Development of Genomic Tools for Almond Rootstock Improvement

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INTRODUCTION

With the phasing out of soil fumigation, reliance on rootstocks with field resistance to soil borne pests and diseases is increasing. As a consequence, the California Almond Industry has identified rootstock development, testing, and commercialization as a priority to deal with serious soil borne pests and pathogens, including the complex replant syndrome. Widely used rootstocks (e.g. 'Nemaguard', peach, and peach x almond hybrids) tolerate or resist the attack of root knot nematodes, but they are susceptible to other soil borne pests and diseases, such as lesion and ring nematodes, bacterial canker, crown gall, *Phytophthora* and *Armillaria*. Countering these soil borne disease-pest pressures on almond rootstocks requires identifying novel sources of resistance for incorporation into current and future rootstocks through conventional breeding programs.

Identifying novel sources of disease resistance necessitates understanding its genetic basis, which is established by linking disease evaluation data (phenotype) with molecular characterization (genotype). Phenotype-genotype linkage is the key to developing an effective marker assisted selection (MAS) strategy for rapid development of rootstocks with disease resistance. Developing effective MAS strategies requires: (1) marker systems for fine scale genotyping of large populations; (2) molecular marker(s) that co-segregate with the disease resistance trait; and (3) reliable disease screening techniques to assay current commercial rootstocks, large breeding populations, interspecific hybrids, and germplasm.

Molecular markers permit rapid and accurate characterization, identification, and selection of desired genes and gene combinations from among large pools of germplasm. They also make it possible to screen large breeding populations and diverse germplasm collections without challenging them with pathogens; the importance of which cannot be overstated in tree breeding where one cycle of trait-based selection can take many years and is exceedingly costly.

OBJECTIVES

1. Develop molecular markers linked to soil borne disease-pest resistance
   - Validate single nucleotide polymorphisms (SNPs) of peach using existing DNA sequence databases.
   - SNP discovery in root-specific genes expressed in commercial rootstock materials and genomic sequences from a diverse set of peach, almond and wild species.
2. Molecular characterization of a genetically diverse collection of commercial rootstocks and mapping populations of newly derived interspecific hybrids.

Table 1. Interspecific *Prunus* hybrids produced in 2012. Of 373 embryos cultured 55 individuals were recently tested, and 42 (76.4%) confirmed as true hybrids. The remaining 318 will be tested in December 2012. (peach = *P. persica*)

Cross composition	No. Fruits produced	No. Embryos cultured
peach x Marianna 26-24	4	4
peach x *P. argentea* (wild almond)	47	45
peach x *P. tangutica* (wild almond)	251	234
peach x *P. dulcis* (almond)	39	24
peach x *P. davidiana* (wild peach)	12	10
peach x *P. arabica* (wild almond)	1	1
peach x *P. fenzliana* (wild almond)	6	4
peach x *P. bucharica* (wild almond)	9	8
peach x [(*P. dulcis* x *P. scoparia*) x OP] (almond hybrid)	2	2
peach x *P. kansuensis* (wild peach)	12	6
peach x *P. kuramica* (wild almond)	7	5
peach x plumcot	10	7
peach x *P. tomentosa*	32	14
peach x *P. salicina* (Japanese plum)	14	9
Totals	446	373

MATERIALS AND METHODS

We are using the following **Three Steps** to develop commercially viable almond rootstocks with resistance to one or more of the key soil borne diseases/syndromes.

Step 1. Identify novel sources of resistance and evaluate existing rootstocks against soil borne diseases (ongoing)

The importance of high quality, reproducible, disease resistance evaluation data for each plant genotype cannot be understated. In a separately funded project, we are screening commercially available rootstocks for disease resistance including replant disorder. To enhance our success of identifying genetic loci mediating disease resistance in currently available rootstocks we have also generated new *Prunus* hybrids with broad genetic backgrounds in novel combinations (Table 1), which we hypothesize contain resistance to multiple diseases. These newly developed interspecific hybrids are undergoing clonal propagation and will be subjected to disease evaluation following standard replicated statistical designs. Approximately 15 wild *Prunus* species from the *Prunus* collections at the USDA-ARS National Clonal Germplasm Repository (NCGR) were used as potential donors of resistance for the production of novel hybrids at the Wolfskill Experimental Orchards (Winters, CA).

Step 2. Genetically map genes mediating disease resistance (ongoing)

While disease resistance evaluations are being conducted as described above we are laying the molecular genetics ground work for *Prunus*, which will allow us to identify candidate disease resistance genes by mapping, further allowing us to rapidly select disease resistant rootstock genotypes.

In addition to identifying SNPs through alignment of genomic and root-specific expressed sequence tags from a diverse panel of wild *Prunus* used in hybrid development and commercial rootstocks, we will use genotyping-by-sequencing (GBS) method (Fig. 1) to identify and validate tens of thousands of SNPs while simultaneously genotyping thousands of SNPs around restriction sites located throughout the genome (Elshire *et al*., 2011). GBS data is highly suited for genome-wide association studies.

Step 3. Identify SNPs associated with resistance (planned for second half of 2013).

Using molecular data developed in Step 2 above, we will identify SNP markers associated with disease resistance/susceptibility. Once confirmed, these SNPS will be used to develop juvenile selection strategies for rootstock improvement. We anticipate performing Step 3 activities towards the end of this funding cycle.

Fig. 2. Embryo-cultured *Prunus* interspecific hybrids at the USDA-ARS NCGR greenhouse ready for disease evaluation

GENOTYPING BY SEQUENCING

Genotyping by sequencing (GBS) is a platform independent genotyping technique that utilizes high throughput parallel sequencing technology, also known as next generation sequencing, to produce thousands of nuclease restriction site-associated fingerprint profiles based on sequencing. Below is a diagram of library construction as described by Elshire et al. (2011).

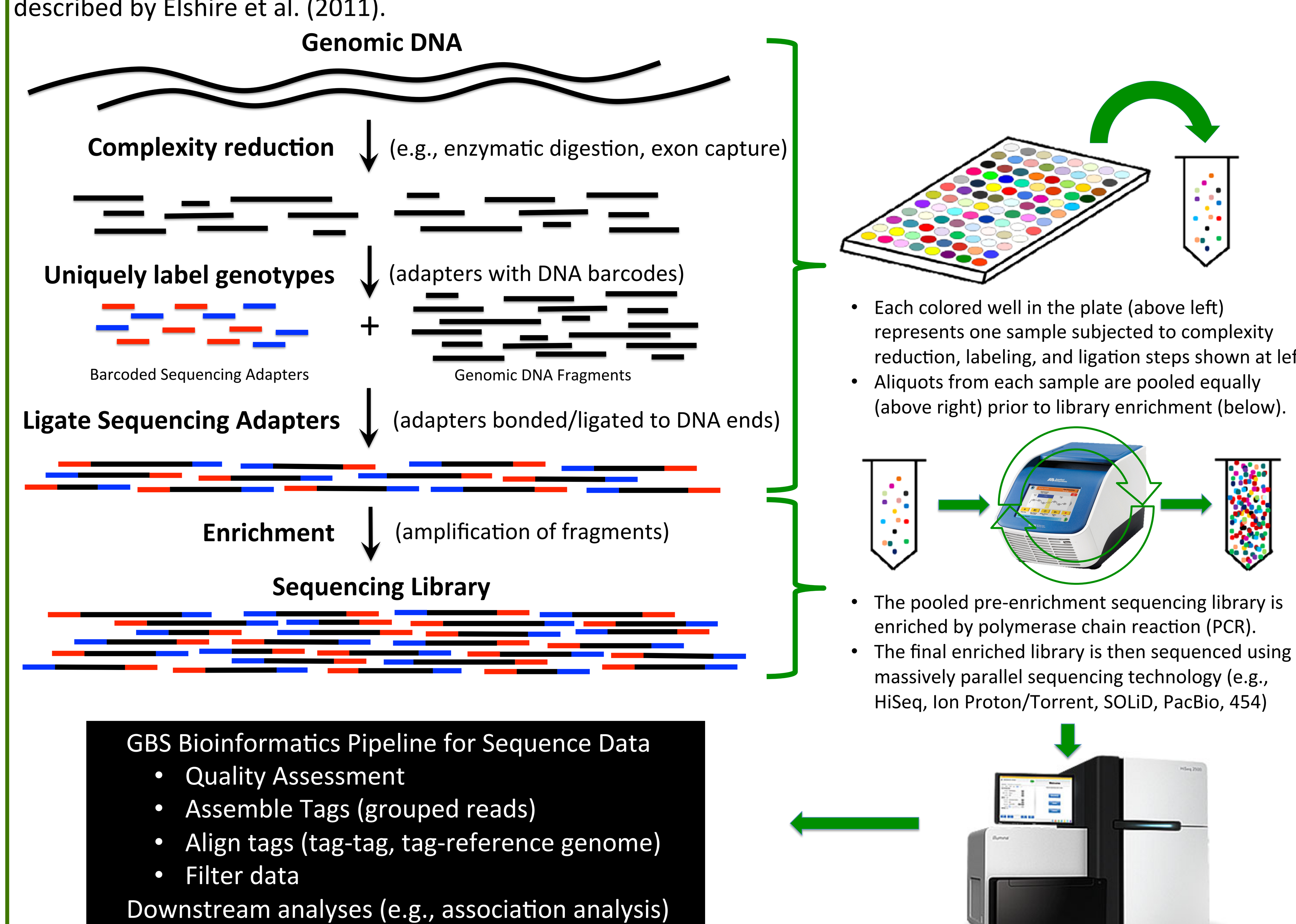


Fig. 1. Diagrammatic overview of the genotyping by sequencing (GBS) procedure.

RESULTS AND DISCUSSION

Disease evaluation and genotyping of existing and novel almond rootstocks (ongoing)

Four hundred and forty-six immature fruit were collected from 20 different interspecific crosses involving 14 different wild *Prunus* species (Table 1) that are potential donors of resistance to soil borne diseases. They are currently undergoing embryo culture and multiplication at the California Seed and Plant Laboratory (CSPL) in Elverta, California. Of the 446 immature fruit delivered, 373 potentially viable embryos were successfully placed into culture where they will be multiplied via micropropagation. Additionally, interspecific hybrids produced in 2010 and 2011 are still undergoing multiplication, and 468 clonal plants representing 23 genotypes from four confirmed crosses were received mid-summer of 2012 (Fig. 2).

Disease evaluation nurseries and replicated experiments have been planted in Davis and Parlier locations by USDA-ARS Crops Pathology group (D. Kluepfel and G. Browne) for crown gall, *Phytophthora*, and replant syndrome evaluations. Both fumigated and non-fumigated treatments have been imposed in the *Phytophthora* and replant disease evaluation experiments.

Molecular data development and association analyses (ongoing)

The DNA from all available rootstocks (e.g., commercial, experimental, and new hybrids produced in this project) has been extracted to be used for genotyping with GBS technology at the Institute for Genomic Diversity, Cornell University, in February-March, 2013. This will produce genotyping data for tens of thousands of loci covering the entire genome and combined with disease evaluation data will permit genome-wide association analysis to identify markers associated with resistance to the major soil borne diseases.

Recent bioinformatic analysis of sequence data from various *Prunus* databases revealed thousands of SNP primer sequences that uniquely aligned to the peach reference genome (Tables 2&3). The uniquely aligned sequences as shown in Table 3 would be useful for developing a genotyping platform in addition to GBS. However, development of a genotyping platform using *in silico* validated SNP markers is dependent on the availability of funding.

As per the time table the genotype data should be ready for analysis in March 2013. In the meantime we anticipate at least one year of disease evaluation data permitting us to proceed with association analysis to identify markers linked to disease-pest resistance/tolerance. We would be ready to present the final results in the annual Almond Board meeting in 2013.

Table 2. SNP primer sequences and EST sequences from databases

Resources	P. dulcis	P. persica	All Prunus
SNPs	109	6,657	8,009
ESTs	3,864	76,824	110,815
Sequence Read Archives	0	15	33

Table 3. Reference-guided alignment of sequences containing SNPs

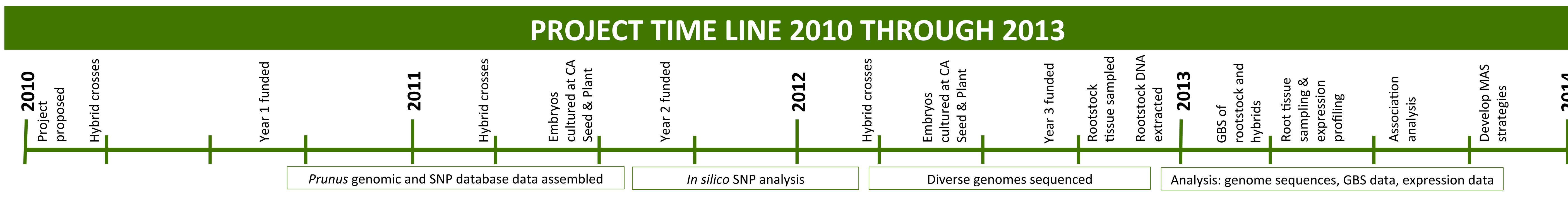
Alignment	Almond[1]	Peach[1]	Peach[2]	Peach/Almond[3]	Almond[1,2]	Almond[1]
Reads	100	6,657	40,794	17,291	6,658	3,864
Did not align	10	0	0	5	5	9
Uniquely aligned	74	4,709	18,083	2,330	1,137	664
Non-uniquely aligned	16	1,948	22,706	14,956	5,508	3,191

[1]NCBI; [2]GDR; [3]ESTree

PLANS FOR 2013

- Perform GBS analysis, assemble data for bioinformatic analyses, identify SNPs and tabulate genotype data.
- Perform genome-wide association analysis combining GBS genotype and disease evaluation data to identify markers linked to resistance/tolerance.
- Develop effective and high throughput marker-assisted selection schemes to increase selection efficiency and trait integration in the genetic improvement of almond rootstocks.
- Present final results at 2013 ABC annual meeting.

PROJECT TIME LINE 2010 THROUGH 2013



REFERENCES

Elshire *et al*. (2011). A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLOS One 6:e19379.

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