FINAL PROJECT REPORT

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

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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.

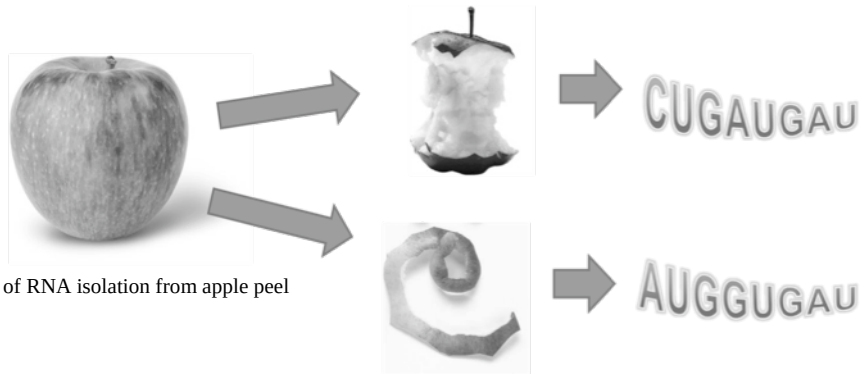

Figure 1. Schematic of RNA isolation from apple peel and core samples.

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.

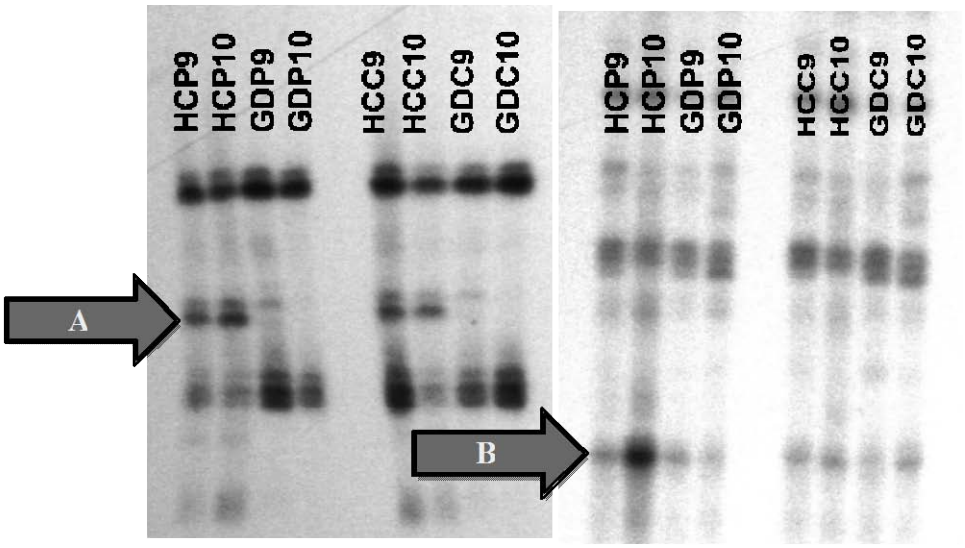

Figure 2: Differential display gel of Honeycrisp and Golden Delicious peel and core RNA samples. HC-Honeycrisp; GD-Golden Delicious; P-Peel; C-Core; 9-September; 10-October. "A" displays a gene expressed in Honeycrisp peel and core but not in Golden Delicious peel and core. "B" displays a gene upregulated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

- 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.
- 34 potentially interesting genes have been directly taken out of the gel and amplified from the experiment.
- 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.