBA software package was obtained from Dr. Marco Bink – FlexQTL. This software has not yet been used. It will first be tested (in early 2009) on firmness/storage data obtained by Drs. Zhu and Barritt for their *Md-ACS1* and *Md-ACO1* study.

4b: Test high-throughput DNA extraction technologies

Three high-throughput DNA extraction methods (TAS, SBM, and MBM, as described for activity 2b) were trialed using leaf samples from each of three germplasm sets (WABP, Geneva, Brogdale) arranged in 96-well plate formats. All three methods produced suitable DNA extracts, the most reliable being TAS. Costs for TAS were estimated at $200,000 for equipment and $2.86 per sample. SBM: $19,000 equipment and $0.24 per sample. MBM: $61,000 equipment and $0.19 per sample. SBM was chosen for future apple high-throughput DNA extraction because of its simple tissue sampling requirements (no freeze-drying needed) and for its lower start-up cost. A research poster on these efforts was presented at the 4th International Rosaceae Genomics Conference (March 2008) by Mr. Daniel Edge-Garza, which garnered interest from many other programs seeking to ramp up their genotyping. The poster was also presented by Mr. Edge-Garza at an research-industry forum in Fresno, CA (September 2008).

4c: Implement Pedigree Based Analysis for the WABP

Several markers have been screened on the WABP, Geneva, and Brogdale 96-sets so far: *Md-ACS1* and *Md-ACO1* (the fruit softening and storability genes that encode critical enzymes of the ethylene biosynthesis pathway), *Md-Exp7* (a cell wall-modifying gene recently implicated in texture determination), *Md-PG2* (the apple version of the gene controlling the rapid softening "melting flesh" of peaches), and SSR markers at genomic regions implicated in the control of acidity, crispness, and juiciness (*Ma* locus), and firmness and columnar growth habit (*Md-PG1* region and the *Co* locus). Results have proven interesting. Polymorphism was recorded for each marker across individuals of the WABP, Geneva, and Brogdale 96-sets, and collated in the database of activity 4a. Allele frequencies were compared among the various apple germplasm categories (1. wild *Malus* species, 2. *Malus* species believed to be the progenitors of cultivated apple, 3. diverse apple cultivars not in the WABP, 4. parent cultivars of the WABP, and 5. WABP selections). Results for the four genes were reported at the Apple Crop Germplasm Committee meeting in October 2008 (Appendix 6). This analysis revealed certain alleles (gene variants) that are apparently increasing in frequency in the WABP (i.e. more frequent in WABP parents than in diverse cultivars, and more frequent in WABP selections than parents), and thus candidates for association with desirable attributes. Some alleles were less frequent in recent selections – candidates for association with undesirable attributes. As would be expected, the low ethylene alleles of *Md-ACS1* and *Md-ACO1* corresponded to the former while the high (normal) ethylene alleles matched the latter. This indicates that phenotypic selection in the WABP for improved firmness and storability is favoring the accumulation of low ethylene alleles. However, these desirable alleles are only at 75% (Md-ACS1) and 35% (Md-ACO1) in the 35 WABP selections surveyed. Thus, the average performance of WABP selections can still be improved (until all selections carry two copies of the desirable alleles), and the gene marker tests can get us there much more efficiently than with phenotypic selection. Marker profiles were also used to verify and deduce parentage for WABP individuals. We are currently filling in missing genotypic data for these germplasm sets, conducting more detailed analyses of what it all means, and targeting further promising genomic regions.

4d: Test high-throughput genotyping technologies

A breeders' MAB Decision Support Tool was developed in the form of an Excel spreadsheet. This tool determines the cost efficiency of MASS using available markers, or hypothetical markers to help guide research directions. Using input parameters that describe aspects of a breeding program – such as the stages involved, costs of each routine operation with traditional phenotypic selection (i.e. without markers), and proportions of seedlings expected to be maintained through each stage – we can determine the potential savings of replacing phenotypic selection with marker selection and the optimum stage for genotyping. So far we have used this for the WABP, and identified, for example, that if only a single genetic marker is available that detects 50% of the seedlings as undesirable, approximately 40% of the total cost after eight years (from crossing to deciding on which seedlings to advance to replicated trials) can be saved by using that marker. Also, we unexpectedly discovered that the optimum stage for genotyping is not always as early as possible. With the availability of more good markers comes greater savings and efficiency, which could be reinvested into larger initial seedling numbers for genotyping.

Use of this economic aid spreadsheet was described at two cherry breeding program meetings in Prosser, WA, by Dr. Peace, for which the tool is also applicable, although the examples used were based on the WABP. As part of his Masters studies, Mr. Daniel Edge-Garza presented results of this work in a paper for a Business class (May 2008), which was slightly amended and distributed to Dr. Jim Luby (October 2008) for use in his genetics course at the University of Minnesota. This document is available to WTFRC members on request. Mr. Edge-Garza also presented two seminars on the topic in September 2008, at WSU Pullman and at California State University Fresno. While the tool is currently in a workable format for the WABP, we plan to update it with further functionalities.

Trial use of high-throughput MASS was conducted for the WABP using *Md-ACS1* and *Md-ACO1* in several thousand seedlings made up of three families: Honeycrisp x Pink Lady, Pink Lady x Crimson Crisp, and Honeycrisp x Crimson Crisp. First, we used the economic aid spreadsheet to determine the optimum genotyping stage for cost efficiency. This was determined to be the 2-3 month window in the middle of Year 3 following powdery mildew selection and prior to fall budding. Effectively, 6250 original seeds became 5000 nursery seedlings after early losses from lack of germination and low vigor, falling to 3800 after powdery mildew infection in the nursery. These 3800 seedlings were genotyped (although only the Crimson Crisp crosses required *Md-ACO1* genotyping). Genotyping costs mirrored spreadsheet expectations, and the proportions of genotypes recommended for culling were consistent with genetic expectations based on parent genotypes: ¾ of each family. If these seedlings were culled, the costs of budding, field planting, maintaining, and evaluating these inferior individuals would be saved (about $18 per tree, totaling more than $50,000), for relatively minor genotyping cost (less than $1 per tree, totaling less than $4000). However, these seedlings are to be retained to allow a direct comparison, once trees are fruiting, of phenotypic evaluation and selection for softening and storability versus *Md-ACS1*/*Md-ACO1* genotyping.

EXECUTIVE SUMMARY

Our overall goal is to integrate genomics with Washington tree fruit breeding programs, to enhance breeding scope and efficiency. The major objective of the project was to lay the foundation for the larger program by developing the capability and technical infrastructure for applying available genomics information and tools to the Washington apple breeding program (WABP). This will allow, in future years, for more genetically-informed and targeted selection of parents and an efficient means of predicting the genetic value of thousands of seedlings in a short time. Such an enhanced breeding program will also be able to quickly capitalize on future genomic discoveries in apple and other fruit crops.

Specific objectives of this proposal were to:

1) Identify reported genes and linked markers for important traits in apple with the most potential value for the WABP.
2) Adapt the Pedigree Based Analysis approach to the WABP.
3) Establish efficient methods of obtaining DNA samples from thousands of plants at a time.
4) Establish efficient means of routine high-throughput genetic testing of potential parents and seedling populations for the WABP.
5) Coordinate between the WABP and internationally-renowned apple germplasm collections for genetic surveys that identify commercially valuable traits, enabling more rapid exploitation of genes for improved or novel traits that reside in germplasm currently outside of the WABP.
6) Obtain expert advice on important issues by exchange visits with key internationally-eminent scientists and institutions.

Significant accomplishments in 2007-2008 include:

- A comprehensive review of opportunities and constraints for integrating genomics into the WABP. Specific opportunities include promising markers for use in marker-assisted breeding. The best candidate for immediate use is the gene *Md-ACS1*, which controls fruit ethylene levels during ripening and thereby influences firmness retention during long term storage.
- Establishment of required technical infrastructure for high-throughput genetic testing of seedlings of the WABP. Equipment was identified, compared, and funds obtained to purchase the best.
- Fostering of strong national and international collaborations for apple genomics, genetics, and breeding. World experts were hosted, other apple programs were visited, and we had leadership and participation in several large federal grant proposals requiring community-wide coordination.
- Identification of novel and useful genetic variation for use in the WABP.
- Development of strategic apple germplasm sets for a WA long-term apple germplasm planting.
- A breeders' Decision Support Tool that determines cost efficiency of marker-assisted seedling selection in the WABP.
- Successful demonstration of high-throughput genetic screening for the WABP.
- A federal award of $400,000 to elucidate apple fruit texture genetics and show industry value.
- Priming the WABP for marker-assisted breeding, so that routine genotyping can start in 2009.

CONTINUING PROJECT REPORT **YEAR**: 1 of 3

Project Title: Crop load and canopy management of apple

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Address: 1719 Springwater Ave.
City: Wenatchee
State/Province/Zip WA 98801

Cooperators: Jim McFerson, Ines Hanrahan, Felipe Castillo, Tom Auvil - WTFRC

Budget 1:
Organization Name: WTFRC **Contract Administrator:** Kathy Schmidt
Telephone: (509) 665-8271 **Email address:** kathy@treefruitresearch.com

Item	**Year 1:** 2008	**Year 2:** 2009	**Year 3:** 2010
Salaries	23,230	26,220	25,000
Benefits	6,770	7,650	7,200
Wages	27,150	25,700	24,000
Benefits	12,750	12,100	11,500
Equipment	2,500	3,000	3,000
Supplies	2,500	3,000	3,000
Travel	2,000	2,000	2,000
RCA rental	1,200	4,200	4,200
USDA facilities fee	750	750	750
Total gross costs	76,850	84,620	80,650
Reimbursements	(27,600)	(25,000)	(25,000)
Total net costs	49,250	59,620	55,650

Footnotes: RCA rental based on fiscal year billing cycle
Travel includes fuel costs for driving to trial sites
USDA facilities fee covers storage space and use of research packing line

NOTE: Budget for informational purposes only; research is funded through WTFRC internal program

OBJECTIVES:

1) Evaluate pre-bloom, bloom, and post-bloom chemical thinning agents and mechanical thinning technologies with particular focus on complete programs to achieve three goals:
   a) Minimize costs of green fruitlet thinning
   b) Maximize fruit quality
   c) Encourage annual bearing
2) Investigate influence of important variables (drying conditions, spray technology, carrier volume) on chemical thinner efficacy and fruit finish
3) Develop practical PGR programs to manipulate floral initiation and promote annual bearing
4) Evaluate horticultural effects of reflective materials (Extenday, mylar products)
5) Profile natural tree-to-tree variation in long-term cropping patterns in a newly planted apple block
6) Expand collaborative efforts with other research programs

SIGNIFICANT FINDINGS:

Effective chemical thinning programs reduce hand-thinning, improve fruit size and quality, and increase return bloom; bloom thinners generally achieve these goals more consistently than postbloom programs (Tables 2, 4)

Oil (dormant, summer, vegetable, fish) + lime sulfur programs are the most efficacious options for bloom thinning; results with Crocker's Fish Oil are most consistent (Table 4)

Thinning efficacy and fruit finish were not clearly affected by variations in spray technology (AccuTech vs. Proptec vs. airblast), carrier volume (100 vs. 200 gal/acre), or drying conditions (dawn vs. noon vs. evening sprays) of chemical thinning programs (data not shown)

BA + carbaryl thinning programs give results equal or superior to NAA + carbaryl or ethephon + carbaryl programs; BA often shows a positive effect on fruit size (Tables 3-4)

Factorial field trials indicate that chemical thinning (bloom and postbloom) and PGR (BA or BA+GA) programs are not affected by the presence/absence of Extenday throughout the growing season, including at time of application (data not shown)

Summer applications of NAA have not increased return bloom in WTFRC trials; GA trials to inhibit return bloom will be evaluated in spring 2009 (data not shown)

Ongoing collaborative efforts across disciplines, institutions, and regions (Greene, McArtney, Hirst, Yoder, Rom, Fallahi, Elfving, Lewis, Toye) increase relevance and impact of all crop load management research

Extenday products improve yields of target fruit in apple by:

1) Increasing fruit set without sacrificing fruit size (Tables 6, 7)
2) Increasing fruit size without reducing fruit set (Tables 6, 7)
3) Increasing fruit color (Tables 6-8)

Trees treated with Extenday products over multiple seasons demonstrate increasing capacity to carry high quality fruit (Tables 6, 7)

Mylar products increase apple fruit color, but not as dramatically as Extenday in WTFRC trials (Table 8)

BACKGROUND:
We have begun scaling back internal research efforts in chemical thinning to accommodate more collaborative work in other areas, but also in part because of the success of earlier work. Many programs and principles put forward by our research, especially aggressive bloom thinning with lime sulfur, are now firmly established across the Washington industry. We plan to continue screening new materials and programs for crop load management, but our focus is now increasingly on collaborative projects exploring mechanical thinning techniques (see Lewis/Schupp technology committee project report for more details), the genetic and physiological basis for cropping, and increasing the precision and predictability of crop load management programs through web-accessible developmental models and decision systems.

We continue to evaluate the relative success of chemical and mechanical thinning programs through three measurable targets which are directly tied to a grower's economic bottom line:

1. Reduction of green fruitlet hand-thinning
2. Improved fruit size and quality
3. Increased return bloom/annual bearing

The degrees to which our chemical thinning programs achieve each of these goals are reflected in our data labeled fruitlets/100 floral clusters, harvest fruit size, and percent return bloom, respectively.

Our protocols generally call for two applications of each bloom thinning program, at 20% and 80% full bloom. Likewise, most postbloom thinning programs are applied twice, typically at 5mm and 10mm fruitlet size. Programs in 2008 are reflected in Table 1; in programs which show a range of possible rates, higher concentrations are typically reserved for cultivars known to be difficult to thin, such as Fuji and Golden Delicious. In most cases, additional chemical thinning treatments were left to the discretion of individual grower-cooperators, provided that each experimental plot receives the same programs.

Table 1. Typical chemical thinning programs evaluated. WTFRC 2008.

BLOOM THINNERS
2 gal Ammonium thiosulfate (ATS)/A
6-8% Lime sulfur (LS)
2% Crocker's Fish Oil (CFO) + 2-3% LS
15% GS Long thinner (unnamed)
1 gal MaxCel, Exilis, Genesis benzyladenine (BA)/A
20% Raynox
6 pts Ethrel (ethephon)/A
4 oz naphthaleneacetic acid (NAA)//A
0.5% Potassium bisulfate
1% Salicylic acid
0.5-1% Clove oil + 1% dormant petroleum oil
0.5-1% Matran EC
POSTBLOOM THINNERS
2-3 qts Sevin (carbaryl)/A
3-4 qts MaxCel, Exilis, Genesis BA (BA)/A
3 oz NAA/A
0.5-1% Matran EC
1% Citric acid
2% Potassium sulfate
1% Clove oil + 1% dormant petroleum oil

BLOOM THINNING:
A 2008 chemical bloom thinning trial produced only one significant result: ethephon applied during bloom reduced fruit set and increased fruit size in Golden Delicious (data not shown). We plan to screen novel chemistries on an ongoing basis, including more ethephon programs in 2009. Despite promising results in 2007, programs developed by Curt Rom and Jason McAfee (U of Arkansas) featuring clove oil and salicylic acid failed to perform in 2008 in either bloom or postbloom chemical thinning trials (data not shown).

Even though we have reduced our work in dedicated bloom thinning trials, we continue to corroborate prior results of ATS and oil + lime sulfur programs in the context of other experiments. No thinning program we have evaluated yet outperforms oil + lime sulfur combinations. Table 2 summarizes results from all apple bloom thinning trials conducted by the WTFRC since 1999, reflecting a very conservative standard by which to assess our most frequently studied programs.

Table 2. Incidence and percentage of results significantly superior to untreated control. Apple chemical bloom thinning trials WTFRC 1999-2008.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
ATS	15 / 56 (27%)	10 / 59 (17%)	4 / 50 (8%)
NC99	15 / 30 (50%)	7 / 32 (22%)	2 / 27 (7%)
Lime sulfur	25 / 54 (46%)	12 / 48 (25%)	9 / 47 (19%)
CFO + LS	59 / 100 (59%)	26 / 91 (29%)	20 / 87 (23%)
JMS + LS	14 / 24 (58%)	8 / 23 (35%)	4 / 22 (18%)
WES + LS	14 / 27 (52%)	4 / 26 (15%)	4 / 26 (15%)
VOE	13 / 29 (45%)	4 / 28 (14%)	2 / 30 (7%)

[1]Does not include data from 2008 trials.
[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

POSTBLOOM THINNING:
The primary focus of our postbloom thinning work is to identify effective programs which do not rely on carbaryl; fruit treated with this insecticide is already prohibited in many foreign markets, and we expect this trend to continue. Results from 2008 grower-applied trials are consistent with prior outcomes which demonstrate that 1) tank mixes of carbaryl and BA outperformed tank mixes of carbaryl and NAA and 2) BA + NAA programs are equal or superior to any standard postbloom thinning programs utilizing carbaryl (Table 3). Table 4 also confirms our past assertions that carbaryl + BA programs are often superior to standard carbaryl + NAA programs. Perhaps most striking about Table 4 is the overall dearth of significant effects from any postbloom chemical thinning program; when compared to the general success rates of bloom chemical thinners (Table 2), it becomes all the more clear that early, aggressive thinning is critical to effective crop load management.

Table 3. Crop load effects of postbloom thinning programs (grower applied). WTFRC 2008.

Trial	Treatment	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russeted fruit
			%	%	g		%
Jonagold/M.7	BA + NAA	53 b	54 ns	40 ns	265 a	72	47 ns
- Royal Slope	Carbaryl + BA	50 b	56	38	256 ab	75	36
	Carbaryl + NAA	60 ab	47	46	240 ab	79	48
	Control	67 a	46	43	226 b	84	35
Gala/M.9	BA + NAA	191 b	25 ab	15 ns	208 ns	92	45 ns
- Grandview	Carbaryl + BA	192 b	25 ab	17	201	95	47
	Carbaryl + NAA	186 b	28 a	16	204	93	56
	Control	215 a	20 b	13	203	94	51

Table 4. Incidence and percentage of results significantly superior to untreated control. Apple chemical postbloom thinning trials WTFRC 2002-2008.

Treatment	Fruitlets/100 blossom clusters	Harvested fruit size	Return bloom[1,2]
BA	2 / 18 (11%)	0 / 19 (0%)	0 / 18 (0%)
Carb + BA	27 / 73 (37%)	9 / 71 (13%)	7 / 63 (11%)
Carb + NAA	10 / 49 (20%)	7 / 49 (14%)	3 / 45 (7%)
Carb + NAA + Ethephon	0 / 5	0 / 5	2 / 5
Carb + NAA + BA	0 / 8	0 / 8	3 / 8
BA + NAA	4 / 9	2 / 9	0 / 6

[1]Does not include data from 2008 trials.

[2] (no. blossom clusters year 2/sample area) / (no. blossom clusters year 1/sample area)

COMBINED BLOOM AND POSTBLOOM PROGRAMS:

Our results continue to demonstrate that comprehensive chemical thinning programs are typically necessary to reduce crop load to appropriate levels. Table 5 details a grower-applied trial in which reductions in fruit set from bloom and postbloom thinning programs were additive when both programs were combined. Treatments from this trial were segregated at harvest and we look forward to comprehensive analysis of all fruit from this block when it is run over a commercial packing line.

Table 5. Crop load effects of chemical thinning programs (grower applied). WTFRC 2008.

Trial	Bloom thinner	Postbloom thinner	Fruitlets/100 floral clusters	Blanked spurs	Singled spurs	Harvest fruit weight	Relative box size	Russeted fruit
				%	%	g		%
Gala/M.9	CFO + LS	none	138 b	28 ab	28 ns	187 b	102	37 ns
- Royal Slope	CFO + LS	Carbaryl + BA	121 b	35 a	27	199 a	96	34
	none	Carbaryl + BA	137 b	30 ab	25	204 a	93	27
	Control		158 a	24 b	24	187 b	102	32

VARIABLES AFFECTING THINNING EFFICACY AND FRUIT FINISH:

While our results have clearly demonstrated the efficacy of several chemical thinning programs, we seek to improve their consistency and predictability. We have conducted a series of trials to investigate the effects of drying conditions, spray technology, and application carrier volume on efficacy of proven chemical bloom and postbloom thinning programs and their impact on fruit finish.

From 2006-2008, we conducted two trials each year in which identical bloom (CFO + LS) and postbloom (carbaryl + BA) chemical thinning programs were applied at different times of the same days. Morning applications were typically during cool (53-58ºF) and damp, but warming conditions; midday conditions featured temperatures continuing to rise from 60-65ºF; evening sprays occurred in relatively dry conditions cooling from 62-67ºF. No consistent effects of spray timing were observed regarding fruit set or finish. While conclusive evaluation of these interactions would require dozens of trials conducted over many years and under varying environmental conditions, we have not found enough evidence of these effects to justify continuing this line of study.

At another site, we concurrently applied identical chemical thinning programs using Proptec vs. AccuTech vs. airblast (Slime Line) sprayers at either of two carrier volumes in both 2006 and 2007; our 2008 trial was cancelled due to the grower's concerns over frost damage. Results suggest that spraying with 200 gal/acre carrier volume slightly increases thinning vs. 100 gal/acre; carrier volume did not affect fruit finish and no clear trends were observed regarding the use of sprayer technology on either fruit set or finish.

RETURN BLOOM PROGRAMS:
2008 results in three trials treated with bloom promotion programs based on NAA and ethephon revealed no treatment effects. After 5 years of work and approximately 20 trials of disappointing results from programs suggested by product reps and East Coast researchers, we took a different tack in 2008: we established two trials (to be evaluated in spring of 2009) applied by growers using an NAA program lifted directly from a large Washington grower's annual spray program. If this approach fails to produce positive results, we plan to discontinue our research in this area.

Three 2007 trials utilizing GA_3 to inhibit return bloom in spring of 2008 were inconclusive. Variation in initial crop levels of sample trees and limbs produced wildly variable results, masking any treatment effects. Based on earlier successful results, we remain confident in the potential of these programs to be viable tools for crop load management and expect cleaner results from six new trials initiated in 2008.

REFLECTIVE MATERIAL TRIALS:
Since 2005, we have conducted more than 20 trials evaluating reflective materials in commercial Washington apple orchards. Products tested have included the woven plastic fabrics Extenday, Daybright, and Daywhite, all distributed by Extenday USA, as well as Brite N'Up, a mylar-based material. The Extenday products are designed for use throughout the growing season and may be reused for 6-8 years with good maintenance, while mylar products cannot be reused and are generally only deployed 2-3 weeks before harvest.

All materials we tested are designed to reflect sunlight striking the orchard floor back up into plant canopies. Increased light saturation as harvest approaches can increase red color development, while increased light saturation throughout the growing season is associated with increased carbon fixation (photosynthesis), cell division, and cell expansion. While all products tested improved apple fruit color when deployed shortly before harvest (Table 8), Extenday products have also consistently increased fruit set and/or fruit size in WTFRC apple (Tables 6, 7), pear, cherry, peach, and nectarine trials. Because these materials promote the production of high yields of large, well-colored, high quality target fruit, they have tremendous potential to significantly improve grower returns.

Table 6 reflects two years of results from a Honeycrisp block treated with Extenday from bloom until harvest. In the first year of the study, no significant yield effects were observed, but Extenday clearly improved fruit color. In the following year (2008), however, Extenday-treated trees set far more fruit in the block's off year of a biennial bearing cycle. The higher crop load likely impaired fruit coloring

as reflected by percentage of yield harvested in the first color pick; this is the only implication we have seen of Extenday-treated fruit having color inferior to untreated controls, and the effect was not corroborated by a packing line color grader.

Table 6. Full-season reflective material effects on fruit yield, harvest sequence, and fruit color. Honeycrisp/Sup.4, Selah, WA. WTFRC 2007-2008.

	YIELD				HARVEST		COLOR GRADE	
	Fruit set	Fruit wt.	Box size	Yield	1st pick	2nd pick	WAXF	WAF
	(#/tree)	(g)		(kg/tree)	(%)	(%)	(%)	(%)
2007								
Extenday	433 ns	227	84	94 ns	89 a	11 b	39	49
Control	438	210	91	87	65 b	35 a	30	46
2008								
Extenday	389 a	209	91	71 a	43 b	57 a	28	69
Control	266 b	204	93	48 b	56 a	44 b	27	70

The increased yield in the second year of the Honeycrisp trial is not unique; we have frequently observed a cumulative increase in yields over the course of multiple year studies. Table 7 summarizes the average effects of Extenday in each season of every full-season apple trial we have conducted since 2005. While modest yield gains are typical in the first year of trials, the effects are more dramatic in subsequent seasons, likely due to increased carbohydrate reserves and renewed fruiting wood, especially in lower, shaded portions of tree canopies.

Table 7. Mean cumulative yield effects relative to untreated controls of full-season multiyear use of Extenday in all WTFRC apple trials. 2005-2008.

Trial age	*n*	Fruit set (harvested fruit/tree)	Individual fruit size (g)	Total yield (kg/tree)
1st year	8	+ 8%	+ 6%	+ 15%
2nd year	5	+ 32%	No effect	+ 32%
3rd year	2	+ 28%	+ 3%	+ 29%

Reflective materials deployed late in the growing season have little effect on apple fruit set or size, but can improve fruit color in red or partially red cultivars. Table 8 shows effects of Extenday and Brite N'Up on Gala fruit color; both materials were deployed at the same timings using equal material widths. While the mylar product improved color, Extenday was more effective in both seasons.

Table 8. Effects of reflective materials deployed 4 weeks prior to harvest on harvest sequence and fruit color. Gala/M.9, Othello, WA. WTFRC 2007-2008.

	TOTAL YIELD HARVESTED				COMMERCIAL COLOR GRADE		
	1st pick	2nd pick	3rd pick	4th pick	WAXF	WAF	US#1
	(%)	(%)	(%)	(%)	(%)	(%)	(%)
2007							
Extenday	39 a	40 ns	19 b	2 b	92	7	1
Brite N' Up	21 b	42	30 a	7 a	82	17	1
Control	16 b	40	35 a	8 a	78	21	1
2008							
Extenday	32 a	59 ns	9 b	na	99	1	0
Brite N' Up	19 b	63	19 b		96	4	0
Control	14 b	56	30 a		95	5	0

CONTINUING PROJECT REPORT **YEAR**: 1 of 2

Project Title: Management of vegetative growth in apple trees with bioregulators

PI: Don C. Elfving
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City: Wenatchee
State/Zip: WA 98801

Cooperators: Tory Schmidt, Research Associate, WTFRC;
Dwayne B. Visser, Agricultural Research Technologist III, WSU-TFREC

Total project funding request: **Year 1:** 13,111 **Year 2: 14,109**

Other funding Sources

Agency Name: BASF
Amount requested/awarded: $5,000 awarded in 2008

WTFRC Collaborative expenses:

Item	2008	2009
Stemilt RCA room rental	0	**0**
Crew labor	8,000	**10,000**
Shipping	0	**0**
Supplies	0	**0**
Travel	500	**700**
Miscellaneous	0	**0**
Total	8,500	**10,700**

Footnotes: Trial establishment and data collection in trials with growth-controlling bioregulators. Harvest 3 Apogee/ethephon/VBC trials in 2008.

Budget 1:
Organization: WSU-TFREC **Contract Administrator:** Mary Lou Bricker; Kevin Larson
Telephone: 509-335-7667 x221 **Email:** mdesros@wsu.edu; kevin_larson@wsu.edu

Item	2008	2009
Salaries	5,442	**5,714**
Benefits	1,959	**2,057**
Wages	1,080	**1,134**
Benefits	130	**204**
Equipment	0	**0**
Supplies	500	**800**
Travel	4,000	**4,200**
Miscellaneous	0	**0**
Total	13,111	**14,109**

Objectives:

1. Explore possible methods for improving the efficacy of prohexadione-Ca (Apogee) for control of unwanted vegetative vigor, including application timing, combinations of Apogee with ethephon and/or other bioregulator products, such as VBC30051, a bioregulator implicated in the initiation of dormancy.
2. Examine whether VBC30051 can be used either alone or in combination with other bioregulators to force terminal bud set in growing shoots, thus controlling growth.
3. Evaluate the potential benefits for stimulation of latent bud growth on "blind wood" with high concentrations of cytokinins (e.g., chlorfenuron, thidiazuron, 6-benzyladenine) with or without supplemental gibberellic acid (e.g., GA_{4+7}).
4. Compare cyclanilide (Tiberon®) with cytokinin/gibberellin products (e.g., Promalin) for induction of desired shoots at trellis wires during canopy development in sleeping-eye apple trees.
5. Examine fruiting in cyclanilide treated sleeping-eye trees to determine if this approach results in the development of better quality fruiting wood.

Significant findings:

1. Three treatments, 1) Promalin (PR, 20,000 ppm, pure formulation), 2) thidiazuron (TDZ) + ProVide (GA_{4+7}) (5,000 ppm and 2,500 ppm + Pentra-Bark 2% v/v), and 3) thidiazuron (TDZ) + ProVide (GA_{4+7}) (both 5,000 ppm + Pentra-Bark 2% v/v) painted on one-year-old vertical leader shoots of 'Cameo'/M.26 trees at trellis wires doubled the number of new shoots forming at the wire compared to no treatment.
2. The Promalin formulation alone (no surfactant) was concentrated enough to assure a branching response in the absence of bark injury and did not cause phytotoxicity.
3. In apple, cytokinin is as important as gibberellin for inducing shoot formation. This approach can help growers developing apple canopies on trellises, such as for "sleeping-eye" systems.
4. In vigorous, grafted 'Fuji'/MM.106 trees, four Apogee (prohexadione-Calcium, P-Ca) sprays did not maintain control of shoot growth (Fig. 1). Shoot growth on Apogee-treated trees eventually equaled that of untreated control trees.
5. When four Apogee sprays were followed by one or two ethephon applications, two VBC applications or one or two tank-mix applications of ethephon and VBC, shoot growth was essentially halted after treatment.

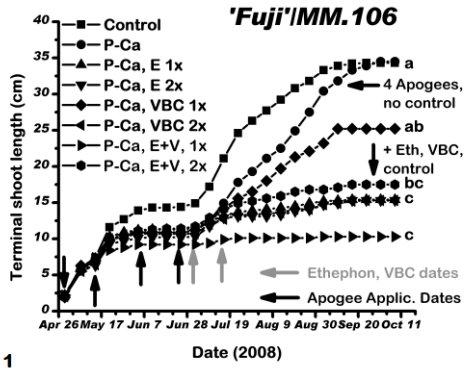

Fig. 1

6. The various treatment combinations of four Apogee sprays with or without follow-up ethephon and/or VBC30051 applications showed minor effects on fruit quality.
7. In 'Fuji'/M.26 trees of roughly comparable vigor, untreated shoots grew normally (Fig. 2). Four Apogee applications were sufficient to control shoot growth. Curiously, Apogee-treated shoots receiving one or two ethephon treatments grew enough to be the same as controls. Additional treatments with VBC alone or VBC+ethephon were equivalent to four Apogees alone.
8. Pretreating 'Fuji'/M.26 apple trees later in the growing season with Apogee followed by ethephon and/or VBC resulted in no overall control of growth (Fig. 3). Waiting until 24 June to start this program was too late.

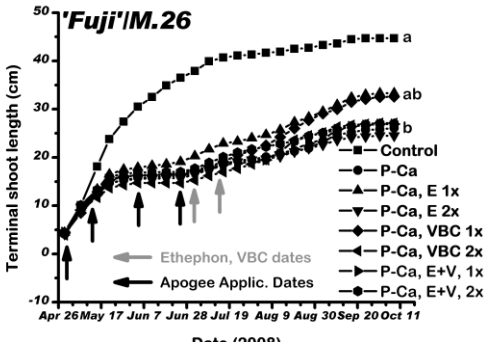

Fig. 2

Nonetheless, shoot growth was less vigorous where ethephon and/or VBC were used.

9. A preliminary trial testing the potential vegetative growth control agent FAL 1210 was carried out on vigorous 'Fuji'/MM.106 apple trees. FAL 1210 was applied at 125 ppm three times and at 250 ppm only once in early spring, while Apogee (125 ppm) was applied four times. Apogee-treated shoots eventually grew as much as untreated control shoots. A single application of 250 ppm FAL 1210 or three applications of 125 ppm were both effective for controlling terminal shoot elongation in this trial. This product shows promise as a vegetative growth control agent for apple. Three applications of the FAL1210 product appeared to reduce fruit grade-out and resulted in lower titratable acidity. These observations should be considered as preliminary.
10. 'Fuji'/M.26 trees were treated with a single late-June application of Apogee followed one week later by single applications of ethephon and/or VBC30051. These treatments were applied too late to significantly reduce terminal growth (Fig. 3), although combination treatments did have somewhat shorter terminal shoots. There were no effects of any treatment on fruit quality.

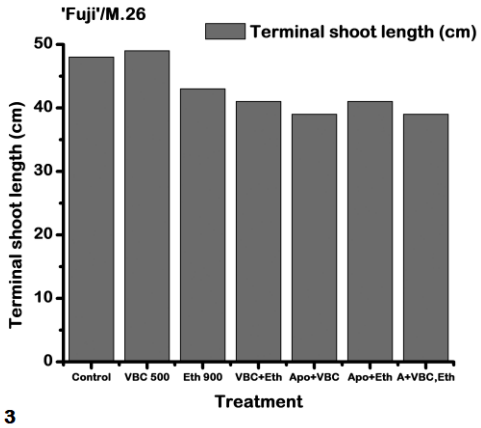

Fig. 3

Methods:
Four trials were initiated in 2008 to examine effects of bioregulators on branch induction at trellis wires and on the potential for control of shoot regrowth in Apogee-treated apple trees. Cytokinin/gibberellin mixtures were applied along with a powerful surfactant to test for branching success without bark injury. Control of apple shoot regrowth focused on summer treatments of either ethephon, VBC30051 (Valent Biosciences, Walnut Creek, CA) or tank mixes of those products.

Results and discussion:
Research in 2008 confirmed observations in 2007 that appropriate surfactants can substitute for cutting the bark in assuring that branch-inducing bioregulator products penetrate into living tissues in shoots. Several questions remain to be explored; perhaps the most important of those has to do with the relative importance of surfactant type vs. applied concentration. It may be that a variety of commonly-used surfactants will work if applied in high enough concentration. We have used up to 4% v/v (6 fl. oz./gallon solution) with good results and no phytotoxicity.

In apple, the Promalin formulation alone, uncut, continues to be effective for stimulation of branch induction. The capacity to place new branches adjacent to trellis wires greatly facilitates proper canopy development in trellised high-density plantings. This type of tree structure has much to recommend it from the physiological point of view, so facilitating this process without the need for pruning is an important development. Although such a process is labor-intensive, our most successful treatments minimize the labor cost involved.

Apogee programs in commercial apple orchards continue to be plagued by the problem of uncontrolled "regrowth" after a successful initial control response to early Apogee applications. The advent of commercially formulated abscisic acid (VBC30051) opens a potential new avenue for approaching a resolution to this problem. In the first two trials of this kind under WA conditions, we have some evidence to suggest that ethephon and VBC 30051 have potential for contributing positively to regrowth control. The results to date are preliminary and no hard conclusions can yet be drawn. Future research in 2009 should help us tremendously in determining the potential of these products for overcoming the limitation of Apogee for season-long vegetative growth control in apple trees.

Acknowledgments:
The assistance and support of the following people and organizations is gratefully acknowledged: Felipe Castillo, Dean Christie, Kevin Forney, Tom Gausman, Dr. Ines Hanrahan, Dr. Chris Ishida, Rick Kamphaus, Dr. Jim McFerson, Eric Monson, Brandon Mulvaney, Ron Moon, Chris Olsen, Tory Schmidt, Tim Scott, Bill Stringfellow, Jim Thornsberry, Dwayne B. Visser, Dr. Sam Willingham, AgriMACS Oxteam Orchard, Apple-Eye Orchards, BASF Corp., Bayer CropScience, Fine Americas, Monson Fruit Co., Scott Orchards, Valent Biosciences, Washington Tree Fruit Research Commission and the WSU Agricultural Research Center.

Publications 2008:

Schmidt, T.R., D.C. Elfving, J.R. McFerson and M.D. Whiting. 2008. Gibberellic acid accelerates 'Honeycrisp', but not 'Cameo', apple fruit maturation. **HortTechnology 18:39-44.**

Lenahan, O.M., M.D. Whiting, and D.C. Elfving. 2008. Gibberellic acid is a potential sweet cherry crop load management tool. **Acta Hort. 795:513-516.**

Elfving, D.C. 2008. Bioregulator sprays. p. 75-87. In: T.J. Smith (coord.), **2008 Crop Protection Guide for Tree Fruits in Washington. EB 0419.**

Elfving, D.C. and D.B. Visser. 2008. Bioregulator effects on growth, flowering and cropping in apple trees. **Poster, WA State Horticultural Association Annual Meeting, Yakima, WA.**

Visser, D.B. and D.C. Elfving. 2008. Bioregulators for managing growth, cropping and fruit quality in sweet cherry. **Poster, WA State Horticultural Association Annual Meeting, Yakima, WA.**

CONTINUING PROJECT REPORT **PROPOSED DURATION**: 2 years

Project Title: Functional genomics and marker development for apple sensory qualities

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Budget 1:

Organization: USDA, ARS		**Contract Administrator:** Charles Myers, Extramural Agreements Specialist	
Telephone:(510) 559-6019		**Email:**cwmyers@pw.ars.usda.gov	
Item	**Year 1: 2007**	**Year 2: 2008**	
Salaries	$33,000	33,000	
Benefits	10,000	10,000	
Wages			
Benefits			
Equipment			
Supplies	10,000	10,000	
Travel	1,500	1,500	
Miscellaneous	500	500	
Total	55,000	55,000	

The **salaries and benefits** are for hiring a postdoc dedicated to this project.
The **supplies** include common reagent for molecular genetics study and gene profiling analysis.
The budget for **travel** includes the cost for visiting Malus germplasm repository at Geneva, New York, for identify the phenotypic extremes on related fruit quality.

Objectives

1. Continue to apply the tested ethylene molecular markers for ACS1 and ACO1 in segregation populations in the WSU Apple Breeding Program to select for low ethylene production.

2. Test and apply a reported apple fruit peel red color marker in the existing WSU segregation population for selection of red color development capacity.

3. Identify potential candidate genes regulating apple firmness and crispness.

4. Elucidate relationships between expression of apple AAT (alcohol acyl transferase) genes and cultivar differences in volatile ester production.

Proposed schedule of accomplishment (August 2008-July 2009):

1. Test peel red color molecular marker to additional selections in the WSU breeding program.

2. Validate the expression patterns of 15-20 genes potentially associated with firmness and crispness traits that were identified in year one.

3. Characterize the promoter region of selected AAT gene-family members to determine if sequence polymorphism is associated with expression levels between two cultivars. Further test the association between gene expression and volatile production in additional germplasm in the WSU population.

Significant findings

1. Test of a published apple skin color marker among cultivars, and in a Pink Lady x Honeycrisp segregation population has complete. It was found that the robustness of this marker still needs to be improved for use in Pink Lady x Honeycrisp population.

2. Most of 5000 seedlings in WSU breeding program have been genotyped for their allelotypes of both climacteric ethylene biosynthesis genes, MdACS1 and MdACO1. The genotype data can be used by breeder for selection process.

3. A large scale gene expression profiling analysis, aimed to uncover the genetic control of apple fruit texture attributes of crispness and firmness as well as fruit ripening in general, has completed; Preliminary data analysis indicated a high quality data set, and the compiled candidate gene list point to several interesting genes, whose role will be validated in the phenotype-characterized segregation population. Microscopic examination also revealed a distinguishable feature at cell wall thickness.

Methods

1. Physiological characterization of textural attributes during ripening: Systematic characterization of apple fruit firmness, crispness during ripening on selected cultivars has been carried out using the Mohr Digi-test instrument. Weekly maturity data including ethylene production, starch index fruit firmness and crispness, was conducted. Such physiological study is critical for selecting tissues at specific developmental stage for gene expression analysis.

2. Gene expression profiling analysis to investigate the genetic factors regulating fruit textural attributes: selected tissues based on physiological study were used to isolate RNA for comparative gene expression profiling (microarray analysis), during fruit ripening and between Pink Lady and Honeycrisp.

3. Scanning electron microscopy (SEM) of apple fruit cortex cellular and cell wall features: High power electron microscope was employed to examine the sub-cellular feature which may contribute to the distinguished texture attributes between Pink Lady and Honeycrisp.

4. Test the robustness of an apple skin color marker: An apple fruit red skin color marker was tested in segregation population and among cultivars for its predictability.

Result and Discussion

1. Evaluation of an apple fruit red skin marker.

An apple red skin color marker was developed based on the cloning and functional analysis of a transcription factor, MdMYB1, in anthocyanin pathways. A derived dCAPS (derived Cleaved Amplified Polymorphic) marker was designed based on the polymorphisms segregated with the inheritance of skin color in progeny from a cross of an unnamed red selection (a sibling of Cripps' Pink or Pink Lady) and the non-red cultivar Golden Delicious. Initial results indicated a good correlation between this apple fruit red skin marker among a group of red or non-red apples, with Granny Smith as an exception (showing a unexpected positive genotype).

Below is the result in evaluating this red skin color maker in a WSU Pink Lady X Honeycrisp segregating population:

From 120 selections tested,

Seedlings with red skin apples: 81

Seedlings non-red skin apples: 39

69 selections +/+*	12 selections +/-
36 selections -/-	3 selections -/+

*(phenotype / genotype)

Overall, the accuracy of predictability is 87.5%, error rate 12.5%.

Assuming the goal is to keep seedlings with red skin color and discard non-red selections, by eliminating the negative genotype based on this marker, then the error rate predicts 18% with red skin color will be discarded, while 8% non-red selections will be retained.

Conclusion: this red skin color marker has a relatively high potential to predict skin color but its robustness could be improved. The results suggest that other genetic factors in addition to this MYB transcription factors may participate in the regulation of apple fruit skin color development.

2. Genotype WSU breeding selections using ethylene gene functional molecular markers.
(Collaborators: Dr. Cameron Peace and Bruce Barritt)

Functional molecular markers, designed from ethylene biosynthesis genes MdACS1 and MdACO1, has been previously tested in the WSU breeding program. There is a good correlation between ethylene synthesis gene genotypes and fruit firmness (as reported last year). Currently, these markers are being

utilized to genotype 5,000 seedlings of diallel crosses between Pink Lady, Honeycrisp and CrimsonCrisp for their MdACS1 and MdACO1 allelotypes.
After eliminating those selections susceptible to fire-blight, leaf samples of 3700+ seedlings were collected and high throughput DNA isolation has been completed. The genotyping for ethylene biosynthesis capability based on the MdACS1 and MdACO1 functional molecular markers is in progress and almost complete.

3. Genetic regulation of apple fruit texture by combined approach of transcriptome analysis, physiological characterization and microscopic study.

(Collaborator: Dr. Dorrie Main, Dr. James Mattheis, Dr. Eric Curry)

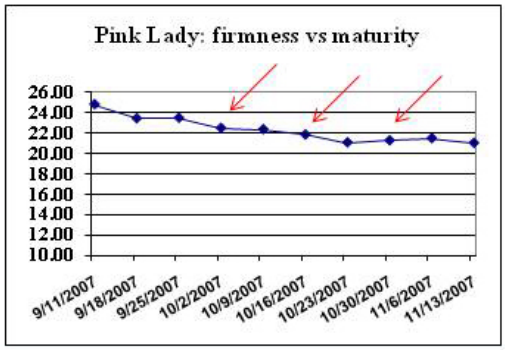
A large scale gene expression profiling experiment has been completed and the data are currently being analyzed and validated. Cortex tissues of Honeycrisp and Pink Lady apples were collected based on defined maturity data. The samples were used for RNA isolation and large scale gene expression analysis. Nimblegen apple microarrays were designed in Dorrie Main's lab. Additionally, preliminary fruit quality phenotype data for 177 (is this the correct number) Pink Lady X Honeycrisp trees has been completed, and fruit from these trees will be used for validating gene-trait association of those candidate genes based on the analysis of expression profile data.

A. Define ripening stage in both cultivars for selecting fruit cortex tissues transcriptome analysis.

For each cultivar, tissues from three time points representing commercial maturity (0), 4 weeks before (-4) or 2 weeks before (-2) the commercial maturity were used for transcriptome analysis, (indicated by stars and arrows in figures below). Four biological repeats were collected for each time point. Data of physiological characterization on fruit maturity and texture changes are shown below in Table 1 and 2.

Table 1. Maturity data and texture characterization for Pink Lady

Pink Lady					-4		-2		0		
	Harvest Date	9/11	9/18	9/25	10/2	10/9	10/16	10/23	10/30	11/6	11/13
	Ethylene	0.00	0.05	0.08	0.81	0.04	0.04	0.15	0.86	1.07	4.19
	Firmness	24.8	23.4	23.5	22.5	22.4	21.8	21.1	21.2	21.5	21.0
	Starch	1.00	1.00	1.01	1.07	1.31	1.60	2.11	2.68	3.59	4.85
	Cn	163	142	168	166	192	173	167	188	160	163
					*		*		*		



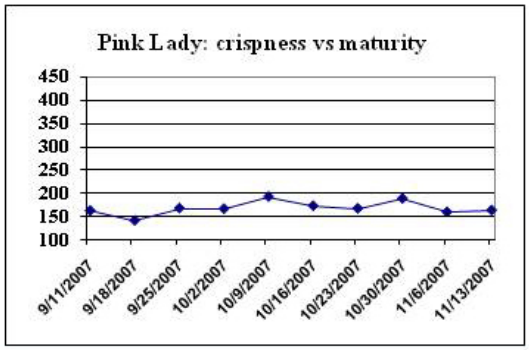
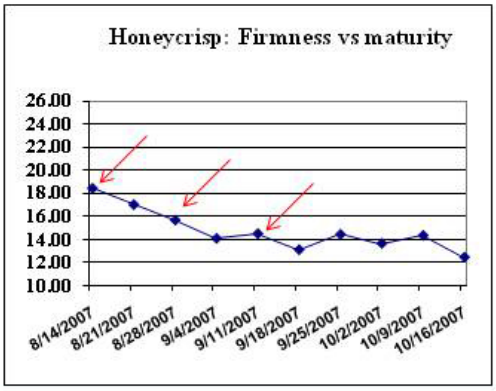
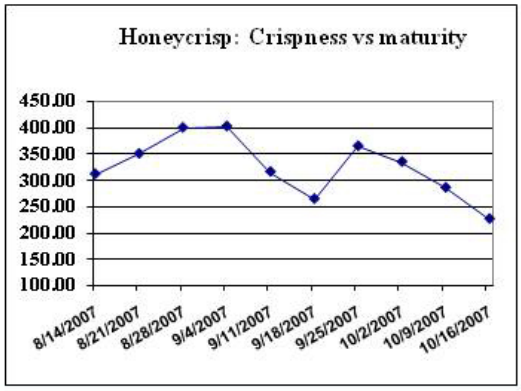


Table 2. Maturity data and texture characterization for Honeycrisp

Honeycrisp		-4		-2		0					
	Harvest date	8/14	8/21	8/28	9/4	9/11	9/18	9/25	10/2	10/9	10/16
	Ethylene	0.01	0.04	0.01	1.15	0.83	1.02	3.68	2.54	3.34	5.14
	Firmness	18.4	17.0	15.7	14.1	14.5	13.1	14.5	13.7	14.4	12.5
	Starch	1.00	1.04	1.36	2.78	4.79	5.87	5.93	5.96	5.62	6.00
	Cn	312	350	399	402	316	265	365	335	286	227
		*		*		*					

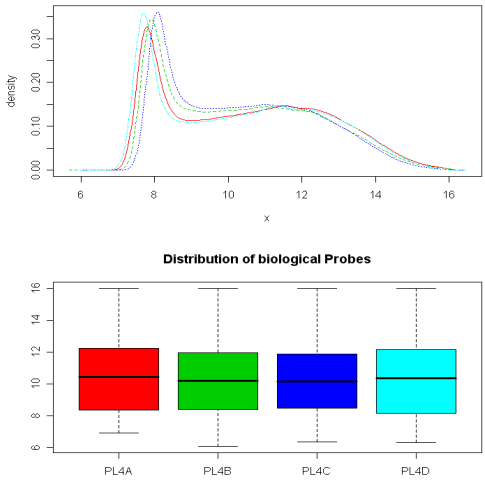




B. Design of Nimblegen apple long oligo microarray and array hybridization.

An apple long oligo microarray was designed in collaboration with Dorrie Main's lab and Nimblegen. EST sequences (updated till December 2007) were assembled using the assembly software CAP3. A 50-70-mer isothermal oligonucleotides (NimbleGen array) corresponding to the sequence within the coding region of 40,166 unigenes was designed using house scripts. 190,135 cross-hybridization-free set of oligos representing 23,997 unigenes was manufactured on a Nimblegen array platform. Total of 24 microarray slides were utilized to analyze the gene expression profiling from tissues of 2 cultivars x 3 developmental stages x 4 biological repeats.

Preliminary analysis on hybridization data indicated a high quality gene expression profiling data set was obtained (left panel: for example, similar pattern of curves in the top section indicates the high repeatability of signal intensity from internal controls among microarray slide). The quality of the hybridization data is critical for selecting candidate genes with confidence.



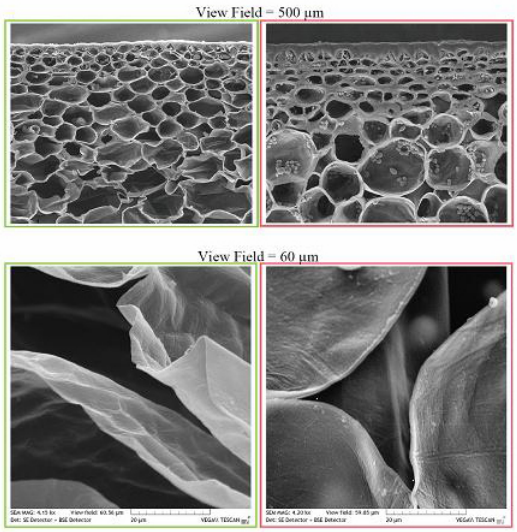
The differentially expressed genes between developmental stage and across cultivars are being compiled and validation of candidate genes will be carried out soon.

C. Phenotypic study of a PL X HC segregation population.

A non-selected Pink Lady x Honeycrisp segregation population included 177- 5 year-old trees on seedling rootstocks were used for phenotype characterization of the segregation of both fruit firmness and crispness. For 100+ selections which have enough number to work with, fruit crispness and firmness were measured at three time points around starch level of 3. The trees have been propagated and will be planted as a resource for longer-term genetic study. This work is continuing to validate the selected candidate genes by Q-PCR.

D. Scanning electron microscopy (SEM) of apple fruit cortex cellular and cell wall features of both Pink Lady and Honeycrisp.

SEM images appear to indicate no differences in cell number or cell size (images in lower power of magnification: top left for Honeycrisp, top right for PinkLady). However, differences in cell wall thickness between the two cultivars are apparent (images in higher power of magnification: bottom left for Honeycrisp, bottom right for PinkLady). The results appear to be consistent with the hypothesis that cell wall metabolism related genes and pathways contribute to phenotypic variation in apple fruit texture.



4. Other activities related to this proposal

A. Two publications in peer reviewed journals

Zhu, Y. and Barritt, B. H. Md-ACS1 and Md-ACO1 genotyping of apple (*Malus x domestica* Borkh.) breeding parents and suitability for marker-assisted selection. Tree Genetics and Genomes 4: 555-562. 2008.

Zhu, Y. and Rudell, D. R., Mattheis, J. P. Characterization of cultivar differences in alcohol acyltransferase and 1-aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening. Posthav Bio. Technol. 49: 330-339. 2008.

B. Submission of a NRI grant proposal directly related to this research

A grant proposal was submitted with the objectives of extending this research to cover longer developmental stages and year to year variation, unravel micro RNA's role (another aspect of genetic regulation) on fruit texture and map validated genes to existing Malus genetic map were submitted, though did not get funded.

CONTINUING PROJECT REPORT **YEAR**: 1 of 2

Project Title: Sensory and consumer acceptance of advanced apple breeding selections

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Total Project Request: **Year 1:** $28,580 **Year 2:** **$28,895**

Other funding Sources: None

Budget 1
Organization Name: WSU **Contract Administrator:** ML. Bricker
Telephone: 509-335-7667 **Email address:** mdesros@wsu.edu

Item	2008	2009
Salaries[1]	14,000	**22,014**
Benefits[2]	9,380	**1,881**
Wages		
Benefits		
Equipment		
Supplies[3]	4,200	**4,000**
Travel	1,000	**1,000**
Total	28,580	**28,895**

Notes:

[1] Salaries: One Ph.D. graduate student will be supported by this research (9- month salary support). The original post-doctoral fellow on the project will be leaving WSU in July 2009.
[2] Employee Benefits: The fringe benefits rate for the graduate student is at WSU standard rates.
[3] Supplies: Sensory panels: Consumables: paper plates, towels, cuspidors, forks, plastic wrap, tape, saltines, photocopies, participation incentives, advertising

OBJECTIVES:
The overall objective of this study was to characterize the sensory properties of newly developed selections from WSU Apple Breeding Program and determine the preference of these various apple selections. The sensory properties of these apple selections will then be related to consumer acceptance. Specific objectives for 2008 and 2009 are to:

Objective 1: To perform trained sensory panel analysis to characterize new selections of WSU Apple Breeding Program.
Objective 2: To perform consumer sensory panel evaluation to determine preference (overall and specific attributes) of new selections of WSU Apple Breeding Program.
Objective 3: To create apple groupings based on taste/flavor and texture. This is a new objective added for 2009 work. This would result in the assignation of apples to specific flavor/texture groupings. The objective of these groupings is to give consumers more information about the sensory properties of the apples at the point of purchase, particularly important for new apple selections as consumers will not be familiar with the sensory properties of these apples. We have performed similar work in generating groupings for sweet cherries.

SIGNIFICANT FINDINGS (2008):

- Apple varieties showed differences in analytical measurements (firmness, Brix and TA) and these differences translated to differences in sensory properties.
- From trained panel evaluations of apple taste/flavor, apples differed in their sensory properties. Fuller 24 was the lowest in sweetness and highest in sourness. Results were confirmed by analytical measurement. Fuller 30 was the highest in sweetness but was not significantly different from Allen 2, Fuller 17, 18, 34 and 36. Fuller 20 was the lowest in sourness. Large differences were not observed between the apple selections in astringency. Apples were similar in apple flavor intensity, with Fuller 20 having the lowest flavor. For texture attributes, firmness showed the greatest variation between the apple selections. Fuller 20 had the lowest firmness, crispness and juiciness and the highest perceived mealiness. Fuller 24 was highest in firmness, crispness and lowest in mealiness.
- From consumer panel evaluations, results showed that apple selections differed based on acceptance of flavor and texture attributes. For overall acceptance, 7 of the 10 apple selections did not significantly differ in overall acceptance from the control Fuji apple. The apple selections significantly lower in acceptance than the Fuji were Fuller 7, Fuller 20 and Fuller 34. Based on sweetness, the two least accepted selections were Fuller 7 and Fuller 20, with Fuller 20 also the least accepted based on sourness and flavor intensity. Based on hardness, Allen 2 and Fuller 17 were more acceptable than the Fuji apple. Based on crispness, Allen 2, Fuller 10, 17 and 36 were more acceptable than the Fuji. For all texture attributes, Fuller 20 and 34 were consistently rated significantly lower in acceptance compared to the control Fuji apple.
- In the consumer panel, there was a strong positive correlation between flavor intensity and overall acceptance, indicating the importance of this attribute.
- Based on the trained panel and consumer panel results, Allen 2, Fuller 10, 17, 18, 24, 30 and 36 show the most promise for commercialization.

METHODS:
1) *Objective 1: Trained panel analysis of new selections of Washington State apples*
Fruit Selection: Fruit selection and chemical/physical analyses will be performed in Wenatchee in Dr. Kate Evans' lab. Analytical data of firmness, titratable acidity (TA) and sweetness will be determined using standard protocols.

Trained Panel: We will identify promising apple selections based on the preliminary evaluation results for eating quality conducted by the three-person breeding team. We anticipate 8 apple selections. We will obtain fruit from selection trials to complete both the trained panel evaluation and the consumer acceptability studies. Fruit from regular cold storage (1-3°C) will be brought up to room temperature 24 hours before analysis. Soluble solids and acidity will be also measured.

A sensory panel of 12-14 panelists will be recruited using advertising in the WSU/Pullman community. Trained panelists who participated in previous trained apple texture panels will also be contacted. Panelists will be screened for any known allergies and anosmias. Panelists will undergo training to identify the flavor/taste attributes of acid, sweet, apple flavor intensity and astringency. Texture attributes will include firmness (hardness), juiciness, mealiness and crispness. These were the same attributes evaluated in 2008. The attributes were selected based upon previous literature (Harker et al.2002; Mehinagic et al., 2004) as well as previous research conducted in the Ross laboratory (Chauvin et al., 2007). For training, published texture scales (Szczesniak, 1963) will be used for the different texture attributes and panelists will be trained to both recognize the attribute and assign it an intensity rating. Fruit of varying texture intensities will be used for the training process. Throughout training, feedback for all attributes will be provided to the panelists. The aspects of performance that will be evaluated will be the panel's accuracy (bias) and precision (variability) (Meilgaard et al., 1999).

The trained panel will develop sensory profiles for each of the new apple selections provided by the WSU Breeding Program. Panelists will be presented with 1/8 of a washed apple for evaluation. Evaluations will take place in individual sensory booths equipped with lap top computers for recording data. During evaluations, panelists will receive 2 pieces of each fruit taken from different sides of the fruit (blushed and unblushed). The apple sections will be randomly presented at room temperature to the panelists. Apple selections will be identified using three-digit codes and presented one at a time to panelists. Each panelist will be provided with water to rinse between samples as well as a cuspidor for sample expectoration. The samples will be scored for intensity of attributes using a 15-cm unstructured line scale, with the left end of the scale corresponding to the lowest intensity (0 mm=absent) and the right end corresponding to the highest intensity (150 mm=extreme). Results will be collected and analyzed using Compusense 6.0 software (Guelph, ON) and sensory data will be quantified by measuring the distance of the mark along the line. The evaluations will be conducted as a balanced complete block design, with each panelist to evaluate 6 apple selections x 2 sides (blushed and unblushed) during each of three sessions.

To determine possible differences in sensory attributes between apples, data will be analyzed using two-way analysis of variance (ANOVA), with multiple comparisons made using Tukey's HSD. Data will also be analyzed using principal components analysis (PCA). We expect to achieve results from this trained panel following harvest of the apple selections, and complete this objective by March 2009. In 2008, we completed the apple selection profile following a similar timeline.

2) *Objective 2: Consumer panel evaluation of new selections of Washington State apples*
A consumer panel will be conducted in the Sensory Facilities at WSU-Pullman. Participants will be screened and selected only if they consume apples more than once a week. Two consumer panels of 100 subjects will be conducted in the sensory evaluation facilities in Pullman. Extensive advertising in the local and campus newspaper will be used to recruit panelists from the community. Samples will be served to each panelist using a random serving order and red lights to disguise color differences between apple selections. Apple selections will be identified using three-digit codes and presented one at a time to panelists.

Participants will evaluate each apple selection and rate each for overall preference, overall flavor, sweetness, acidity and texture (hardness, crispness and juiciness) using a 7-point hedonic scale, anchored with "dislike extremely", "neither like nor dislike" and "like extremely". A standard apple variety (Braeburn) will be included to provide a comparison between the two panel days. Data will be analyzed using analysis of variance (ANOVA), with multiple comparisons made using Tukey's HSD. Relationships will be examined between: (1) Overall consumer preference and consumer evaluation of other individual sensory attributes (aroma, flavor, texture and appearance), (2) Consumer preference and apple sensory properties determined using the trained sensory evaluation panel. We expect to achieve results from the consumer panel by March 2009.

Objective 3: Creation of apple flavor/texture groupings. Following the evaluation of the apple selections, panelists will then assign the apple to one grouping. For flavor: (1) high sugar, high acid, (2) high sugar, low acid, (3) balanced. For texture: (1) high firmness, high juiciness, (2) low firmness, low juiciness, (3) balanced. The use of appropriate attributes for the development of the groups will be further explored with the trained panelists.

2008 RESULTS:

Analytical Characterization of Apple Selection:
The analytical characterization of the apples is shown in Table 2. Results showed that the apple selections did vary in firmness, Brix and TA.

Table 2. Apple genotypes and analytical data of firmness, brix, and titratable acidity (TA).

Apple Variety	Allen 2	Fuller 7	Fuller 10	Fuller 17	Fuller 18	Fuller 20	Fuller 24	Fuller 30	Fuller 34	Fuller 36
Description	Splendour x Open	NJ90 x Goldrush	Honeycrisp x Cripps Pink	Honeycrisp x Chinook	Honeycrisp x Enterprice	Coop 25 x Goldrush	Gala x Cripps Pink	Fuji x BC 8S-27-2	Honeycrisp x Delicious	Honeycrisp X Chinook
Firmness (N)	74.80	70.30	79.80	89.00	69.50	67.30	91.40	62.90	79.30	71.80
Brix (%)	17.90	15.00	15.60	16.10	15.70	14.80	16.00	17.60	16.50	16.20
TA (ml added)	0.52	0.58	0.52	0.45	0.65	0.61	0.80	0.46	0.62	0.52

Trained Panel Evaluation of Apple Selections
The trained sensory panel was composed of 9 panelists (7 females and 2 males) between the ages of 25 and 64 (average age of 41). Results from the analysis of variance indicated that the sensory attributes were significantly influenced by the apple selection.

In **Table 3**, the separation of the different apple selections based on specific sensory attributes is shown. Results indicated specific attribute differences between selections. Based on range of intensity, the smallest differences between apple selections were observed with astringency, flavor intensity and juiciness while the largest differences between selections were observed with sourness, crispness and mealiness.

Table 3. Mean separation (Tukeys HSD) for all apple selections and sensory attributes as analyzed by the trained panel (n=9) along a 15-cm line scale. Within each attribute, different letters indicate a significant difference ($p<0.05$).

Apple Attribute	**Allen 2**	**Fuller 7**	**Fuller 10**	**Fuller 17**	**Fuller 18**	**Fuller 20**	**Fuller 24**	**Fuller 30**	**Fuller 34**	**Fuller 36**
Sweetness	9.75^{abc}	7.94^{cd}	10.32^{ab}	9.75^{abc}	9.14^{abcd}	8.88^{bcd}	7.05^{d}	11.38^{a}	9.42^{abc}	9.11^{abcd}
Sourness	5.94^{cd}	7.54^{bc}	6.43^{cd}	6.61^{cd}	9.29^{ab}	4.69^{d}	11.71^{a}	7.08^{bcd}	6.59^{cd}	7.26^{bcd}
Astringency	4.79^{ab}	4.23^{abc}	4.29^{abc}	5.34^{a}	4.79^{ab}	2.75^{c}	5.71^{a}	4.59^{ab}	3.45^{bc}	4.32^{abc}
Apple Flavor Intensity	6.39^{ab}	6.33^{ab}	6.85^{ab}	7.73^{ab}	7.12^{ab}	5.12^{b}	6.69^{ab}	8.65^{a}	6.01^{ab}	6.39^{ab}
Firmness	10.84^{abc}	8.11^{de}	11.58^{ab}	11.93^{a}	8.49^{de}	6.07^{f}	12.11^{a}	9.74^{bcd}	6.71^{ef}	9.31^{cd}
Crispness	10.57^{abc}	8.62^{d}	11.84^{ab}	11.59^{ab}	9.35^{cd}	6.24^{e}	12.36^{a}	10.08^{bcd}	6.56^{e}	9.94^{bcd}
Juiciness	8.06^{a}	8.07^{a}	7.99^{a}	7.74^{a}	8.28^{a}	5.09^{b}	8.19^{a}	8.63^{a}	7.14^{ab}	7.89^{a}
Mealiness	3.05^{e}	6.87^{bc}	3.39^{de}	3.35^{de}	5.82^{bcd}	9.68^{a}	3.31^{de}	4.09^{de}	7.21^{ab}	4.49^{cde}

Table 4 shows the correlations between the perceived sensory attributes of the apple selections. These results showed a strong positive relationship between crispness and firmness, indicating that as perceived crispness increased, perceived firmness did as well. A negative relationship was observed between perceived firmness and mealiness, and crispness and mealiness, indicating that as perceived firmness and crispness increased, perceived mealiness decreased.

In order to provide a visual representation of the relationship of the sensory attributes evaluated, a spider plot was generated. **Figure 1** shows the separation of the ten apple selections based on sensory attributes. These results indicated the differences between the different selections based on the attributes as evaluated by the trained panel.

Table 4. Pearson correlation between perceived sensory attributes for the apple selections.

Variables	Sweetness	Sourness	Astringency	Flavor Intensity	Firmness	Crispness	Juiciness	Mealiness
Sweetness	1	-0.076	0.061	0.371	0.161	0.153	0.161	-0.213
Sourness	-0.076	1	0.292	0.278	0.301	0.324	0.338	-0.196
Astringency	0.061	0.292	1	0.043	0.344	0.284	0.070	-0.249
Apple Flavor Intensity	0.371	0.278	0.043	1	0.111	0.207	0.409	-0.249
Firmness	0.161	0.301	0.344	0.111	1	0.913	0.284	-0.702
Crispness	0.153	0.324	0.284	0.207	0.913	1	0.403	--0.710
Juiciness	0.161	0.338	0.070	0.409	0.284	0.403	1	-0.389
Mealiness	-0.213	-0.196	-0.249	-0.249	-0.702	-0.710	-0.389	1

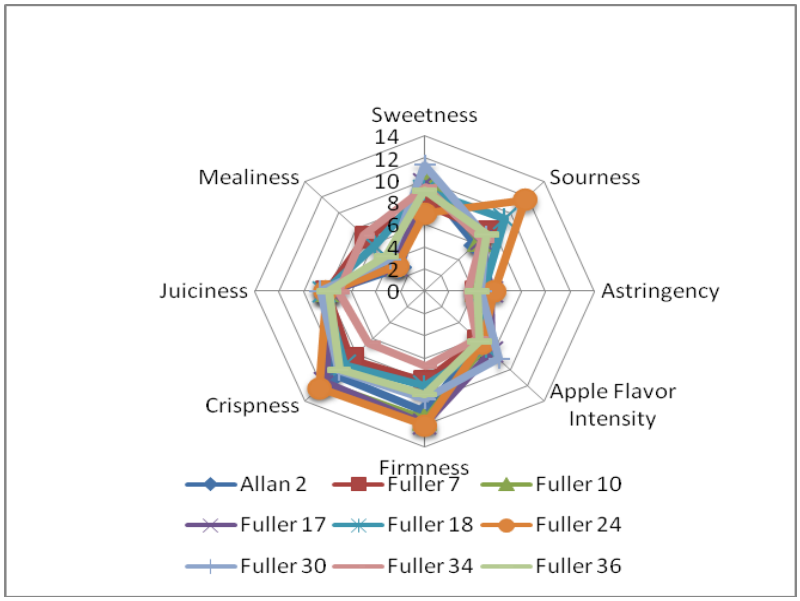

Figure 1. Spider plot of the apple selections (n=10) evaluated by the trained panel (n=9) for all sensory attributes.

Consumer Panel

The consumer panel was composed of 162 panelists (99 females and 63 males). Results from the analysis of variance showed that apple selections differed in their acceptance of the different sensory attributes. Fuji was included with the other 5 apple selections on both consumer panel evaluation days in order to serve as a control sample. No significant differences between Day 1 Fuji and Day 2 Fuji were observed for any attribute, indicating the consistency of the panel between days. In addition, the evaluation of Fuji allows the researchers to make comparisons between the evaluation of a well-known apple variety and the new apple selections.

In **Table 6,** the separation of the different apple selections based on acceptance of sensory attributes is shown. Based on the acceptance of all attributes, including overall acceptance, Fuller 17 appeared to

be the most accepted selection, followed by Allan 2. Compared to Fuji (control), the apple selections shown to be significantly lower in overall preference were Fuller 34, Fuller 7 and Fuller 20.

Table 6. Mean separation (Tukeys HSD) for all apple selections and sensory attributes as analyzed by the consumer panel (n=162). Values represent acceptance along a 7-pt hedonic scale (1=dislike very much and 7=like very much).

Apple Varietal/ Apple Attribute	Allan 2	Fuller 7	Fuller 10	Fuller 17	Fuller 18	Fuller 20	Fuller 24	Fuller 30	Fuller 34	Fuller 36	Fuji (Control)
Overall Acceptance	5.47^{ab}	4.40^{e}	5.17^{abc}	5.70^{a}	4.57^{cd}	3.32^{e}	5.00^{abcd}	4.95^{bcd}	4.41^{d}	5.36^{ab}	5.24^{abc}
Sweetness	5.40^{ab}	4.47^{de}	5.14^{abc}	5.63^{a}	4.68^{cde}	4.23^{e}	4.73^{cde}	5.11^{abcd}	4.82^{bcde}	5.22^{abc}	5.25^{abc}
Sourness	5.04^{a}	4.36^{cde}	4.80^{abc}	5.09^{a}	4.51^{bcd}	3.95^{e}	4.70^{abcd}	4.54^{bcd}	4.32^{de}	4.94^{ab}	4.60^{abcd}
Flavor Intensity	5.32^{ab}	4.60^{bc}	4.83^{abc}	5.50^{a}	4.34^{cd}	3.68^{d}	4.91^{abc}	4.68^{bc}	4.41^{c}	5.16^{ab}	4.94^{abc}
Hardness	5.77^{a}	4.36^{cd}	5.48^{ab}	5.74^{a}	4.41^{cd}	2.62^{e}	5.70^{a}	4.84^{bc}	3.73^{d}	5.49^{ab}	4.93^{bc}
Crispness	5.75^{a}	4.21^{bc}	5.91^{a}	6.07^{a}	4.55^{b}	2.63^{d}	5.87^{a}	4.83^{b}	3.70^{c}	5.67^{a}	4.83^{b}
Juiciness	5.52^{a}	4.85^{bcd}	5.43^{ab}	5.50^{a}	4.72^{cd}	3.49^{e}	5.21^{abc}	5.37^{ab}	4.39^{d}	5.43^{ab}	5.27^{abc}

Table 7 shows the correlation between the acceptance of the different sensory attributes of the apple selections. Strong positive correlations were observed between flavor intensity and overall preference (0.791) and hardness and crispness (0.892).

Table 7. Pearson correlation for apple selections as evaluated by the consumer panel (n=162).

Variables	Overall Preference	Sweetness	Sourness	Flavor Intensity	Hardness	Crispness	Juiciness
Overall Acceptance	1	0.714	0.654	0.791	0.667	0.657	0.672
Sweetness	0.714	1	0.656	0.731	0.455	0.451	0.576
Sourness	0.654	0.656	1	0.674	0.437	0.436	0.521
Flavor Intensity	0.791	0.731	0.674	1	0.555	0.572	0.644
Hardness	0.667	0.455	0.437	0.555	1	0.892	0.621
Crispness	0.657	0.451	0.436	0.572	0.892	1	0.643
Juiciness	0.672	0.576	0.521	0.644	0.621	0.643	1

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CONTINUING PROJECT REPORT **YEAR**: 1 of 2

Project Title: Washington apple varieties for management of Type 2 Diabetes

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Cooperators: NA
Total project funding request: Year 1: **$45,000** **Year 2**: $ 47,850 **Year 3**: NA

Other funding Sources: NA

Budget 1
Organization Name: University of Massachusetts at Amherst
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Item	**2009**	
Salaries (50% Research Associate**)**	$ 21,000	
Benefits 35% of Salary	$ 7,350	
Wages	NA	
Benefits	NA	
Equipment: (HPLC Columns & Maintenance)	$ 4,000	
Supplies (Reagents & Enzymes)	$ 8,000	
Travel	NA	
Miscellaneous:(Orchard needs-preparation & extraction of samples)	$ 7,500	
Total	**$ 47,850**	

OBJECTIVES:

1) To evaluate the health benefits of major fresh varieties of apples grown in Washington State for combating early stages of Type 2 diabetes and to better advance a fruit and vegetable rich healthy diet based on this information (Year 1).
2) To evaluate the health benefits of post-harvest stored apples under CA and 1-MCP conditions of important varieties grown in Washington State to determine if the potential evaluated under Objective 1 for combating early stages of Type 2 diabetes is maintained during storage (Year 2).

Goals and activities: During the 1st year, 10 popular varieties of apples of importance to the grower network of WTFRC (Ginger Gold; Honeycrisp; Gala; McIntosh; Jonagold; Empire; Braeburn; Golden Delicious; Fuji; Delicious) were selected to be evaluated for determining the potential for management of early stages of type 2 diabetes using *in vitro* biochemical and enzyme assay models. The findings are reported in this report. Many of these varieties are maintained and managed locally at the University of Massachusetts orchards under the guidance of Dr. Duane Greene. During the 2nd year this study will be repeated to address seasonal changes in bioactives and 4-5 varieties will be regular air stored (0°C or 32 °F) for 3 months. Further since all Washington apples are sold after long-term post-harvest storage, apples of 4-5 important varieties under CA and 1-MCP storage will be evaluated and compared during the 2nd year for documented health benefits relevant to type 2 diabetes management using *in vitro* biochemical and enzyme models based on the 1st year study from freshly harvested apples. **The goal is to confirm results from this first year's research that fresh whole apples have enhanced health benefits at various stages from fresh to stored conditions.**

Schedule of activities will include 1) post-harvest air stored and long term CA and 1-MCP stored apple evaluation of type 2 relevant functionality over the year with 4 replications. Different stages of storage will be compared to fresh harvested apples from 1st year and to determine whether the bioactive phenolics and their functional benefits are maintained. We will also evaluate apple varieties in the commercial markets along the east coast that are sold after long term storage in Washington State to determine if the health benefits are maintained at the time of consumption. Some guidance for the history of these apples will be sought from WTFRC.

The ***anticipated benefits*** will include the use of innovative *in vitro* biochemical and enzyme bioassays have been developed in PI's laboratory and tested in fresh and post-harvest stored stages to determine the biochemical relevance of apples as a part of better diet for management of early stage type 2 diabetes. These *in vitro* studies will provide key biochemical rationale for future animal and clinical studies. Results also indicate that the whole apple has more complete and enhanced health benefits.

Deviations from original objectives: Based on the suggestions of WTFRC we also included 3 pear varieties (Bosc; Seckel and Bartlett) in our study, which was outside the original proposal. During the 2nd year these pear varieties will also be studied following cold storage using selected varieties. We have also developed *in vitro* bioassays for long term hypertension complications of type 2 diabetes and these will also be evaluated in various extracts in the second year of the study.

SIGNIFICANT FINDINGS:

The rationale for this study is to better biochemically define the well-known health benefits of apple that have been attributed in part to their polyphenolic metabolite content and related antioxidant capacity, as well as other bioactive components such as dietary fiber. The consumption of apple could provide health benefits by lowering the risk for chronic diseases such as metabolic syndrome diseases including type 2 diabetes. This chronic disease affects 20 million Americans and 180 million globally and is projected to increase to 400 globally by 2030. The objective of this study is to investigate the phenolic-linked anti-hyperglycemia bioactive factors in apple and pear varieties.

Overall, <u>whole apple consumption including peel and pulp</u> has more complete health benefits potential relevant for dietary support for managing type 2 diabetes and its complications and these cannot be obtained from apple juice or other common fruits such as banana and orange.
Ten different apple varieties and three different pear varieties were analyzed in relation to peel and pulp fractions of each variety separately and extracted in distilled water and 12 % ethanol. These extracts were analyzed using *in vitro* biochemical and enzyme analysis in the context of relevance and benefits to managing early stages of type 2 diabetes.

1) **Peel** sample was shown to have higher total soluble phenolic content and related antioxidant activity than pulp sample (Figures 1 & 2).
2) **Honeycrisp and Delicious varieties have the highest total phenolic content (>800 ug/g FW)** this is correlated well to high (>70%) antioxidant activity (Figures 1 & 2).
3) Preliminary results indicate all 5 of the 10 varieties (Figure 3) evaluated so far showed high (>70%) α-amylase inhibitory activity and of these only 3 varieties **(Jonagold, Golden Delicious and McIntosh)** moderate (25%-30%) α-glucosidase inhibitory activity and **pulp is superior to peel.** This provides the biochemical rationale that if apple is consumed with a soluble carbohydrate (high glycemic index) diet it has the potential to slow digestive process and reduce degradation of starch (alpha amylase inhibition) or sugar and moderately slow down glucose absorption (alpha-glucosidase inhibition) and therefore slow accumulation of high levels of glucose in the blood that can otherwise result in the condition of hyperglycemia relevant to increased type 2 diabetes (Figure 1 below for **summary of mechanism of action**).
4) **For complete bioactive benefits whole apple consumption (peel and pulp) is superior.**
5) Overall all 10 apple varieties had high phenolic-linked and antioxidant activity in **peel** and 5 so far have alpha-amylase inhibitory activity (resistance to starch breakdown) **in the pulp** and therefore have overall potential for being part of an improved diet for managing glycemic index in the context of type 2 diabetes and complications of oxidative stress.
6) Some of alpha-amylase inhibitory (resistance to starch breakdown) potential may be due to insoluble polysaccharide and oligosaccharide (fiber) fractions of apple pulp and similar to standard drugs (acarabose) with **whole apple likely having potential of resulting in less side effects of undigested starch than drugs.**
7) The **additional benefits of high phenolic-linked antioxidant activity in the apple peel** has potential to contribute to the reduction of microvascular complications of late stages of type 2 diabetes such as wound healing, macular degeneration and kidney dysfunction.
8) Quercetin and related flavanoids are important phenolics from apple peel that are linked to bioactive benefits. However, overall profile of phenolics and fiber are likely more important.
9) The bioactive benefits are clear based on *in vitro* biochemical and enzyme assays and this only provides the biochemical rationale for future animal and clinical studies.
10) The phenolic-linked antioxidant benefits of apple in the diet have potential for radiation and chemotherapy benefits by reducing the side effects. This is being further investigated using yeast models to reduce radiation damage in the presence of apple biaoctives.
11) All pulp of the 3 varieties (Bartlett, Seckel and Bosc) evaluated have moderate phenolics and antioxidant activity and high alpha-amylase inhibitory activity with Seckel the only one having alpha-glucosidase inhibitory activity.

Summary Conclusions

This study indicates that whole apple in general and some varieties like Honeycrisp and Delicious are even better; have medium to high phenolics and free radical scavenging-linked antioxidant activity in the **peel.** Several varieties have moderate profile of α-glucosidase inhibition and high α-amylase inhibition in the **pulp**. **This indicates that a complete whole fruit** offers the best potential for good postprandial blood glucose management linked to hyperglycemia associated with type 2 diabetes and its oxidative stress complications without the common side-effects associated with very high α-amylase inhibition in drugs such as acarbose. Compared to drugs, whole apple have free radical scavenging-linked antioxidant activity which can help maintain the redox balance in susceptible cells.

This study provides a strong biochemical rationale for future animal and clinical studies to include apple as an important part of the overall diet and medicinal therapy for better management of type 2 diabetes and its complications. This also helps basis for breeding of better apple varieties for health.

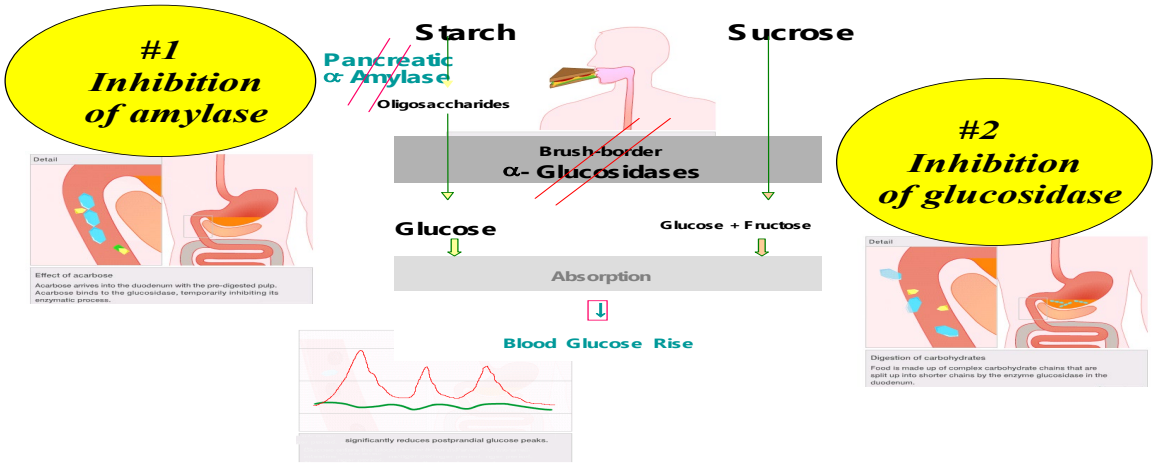

Figure 1: How alpha-amylase and alpha-glucosidase from apple work?

METHODS:

The bioactive potential of post-harvest stored apples will be determined by analysis of total soluble phenolics, DPPH-linked antioxidant activity and their associated *in vitro* enzyme, α-glucosidase and α-amylase inhibitory activity.

Sample Extraction

Soluble phenolics will be extracted from the peel and pulp of each variety separately. Phenolics will be extracted using distilled hot water and 12% ethanol. The water extractions will be done with 20 g of peel in 50 ml of water and 100 g of pulp in 50 ml of water. The ethanol extractions will be done with 5 g of peel in 15 ml of 12% ethanol and 10 g of pulp in 20ml of 12% ethanol. Apples will be first peeled then cut and weighed. Peel and pulp will then be mixed with either distilled water or 12% ethanol. This will be then homogenized for 2 min using a blender. Resulting mix will be collected and centrifuged for 5 min. Supernatant will be collected and stored at -20°C. This will be done every month until the 3rd month of storage under both air room temperature storage and 4 ºC storage for freshly picked apples and as when it arrives for CA and 1-MCP stored apples.

Total Phenolics Assay

The total phenolic content will be determined by an assay modified from Shetty *et al*. (1995). Briefly, one milliliter of extract will be transferred into a test tube and mixed with 1 ml of 95% ethanol and 5 ml of distilled water. To each sample 0.5 ml of 50% (v/v) Folin-Ciocalteu reagent will be added and mixed. After 5 min, 1 ml of 5% Na_2CO_3 will be added to the reaction mixture and allowed to stand for 60 min. The absorbance will be read at 725 nm. The absorbance values will be converted to total phenolics and were expressed in micrograms equivalents of gallic acid per grams fresh weight (FW) of the sample. Standard curves will be established using various concentrations of gallic acid in 95% ethanol.

Antioxidant Activity by 1, 1-Diphenyl-2-Picrylhydrazyl Radical (DPPH) Inhibition Assay

To 3 ml of 60 µM DPPH in ethanol, 250 µl of each extract will be added, the decrease in absorbance will be monitored at 517 nm until a constant reading is obtained. The readings will be compared with

the controls, which will contain 250 µl of 95% ethanol instead of the extract. The % inhibition will be calculated by:

$$\%\ \text{inhibition} = \left(\left[\frac{A_{517}^{Control} - A_{517}^{Extract}}{\left[A_{517}^{Control} \right]} \right] \right) x100$$

α-Glucosidase Inhibition Assay

α-Glucosidase (EC 3.2.1.20) will be purchased from Sigma Chemical Co. A volume of 50 µl of sample solution and 100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ml) will be incubated in 96 well plates at 25°C for 10 min. After pre-incubation, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) will be added to each well at timed intervals. The reaction mixtures will be incubated at 25°C for 5 min. Before and after incubation, absorbance readings will be recorded at 405 nm by micro-array reader (Thermomax, Molecular device Co., Sunnyvale, CA) and compared to a control which will have 50 µl of buffer solution in place of the extract. The α-glucosidase inhibitory activity will be expressed as inhibition % and will be calculated as follows:

$$\%\ \text{inhibition} = \left(\left[\frac{\Delta A_{405}^{Control} - \Delta A_{405}^{Extract}}{\left[\Delta A_{405}^{Control} \right]} \right] \right) x100$$

We are also isolating a porcine enzyme as this is closer to human enzymes and will be included in the studies when relevant.

α-Amylase inhibition assay

Porcine pancreatic α-amylase (EC 3.2.1.1) will be purchased from Sigma Chemical Co. A total of 500 µl of extract and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) will be incubated at 25°C for 10 min. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) will be added to each tube at timed intervals. The reaction mixtures will then incubated at 25°C for 10 min. The reaction will be stopped with 1.0 ml of dinitrosalicylic acid color reagent. The test tubes will then be incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture will then be diluted after adding 10 ml distilled water and absorbance will be measured at 540 nm.

$$\%\ \text{inhibition} = \left(\left[\frac{A_{540}^{Control} - A_{540}^{Extract}}{\left[A_{540}^{Control} \right]} \right] \right) x100$$

High Performance Liquid Chromatography (HPLC)

HPLC analysis of best and relevant varieties in terms of best anti-diabetes potential will be further analyzed for their most important phenolic components to develop markers for selection, breeding, post-harvest preservation strategies and further clinical and health recommendations. The phenolic profiles and ratios will be matched to antioxidant potential and anti-hyperglycemia potential.

Two ml of apple extracts from various variety samples will be filtered through a 0.2 µm filter. A 5 µl volume of sample will be injected using Agilent ALS 1100 auto sampler into Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for gradient elution will be (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration will be increased to 60% for the first 8 min and to 100% over

the next 7 min, then decreased to 0% for the next 3 min and than maintained for the next 7 min (total run time, 25 min). The analytical column used will be Agilent Zorbax SB-C18, 250 x 4.6 mm i.d., with packing material of 5 µm particle size at a flow rate of 1 ml/min at ambient temperature. During each run the chromatogram will be recorded at 306 nm and 333 nm and integrated using Agilent Chemstation enhanced integrator. Pure standards of quercetin, caffeic acid, chlorogenic acid and range of other common phenolics known to be found in apple (purchased from Sigma Chemical Co., St. Louis, MO) in 100% methanol will be used to calibrate the standard curve and retention times. Variety and stage specific profiles of promising extracts will be matched to the health-relevant functionality described above.

Statistical Analysis

Analysis at every time point will be carried out in triplicates. Means, standard errors and standard deviations were calculated using Microsoft Excel 2003.

RESULTS & DISCUSSION:

Apples were harvested from August 28, 2008 (Ginger Gold) to October 23, 2008 (Braeburn) at the University of Massachusetts Orchards in Belchertown, MA under supervision of Dr. Duane Greene based on standard harvest index standards (data available) based fruit weight, red color, circumrance, firmness, starch, soluble solids and percent water core.

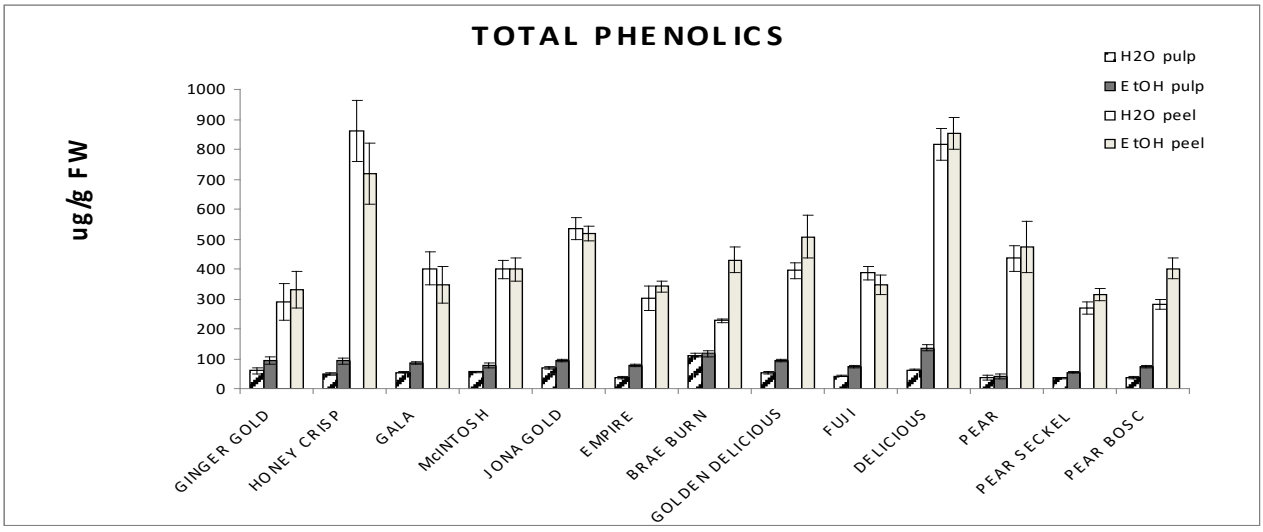

Figure 2: Total soluble phenolic content of evaluated apple WA apple varieties

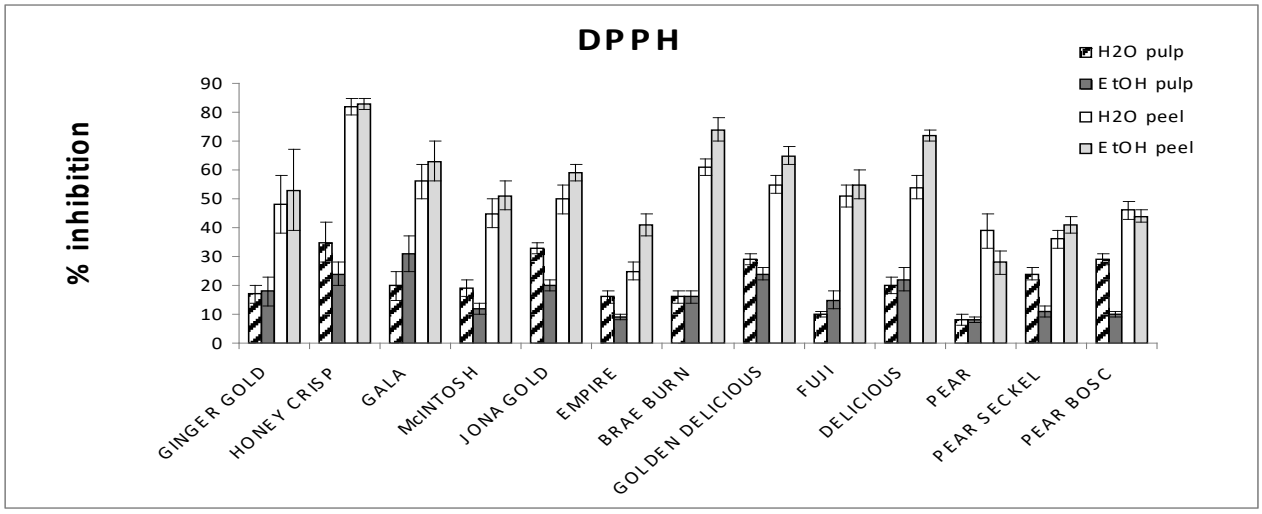

Figure 2: Free radical scavenging-linked antioxidant activity of evaluated WA apple varieties

The **Peel samples have higher total soluble phenolic content and related antioxidant activity than pulp** sample (Figures 1 & 2). Among these Honeycrisp and Delicious varieties have the highest total phenolic content (>800 ug/g FW) this is correlated well to high (>70%) antioxidant activity (Figures 1 & 2). **Therefore for health benefits derived from phenolics whole apples with peel would be superior.** This is particularly relevant for microvascular complications of type 2 diabetes due to cellular oxidative breakdown such as wound healing, macular degeneration and improved kidney function.

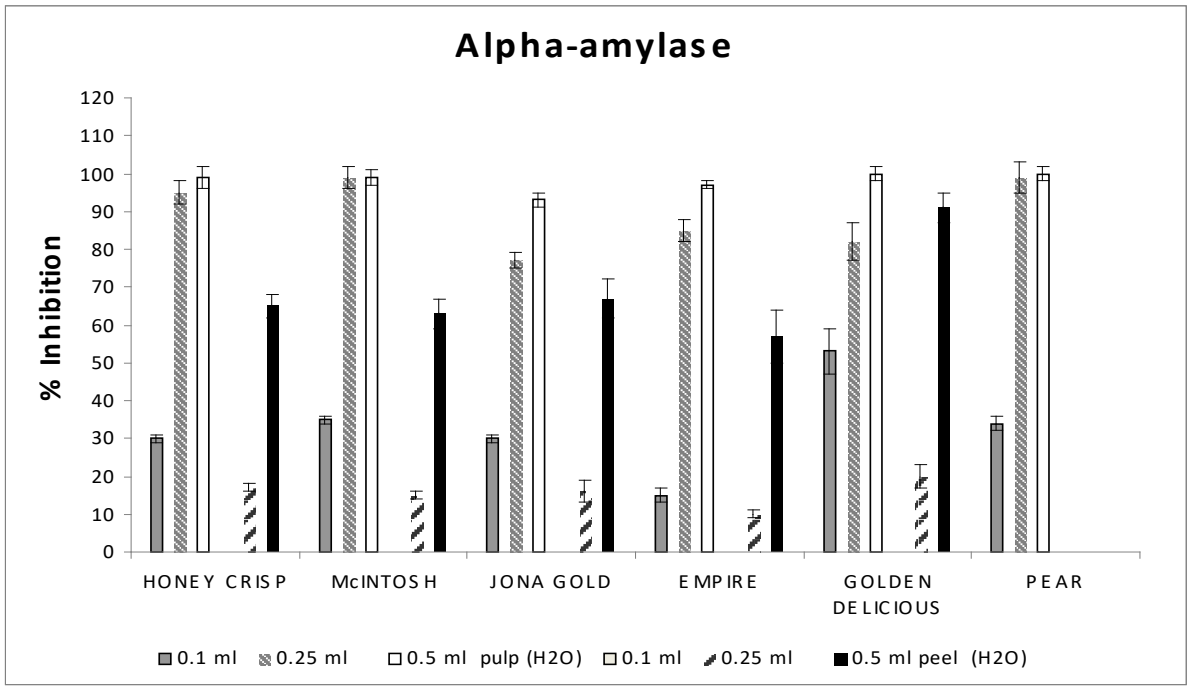

Figure 3: Alpha-amylase inhibitory activity

Among the 5 of 10 apple varieties and 1 of the 3 pear variety (Bartlett) in general have high alpha-amylase inhibitory benefits in the pulp and therefore have **potential to control (resist) the breakdown of starch.** Among these only Jonagold, Golden Delicious and McIntosh have moderate alpha-glucosidase inhibitory bioactives (data not shown) and there have the potential for managing gut absorption of glucose after starch breakdown. **Overall whole apple with pulp and peel have the best combined bioactives for maximum potential for use in diets for managing type 2 diabetes**

and its complications. Bioactive compounds beyond phenolics in the fiber fractions are also important and need to be investigated.

Significance to apple industry and economic benefits:
Clearly for maximum health benefits in the context of better diet for type 2 diabetes management **whole apple including pulp and peel has potential when compared to other common fruits such as banana and orange.** These bioactives from whole apple have the potential to influence positively multiple physiological pathways from soluble carbohydrate utilization control to oxidative breakdown of this chronic disease. This biochemical rationale can be basis of better fruit and vegetable diet that could include 1-3 apples per day. The results from these current studies can provide the **key biochemical rationale** for future clinical studies that can be the basis for how many apples/day may be relevant and for what stages of type 2 diabetes such as pre-diabetes, when diet and exercise are most effective. Therefore better defining the biochemical basis of health benefits of apple contributes to enhanced fresh apple consumption globally. Further, based on biochemical rationale from this study and further future clinical studies as the next step could show that **whole apple with peel and pulp is potentially superior** to juice from pulp, where high phenolic skin and fiber are discarded.

CONTINUING PROJECT REPORT **YEAR**: 2008

Project Title: Apple rootstock and scion evaluation

PI: Tom Auvil
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Collaborators: Dr. Bruce Barritt, WSU-TFREC, Wenatchee,
Dr. Kate Evans, WSU-TFREC, Wenatchee,
Dr. Gennaro Fazio, USDA-ARS, Geneva, New York

Cooperators: Dave Allan, Bob Brammer, Ray Fuller, John Verbrugge, Del Feigal, Ron Wilcox, Dale Goldy, Tim Welsh and Gus Heinecke

Total project funding request: **Year 1**:85,574 **Year 2**: 77,415

WTFRC Collaborative expenses:

Item	(2008)	(2009)
Salaries[3]	37,270	34,925
Benefits[3]	12,104	11,390
Crew Wages	19,940	18,022
Crew Benefits	6060	5478
Stemilt RCA room rental	1,200	4,200
Shipping		
Supplies[1]	7,300	600
Travel[2]	1,700	2,800
Miscellaneous		
Total	85,574	77,415

Footnotes: [1]Scion test trees.
[2]Fuel and maintenance
[3]Salaries and benefits for Auvil, Schmidt, Castillo apportioned to this project.

OBJECTIVES:

1. Evaluate apple rootstocks, particularly disease resistant rootstocks, in commercial settings in Washington State with known replant conditions.
2. Integrate the processes of evaluation and industry adaptation.
3. Extend procedures for rootstock evaluation into scion breeding program.
4. Establish protocol for scion evaluation program.

Table 1: Rootstock and scion trials in the Northwest

Location	Term	Tree # / Acreage	Apple Scion	Apple Root	Pear Root	Cherry Scion	Cherry Root
Brewster	5 - 8 years	234 / 0.2	**x**				
Mattawa	5 - 8 years	234 / 0.2	**x**				
Quincy/Babcock	5 - 8 years	234 / 0.2	**x**				
Prosser	5 - 8 years	234 / 0.2	**x**				
Brewster	2006-2012	837 / 1		**x**			
Chelan	2004-2010	1147 / 0.83		**x**			
Chelan	2003-2009	231 / 0.18		**x**			
Frenchman Hills	2003-2009	275 / 0.25		**x**			
Naches	2004-2010	728 / 0.41		**x**			
Vantage	2006-2012	731 / 0.31		**x**			
Wapato	2004-2010	970 / 0.86		**x**			
Wapato	2006-2012	768 / 0.7		**x**			
Wapato	2006-2012	768 / 0.7		**x**			
Royal City	2008-2016	300/.5		**x**			
Cashmere[1]	2002-2012	na			**x**		
Hood River[1]	2002-2012	400 / 1.5			**x**		
Hood River[1]	2004-2010	300 / 0.33			**x**		
Hood River[1]	2006-2014	1000 / 2.2			**x**		
Tonasket[1]	2002-2012	Na			**x**		
Hood River [1]	5 years	Na				**x**	
Manson	5 years	Na				**x**	
Mattawa	5 - 8 years	Na				**x**	
Prosser[1]	5 years	Na				**x**	
Manson	8 years	700 / 1.75					**x**
Mattawa	5 - 8 years	700 / 1.75					**x**
Mosier[1]	8 years	700 / 1.75					**x**
Prosser[1]	8 years	700 / 1.75					**x**

[1]Trials conducted by WSU / OSU faculty

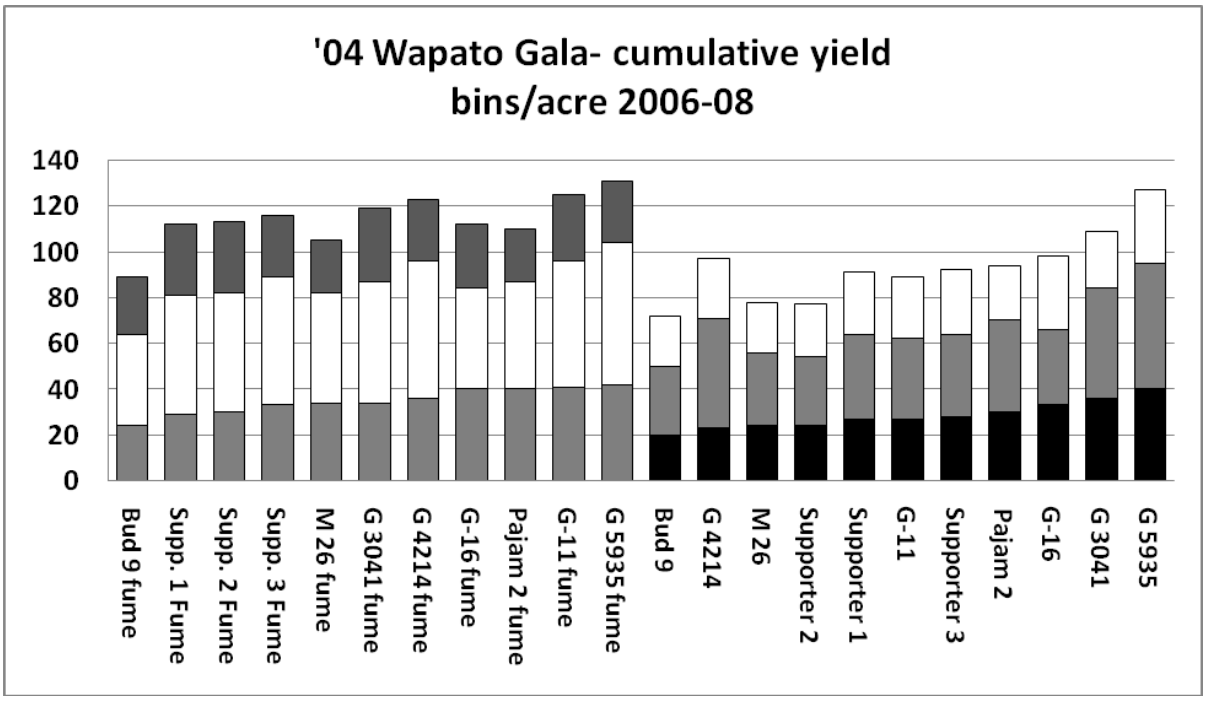
Apple scion accomplishments:

- Nine advanced selections have been planted in four sites in Washington State.
- Protocols and timelines for fruit and tree evaluation have been developed.

Significant rootstock findings:

- G.935, G.41, CG4214 are performing better in most unfumigated replant sites than the M.9 or M.26 class apple rootstocks commercially available.

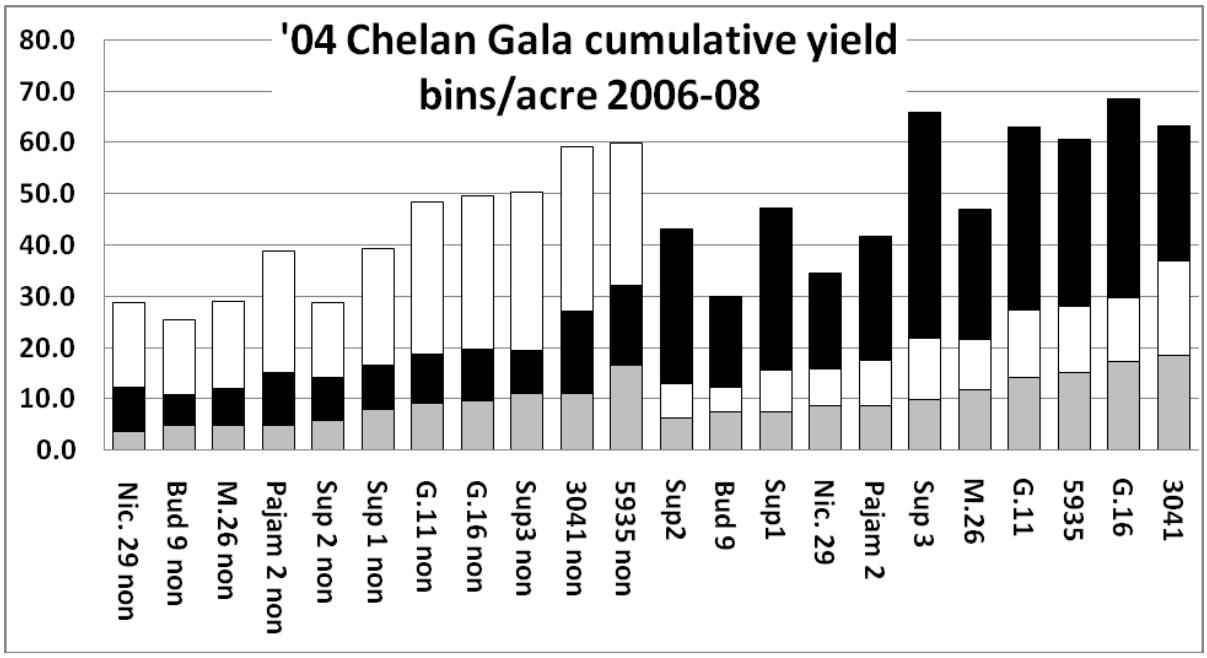
- In fumigated replant trials: G.935, G.41, CG4214 and G.11 all out perform commercial apple rootstocks planted as standards of comparison in the trials in terms of yield, fruit size and consistenancy of tree canopy.
- CG.4011, 2034 and 5890, introduced to Washington in 2006, are showing promise. 4011 is an M.9 size tree with excellent yields. 2034 is smaller than Mark with equal spur density. 2034 could be useful in formal training systems with vigorous scions. 5890 has high vigor in the first two seasons, and bloomed and set a significant crop of large fruit in third leaf.
- G.16, CG.3007, 4002, 4019, 5046, 4013 and 6874 are too virus sensitive for commercial production.
- G.30 is a very productive rootstock but is too large for dwarf systems and is very difficult to manage in the nursery.
- CG5463, 7707, 7037, 7480, 6976 and 6879 are too vigorous or too low in precocity to be efficient in modern plantings.
- Supporter 1,2,3 and 4 performance varies site to site and offer no significant advantage over current commercial rootstocks
- JTE B and C offer no advantage over current commercial rootstocks.



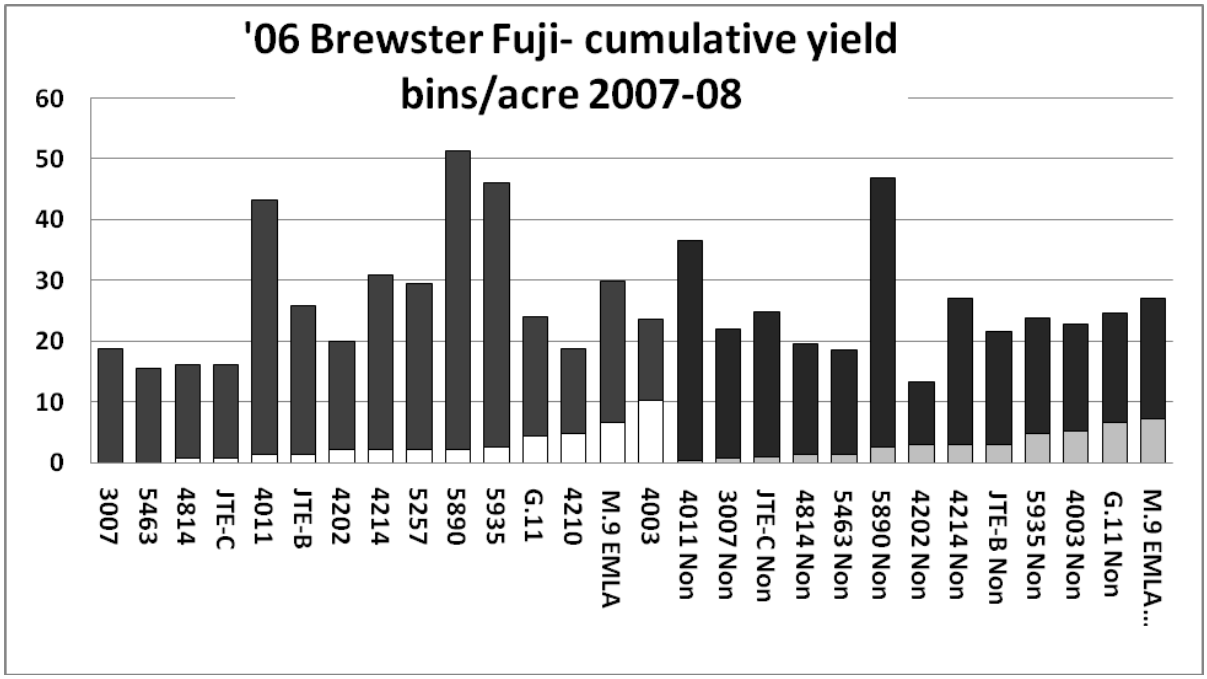
'04Wapato Gala had a small crop in 2008 with yields ranging between 20 and 30 bins per acre in fumigated and unfumigated trials. The trees were shortened to 11 feet, a large branch or two were pruned out followed by frost damage and cold weather during bloom. Fruit size was 100 to 113 count across rootstocks.

G.935 is very precocious and can overset, especially as a young tree. The high fruit count will suppress fruit size. Fruit size with G.935 is equal to M.9 in mature trees. G.11, G.41 and CG.4214 often will produce better fruit size with higher yields than M.9 clones.

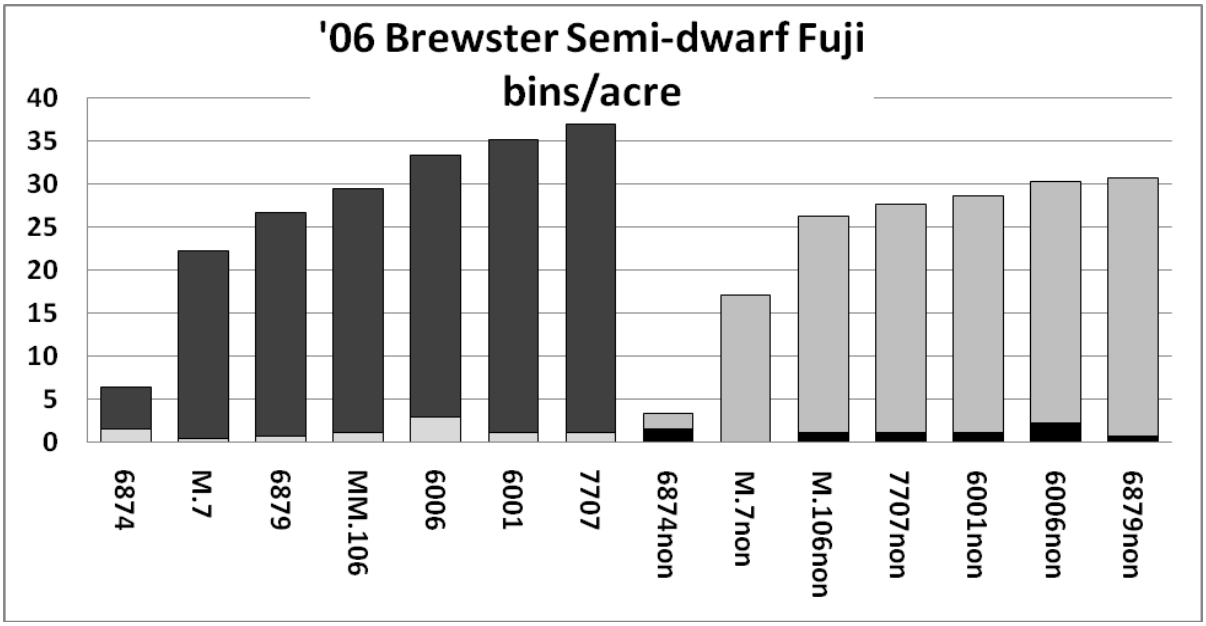
The canopy volume has been filled in both fumigated and unfumigated trials in the '04 Wapato Gala trial. The yield and fruit quality characteristics are going to be heavily influenced by the cultural practices maintaining canopy size. The differences observed to date will remain similar or diminish as the canopy volumes become more similar.



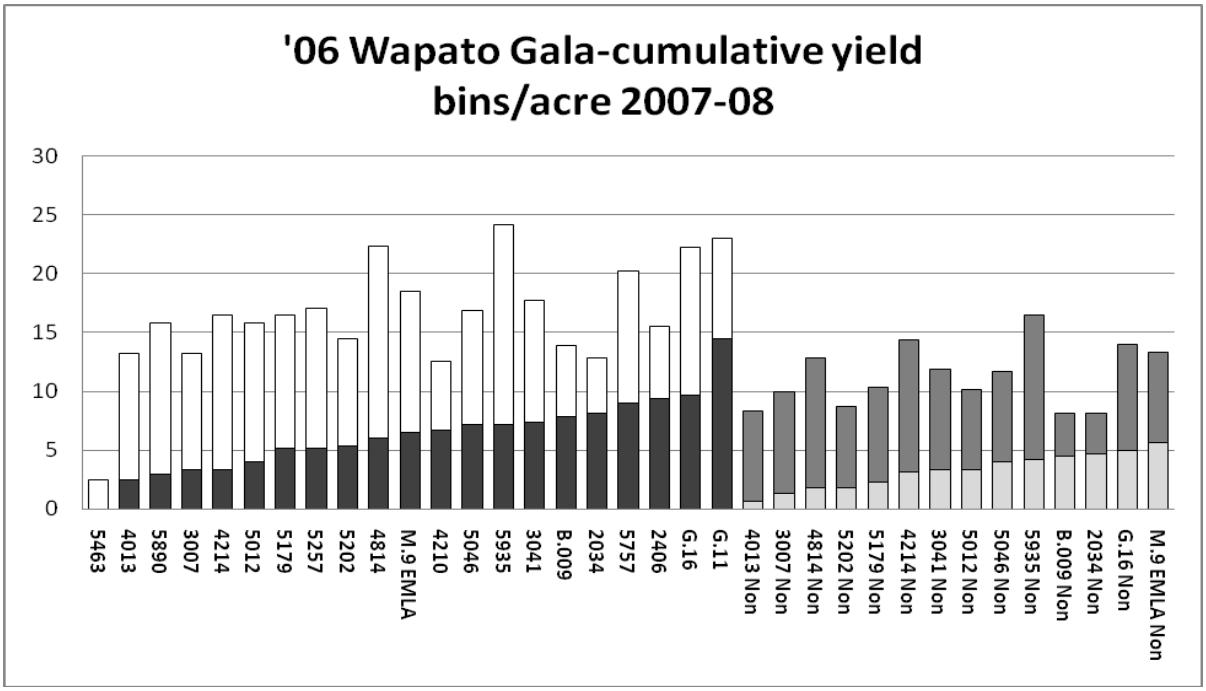
’04 Chelan Gala is planted in a severe replant site. Note the yield is about half of ’04 Wapato. The trees in this trial were reheaded after the first growing season. The commercially available rootstocks all did poorly in this trial .



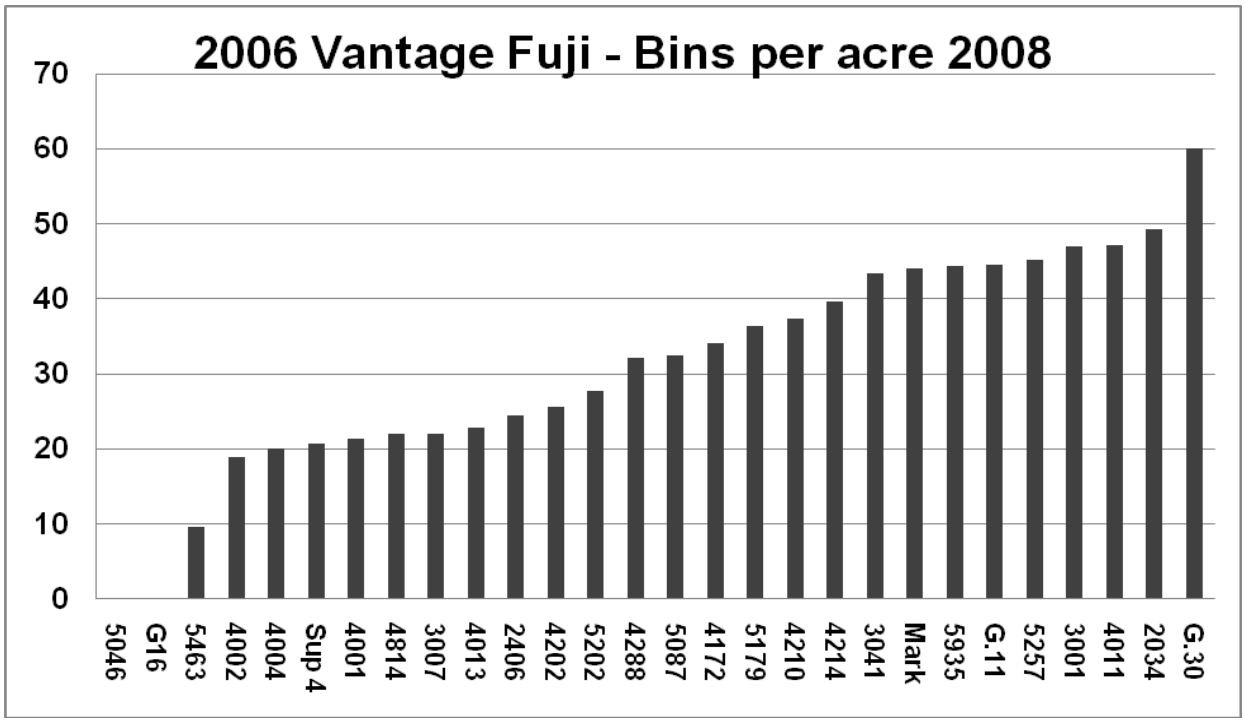
’06 Brewster Fuji is planted in a severe replant site. CG.4011 and CG.5890 both perform very well in fumigated and non-fumigated trials. CG.5890 grew more similar to the vigorous semi-dwarf trees, but fruited in the third leaf like the dwarf rootstock trees.



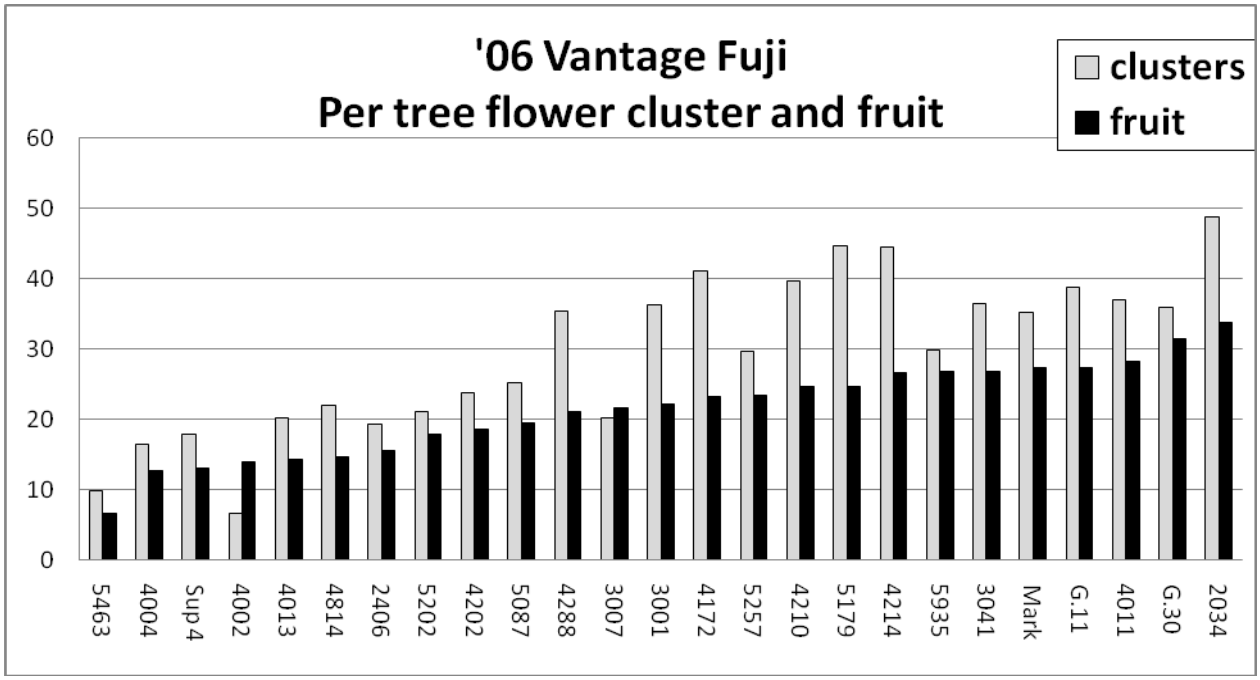
’06 Brewster Fuji semidwarf has three selections to watch; 6879,6006 and 6001. Their second leaf yields were very low and the third leaf yield did not compete with the best of the dwarf rootstocks. M.7 is not very efficient. The semi dwarf trees grew about 30% less fruit and have more than double the pruning.



’06 Wapato Gala chart has 2nd and 3rd leaf yields. The ’04 trials did not crop in 2nd leaf. The’06 trial has more replant challenges than the 2004 trial. CG.4810 looks promising in this data set, but it is not a best choice in other trials. It encourages upright growth with steep branch-crotch angles.



The Vantage Fuji plant-in-place trial with bench graft trial has mostly filled the canopy at the end of the second growing season. The standard for this trial is Mark. The third leaf yield target is less than 40 bins per acre to reduce the risk of biennial bearing. Most promising in this trial: 4214, G.41, G.935, G.11, 4011 and 2034. G.30 and 5463 are too vigorous and may need to be removed.



In this precision management system, it is important for management to understand the relationship of the number of flower clusters needed to yield a targeted number of fruit. Excess flower cluster counts can be adjusted by pruning or by spacing at hand blossom thinning.

CONTINUING PROJECT REPORT **YEAR**: 1of 2

Project Title: Mapping *M. sieversii: A Valuable Genetic Resource for Apple Breeding*

PI:	Michael Wisniewski	**Co-PI(2):**	Donnal Lalli
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Co-PI(3):	Gennaro Fazio	**Co-PI(4):**	Jay Norelli
Organization:	USDA-ARS	**Organization:**	USDA-ARS
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City:	Geneva	**City:**	Kearneysville
State/Province/Zip	NY 14456	**State/Province/Zip:**	WV 25430

Cooperators: Dr. Michael Malnoy, IASMA., Italy (SNP Analysis): Dr. Sue Gardiner, Plant & Food Research, NZ (Mapping of GMAL 4591), Dr. Charles Simon, USDA-ARS, Geneva, NY (Consultant on Mapping), Dr. Phil Forsline and Dr. Herb Aldwinckle (USDA-ARS and Cornell University (Initial Assessment of Fire Blight Resistance).

Total Project Request: Year 1: $25,500 **Year 2:** $28,500

Other funding Sources: None

Budget 1:

Organization:USDA-ARS	**Contract Administrator:**Ingrid Charlton
Telephone: (215) 233-6554	**Email:** Ingrid.Charlton@ars.usda.gov

Item	Year 1 2008	Year 2 2009	
Salaries	10,000	10,000	
Benefits	3,500	3,500	
Wages			
Benefits			
Equipment			
Supplies	12,000	15,000	
Travel			
Miscellaneous			
Total	25,500	28,500	

Footnotes: Year 1 - $25,500 Awarded First Year: Received $21,564.72 – Remaining funds drawn from unspent funds in expired grants ($3,935.28). Year 2 – Additional funds requested to cover synthesis of additional labeled primers for markers, SNP analysis, increased cost of mapping supplies (TAQ-Polymerase, pipette tips, ABI plates, etc.), and establishment of mapping population at the USDA-ARS-AFRS in Kearneysville, WV.

Objectives

The objectives of the present proposal addresses the following high priority research area defined by the Washington Tree Fruit Research Commission: Identification of genes and improvement of gene marker tools for continued increase in the efficiency of apple breeding program as well as improved disease and pathogen resistance. The project also brings together the expertise of researchers nationally and internationally to work together as a team.

The objectives are:

1) Produce genetic marker data for a family (**GMAL 4593)** of 190 seedlings derived from a Gala' x *M. sieversii* cross using a collection of 159 SSR markers. – **Year 1 and Year 2.**
2) Use the resulting marker data to produce a basic "framework" map for *M. sieversii*. **– Year 1 and Year 2.**
3) Identify additional SSR markers to fill any gaps in the evolving map and enrich regions of interest with a high density of markers. **– Year 2**
4) Anchor the generated map to other apple maps by using identical SSR markers. In particular, exchange markers with Plant & Food Research (previously HortResearch), New Zealand who will be mapping another family (**GMAL 4591**) from a different 'Gala x *M. sieversii* cross. **– Year 2**
5) 200 SNP markers will be identified by IASMA and placed on the genetic map for the GMAL4593 population. – **Year 2.**
6) Collaborate with others to define QTLs for important traits as the population is characterized. Specifically, **GMAL 4593** will be assessed for fire blight resistance in 2008 and 2009. **– Year 1 and 2.**

Note: **All the maps and marker data will be deposited in the Genomic Database for the Rosaceae (GDR) in order for it to be available to the entire apple breeding and research community.**

<u>YEAR 1 ACCOMPLISHMENTS</u>

- DNA has been extracted from all 192 trees of the GMAL 4593 mapping population of *M. sieversii* x Royal Gala.
- 196 SSR markers have been compiled to map the population. Each marker has been or will be screened on each of 182 individuals.
- **54 SSR markers have been run on the entire population and individuals have been successfully scored for mapping.**
- **37 of 44 SNPs evaluated have been genotyped for the population. They are in the process of being placed on the map and will be presented in the PowerPoint presentation in January.**
- 80 additional markers have been evaluated on a 32 individual sub-set of the population and are in line to be run on the entire population and 60 markers are awaiting evaluation.
- **All individuals in the population have been scored for fire blight resistance.**
- **Initial linkage map has been constructed (see Results and Discussion).**
- **Mapping population has been established at USDA-ARS, Kearneysville, WV for further fire blight resistance evaluation and scoring in order to locate fire blight resistance QTL(s).**
- Initial evaluation of cold hardiness has been initiated in parents of the mapping populations GMAL 4593 and GMAL 4591.

Methods

Plant Material:

The family GMAL 4593 consisting of 190 seedlings derived from an F1 cross, 'Gala' x *M. sieversii,* will be used as the mapping population in this study. Leaf and bark tissue will be obtained from the 2 parents ('Gala' and *M. sieversii*) and the 190 seedlings. This tissue will serve as the source of DNA for genetic analysis. The seedlings are entering into their 5th leaf.

DNA Extraction and SSR Analysis

DNA for SSR marker analysis will be extracted from apple leaf tissue using plate format Qiagen DNA extraction kits. Our lab has an extensive and successful history using this system with apple. DNA will be dissolved and stored in sterile TE buffer.

Initially, a subset consisting of the 2 parents and 30 individuals from GMAL 4593 will be screened with a total of 159 SSR markers to determine which SSR markers will be informative for mapping in the entire population. Only the most informative SSR markers will be evaluated in the entire population. GENEPOP (ver. 3.4) will be used to test the utility of each SSR marker for distinguishing the population.

PCR amplification will be performed with a single pair (forward + reverse) of PCR primers for each SSR marker. The forward primer will be labeled with one of three fluorescent dyes (6-FAM-blue, HEX-green, or NED-yellow) and used to PCR amplify the SSR DNA loci in individuals from the GMAL 4593 mapping population.

PCR products will be confirmed by running 5 µl samples on 2% agarose gels. PCR products will be multiplexed (based on product size) whenever possible to reduce total costs. Up to 3 different fluorescently labeled PCR products can be ran per capillary on an automated laser fluorescence sequencer, ABI Prism 3730. Appropriate size standards will be employed. GeneMapper version 3.7 (ABI, Foster City, CA, USA) or other similar mapping software will be used to analyze trace files, determine PCR product size and assign genotypes to individuals.

After the initial framework is established, additional markers will be used to provide additional coverage where large gaps and/or regions of interest exist and anchor the map to other apple maps. In particular, markers will be exchanged with Plant & Food Research (New Zealand) who will be mapping a population (GMAL 4591) also derived from a 'Gala' x *M. sieversii* cross where the *M. sieversii* pollen parent represents a different elite line than the pollen parent used in GMAL 4593.

Linkage analysis:

The segregation of each marker will be tested first for goodness-of-fit to a 1:1 segregation ratio using a chi-square test. A double pseudo-testcross approach will be used as is typical for analyses in F1 populations where both parents are heterozygous (Grattapaglia and Sederoff 1994). With this approach, linkage maps are generated for each parent of a cross. JoinMap (Kyazma) version 4.0 (Stam 1993, 1995) will be used to establish a framework map for each individual parent of the F1 population GMAL4593 (*Malus sieversii* x 'Gala'). Linkage groups were established at a recombination fraction of 0.40 a LOD score $\geq$ 4.0 with the exception of LG1 of 'Gala.' The Kosambi mapping function was used for the calculation of map distances. .

QTL Mapping of Fire Blight Resistance

The fire blight resistance trait evaluation (performed by P. Forsline and H. Aldwinckle) of GMAL 4593 will be repeated for a second year in the field by Dr. Jay Norelli. The results will be placed on the framework map in order to define QTLs for fire blight resistance derived from *M. sieversii*.

Coordination:

Plant material will be collected from the GMAL 4593 population of apple trees located in Geneva, NY. DNA extraction and SSR primer screening will be conducted in Kearneysville, WV using an ABI-3730 genotyper located at the USDA-ARS facility in Leetown, WV. Dr. Donna Lalli, who has experience developing DNA markers, mapping disease resistance genes in peach, and constructing a genetic linkage map for an apricot BC1 population segregation for plum pox virus resistance will assist Dr. Michael Wisniewski in screening the GMAL 4593 population using the SSR primers. Interpretation and mapping of the SSR marker data will be conducted with the assistance of Gennaro Fazio. Dr. Jay Norelli will continue to evaluate the population for resistance to fire blight and assist in transferring the fire blight analysis to the framework map. Additionally, Norelli will help to coordinate mapping efforts of the GMAL4591 population being mapped by Plant & Food Research (New Zealand).

Results and Discussion

Genetic Map Construction for GMAL4593

Genetic markers were established in the GMAL4593 population for 14 of the 17 linkage groups of apple (Fig 1). Fifty-three SSR markers were screened in the entire F1 population GMAL4593. Forty-nine (92%) SSRs were polymorphic and were analyzed for the genetic map construction. Of the 49 SSRs, 26 segregated in both *Malus sieversii* (MS) and 'Gala' (G), 6 segregated in MS only and 13 in G only. Seventeen SSRs were assigned to 7 of the 17 linkage groups of MS and 27 SSRs were assigned to 13 of the 17 linkage groups of RG.

During the second year of this project, numerous additional SSR and SNP markers will be added to the map in order to complete a genetic framework map for both parents covering all 17 linkage groups. The continuation of the project will greatly enhance the number of markers that are segregating in the *M. sieversii* parent and allow for a more detailed connection to other genetic maps. Additionally, the information obtained from our mapping work will be combined with the mapping work of an additional *M. sieversi*i x Royal Gala mapping population (GMAL 4591) being generated by Plant & Food Research, New Zealand.

The construction of a framework map for *Malus sieversii* is the first step in enabling the identification of regions of the genome that control important disease resistance and fruit quality traits. Initially, this map will be used to place QTLs for fire blight resistance (USA) and apple scab resistance (New Zealand). Identifying molecular markers in these regions will help breeders streamline the breeding process by allowing the breeders to make early selections of resistant material. The preliminary map presented in Figure 1 will not only serve to identify genomic locations of important traits in *Malus sieversii*, but by using molecular markers common in other apple maps, allow the comparison of these locations in the *Malus sieversii* genome to other apple genomes. Figure 2 illustrates the cross comparison of *Malus sieversii* linkage groups with the corresponding linkage groups from the apple consensus map recently constructed by N'Diaye et al. (2008) and the 'Fiesta' x 'Discovery' map (www.rosaceae.org and Silfverfberg-Dilworth et al. 2006).

Development of genomic tools in order to enhance the efficiency and timely development of new apple cultivars has been highlighted as a primary goal in the Apple Technology Roadmap and the White Paper of the Rosaceae Genomics Community. Providing a molecular-marker-based framework map of the GMAL 4593 and subsequent enhancement of the map with QTLs will provide a valuable tool to apple breeders. Apple growers will benefit through the development of new apple cultivars that address key production concerns, as well as consumer preferences

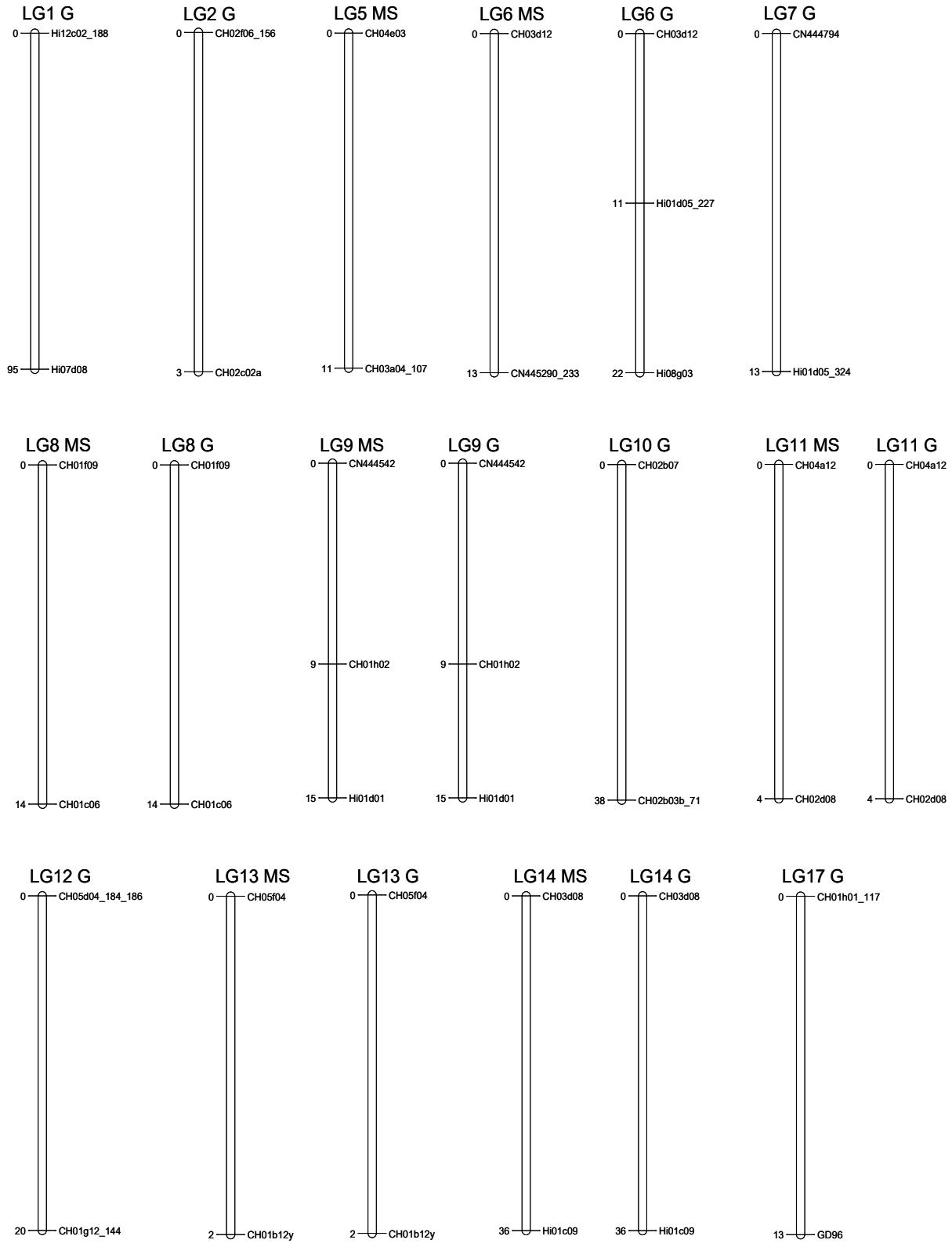

Fig. 1 A framework genetic linkage map for the F1 apple population GMAL4593 (*Malus sieversii* x 'Gala'). Thirteen of the seventeen chromosomes of apple are illustrated as linkage groups (LG).'G' indicates the parent 'Gala' and MS indicates the parent *Malus sieversii* . The numbers to the left of the linkage groups represent the relative distances of markers shown on the left.

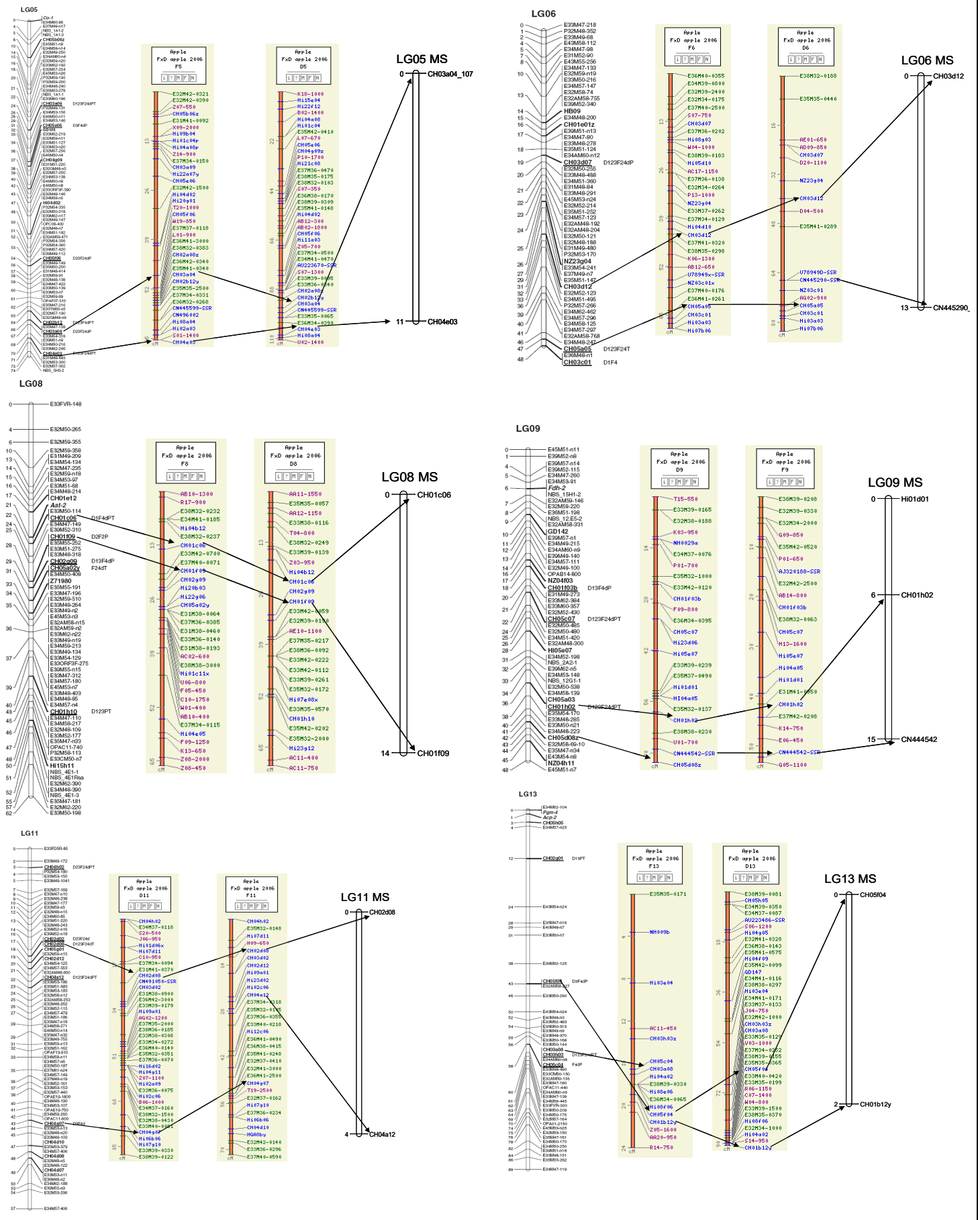

Fig. 2 Comparitive mapping of the apple consensus map (N'Diaye et al. 2008), 'Fiesta' x 'Discovery' (Silfverbeg-Dilworth et al., 2006), and GMAL4593 (*Malus sieversii*, MS).

CONTINUING PROJECT REPORT **YEAR**: 1 of 3

Project Title: Integration of storage technologies for fruit quality management

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Cooperators: Dr. Yanmin Zhu, USDA, ARS, TFRL, Wenatchee
Dr. Chris Watkins, Dept. Horticulture, Cornell University, Ithaca, NY

Total Project Request: Year 1: $27,075 **Year 2: $27,863** **Year 3:** $28,675

Other funding Sources

Agency Name: USDA-CSREES
Amount requested: $4,452,540.00 (total), $370,535.60 (for ARS-Wenatchee metabolomics)
Notes: D. Rudell was Co-Project director, J. Mattheis was Co-PI. The Coordinated Agricultural Project (multi-institutional), RosTRAIT, was not funded.

Agency Name: AgroFresh, Inc.
Amount requested or awarded: $12,000
Notes: Funding supports 'Honeycrisp' storage research.

Budget 1:

Organization: USDA, ARS	**Contract Administrator:** Chuck Myers		
Telephone: (510)559-5769	**Email:** Chuck.Myers@ARS.USDA.GOV		
Item	**2008**	**2009**	**2010**
Salaries	$19,605	$20,198	$20,808
Benefits	$6,470	$6,665	$6,867
Wages	0	0	0
Benefits	0	0	0
Equipment	0	0	0
Supplies	$1000	$1000	$1000
Travel	0	0	0
Miscellaneous	0	0	0
Total	$27,075	**$27,863**	$28,675

Objectives:

1. Identify postharvest protocols to maximize storage life of 'Honeycrisp' and other softscald-sensitive cultivars. This information will contribute to development of postharvest protocols to successfully handle and store the increasing volume of 'Honeycrisp' produced in Washington.
2. Characterize physiological events that result in softscald symptom development. The goal is to identify metabolic and/or molecular markers that could be used for cultivar improvement and/or identify in-season susceptibility to softscald development.
3. Identify minimum storage duration required to control superficial scald by use of oxygen setpoints below 1%. This information would provide warehouses an additional means to operate CA rooms to optimize quality of conventional and organic fruit.
4. Identify what if any limits for CO_2 exist during apple storage in oxygen below 1%. CA disorders related to CO_2 may be manageable using lower O_2 due to less CO_2 accumulation in the low O_2 environment.

Significant Findings:

1. Symptoms of 'Honeycrisp' soft scald can be induced within 2 weeks of harvest when fruit are rapidly cooled to 33°F.
2. Oxygen concentrations where fluorescence changes were detected for 'Delicious', 'Granny Smith', and 'Honeycrisp' apples were 0.2, 0.3 and 0.2%, respectively.

Methods

Fruit from commercial orchards were used for all experiments. 'Honeycrisp' apples were used for softscald studies with 'Delicious' and 'Granny Smith' apples used for low O_2 studies for superficial scald. All fruit have been stored utilizing existing cold storage and controlled atmosphere facilities in our laboratory. All fruit quality analyses (color, firmness, texture, soluble solids content, titratable acidity, volatile production) will be conducted using established methods and existing equipment. Fruit firmness/texture assessment is conducted using a recording penetrometer. Ethylene, CO_2, and other volatiles are analyzed using gas chromatography. Monitoring of both volatile (ethylene, CO_2, aldehydes, alcohols, esters, other gases) and non-volatile compounds will be the means used to characterize physiological events during the onset of softscald symptom development. Non-volatile compounds will be analyzed using GC-MS with a focus on respiratory pathway components in the outer cortex and peel. Genetic analysis of the same tissues will utilize subtractive hybridization to identify uniquely expressed genes prior to symptom development in fruit stored under conditions known to induce the disorder. Peel fluorescence of fruit stored in low (less than 1%) O_2 is monitored using existing fluorescence sensors.

Results and Discussion

Multiple experiments using 'Honeycrisp' apples were initiated.

Experiment 1. Fruit obtained at commercial maturity were stored continuously in air at 33°F, or at 50°F for 7 days followed by 33°F. Apples were rated for softscald development after 1, 2, 3, 4, and 8 weeks. Softscald symptoms were evident beginning at 2 weeks in fruit stored continuously at 33°F indicating changes in fruit physiology that lead to disorder development are rapidly induced by cold temperature. No fruit held at 50° prior to 33°F developed softscald. Peel and cortex tissues collected at each evaluation date are being analyzed to characterize metabolic changes associated with softscald development.

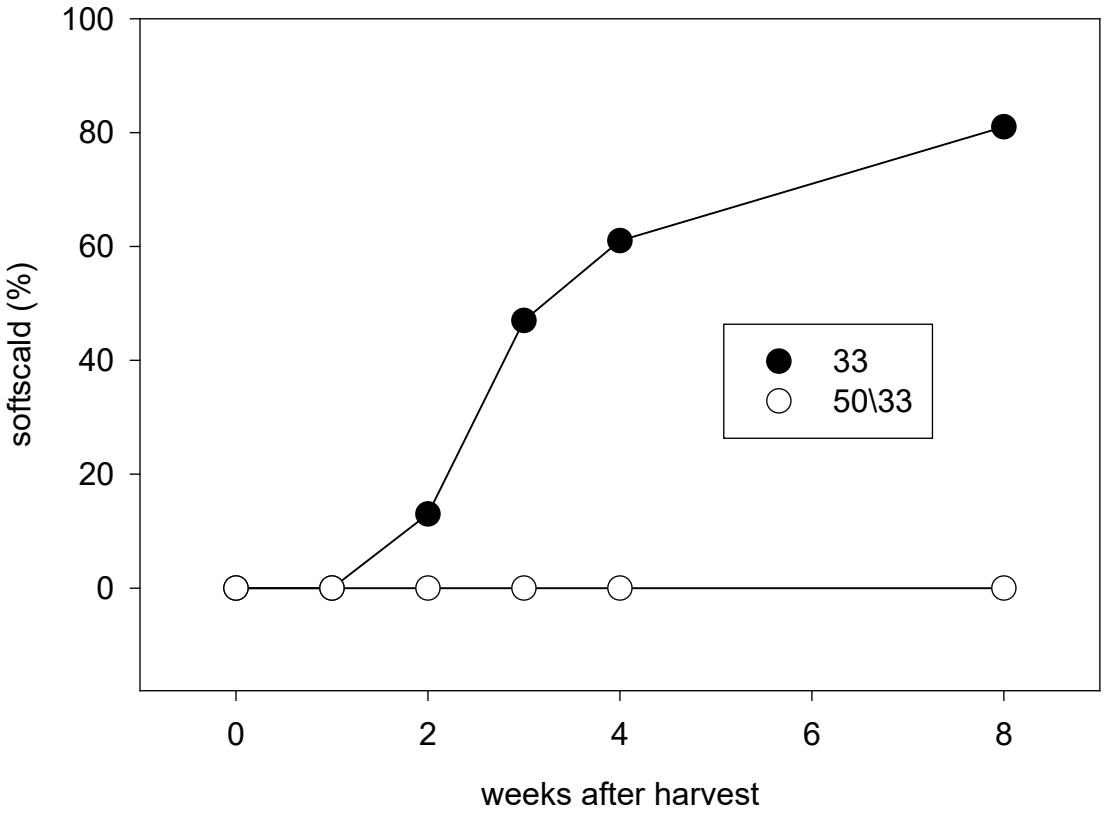

Figure 1. Incidence of 'Honeycrisp' softscald. Fruit were stored in air at 33°F continuously or at 50°F for 7 days then 33°F.

Experiment 2. Influence of CA and/or SmartFresh on 'Honeycrisp' quality. Fruit were stored at 50°F for 7 days then were transferred to 33°F. SmartFresh was applied the day of harvest while fruit were held at 50°F. Two days after transfer to 33°F, controls and SmartFresh treated fruit were placed into CA chambers and an atmosphere containing 1.5% O_2 and 1% CO_2 was established. Fruit from each treatment/storage combination (control/air; SmartFresh/air; control/CA; SmartFresh/CA) is evaluated monthly for appearance of softscald and other disorders (decayed fruit are removed). At 5 and 7 months fruit will be removed from storage and held for 7 days prior to assessment of quality (firmness, soluble solids, titratable acidity, color, disorders).

Experiment 3. This study is evaluating the final storage temperature in air and CA for 'Honeycrisp'. All fruit were held at 50°F for 7 days followed by transfer to 33, 35, or 37°F. After two additional days, CA (1.5% O_2, 1.0% CO_2) was imposed on some fruit at each of the 3 temperatures. As in experiment 2, fruit is evaluated monthly for external disorders, and quality assessed after 3,5 and 7 months plus 7 days at 70°F.

Experiment 4. Oxygen concentration during storage is the focus of this experiment. Fruit are being stored in air or in 1% CO_2 with 0.5, 1.5, or 2.5% O_2 at 33°F. All fruit was held at 50°F for 7 days prior to transfer to 33°F. Quality evaluations will be conducted after 3 and 6 months.

Experiment 5. An evaluation of an expanded low O_2 range is also being conducted. Fruit were held at 50°F for 7 days then were transferred to 33°F. After two additional days, a CA chamber was sealed and the low O_2 concentration at which a change in fluorescence was identified to be 0.2%. CA O_2 concentrations of 0.1, 0.2, 0.5 and 1.5% were established (each with 0.5% CO_2). Evaluations of quality and disorders will be made after 4 and 6 months.

Experiment 6. A trial similar to 'Honeycrisp' Exp. 5 is in progress using 'Delicious' and 'Granny Smith' apples. Fruit harvested at commercial maturity were cooled to 33°F and held for 2 days. CA conditions were established (CO_2: 2% 'Delicious', 1% 'Granny Smith') and a change in fluorescence was observed at 0.2% ('Delicious') and 0.3% ('Granny Smith') O_2. The plots for 'Delicious', 'Granny Smith', and 'Honeycrisp' fruit used in these studies varied in the amount of deviation around the running average, 'Delicious' and 'Honeycrisp' with some deviation compared to that for 'Granny Smith' (Figures 2-4). A reduction in fluorescence was observed as 'Honeycrisp' apples were held at the low O_2 concentration where the initial change in F-alpha occurred. This reduction was not observed for 'Granny Smith', indicating a potential impact of anthocyanin or other components in red versus green peel tissues. Final O_2 setpoints for 'Delicious' were 0.2, 0.4, 0.7, and 1.2%; and for 'Granny Smith 0.5 and 1.1%. 'Delicious' fruit will be evaluated after 4 and 8 months plus 7 days at 70F, 'Granny Smith' after 3,5,7, and 9 months plus 1,2, or 3 weeks in RA prior to 7 days at 70F.

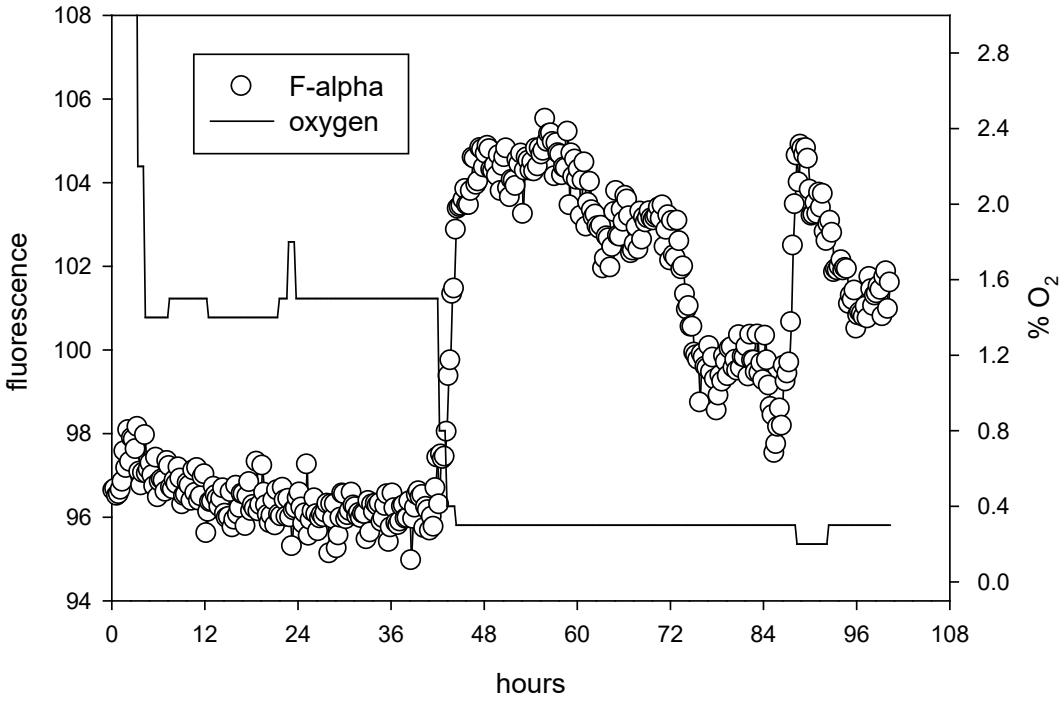

Figure 2. 'Honeycrisp' apple fluorescence during CA storage at 33°F. Fruit were stored 7 days at 50°F, then 2 days at 33°F prior to establishment of low oxygen. CO_2 was maintained at 0.5%.

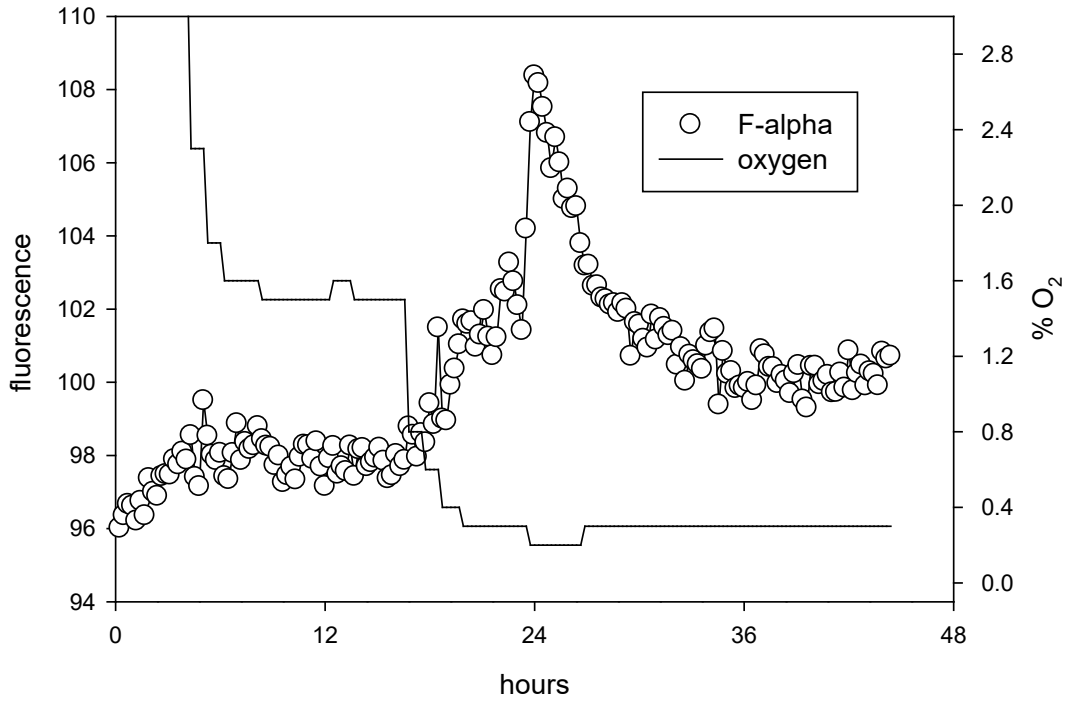

Figure 3. 'Delicious' apple fluorescence during CA storage at 33ºF. Fruit were stored 2 days prior to establishment of low oxygen, and CO_2 was maintained at 2%.

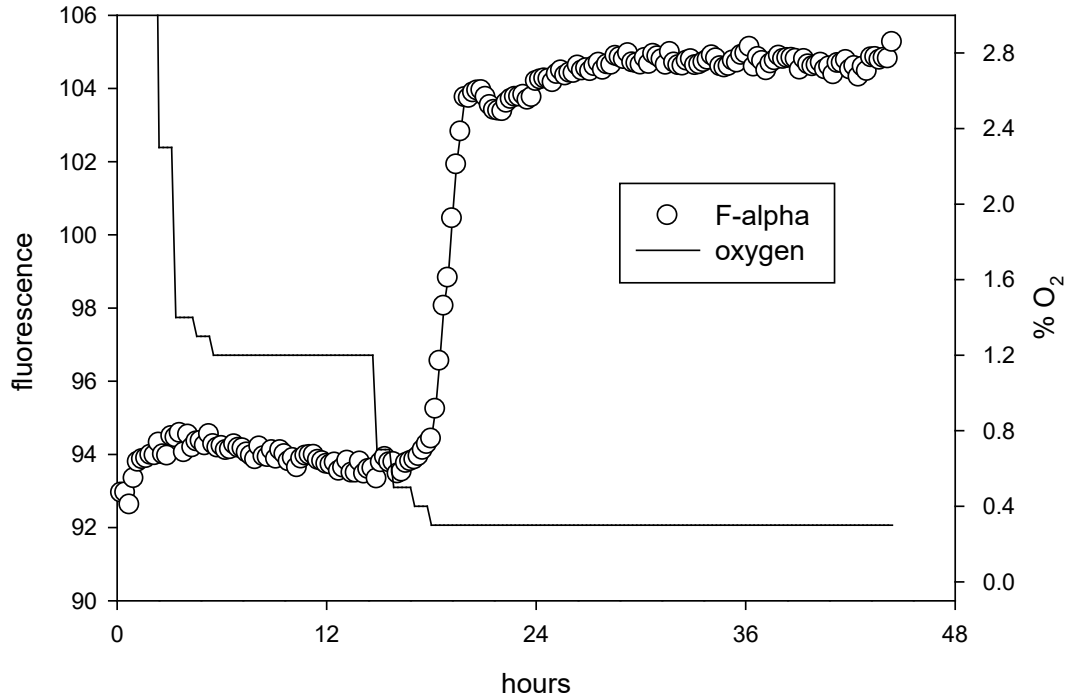

Figure 4. 'Granny Smith' apple fluorescence during CA storage at 33ºF. Fruit were stored 2 days prior to establishment of low oxygen, and CO_2 was maintained at 1%.

CONTINUING PROJECT REPORT **YEAR**: 2008/2009

Project Title: Programs to increase packouts of apples

PI: Ines Hanrahan
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State/Province/Zip WA, 98901

Cooperators: Jim McFerson, Tom Auvil, Felipe Castillo, Tory Schmidt, WTFRC, Wenatchee, WA

Budget 1:
Organization Name: WTFRC **Contract Administrator:** Kathy Schmidt
Telephone: 1 509 665 8271 **Email address:** Kathy@treefruitresearch.com

Item	Year 1: 2008	Year 2: 2009
Salaries	16,847	19,860
Benefits (32%)	7,929	9,346
Wages	30,464	36,557
Benefits (32%)	14,336	17,203
Equipment + supplies	5,000	6,000
RCA rental	9,600	33,800
USDA rental	750	750
Travel	2,000	2,000
Reimbursements	9,400	14,000
Total	**77,526**	**111,516***

*potential budget reduction of $12,800 if sunburn and LB work is reduced

Salaries: include proportional time spent on projects for Hanrahan, Castillo, Schmidt, Auvil
Wages: covers timeslip expenses, increase in 2009 based on projected project expansion for Honeycrisp; if sunburn work gets suspended in 2009, budget will be reduced by $6,400; based on 2008/09 results LB trials will be cut by 50% or $6,400
RCA rental: numbers based on fiscal year (80% of 2 rooms 2008, 80% of 6 rooms 2009)
USDA rental: access to packingline and storage space for equipment
Travel: fuel costs to travel to and from trial sites
Reimbursements: monetary contributions by chemical suppliers
Other: all chemicals were donated by industry suppliers

Acknowledgement: We would like to thank Pace Intl., BASF, Fine Americas, Valent, Monterrey Ag, Wilbur Ellis, Brandt Consolidated, Globachem, NW Wholesale, GS Long, Wilson Irrigation and D & M Chemicals for graciously donating chemicals.

Special thanks to our cooperating growers: Stan Olson, Clyde Buchler, Rick Kamphaus, McDougall & Sons, Jason Matson, Ed Tradeup, Bill Clark, Broetje, Del Feigal, Dave Silvernail, Jim Thornsberry, Mike van Pelt, Mike Young and Mike Copley.

OBJECTIVES:

1. Investigate chemical programs to improve fruit finish of 'Fuji' and 'Golden Delicious' apples.

2. Compare sunburn protectant efficacy in apple and evaluate ease of cleanup in the warehouse.

3. Facilitate field testing of promising approaches to mitigate lenticel breakdown in apples.

4. Can Honeycrisp apple storage performance be improved when utilizing DCA (dynamic controlled atmosphere) storage?

SIGNIFICANT FINDINGS:

Russet: No treatment significantly reduced Fuji flecking or Golden Delicious russet in 2008.

Sunburn: All materials tested increased the percentage of sunburn-free fruit. Most materials cleaned easily off fruit flanks. Residues of particle films remained visible in the stem bowl after drying.

LB: No consistent treatment effect was noted after preharvest application of hydrophobic spray emulsions (summer supreme oil, soybean oil, SylTac) in the 2007/08 season. 2008/09 trials are in storage.

Honeycrisp: Trials in storage.

METHODS

Russet suppression: In 2008, we conducted 8 fruit finish trials (3 x Golden Delicious, 5 x Fuji). *(A)* Trials (1 x Golden, 2 x Fuji) evaluating standard GA programs vs. alternatives were sprayed with a PropTec sprayer at 200 gal/acre using a randomized complete block design with 4 replications and 6-7 trees/treatment/rep. We tested the following materials: ProVide (GA_{4+7}) at 1.4 oz/acre, Raynox Plus at 1.25 gal/acre, Platina at 0.11 gal/acre, BlueStim at 4 lbs/acre and 8 oz/acre surfactant, SylTac at 16 oz/acre, Sylgard 309 at 4 oz/acre, and EpiShield as 2.5% solution (Table 1). Materials were applied at five weekly timings starting at petal fall, reflecting standard industry practice.

Table 1. Commercial products utilized in WTFRC fruit finish trials in 2008.

Active Ingredient	Commercial product(s)
Gibberellic acid mixture	ProVide
Plant wax	Raynox Plus
Lipid emulsion	EpiShield
Silicone surfactant	SylTac, SylGard 309
Glycine Betaine (osmoregulator)	Bluestim
L-Tryptophan (auxin synthesis)	Platina

(B) Trials (1 x Golden, 2 x Fuji) were conducted utilizing cooperators spray equipment and covering several acres depending on row length. General trial layout was a randomized complete block design with 4 replications. BlueStim (4 lbs/acre and 8 oz/acre surfactant) and Platina (0.11 gal/acre) were applied at five weekly timings starting at petal fall.

(C) Trials (one each: Golden and Fuji) evaluating Platina timings were sprayed with a hand-held sprayer to run-off. A completely randomized design was applied using 6 single tree reps/trt. Platina was applied in 5 timing combinations (PF, PF + 14, PF + 14 + 28, 14 + 28, 5 x weekly, starting at PF) at 0.11 gal/acre.

Sunburn suppression: Three trials were established near Manson, WA, (Granny Smith/M.106, Golden Delicious/M.26 Manson, Braeburn/M.26) testing a variety of commercially available sunburn protectants (Cocoon, Eclipse, Fruit Shield, Invelop, Raynox Plus, Sun Guard, Surround WP). All materials were applied starting on July 3 four times according to each product's respective labeled rate. At harvest, individual fruit was graded for sunburn according to the Schrader/McFerson system (0 = clean, 6 = necrosis). The ease of cleanup was evaluated by running fruit over the USDA-ARS packing line in Wenatchee. No wax was applied. Fruit was allowed to dry for 24 hours before evaluation.

Lenticel breakdown: In 2007 we conducted 2 trials sprayed with a PropTec at 100 gal/acre in Desert Aire & Royal City testing the following materials: Summer Supreme Oil (2%), soybean oil (2%), and SylTac (2 pt/acre). Timings were: 4, 2, 1 weeks before anticipated harvest alone or in combination. Samples were stored under CA conditions and evaluated for LB incidence after 3 and 6 months of storage. Secondly, we applied BlueStim (at 4 lbs/acre and 8 oz/acre surfactant) to Galas and Fujis (both in Orondo) using a handgun sprayer and utilizing a completely randomized design with 6 single tree replications/treatment. Treatments were: mid-season, one week preharvest, postharvest dip. In addition, Gala apple samples were taken from several Extenday trials and a rootstock trial. Lenticel breakdown was induced by running fruit over the ARS packingline.

In 2008 we conducted 2 trials sprayed with a PropTec (100gal/acre unless otherwise specified) using a randomized complete block design with 4 replications and 20 trees/treatment/rep. We tested the following materials: EpiShield as 2.5% or 1.5% solution with one or two weekly applications, Safe-T-Side at 32 oz/acre, BlueStim at 4 lbs/acre and 8 oz/acre surfactant and Platina at 0.11 gal/acre. Materials were applied at 2 weekly timings starting at 2 weeks before anticipated harvest. We also conducted 4 grower-applied trials (3 Gala, 1 Fuji) utilizing whole rows. Trial layout was a randomized complete block design with 4 replications. EpiShield was applied once or twice as 2.5% solution, starting 2 or one week prior to anticipated harvest. All samples are currently stored under CA conditions and will be evaluated for LB incidence after 6 months.

Honeycrisp storage: We selected 3 orchards (Prescott, Brewster, Manson) based on the following criteria: even crop load with minimal alternate bearing, trees being at least 4 years old. Harvest timings were a) one week prior to anticipated first pick, and b) first pick (or best-storing pick). Fruit was transported to Stemilt RCA facility and held for 1-3 weeks at 50F before being stored at 38F in RA, CA (0.5% CO_2, 1.5% O_2) or DCA (0.5% CO_2, 0.7% O_2) until the end of February 2009. Monthly pulls will evaluate storage performance.

RESULTS AND DISCUSSION

Russet suppression: Fruit russet is typically induced early in the growing season and is likely aggravated by a combination of weather conditions, spray chemicals, and/or topical biotic pests. Few practical options are available to orchardists to suppress russet. Standard gibberellic acid programs include up to five weekly applications starting around petal fall and amount to considerable spray material costs ($100-300/acre). After encouraging results in 2007, we continued testing commercial spray materials with novel chemistries (Table 2).

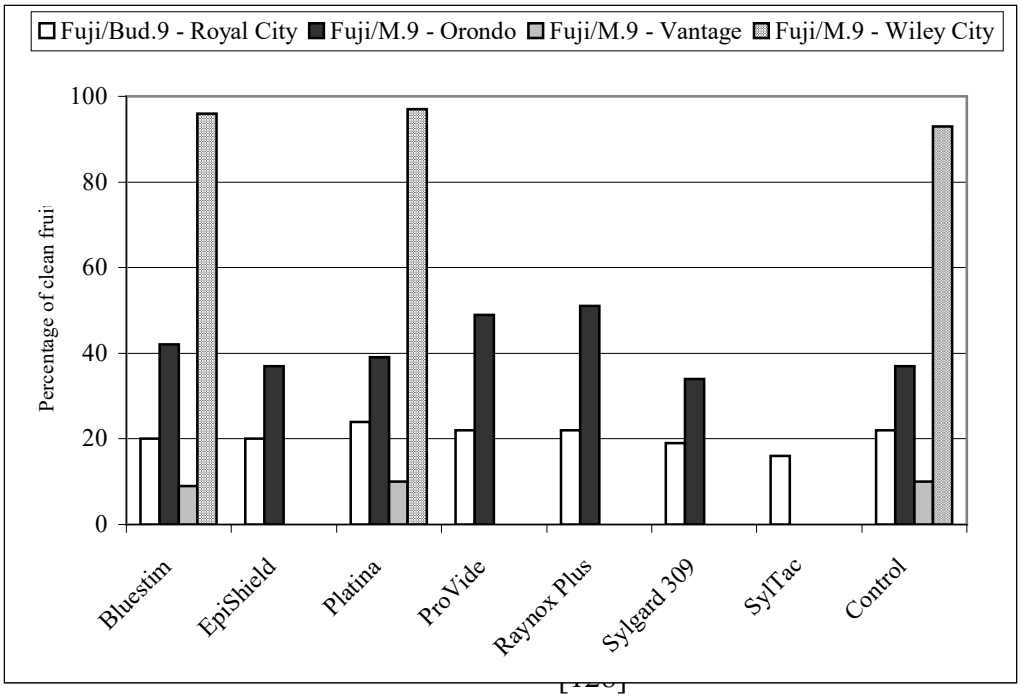
<u>*Golden Delicious:*</u> No product evaluated in 2008 increased the percentage of premium grade fruit or influenced the degree of russet development (Table 2). Just like 2007, low russet pressure as

indicated by 83-94% WAEXF in highly russet prone orchards, has significantly impacted the outcome of our trials.

Table 2. Field russet incidence and commercial grades of Golden Delicious after application of standard GA and alternative spray programs for russet suppression in 2008.

	FIELD RUSSET INCIDENCE				CHANGE IN GRADE			
TREATMENT	CLEAN (%)	BOWL (%)	SHOULDER (%)	NET 10% (%)	WAEXF (%)	WAF (%)	US#1 (%)	CULLS (%)
Golden Delicious / M.111 - Wapato (handgun)								
Platina PF	33 ns	55 ns	8 ns	4 ns	88 ns	12 ns	0 ns	0 ns
Platina PF + 14	31	63	4	3	93	7	0	0
Platina 14 + 28	30	60	5	4	91	9	0	0
Platina PF + 14 -	28	62	7	3	89	11	0	0
Platina 5 times	35	56	5	4	92	8	0	0
Control	27	66	2	4	94	6	0	0
Golden Delicious / M.111 - Wapato (PropTec)								
Bluestim	42 ns	41 ns	12 ns	5 ns	83 ns	17 ns	2 ns	0 ns
EpiShield	48	37	14	2	84	16	1	0
Platina	41	45	11	4	86	15	1	0
ProVide	42	45	11	3	87	13	1	0
Raynox Plus	46	38	14	2	85	16	1	0
Sylgard 309	48	39	11	2	87	13	0	0
SylTac	42	41	15	4	84	19	1	0
Control	45	38	14	3	83	17	0	0
Golden Delicious / Selah (grower applied)								
Bluestim	62 ns	34 ns	4 ns	1 ns	95 ns	5 ns	0 ns	0 ns
Platina	60	36	4	1	96	4	0	0
ProVide	59	35	6	0	94	6	0	0

Figure 1. Influence of early season spray programs on prevention of Fuji flecking in 2008.



Fuji: Fuji flecking was not influenced by any spray program tested in 2008 regardless of orchard susceptibility (Figure 1).
Conclusion: Standard GA programs are still the most reliable strategy for improving apple fruit finish. GA alternatives (BlueStim, Raynox, Platina, SylTac) have shown some effectiveness for improving Fuji fruit finish in previous years, but year-to-year consistency is lacking.
2009 trials: We are planning on a third year of trials aiming to verify GA alternative product efficacy. In addition, we are moving towards more basic work such as determining the actual position of fruit affected by russet/flecking within the tree canopy and the determination of the onset of Fuji flecking.

Sunburn suppression: Sunburn is the primary physiological cause of cullage, sometimes damaging up to 50% of the fruit in a given orchard. Previously, WTFRC trials have shown calcium-based products (Eclipse, FruitShield) to perform as well as industry standards (Raynox, Surround WP). We revisited the question of sunburn protection product efficacy in 2007 and repeated the trials in 2008 (Table 3).

Table 3. Sunburn protectants used in 2008 WTFRC comparative trials.

Type	Product(s)
Plant wax	Raynox Plus
Kaolin clay	Surround WP, Cocoon
Talc	Invelop
Calcium carbonate	SunGuard, Eclipse, FruitShield

All materials tested increased the percentage of sunburn-free fruit (Table 4). A common concern with sunburn protectants is the ease of cleanup in the warehouse. Ideally, fruit emerges free of residue after a standard washing and rinsing. We simulated this process by running fruit over the USDA-ARS packingline in Wenatchee. Visible residues were observed before placing fruit on the line and after 24 hours of drying time. All materials cleaned easily off fruit flanks. Residues remained in the stem bowls at significantly higher levels for kaolin clay and calcium carbonate-based products (Table 5). Our results on product efficacy and ease of clean-up have been very consistent over the past few years and indicate no further study.

Table 4. Sunburn severity readings at harvest in Braeburn and Granny Smith apples. WTFRC 2008.

	FIELD SUNBURN INCIDENCE[a]					
TREATMENT	Clean (%)	Y1 (%)	Y2 (%)	Y3 (%)	Tan (%)	Black (%)
Braeburn / M.26 - Manson						
Cocoon	54 ns	20 ns	11 ab	6 ns	4 ns	5 ns
Eclipse	57	20	14 ab	3	4	3
Fruit Shield	55	23	9 b	6	4	3
Invelop	50	20	12 ab	10	6	2
Raynox Plus	52	23	11 ab	8	6	1
Sun Guard	54	22	13 ab	5	5	2
Surround WP	59	22	11 ab	6	3	1
Control	48	25	15 a	6	3	4
Granny Smith / MM.106 - Manson						
Cocoon	51 ab	17 b	14 ab	10 ns	6 ns	4 ns
Eclipse	50 ab	23 ab	13 ab	8	3	3
Fruit Shield	55 a	21 ab	10 ab	7	5	3
Invelop	51 ab	22 ab	12 ab	10	4	1
Raynox Plus	50 ab	21 ab	15 ab	9	2	3
Sun Guard	49 ab	22 ab	16 ab	8	4	2
Surround WP	57 a	20 ab	9 b	6	5	4
Control	40 b	24 a	17 a	10	4	5

[a] based on 'Schrader-McFerson' scale

Table 5. Ease of sunburn protectant product clean-up when submitted to commercial packing line.

	SPRAY RESIDUE PRE WASH				SPRAY RESIDUE POST WASH			
TREATMENT	Clean (%)	Side (%)	Bowl (%)	Caylx (%)	Clean (%)	Side (%)	Bowl (%)	Caylx (%)
Braeburn / M.26 - Manson								
Cocoon	1 b	86 a	79 a	81 a	70 ab	0 ns	30 abc	0 ns
Eclipse	0 b	86 a	77 a	68 bcd	70 ab	0	28 bc	2
Fruit Shield	0 b	78 a	76 a	79 ab	71 ab	0	27 bc	2
Invelop	0 b	56 b	56 bc	64 cd	73 ab	1	25 bc	2
Raynox Plus	1 b	63 b	58 bc	60 cd	75 a	0	23 c	2
Sun Guard	2 b	84 a	70 ab	73 abc	64 bc	0	35 ab	1
Surround WP	0 b	82 a	82 a	83 a	56 c	0	41 a	3
Control	5 a	61 b	52 c	58 d	77 a	0	22 c	1
Golden Delicious / M.26 - Manson								
Cocoon	3 c	69 ab	50 b	49 bc	75 abc	3 ns	20 cd	2 b
Eclipse	8 bc	56 b	53 b	60 ab	66 bcd	2	30 bc	3 b
Fruit Shield	12 bc	58 b	34 b	68 a	78 abc	1	18 cd	5 b
Invelop	19 bc	29 c	42 b	56 ab	68 abcd	0	18 d	16 a
Raynox Plus	27 ab	25 c	30 b	34 cd	80 ab	1	15 d	4 b
Sun Guard	5 c	66 ab	55 b	70 a	64 cd	0	33 b	3 b
Surround WP	2 c	81 a	81 a	70 a	51 d	0	45 a	7 ab
Control	38 a	20 c	35 b	21 d	83 a	0	17 d	0 b
Granny Smith / MM.106 - Manson								
Cocoon	0 b	87 ns	75 a	83 ns	65 a	1 ns	28 b	7 ns
Eclipse	0 b	82	53 b	81	59 ab	1	30 b	10
Fruit Shield	0 b	93	75 a	81	58 ab	0	37 ab	6
Invelop	0 b	86	81 a	77	63 a	1	28 b	9
Raynox Plus	0 b	84	75 a	74	65 a	0	28 b	8
Sun Guard	0 b	87	87 a	82	63 a	1	33 b	3
Surround WP	0 b	92	84 a	83	46 b	0	46 a	8
Control	2 a	84	82 a	78	69 a	0	29 b	3

Lenticel breakdown: The complete data set for the 2007 field trials will be discussed, since it was not yet available at last year's research review. The 2008 data set will be available in March 2009. In 2007 we set up 2 trials to determine if the application of hydrophobic materials within 3 weeks of harvest would alleviate LB development after storage.

All fruit was harvested at commercial maturity suitable for long term CA storage. We found no differences for common maturity parameters at harvest between control and treated fruit (data not shown). Fruit from both orchards expressed symptoms after 3 and 6 months of CA storage at about the same level (Table 6). No significant treatment effect was seen regarding oil type or spray frequency (Table 6). Preliminary results comparing fruit from Extenday plots with untreated fruit indicated no significant effect on LB development of Gala apples after storage (data not shown).

Utilizing the existing rootstock evaluation trial planted in 2004 in Wapato, we assessed fruit susceptibility to LB in relation to the rootstock used (Figure 2). Compared to our orchard trials, we observed higher LB symptom expression after 6 months of CA storage, possibly due to advanced maturity at harvest. When comparing new rootstocks against M.26 in terms of susceptibility to LB

development after storage, no significant treatment effect could be established. This finding has been consistent over 2 seasons. Perceived differences within the industry regarding LB susceptibility among rootstocks may be due to secondary effects caused by crop load and overall tree structure. *2009 plan*: Provided the 2008 data confirms the ineffectiveness of hydrophobic materials, we will discontinue trial work. We have started using EpiShield, a commercial product known to reduce LB expression after storage when applied preharvest. We are planning on continuing our work on the Wapato rootstock trial and will feed data generated into the Hoheisel/Olmstead database of fruit quality and production parameters.

Table 6. Effects of preharvest application of hydrophobic materials on LB development of Gala apples after 3 and 6 months of CA storage during the 2007-08 storage season.

	LENTICEL READINGS				LENTICEL READINGS			
TREATMENT	CLEAN (%)	SLIGHT (%)	SEVERE (%)	TOTAL LB (%)	CLEAN (%)	SLIGHT (%)	SEVERE (%)	TOTAL LB (%)
	3 months CA				6 months CA			
Galaxy Gala / M.9 Royal Slope								
Soybean once	82 abc	18 bc	0 ns	18 bc	82 ab	18 c	0 b	18 b
Soybean twice	92 a	8 c	0	8 c	90 a	9 c	1 b	10 b
Soybean thrice	34 d	37 ab	29	66 a	31 c	51 a	18 a	69 a
Summer oil once	88 ab	13 bc	0	13 bc	86 a	13 c	1 b	14 b
Summer oil twice	60 bcd	40 ab	0	40 abc	59 abc	37 abc	4 ab	41 ab
Summer oil thrice	80 abc	20 bc	0	20 bc	80 ab	19 c	1 b	20 b
SylTac once	94 a	6 c	0	6 c	88 a	13 c	0 b	13 b
SylTac twice	78 abc	21 bc	1	23 bc	76 ab	23 bc	1 b	24 b
SylTac thrice	47 cd	53 a	0	53 ab	48 bc	48 ab	1 b	49 ab
Control	83 ab	16 bc	1	18 bc	81 a	15 c	4 ab	19 b
Imperial Gala / M.26 Desert Aire								
Soybean once	78 ns	22 ns	0 ns	22 ns	71 ns	27 ns	2 ns	29 ns
Soybean twice	85	13	3	15	82	13	5	18
Soybean thrice	78	21	1	23	76	21	3	24
Summer oil once	72	24	4	28	68	20	11	32
Summer oil twice	85	15	0	15	85	14	1	15
Summer oil thrice	65	29	6	35	65	25	10	35
SylTac once	75	25	0	25	74	25	1	26
SylTac twice	59	41	0	41	58	40	3	43
SylTac thrice	62	34	4	38	61	35	4	39
Control	81	16	3	19	80	15	5	20

Figure 2. Rootstock effects on lenticel breakdown (LB) in Gala after 6 months of CA storage. 2006-2007.