FINAL PROJECT REPORT

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

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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

<u>**Pending**</u>
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.

<u>**To be submitted (early 2009)**</u>
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)	MBM – Metallic Bead Method
MAB – marker-assisted breeding	PBA – Pedigree Based Analysis (formerly
MAPS – marker-assisted parent selection	known as PG – Pedigree Genotyping)
(using markers to "map" out the populations	PRI – Plant Research International (Netherlands)
to be created)	SBM – Silica Bead Method
MASS – marker-assisted seedling selection	TAS – Theonyx Automated System
(using markers for mass genotyping of	WABP – Washington apple breeding program
many progeny seedlings)	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.
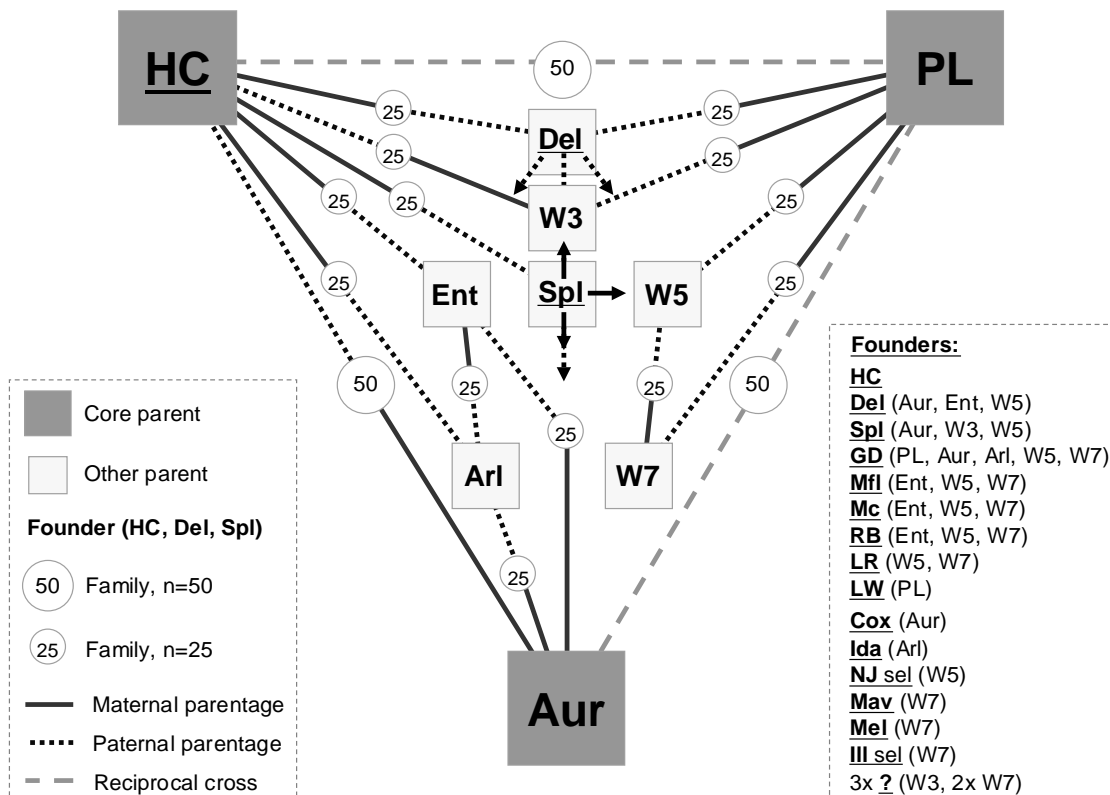

Figure 1: Schematic of the composition of the WABP Pedigree Set. Eighteen founders are represented (15 known and three unknown). Pedigree Set parents derived from each founder are shown in the bottom right section. Three founders (Honeycrisp, Delicious, and Splendour) are immediate parents of the Pedigree 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.