Development of Genomic Tools for Almond Rootstock Improvement

Project No: **10-HORT16-Aradhya/Ledbetter**

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Objectives:

1. Develop a set of molecular markers linked to disease-pest resistance so that they can be incorporated in rootstock breeding programs
    a. Assemble and validate *in silico* (from computer databases) discovered single nucleotide polymorphisms (SNPs) of peach.
    b. SNP discovery based on EST (Expressed Sequence Tags of cDNA) resources developed from a set of diverse peach, almond and wild species
2. Produce and test a number of diverse *Prunus* interspecific hybrids involving potentially useful species known to possess disease resistance attributes.
    a. Molecular characterization of peach and almond wild relatives.
    b. Production and testing of interspecific hybrids to identify novel sources of resistance to soil borne diseases.

Interpretive Summary:

Development of improved almond rootstocks possessing field resistance/tolerance to soil borne pests and pathogens is a top priority for the California Almond Industry. Although some of the widely used rootstocks such as 'Nemaguard', peach and peach x almond derived hybrids, and other complex species hybrids resist infestation of root knot nematodes they are susceptible to other soil borne pests and diseases such as *Phytophthora*, crown gall, *Armillaria* and lesion nematodes. Development and testing of diverse interspecific hybrids utilizing the wild *Prunus* spp. known to possess resistance to soil borne diseases is by far the best approach. However, for far too many species, the information on disease-pest reactions is simply missing rendering selection of species for hybridization difficult.

This project has two main goals: (1) production and testing of diverse *Prunus* interspecific hybrids involving potentially useful species known to possess disease resistance attributes. The wild *Prunus* germplasm was selected for hybrid production based on extensive literature survey and observations in their natural habitats made during germplasm collection trips by the project leaders. We strongly believe that evaluation of diverse hybrids would permit identification of rootstocks with durable resistance or tolerance to a wider array of soil borne diseases including the replant syndrome. This objective mainly focuses on deliverables to the industry in the form of identifying and establishing field testing trials of selected hybrids with improved levels of resistance to diseases; (2) in conjunction with the above mentioned goal, we are aiming to develop a set of molecular markers linked to disease-pest resistance so that they can be incorporated in rootstock breeding programs.

Materials and Methods:

Objective 1a.

Assembly of *in silico* SNPs.
SNPs from various databases such as ESTree (http://www.itb.cnr.it/estree/), Genome Database for Rosaceae (GDR; http://www.rosaceae.org/), and the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) have been downloaded into a local SNP database.

Validation of *in silico* SNPs
The *in silico* SNPs will be validated by verifying the SNP primer sequences in the genomic sequences of peach, almond, and related wild species. The genomic DNA of genotypes representing different *Prunus* species used for interspecific hybrid production (**Table 1**) and the parents of a mapping population from an interspecific cross [*P. persica* cultivar 'Andross' by *P. argentea* (DPRU 194)] from Dr. Tom Gardziel's UC Davis peach/almond breeding program have been collected from the UC Davis Plant Sciences Breeding Block, Davis and Wolfskill Experimental Orchard (Winters, Calif.). Isolation of nuclei is in progress for extracting pure genomic DNAs without any contamination from the cytoplasmic sources for library preparation and sequencing. The nuclei are isolated using a woody plant buffer (Loureiro et al., 2007) and density gradient centrifugation (Henfrey and Slater, 1988). DNA from isolated nuclei is then extracted using a modified CTAB buffer (Doyle and Doyle, 1990). Next generation sequencing

libraries will be prepared and barcoded for multiplex sequencing using an Illumina HiSeq sequencing platform at the UC Davis genome center. Bioinformatic analysis of sequence data for SNP discovery and verification of *in silico* SNP primer sequences will be performed as soon as the sequence data are available in late summer or early Fall, 2011.

Objective 1b.

This objective is currently under progress. Root samples from contrasting rootstock cultivars that show either susceptibility or tolerance/resistance to the major soil borne pests and diseases such as nematodes, *Phytophthora*, and crown gall will be collected within the next two months and mRNA extracted using RNeasy RNA extraction kit (Qiagen USA, Valencia, Calif.). The potential rootstock cultivars under consideration are listed in **Table 2** along with their reaction to soil borne pests and pathogens. The cDNA libraries will be constructed using SMARTer™ Ultra Low Input RNA for Illumina Sequencing Kit (Clonetech Laboratories, Mountain View, Calif.) and sequenced on an Illumina HiSeq sequencer at the UC Davis genome center.

Objective 2a.

All interpsecific hybrids produced in the years 2010 and 2011 will be genotyped using a standard set of 18 microsatellite markers identified in the repository laboratory to verify their hybridity. True hybrids will be clonally multiplied for both SNP genotyping as well as disease testing during 2012 Spring.

Objective 2b.

During 2010 and 2011 Spring seasons, several thousands of crosses were made in the repository *Prunus* germplasm blocks to produce a diverse set of hybrids for disease evaluation and genotyping. Fully matured seeds harvested from the 2010 crosses were surface sterilized and embryos were cultured in an embryo culture medium (Ledbetter et al., 1998). Once germination initiated embryos were transferred to shoot initiation medium (Rajsheker et al., 1995). When shoots were established plantlets were then transferred to the proliferation medium (Hammerschlag et al., 1987). Shoots were the subcultured on proliferation medium. Germinated embryos large enough for leaf collection were genotyped to confirm their hybridity. These confirmed plants will undergo further multiplication at California Seed and Plant Laboratory. The immature fruits from the 2011 crosses were harvested and delivered to California Seed & Plant Laboratory for culturing in June 2011. The immature embryos will be excised from the seeds and cultured in an embryo culture medium to grow them into seedlings and clonally propagate them into 50 copies for disease evaluation.

Semi-hardwood cuttings were collected from the parents and F_1 hybrid of a *P. persica* 'Andross' by *P. argentea* (DPRU 194) cross during summer 2011 for clonal propagation by rooting. The peach cuttings were dipped in 2000 ppm IBA for 5 sec. and the F_1 hybrid had two treatments; (1) cuttings were dipped in 2,000 ppm Indole-3-butyric acid (IBA) and (2) a commercial rooting hormone formulation Dip 'N Grow, both treatments were for 30 sec. The *P.*

argentia cutting were dipped in 10,000 ppm IBA + 5,000 ppm NAA and planted in vermiculate-perlite medium and placed in a greenhouse under mist.

Results and Discussion:

Objective 1a.

A total of 67,194 SNPs from different publicly available sources have been assembled. Of the total, 17,291 are from peach and almond from the ESTree database, 40,794 are from peach from GDR, 109 are from almond and a 9,000 peach SNP genotyping chip from NCBI. The data has not been evaluated for duplicate SNPs, which can potentially reduce the total number of putative SNPs to between 40,794 and 67,194. The best way of validating the *in silico* SNPs is by verifying the presence of primer sequences in the genomic DNA sequences of species involved in hybrid production. Contig assembly of genomic DNA sequences generated from a set of diverse peach, almond, and wild *Prunus* spp. will be searched for the presence of primer sequences using readily available bioinformatic tools.

Currently the genomic libraries of selected diverse *Prunus* spp. involved in hybrid combinations (**Table 1**) are under construction and subsequently sequenced using next generation sequencing technologies. The process of library preparation involves nuclei isolation and extraction of pure genomic DNA from isolated nuclei to enable sequencing of primarily nuclear DNA reducing the contamination of cytoplasmic organelle DNA sequences. The sequencing library preparation is in progress and will include barcoding individual genotypes/species to allow for sequencing multiple individuals/species in the same sequencing reaction on the Illumina HiSeq while still producing approximately 15X coverage of each genome, based on an approximate genome size of 250-300 Mb. The *in silico* SNP primer sequences will be aligned against the genome sequence data for validation.

Objective 1b.

The RNA profile differences between rootstock genotypes that are contrasting in disease reaction (susceptible vs. resistance) to various soil borne pests and pathogens will provide useful information on the genes differentially expressed in the roots between susceptible and resistant rootstocks. This facilitates identifying SNPs that are located well within the fictional genes called candidate genes thus providing potentially useful SNPs that are linked to genes that confer resistance. These SNPs along with others identified using genomic sequences will allow us for developing fine scale mapping of resistant genes. Input was requested from stakeholders regarding rootstock cultivars generally identified as tolerant or susceptible to soilborne pests and pathogens for preparing cDNA libraries for sequencing and RNA profiling. Some of the candidate rootstocks under consideration for cDNA library construction are listed in **Table 2** along with their known disease evaluation scores from various sources. Sequencing of both genomic and cDNA libraries should be complete by the end of summer 2011.

Objective 2a and 2b.

During 2011 Spring season, several thousands of pollinations were made to produce interspecific hybrids involving a number of wild *Prunus* spp. potentially useful for rootstock development based on the available scientific literatures. About 150 pollinations yielded hybrid seeds in nine cross combinations (**Table 4**). Previously, during 2010, 116 hybrid seeds were produced from twenty crosses (**Table 3**). The seeds from 2010 crosses were put into culture at the Davis repository at the end of 2010 after stratification. The immature fruits resulting from 2011 crosses were harvested and delivered to California Seed & Plant Laboratory for culturing in June 2011. The immature embryos will be excised out of seeds and cultured in an embryo culture medium to grow them into seedlings. These seedlings, when sufficiently grown, will be excised and transferred into a shoot multiplication medium and subsequently into a rooting medium to produce 50 clonal copies of each of these hybrids for greenhouse evaluation of resistance to the three major soil borne diseases. Some of the 2010 hybrids in culture at Davis repository are growing (**Figure 1**) and are entering the shoot multiplication stage. A number of open pollinated seeds collected in 2010 from self incompatible wild almond species in the NCGR collection are also in culture, and are so far the best performers of all the cultured material. The most recalcitrant embryos are those from the peach x plumcot cross (see **Table 2** for accession details). Most of the *P. dulcis* ('Tardy Nonpareil') X *P. argentea* hybrids successfully germinated (**Table 2**). Contamination is problematic and most genotypes have been rescued through subculturing of non-contaminated shoots as well as additional disinfestations.

Semi-hardwood cuttings were collected from the parents and F_1 hybrid of *P. persica* 'Andross' by *P. argentea* (DPRU 194) during summer 2011 for clonal propagation by rooting. The cuttings were dipped in rooting hormone, planted in vermiculate-perlite medium and placed in a greenhouse under mist for rooting.

Research Effort Recent Publications:

None

References Cited:

Doyle, JJ and JL Doyle (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Hammerschlag, Bauchan, and Scorza (1987). Factors influencing in vitro multiplication and rooting of peach cultivars. Plant Cell, Tissue and Organ Culture *:235-242.

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Rajashekar, Palmquist, DE and Ledbetter, CA (1995). In vitro screening procedure for osmotic tolerance in *Prunus*. Plant Cell, Tissue and Organ Culture 41: 59-164.

Table 1. Seed and pollen parents used in interspecific hybrid production - The parents are used for genome sequencing.

Pollen Parent		Seed Parent						
Species	**Accession**	***P. dulcis*** **Pomology (UC) Tardy Non-Parei F5 3-5***	***P. persica*** **DPRU 2261B V 68 101 P/12/12**	***P. persica*** **DPRU 2160A Lord Napier' P/11/01**	**Hybrid Pomology Nemared' PG 7-3**	**Hybrid Pomology Nemaguard' PG 7-16**	***P. cerasifera*** **DPRU 1511 de Caradeve' E/05/29**	***P. persica*** **DPRU 2267A Xin Dai Jiu Ba P/12/13**
P. argentea	DPRU 194	X			X	X		
P. bucharica	DPRU 1871.1		X			X		
P. cerasifera	DPRU 1511				X			
P. davidiana	DPRU 581	X	X					
P. fenzliana	Pomology	X	X		X	X		
P. hybrid	DPRU 1060 & 1065							X
P. hybrid	FPS						X	
P. kansuensis	DPRU 582	X		X		X		
P. kuramica	DPRU 1467.x	X				X		
P. pedunculata	DPRU 2329.21			X		X		
P. tangutica	DPRU 2327.x	X	X		X			
P. tomentosa	DPRU 2316.5					X		
P. triloba	DPRU 2312.2		X					
P. webbii	DPRU 196		X			X		

Table 2. Potential almond rootstocks under consideration for root-specific expression profiling (cDNA library preparation and sequencing)

Variety	Type	Root Knot nematode	Ring nematode	Root lesion nematode	Crown gall	P. cinammoni[2]	Replant[2]	Armillaria[1]	Bacterial canker[2]
Alnem 201	Almond	R?[3]			S				
Cadaman	Peach	R	S[3]	HS[2]					
Empyrean#1 (Barrier 1)	Peach	R?		S >Ng[2]			Adapted		
Lovell	Peach	S[3]	MR[3]	MR[3]	S[3]	S?[3]		S[3]	R
Nemaguard (FV 234-1)	Peach	R[3]	S[3]	MR[3]	MR[3]	MR[3]		S	S[3]
Guardian (BY520-9 or SC-17)	Peach	R[3]	MR[3]	MR[3]				S	R[3]
GxN 9 (Monegro)	Pe x Ald	HR							
GxN 15 (Garnem)	Pe x Ald	HR		MR[2]			Well Suited		
GxN 22 (Felinem)	Pe x Ald	MR	MR						
Paramount (=GF677)	Pe x Ald	S	S[3]	MR[3]	S[3]	S[3]	Well Suited	S[3]	
IS-29-5	Pe x Ald	S?	S?	S?					
Krymsk#86 (Kuban 86)	Plu hyb	S	S[3]	R (S?[2])	R		Performs Well	S[3]	
Ishtara	Plu hyb	R[2]		R[2]				R to A. mellea[2] S to A tabescens[2]	HS
Empyrean#2 (Penta)	Plu hyb	R[2,3]	MR[3]	MS[2]		R			
Marianna 2624	Plu hyb	R[2]	S[3]	S[3]	MR[3]	MR?[3]		R	S[3]
Empyrean 101 (Adesoto 101)	Plu hyb	R	S[3]	S[3]				R[2]	HS
Mr. S 2/5	Plu hyb	R[3]	S[3]	S[3]	R[3]	MR?[3]		MR[3]	R
Pumiselect (Rhenus 2)	Plu hyb	HR[2]	MR[3]	MS[2] (MR?[3])					R
Tetra (Empyrean 3)	Plu hyb	R[2]		HS[2]		R			

[1] Armillaria based on K. Baumgartner selected controls (personal communication). [2] Bacterial canker, P. cinammomi, replant, some *P. vulnus*, and other susceptibility/tolerance data based on additional notes in G. Browne spreadsheet. [3] Agriculture and Natural Resources, University of California (http://ucanr.org/sites/fruitreport/Rootstocks/). Green highlighted varieties are almond, peach, or peach x almond varieties. Gray highlighted varieties are plum or plum hybrids.
Abbreviations: Ng, Nemaguard; HR, High Resistance/Tolerance; HS, High Susceptibility; MR, Moderate Resistance/Tolerance; MS, Moderate Susceptibility; S, Susceptible.

Table 3. Current status of interspecific *Prunus* crosses from 2010.

Seed parent	Pollen Parent	Source of Pollen Parent	# Seeds	# alive	# Lost
P. dulcis (Tardy Non-Parell)	*P. argentea*	DPRU 194	22	4	13
P. dulcis (Tardy Non-Parell)	*P. fenzliana*	Pomology	3	2	1
P. dulcis (Tardy Non-Parell)	*P. kansuensis*	DPRU 582	2		1
P. dulcis (Tardy Non-Parell)	*P. kuramica*	DPRU 1467.x	5		3
P. dulcis (Tardy Non-Parell)	*P. tangutica*	DPRU 2327.x	3		
*P. persica (*DPRU2261B)	*P. bucharica*	DPRU 1871.1	2		
*P. persica (*DPRU2261B)	*P. davidiana*	DPRU 581	2		2
*P. persica (*DPRU2261B)	*P. fenzliana*	Pomology	3		1
*P. persica (*DPRU2261B)	*P. tangutica*	DPRU 2327.x	2		
*P. persica (*DPRU2261B)	*P. triloba*	DPRU 2312.2	1		
*P. persica (*DPRU2261B)	*P. webbii*	DPRU 196	4		
P. persica (Lard Napier*)*	*P. kansuensis*	DPRU 582	2	1	
P. persica (Lard Napier)	*P. pedunculata*	DPRU 2329.21	2	1	1
Nemaguard	*P. argentea*	DPRU 194	2		1
Nemaguard	*P. bucharica*	DPRU 1871.1	1		
Nemaguard	*P. fenzliana*	Pomology	1		1
Nemaguard	*P. kansuensis*	DPRU 582	1	1	
Nemaguard	*P. kuramica*	DPRU 1467.x	1	1	
Nemaguard	*P. pedunculata*	DPRU 2329.21	1		1
Nemaguard	*P. tomentosa*	DPRU 2316.5	1		
Nemaguard	*P. webbii*	DPRU 196	1	1	
P. persica (Xin Dai Jiu Bao)	Plumcot	DPRU 2267A	54		6
Total			**116**	**11**	**25**

*Most of these did not have fully formed embryos from the outset.

Table 4. Interspecific *Prunus* crosses from 2011.

Seed Parent	Pollen Parent	Seeds
Tardy Non-Pareil (UCD)	*P. argentea DPRU 194*	4
Tardy Non-Pareil (UCD)	*P. kuramica DPRU 1467.x*	9
Tardy Non-Pareil (UCD)	*P. tangutica DPRU 2327.x*	2
Tardy Non-Pareil (UCD)	*P. davidiana DPRU 581*	1
Nemared (UCD)	*P. cerasifera DPRU 1511*	9
Nemared (UCD)	*P. tangutica DPRU 2327.x*	23
Nemared (UCD)	*P. fenzliana Pomology*	71
Nemared (UCD)	*P. argentea DPRU 194*	23
P. cerasifera (DPRU 1511)	Nickels DPRU 1511	3
Total		145

Figure 1. Interspecific *Prunus* hybrid ('Nemagard' x *P. kansuensis)* undergoing shoot multiplication.