Australian Almond Breeding Program Stage 2 - Secondary Evaluation and Commercialisation

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The University of Adelaide

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AL08000 (1 June 2013)

Dr Michelle Wirthensohn

Final Report

THE UNIVERSITY OF ADELAIDE

AL08000

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Final Report - This Horticulture Australia research report details results of research into breeding new almond cultivars for the Australian almond industry and the investigation of molecular techniques, fatty acid and Vitamin E profiles and water use efficiency to help with this aim.

Date: 1st June 2013

This project has been funded by HAL using the almond industry levy and matched funds from the Federal Government.

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1. Media Summary

This 2008-2013 study discusses progress with the breeding and evaluation of new almond varieties for the domestic and export markets and molecular techniques used to enhance the program. It outlines:

- progress with primary evaluation of imported cultivars and breeding progeny
- almond crosses achieved during 2008-2013
- progress with secondary evaluation of superior breeding lines
- investigation of fatty acid profiles of almond breeding progeny and cultivars
- fingerprinting and molecular techniques used for almond breeding
- investigation into water use efficiency of some breeding lines and cultivars

Field trials were established in the major inland almond-growing areas of Lindsay Point, Monash and Dareton to investigate the potential for new almond cultivars to improve market opportunities. Over 34,000 almond seedlings were produced and planted at these test sites in the Riverland and Sunraysia regions. The fruit is being evaluated for export and domestic appeal.

At the time of this report, the primary evaluation trials were well established and five years of cropping data and kernel evaluation has been collected from superior selections in secondary trials. The research has identified superior varieties with self-fertility, improved kernel quality and increased yield, with more to be evaluated in the continuing project.

This project was funded by the Almond Industry levy with matched funding from the Australian Government facilitated through HAL.

2. Technical Summary

This five-year project followed directly on from project AL07000 and continued with the classical hybridisation approach followed by secondary evaluation. The initial component of the program concentrated on controlled hybridisations using overseas breeding lines and selections and material available in Australia. This approach has been used since the almond (*Prunus dulcis*) breeding program began in 1997 and to date the program has used 84 parents from Australia, Europe and USA and produced 34,177 progeny for evaluation from 315 different crosses. It was replaced in 2008 by a directed breeding method. Using directed hybridisation the breeder used the information generated from the analysis of heritabilities and breeding values of the parents and progeny from the previous ten years of data to achieve greater response to selection in the progeny. The data generated from the evaluations was used to calculate heritability estimates and estimated breeding values of the parents used. To date, only data generated from the 1997-2002 progeny has been analysed. Controlled pollinations continued until 2010. Hybridisations were conducted at the Waite Campus (Adelaide) and the Northern Adelaide Plains. The seedling progeny have been planted in the Riverland region at Monash, SA and in the Sunraysia region at Dareton, NSW. At the time of this report, the primary evaluation trials were well established and kernel evaluation is continuing. Two secondary evaluation sites have been planted and evaluations have been made on the first site for five years. Five superior selections have been chosen to date to enter the tertiary phase of evaluation. The research is expected to identify superior cultivars for each growing area, with self-fertility, improved kernel quality and increased yield. The fruit is being evaluated for export and domestic appeal.

A technique based on the reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect the presence of *Prunus* necrotic ringspot virus (PNRSV), prune dwarf virus (PDV), Apple mosaic virus (ApMV) and Apple chlorotic leaf spot virus (ACLS) in almond. The RT-PCR technique has the added advantage that plant material can be tested at any time throughout the growing season. Each year from 2008 to 2011 a sub sample of the trees at Monash budwood repository and all parents used for breeding were tested for the presence of these viruses.

Lipophilic anti-oxidant profiles of 28 almond samples sourced from several regions in Australia were analysed to determine the influence of variety, year and region on fatty acids and oleic and linoleic acids in particular. Considerable variation was observed not only in linoleic acid (12.2% – 29.9% of total lipids) but also lipid (42.2 – 63.5 g/100 g), oleic acid (58.5% – 78.3% of total lipids), palmitic acid (5.6% – 8.0% of total lipids) and stearic acid (1.0% – 2.47% of total lipids) content. The influence of genotype and climate were evaluated and enabled identification of several selections, as being well suited to growing conditions in Australian regions.

The origin of Australian almond cultivars is mostly unknown and therefore one aim of this study is to characterise them for their incompatibility groups and their genetic diversity. The self incompatibility (SI) genotypes of 25 Australian almond cultivars were determined by PCR analysis of genomic DNA using a combination of specific primers based on the intron regions and primers based on the conserved regions of Rosaceous *S*-RNase genes. DNA fingerprinting of the cultivars was achieved through microsatellite fragment analysis and comparison with European and American cultivars, to determine the genetic diversity within Australian almond accessions. Results showed a diverse range of incompatibility groups within Australian cultivars and fingerprinting reflected their ancestry, a combination of American and European backgrounds. A germplasm collection has been established for conservation and further characterisation of local cultivars.

Despite a high genetic similarity to peach, almonds have a fleshless fruit and edible kernel, produced as a crop for human consumption. While the release of peach genome v1.0 provides an excellent opportunity for almond genetic and genomic studies, well-assessed segregating

populations and the respective saturated genetic linkage maps lay the foundation for such studies to be completed in almond. Using an almond intraspecific cross between 'Nonpareil' and 'Lauranne' (NxL), we constructed a moderately saturated map with SSRs, SNPs, ISSRs and RAPDs. The NxL map displays high synteny and colinearity with the *Prunus* TxE reference map in all eight linkage groups (G1-G8). The positions of 14 mapped SNP-anchored genes corresponded approximately with the positions of homologous sequences in the peach genome v1.0. Segregation analysis showed that an average of 17.9% markers segregated in skewed ratios at the level of $P < 0.05$. Due to the large number of skewed markers in the linkage group 7, the potential existence of lethal gene(s) was assessed in the group. In addition, integrated maps produced by two different mapping methods using JoinMap® 3 were compared, and their high degree of similarity was evident despite the positional inconsistency of a few markers. We presented a moderately saturated Australian almond map, and the map is highly syntenic and collinear with the *Prunus* reference map and peach genome v1.0. Therefore, the well-assessed almond population reported here can be used to investigate the traits of interest under Australian growing conditions, and provides more information on the almond genome for the international community.

Almond is a nut tree in the family Rosaceae which compared to other nut crops, grown in Mediterranean climates, is relatively drought resistant. Due to the lack of, or high cost of water, almond growers are more inclined to improve gross production water use index WUI by adopting water saving irrigation strategies. Meanwhile, intrinsic water use efficiency (WUE_{intr}) can also be used as a criterion for estimating the crop water use index in breeding programs. To study the water relations under non-stress conditions, the WUE_{intr} and water relations in 5 mixed crosses of almond were examined. After exposing the plants to the same environmental conditions for 1 week, the photosynthesis (*A)*, transpiration (*E)*, leaf hydraulic conductivity (k_{leaf}) and the total leaf area (*LA*) were measured. There were significant differences in $k_{leaf,}$ *E* and *A* between different varieties, especially between Johnston x Lauranne and Nonpareil x Lauranne. For WUE_{intr}, significant differences were only observed between Carmel x Tarraco and Nonpareil x Lauranne with Carmel x Tarraco having the highest WUE. According to numerous reports, any decrease in k_{leaf} can lead to the reduction of stomatal conductance g_s and *E*. However, under well watered conditions, stomatal functioning cannot have a large effect on light-saturated carbon assimilation rates; hence, different values of *A* were probably controlled by non-stomatal parameters that can be affected by genotype variations. To study the non-stomatal limitations of photosynthesis, the g_s of leaves of three almond varieties (Nonpareil, Carmel and Masbovera) were recorded under well-watered conditions when plants were near field capacity. There were significant differences for g_s between varieties, the low g_s reading of Masbovera and the cross Johnston x Lauranne may indicate drought tolerance compared to other cultivars and the other families.

3. Introduction

Almond (*Prunus dulcis* Miller [D.A. Webb] syn. *P. amygdalus* Batsch) is one of the oldest domesticated crops in civilization and is the largest tree nut crop produced worldwide. They are grown in Mediterranean-type climates with mild wet winters and warm, dry summers. In 2011 world production of almonds was 1,063,349 tonnes kernel, with the Australian almond industry producing 37,626 tonnes of kernel, which returned over $200 Million (ABA, 2012). Large increases in production have occurred in Australia since 2006, and production will continue to increase as large areas of young trees come into bearing. The Australian almond industry has plantings of 30,000 ha up till 2011, with only 9% of this area being non-bearing and 51% yet to reach full maturity (i.e. ≥8 years old). Current indications are that production will reach 89,432 tonnes by 2017. This will put Australia as the second largest producer worldwide after the USA.

Australian production is based on three main cultivars, 'Nonpareil' being the dominant cultivar followed by pollinisers 'Carmel', and 'Price'. The majority of Australian almond production is based on these cultivars developed over 100 years ago in California and many older orchards will require replanting soon. There is a need for new improved cultivars and especially pollinators, adapted to local environments, to remain competitive locally and overseas. In order to obtain new almond varieties, intervarietal crossing is used which involves research into germplasm collections, designing crossing matrices with suitable parents, evaluation of progeny derived, and finally selection of superior genotypes and their multiplication. There are several almond breeding programs currently underway worldwide. Australia along with other countries such as Spain, Italy, Iran, France and the USA have active programs for scion and/or rootstock production (García et al., 1994; Batlle et al., 1998; Wirthensohn and Sedgley, 2002; Gradziel, 2003; Lopez et al., 2005).

The main research objectives and breeding strategies in almond breeding programs are:

Yield: high kernel yield, no alternate bearing, early bearing.
Self-fertility: reduce need for bee pollinisers and allow for single-cultivar orchards.
Late blooming: to avoid spring frost.
Resistance to drought.
Resistance to diseases: Bacterial (Pseudomonas, Xanthomonas, Agrobacterium); Fungus (Colletotrichum, Botrytis, Wilsonomyces, Fusicoccum, Monilinia);
Insect (Navel orangeworm in USA, Carob moth, mites)(ABA, 2006).
Improved processing and nutritional characteristics (improved human nutritional composition, improved storage characteristics).

In response, the University of Adelaide, in collaboration with the Almond Board of Australia (ABA) and supported by the Horticulture Australia Ltd (HAL) and the Australian Research Council (ARC), commenced an almond improvement project in 1997. The program focussed on three areas: breeding and evaluation; tissue culture; and virus detection and elimination. The current almond breeding program (AL08000) continues and expands from there with emphasis also on molecular markers for breeding. Breeding objectives were developed in consultation with industry and the resultant 5 superior selections have been identified to date from primary evaluation trials. They are now ready for tertiary evaluation and commercialisation. Molecular markers are in regular use to screen for self-fertility and virus detection. Quantitative genetics have been applied to the unique data set generated on more than 18,500 progeny to give us heritability values of important almond traits and breeding values of the parents and progeny. These techniques give us valuable breeding tools to use in the second stage of the breeding program. Continued research will provide ongoing cultivar improvement, which will give the opportunity to diversify from the traditional Nonpareil and the current reliance on two pollinators, Carmel and Price, and allow access to alternative markets and reduce the reliance and risk of single cultivar, export markets. Market targets aimed at include overseas and local markets, both in shell and shelled products. Within the shelled market we will aim for premium products including natural kernels graded into the current categories of Fancy, and Extra Supreme both for the 'Nonpareil' and the 'California' market specifications.

The specific objectives of this project were:

1. Performance evaluation of local and imported selections and rootstocks.
2. Increased production estimated at 15% and decline in reliance on existing cultivars over a period of ten years.
3. Increased range of cultivars to more than six to target niche markets such as European style kernels.
4. Controlled crossing using a targeted breeding approach to produce seedlings for performance evaluation, and selection of new improved scion cultivars with self-fertility, larger kernels, and improved flavour.
5. Virus detection in breeding stocks, selections and budwood repository.

6. Analysis of Free Fatty Acid profiles in almond selections and breeding stock.
7. Adoption of molecular techniques to facilitate the breeding program by use of markers associated with traits of interest such as taste, kernel size, self fertility, which will facilitate the process of evaluation, and by use of virus testing markers to ensure a clean supply of budwood.
8. Estimation of water use efficiency of breeding stock.

4. Material and Methods

4.1 Performance evaluation of local and imported selections and rootstocks

This process was initially begun with project NT96001 with the importation of a range of cultivars from California. Ten cultivars were released up to March 2000 and a further 40 cultivars including several rootstocks from Europe and Israel were released up till February 2012. They were immediately budded to bulk-up the mother trees for bud supply to produce trees for evaluation under commercial conditions. Mother trees are kept at the central repository at Monash. Currently there are three cultivars from Europe in quarantine at Knoxfield, Belona, Mardía and Soleta, and these are due for release in March 2015.

4.1.a Rootstock trial

In 1997/98 the industry identified a range of potential rootstocks not yet evaluated for almonds in Australia. These were GF677, Cadaman, Citation, Hansen 536, Hansen 2168, Atlas, Viking, and Nemasun. Since that time additional rootstocks have been imported with the aim of evaluating them in Australian conditions. These include GF557, GF749, Adafuel, Cornerstone, Nickels, Krymsk 86, Penta, Tetra, Felinem, Monegro and Garnem. Various nurseries have been contacted to provide 30 Nonpareil almond trees budded to each of these rootstocks for the field evaluation on a trial block to be selected.
The rootstocks will be evaluated against the current industry standards, Bright's hybrid and Nemaguard peach. The trial will be planted in 2013 as part of project AL11012.

4.1.b Scion trial

Cultivars imported up to March 2000 from California include Padre, Livingston, Butte, Sonora, Monterey, Avalon, Savanna, Morley, Sauret #1 and Wood Colony. Cultivars imported since 2000 from France via INRA include Glorieta, Masbovera, Ferrastar, Mandaline, Marcona, Supernova, R1066, R1065, Steliette, Aï, Guara, Ferraduel, R1049, Francolí and Ferragnès. Cultivars imported from Israel include R1148, R887, and R1146. Cultivars imported from Spain include Antoñeta, and Marta from CSIC; Masbovera, Glorieta, Francolí, Felisia, Desmayo Largueta, 155, 12-350, Tarraco, Vayro, 21-332, Marinada and Constantí from IRTA. Cultivars imported from Italy include Fascionello and Cristomorto and one cultivar from Iran, White. Most of these cultivars have been planted at Monash as a source of budwood. These original trees are cut back in winter each year and the resulting regrowth is used to summer bud trees the following November for planting the following winter. Two groups of Californian cultivars have been planted for evaluation at Lacton and Nutwood Orchards at Lindsay Point, Victoria. Nine Spanish cultivars were planted at Omega Orchards, Murtho South Australia in 2009; R1065, Marcona, Francolí, Glorieta, Masbovera, 21-332, Vayro, Tarraco and Constantí. Nonpareil, Carmel and Monterey are also planted for comparison. A tenth cultivar Marinada is currently in quarantine. Although the best prospects have been selected, we do need to be cautious and plant only limited areas for evaluation until we have a better idea of their performance under our conditions.

4.2 Controlled crossing to produce seedlings for performance evaluation, and selection of new improved cultivars

This breeding project aims to produce new cultivars with higher kernel yield and quality, self-fertility, drought and disease tolerance, economic gain, and diversity of products/markets for the Australian almond industry. The program focuses on classical breeding via controlled hybridisation, using both Australian and overseas cultivars as breeding stock. Local varieties were used as these are adapted to our environment and have good qualities such as kernel size and tree habit. Self-fertile genotypes were imported from INRA, IRTA and CEBAS-CSIC to achieve some self-fertile progeny. Since 1997 to 2010, when hybridisations ended, 84 different cultivars have been used as either female or male parents (Tables 4.2.1 and 4.2.2).

Hybridisations were conducted at two sites including The University of Adelaide Waite Campus (Urrbrae, 34^{o} 58' S, 138^{o} 38' E), and the Northern Adelaide Plains (Munno Para Downs, 34^{o} 38' S, 138^{o} 40' E). The parent trees at Waite are enclosed in a bird proof cage and each branch is covered with an insect proof net prior to hand pollination. Pollen was collected from unopened flowers (balloon stage) from virus-tested cultivars. Anthers were removed and allowed to dry for 24 hours at 25 degrees Celsius. Dried anthers were then rubbed over a 250 micron sieve to separate the pollen. Pollen of each cultivar was then stored in vials at 4 degrees Celsius if required within 7 days or at -20 degrees Celsius for longer-term storage.

All hybridisations were carried out by hand. Pollen was applied using a small paint brush to transfer pollen to the stigma of each flower. Nuts were harvested at maturity when the hull dehisces. Seeds were germinated by firstly placing them in a solution of fungicide (Captan) overnight following manufacturers protocols, followed by cold-moist stratification (in vermiculite) at 4 degrees Celsius for 8 weeks. By this stage the seeds have developed a radicle and they are potted on in the greenhouse. The potting soil consisted of 2/3 peat, 1/3 sand with additional fertiliser added including Osmocote, pH 6-6.5. Seedling trees were hardened off when they reached 40 cm in height or after 15 weeks. They were then planted in the field for evaluation. The seedling progeny were planted in the Sunraysia region at Dareton, NSW (34^{o} 5' S, 142^{o} 1' E). The climate in Dareton is warm to hot in summer and cool in winter with maximum temperatures in the range 30^{o} – 33^{o} C in January and 15 – 17^{o} C in July. Trees were planted at 1 x 4 m spacing with drip irrigation and fertigation. Earlier plantings were established in the Riverland region at the Riverland Vine Improvement Centre, Monash SA (34^{o} 14' S, 140^{o} 33' E).

Table 4.2.1. Cultivars, breeding lines and their sources used as parents in the breeding program since 1997.

Cultivar	Source	Cultivar	Source	Cultivar	Source	Cultivar	Source
11aT44	BL	UA-6	BL	Lauranne	INRA	UA-1	BL
155	IRTA	A-2-2	CITA	LeGrand	INRA	R14T17	BL
12-350	IRTA	Antoñeta	CSIC	Mandaline	INRA	R21T70	BL
1aT26	BL	Carmel	ABA	Marcona	CSIC	UA-4	BL
1aT30	BL	Chellaston	Local	Marta	CSIC	R29T105	BL
1bT30	BL	Cristomorto	IRTA	McKinlays	Local	R30T25	BL
		Desmayo	CSIC				BL
1aT4	BL	Largueta		Mission	ABA	R30T45	
1aT40	BL	Falsa Barese	IRTA	ModAlnem 6	ABA	R33T48	BL
1aT9	BL	Felisia	CITA	Moncayo	INRA	R38T63	BL
UA-3	BL	Ferraduel	INRA	Ne Plus Ultra	ABA	R42T106	BL
UA-2	BL	Ferragnès	INRA	Nonpareil	ABA	R5T19	BL
1bT42	BL	Ferralise	INRA	Parkinson	Local	UA-7	BL
1bT47	BL	Ferrastar	INRA	Pearce	Local	Sauret#1	ABA
1bT51	BL	Francoli	IRTA	Peerless	ABA	Somerton	Local
21-169	IRTA	Frenzy	Local	Price	ABA	Steliette	INRA
21-323	IRTA	Genco	IRTA	Primorskiy	IRTA	Strout	Local
22-120	IRTA	Glorieta	IRTA	P. webbii	CITA	Supernova	INRA
						Tardy	ABA
2bT33	BL	Guara	INRA	R1065	INRA	Nonpareil	
3aT31	BL	Iranian seedling	INRA	R1146	INRA	Thompson	ABA
3bT45	BL	Johnston's P.	Local	UA-5	BL	Tuono	IRTA
4bT1	BL	Keanes	Local	R12T28	BL	White	Iran

ABA, Almond Board of Australia; BL, breeding line from current program; CITA, Centro de Investigacion y Tecnologia Agroalimentaria de Aragon (Spain); CSIC, Consejo Superior de Investigaciones Cientificas (Spain); INRA, Institut National de la Recherche Agronomique (France); IRTA, Institut de Recerca i Tecnologia Agroalimentàries (Spain).

Table 4.2.2. Characteristics of parents used in the 2008 – 2010 crosses.

Parent	Origin	S-alleles	Kernel size (g)	Crackout percentage
4bT1	Australia	$S_8S_?$	1.73	34
Chellaston	Australia	S_7S_{23}	1.77	29.5
Johnston's Prolific	Australia	$S_?S_{23}$	1.95	23.5
R30T45	Australia	S_5S_8	1.59	36
R42T106	Australia	S_7S_8	1.71	31
Somerton	Australia	S_1S_{23}	1.52	36.1
Ferraduel	France	S_1S_4	1.23	26.3
Ferrastar	France	S_2S_{10}	1.56	32.9
Lauranne	France	S_3S_f	1.56	36.3
R1065	France	unknown	1.45	15.2
R1146	France	unknown	0.9	na
White	Iran	unknown	na	na
12-350	Spain	S_1S_f	0.95	25
21-169=Tarraco	Spain	S_1S_9	1.64	na
21-323=Vayro	Spain	S_9S_f	1.14	24.3
22-120=Constanti	Spain	S_3S_f	0.95	16.1

Antoñeta	Spain	S_1S_f	1.95	na
Carmel	USA	S_5S_8	1.45	28
Nonpareil	USA	S_7S_8	1.36	40.7

na, not available

Primary evaluation of progeny from breeding program

Primary evaluation is based on nut and kernel characteristics. Nut characters included in the evaluation process are percentage double kernels, shelling percentage, fruit weight, kernel weight, shell weight, kernel taste, testa colour, shell seal and appearance.

The selection criteria for kernel evaluation are shown in the following table (Table 4.2.2). Initially the primary selection is done on kernel characteristics followed later by evaluations on tree characteristics. Tree characters scored include yield, vigour, presence of pests and disease, ease of knocking, and architecture. Detailed evaluation of tree characters occur on selected trees only.

Table 4.2.2. Evaluation criteria and selection standards for some kernel characteristics.

Character	Records	Selection			Weighted Score[¥]
		High	Medium	Low	
Sweet kernel	Sweet or bitter	sweet	sweet	bitter*	pass/fail
Kernel size	Kernels per ounce or (g)	≤18/20 ≥ 1.4	20/22 to 23/25 < 1.4 or ≥ 1.1	>23/25** < 1.1	1-5
Double kernel	% double kernels	< 5%	≥ 5% or ≤ 15%	> 15%***	1-5
Kernel colour	Light, medium, dark	light	medium	dark	1-5
Shell hardness	Stone, hard, semihard, soft, paper	hard, semihard, soft, paper		stone	1 or 5
Shell seal	Closed/open seal	closed	closed	open	pass/fail
Kernel appearance****	Score (1-10)	7-10	5-6	1-4	1-10

[¥] The higher the rating the better the tree's performance.
*All progeny with bitter kernels are eliminated from further evaluation.
**All progeny with kernels less than 1.1 g or greater than 23-25 kernels per ounce are eliminated from further evaluation.
***All progeny with double kernels greater than 15 percent are eliminated from further evaluation.
****Kernel appearance takes into account kernel shape, smoothness, and uniformity.

For each tree 30 kernels were evaluated and each tree given a score for each character based on their performance and the individual characters were given a weighted score according to their importance or ranking (Section 9.2.1 of the Almond Breeding Strategic Plan 2001-2005). Trees with the highest total score were selected, however each character is also considered separately. Sensory evaluation is conducted on remaining progeny. Sensory evaluation was undertaken by a panel of 10 tasters to verify the initial categorisation of the almond kernels and to determine preferences for the different kernel flavours. Training was provided to familiarize panelists with the taste of sweet and semi-bitter (marzipan-like) almonds. Classification of kernel flavour was made

using two five-point scales: one for sweetness and the other for marzipan flavor. Another five-point scale was used for the overall taste preference and also for overall appearance of the almond kernels. All sensory data were analysed by ANOVA and the best trees were selected for second stage. *S*-allele identification is conducted on progeny with positive sensory attributes. Superior progeny are evaluated for 2 years prior to grafting of the best for second stage evaluation.

The data generated from the evaluations was used to analyse the heritability of the traits measured, and to give estimated breeding values for all trees. This was achieved using a statistical package, ASREML.

Secondary evaluation of progeny from breeding program
For project AL08000, a secondary evaluation trial was set up in 2006 as a full PBR trial consisting of five budded trees of each selected progeny, five trees of each comparator and reference cultivars. All trees were grafted onto Nemaguard rootstock and planted on a 7 x 5 m grid at Lindsay Point, VIC. Trees were maintained as a commercial planting. Second stage testing on the trees included assessment of disease tolerance to bacterial spot, tree habit, yearly productivity, flowering time, crackout percentage, ease of shaking and harvest time. Traits listed in the PBR descriptors were measured for selections which showed promise, and relevant comparators.

Selection criteria for new almond cultivars are:
High yield
Self pollinating (self-fertility)
Nonpareil type and shape
Compatibility with Nonpareil
White kernel colour and golden testa
Large kernel size ie minimum kernel weight 1.24 g
Double kernels at < 5%

Assessment of Bacterial spot tolerance
Four almond cultivars, Nonpareil, Fritz, Butte and Padre, and five breeding selections, UA-2, UA-3, UA-5, UA-1 and UA-4 were used in this study. Plant materials were obtained from Waite Campus, University of Adelaide, and Lindsay Point field trials. Several branches with fully expanded leaves were cut and brought into the laboratory. Leaves of each cultivar were collected and washed under running tap water to remove any contaminants. After sterilization with 70% ethanol for 90 seconds at room temperature, the leaves were rinsed with MilliQ water. These leaves were used immediately for inoculation.

Xanthomonas campestris pv. *pruni.* Isolates from almond (strain number DAR 64858) were obtained from the Australian Collection of Plant Pathogenic Bacteria. The bacteria were grown on nutrient agar containing 0.25% glucose (NAG) for 2 days at 28^{o}C and a loopful of growth suspended in sterile water. These suspensions were diluted serially to obtain the specified concentration of inoculum suspension using a haemocytometer. The detached leaves or leaf portions were placed abaxial side up on four layers of sterile tissue. Inoculum suspension was held in a sterile plastic 10ml syringe with soft plastic tube over the end of the syringe. Gentle and steady pressure was applied while pressing the open end of the syringe against the leaf until a 4-5mm diameter area of tissue was water-soaked. During the infiltration, 10 sites of each leaf were inoculated. Any excessive inoculum suspension on the leaf was removed by using tissue. The inoculated detached leaves were placed on 0.5% water agar and incubated at 28^{o}C for up to two weeks under dark conditions. The control leaves were infiltrated with MilliQ water in a similar manner. The percentage of inoculated areas with water-soaked spots and diameter was recorded after 14 days. A one-way analysis of variance was used to analyse differences between three almond cultivars.

Development of Breeding Cage and Germplasm Collection.
The almond breeding cage at Waite campus contains 51 cultivars that are used for breeding and research purposes. Imported cultivars were planted in the breeding cage after their release from Knoxfield plant quarantine nursery. Two trees per cultivar were budded in spring onto rootstocks. The cultivars include Aï, All in One, Antoñeta, Atkinson's hardshell, Bright's hybrid, Brown Brandis, Butte, Carmel, Chellaston, Constantí, Cristomorto, Desmayo Largueta, Fascionello, Felisia, Ferraduel, Ferragnès, Ferralise, Ferrastar, Francolí, Frenzy, Fritz, Glorieta, Guara, Johnston's Prolific, Livingston, Mandaline, Marcona, Marta, Masbovera, McKinlay's, Monterey, Ne Plus Ultra, Nonpareil 15-1, Padre, Parkinson, Peerless, Sauret #1, Somerton, Steliette, Supernova, Tardy Nonpareil, Tarraco, Vayro, White and Wood Colony. Breeding lines from Europe such as R1065, R1049, R1146, 21-332, 12-350, and 155 have also been planted.

4.3 Virus detection in breeding stocks, selections and budwood repository

Plant material
Leaf samples were collected randomly from around the canopy of the almond cultivars at the Monash bud wood repository (Riverland Vine Improvement Centre, Monash, South Australia) in each of the years from 2008 to 2011. Leaf samples were also taken from each parent tree used in the breeding program. The leaves were transported on ice to the Waite campus of The University of Adelaide and used either fresh or after storage at −80 °C. Leaves of CEBAS1, a Spanish cultivar, were supplied from trees grown in Spain by CSIC-CEBAS, Murcia, Spain, for comparison with leaves from the Nonpareil and Sauret trees grown in Australia. Leaves of cherry (*Prunus avium*), known to be infected with PDV, were used as a positive control for PDV. Positive controls for ApMV and ACLSV were supplied by DPI Victoria Knoxfield.

Isolation of RNA
RNA was isolated from leaves of almond by using a commercially available extraction kit (Isolate Plant RNA mini kit, Bioline, Aust.). RNA was extracted using the Isolate plant RNA mini kit using the following protocol. Approximately 100 mg of fresh leaf tissue was pulverised in a small plastic bag 450 µl of Lysis buffer APR (from kit). The homogenate was transferred (using a disposable plastic transfer pipette) to a 1.5 mL microcentrifuge tube and centrifuged for 1 min at top speed to remove debris. The supernatant was transferred to Spin Column PR1 and spun at 12,000 rpm for 2 min. The filtrate was saved and 1 volume (400 µl) of 70% ethanol was added to it. This was transferred to Spin Column PR2 in a new collection tube and spun for 2 min at 12,000 rpm. The flowthrough was discarded (the tube was drained on toilet tissue), and 500 µL of Wash Buffer APR was applied to the filter and washed through by centrifugation at 12,000 rpm for 2 min. 650µl Wash Buffer BPR was added to the column and centrifuged at 12,000 rpm for 1 min. After discarding the flowthrough (the tube can be drained on toilet paper, or a new 2 mL microcentrifuge tube can be used), the column (sitting on 2 mL tube) was centrifuged at 12,000 rpm for 2 min to ensure removal of all the alcohol. The column was then transferred to a new 1.5 mL elution tube and total RNA eluted with 70 µL RNase free Water. This was incubated for 1 min then centrifuged at 8,000 rpm for 1 min. RNA was stored at −20 °C (or −80 °C for long-term storage).

Primers
Oligonucleotide primer sequences reported by Raquel et al., 1998 were used to detect PDV. Primer sequences to detect PNRSV were developed by Mekuria et al (2003). Primer sequences to detect ApMV were developed by Petrzik and Svoboda (1997). Primer sequences to detect ACLSV were developed by Candresse et al (1995). The expected amplification products are shown in Table 4.3.1.

Table 4.3.1. Primers to detect PNRSV, PDV, ApMV and ACLSV in almond leaves.

Virus	Primers	Primer length	Primer sequence 5' – 3'	Amplification product size
PNRSV	PNRSVF	19	CTTGAAGGACCAACCGAG	351 bp
	PNRSVR	19	ATCTGCTAACGCAGGTAAG	
PDV	PDVF	22	CCAATTTACTTCCAACTTTCGA	722bp
	PDVR	21	GCACAATCAAATGATGGATCA	
	PDV241F	20	GTATGATATCTCGTACCGAG	241 bp
	PDV241R	20	CTGGCTTGTTTCGCTGTGAA	
ApMV	ApMV1F	21	TGGATTGGGTTGGTGGAGGAT	261 bp
	ApMV2R	21	TAGAACATTCGTCGGTATTTG	
ACLSV	A52	17	GGCAACCCTGGAACAGA	358 bp
	A53	21	CAGACCCTTATTGAAGTCGAA	
Ribosomal RNA – internal control	RNAGCF RNAGCR	19 20	GAAACCTGCCTAGCAGAAC CGAGGACTTGGTATTTATGC	480 bp

RT-PCR

RT-PCR was carried out in a volume of 20 µl containing 70 ng of total RNA, 1.5 mM $MgCl_2$, 0.5 µM of appropriate primers, 200 µM each of dNTPs, 1× PCR buffer, 1× sucrose–cresol red dye (20% (w/v) sucrose containing 1 mM cresol red), 1 U/µl RNase inhibitor, 0.5 U/µl Superscript II and 1.1 U Taq DNA polymerase. The PCR cycle consisted of cDNA synthesis at 50 °C for 45 min, followed by 94 °C for 1 min, 34 cycles for 30 s at 94 °C, 45 s at 56 °C, 1 min at 72 °C and a final extension step of 5 min at 72 °C. PCR products were visualised by electrophoresis on 1.5% agarose gels in 1× TBE buffer (89 mM Tris–HCl, 89 mM boric acid, 5 mM EDTA, pH 8.0) at a constant current of 100 mA. After electrophoresis, gels were stained with ethidium bromide (0.5 µg/ml), destained with sterile water, and visualised under UV light.

Nested PCR for detection of PDV

A second set of primers, internal to the primers used in the first-round of RT-PCR, was designed to perform nested PCR. The PCR products from RT-PCR were diluted 1:100 for almond. Two microlitres of the diluted first-round PCR products were subjected to nested PCR with primers PDV241F/PDV241R using the same PCR mixture and PCR program as for the first-round PCR except that there was no cDNA synthesis step. The expected amplification product was 241 bp for PDV (Table 4.3.1). PCR products were visualised as for the RT-PCR methods.

Cloning and sequencing of the amplification products

The RT-PCR products for PNRSV (351 bp), PDV (722 bp), PDV nested PCR product (241 bp), ApMV (261 bp) and ACLSV (358 bp) were inserted into the pCR 2.1-TOPO 3.9 kb vector using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's instructions, and sequenced to confirm that the amplified products were from the coat proteins of PNRSV, PDV, ApMV and ACLSV.

4.4 Analysis of free fatty acid profiles in almond selections and breeding stock

Sample Pre-treatment
After liquid nitrogen cryo-treatment to peel off the almond skins, the kernels were dried at 50^{o}C for 48 hours. The dried kernels were ground to a powder and sieved through a 1000 μm mesh, and stored in nitrogen flushed glass vials until required.

Fatty Acid Extraction and Determination
The lipid extraction and fatty acids determination were based on chloroform-methanol extraction and methanol-sulphuric acid FAME formation. The method was initially based on Folch et al. (1957) and modified by Makrides et al. (1996). Briefly, sampled almond powder (0.05 g) was mixed with 0.9% NaCl (2 mL), Me-OH (3 mL with 0.005% BHA), FFA (400 μL of 0.16% in Me-OH) as an internal standard, and chloroform (6 mL), vortexing after each addition step, and then left standing for 1 hour. The samples were then centrifuged (3000 x g for 10 min) (performed twice), the lower phase was pipetted to a pre-weighed empty vial and the solvent evaporated using a nitrogen evaporator at 45^{o}C. After drying, the vials were re-weighed to obtain the lipid content. Methylation was achieved by adding chloroform:methanol (v/v, 9:1)(1 mL) with 0.005% BHA, and methanol with 1% H_2SO_4, (5 mL) methylating for 3 hours at 70^{o}C. After the samples cooled, *n*-heptane (2 mL) and Millipore water (0.75 mL) was added and mixed thoroughly. The top layer was transferred to GC vials. The determination of fatty acid composition was performed using a HP 6890GC equipped with flame ionization detector (FID), HP 7683 autosampler, HP Chemstation software, split/splitless injection and capillary GC column SGE BPX (50 m, 0.32 mm ID, 0.25 μm). Helium was the carrier gas and the split-ratio was 20:1, the injector temperature was set at 250^{o}C and the detector temperature at 300^{o}C, the initial oven temperature was 140^{o}C increasing to 220^{o}C at 5^{o}C/min, and then held at this temperature for 3 min. FAMEs were identified based on the retention time of internal standard free fatty acid C17.

4.5 Adoption of molecular techniques to facilitate the breeding program

4.5.a DNA fingerprinting and identification of self-incompatibility genotypes
The aim of this study was to compare the DNA fingerprints of a number of important Australian and overseas cultivars, using the SSR technique, to aid in fingerprinting these cultivars for future reference.

Plant material
Local cultivars were sourced throughout Australia after surveying the industry (Wirthensohn & Sedgley, 2003). Foreign cultivars were sourced from the germplasm collection of the Almond Board of Australia located at Monash Budwood Repository, Monash South Australia.

DNA extraction and S-allele primers
DNA was extracted from 28 accessions (Table 4.5.1) using the CTAB method described by Woolley et al., (2000). To determine the *S*-alleles of the cultivars, the primers designed from the second and third introns, EMPCons2-F and EMPCons3-R (Sutherland et al., 2004) and primers for the first intron, PaCons1–F and EMPCons1-R (Sonneveld et al, 2003; Ortega et al., 2005) were initially used, after which specific primers were used to verify the alleles where primers were available (Channuntapipat et al., 2003). PCR conditions were as stated in the relevant papers. PCR products were electrophorised in either 1.5% Agarose gels in 1x TBE buffer or 2% Metaphor Agarose in 1 x TBE Buffer at 100V for 5 hours and the products were visualised using SybrSafe DNA stain. Gel images were analysed using Gel Pro Analyser (Version 3.1, Media cybernetics, Maryland USA) to detect product sizes.

Fingerprinting using SSR markers

For fingerprinting and genetic diversity analysis, initially 33 sets of SSR primers were screened. Of these, 13 sets of fluorescently labelled SSR primers (PET, NED, VIC and 6-FAM) were finally used as they showed polymorphism at a single locus. Each reaction was repeated and analysed twice for confirmation and the size standard used in the sequencer was Gene Scan™ 500 Liz® (Applied Biosystems). Sequencing was done using an AB3730 DNA analyser and fragment analysis data was analysed using AB GeneMapper software v4.1 (Applied Biosystems, CA). Table 4.5.2 shows the details of the SSR primer pairs. An UPGMA dendrogram was constructed from the SSR marker data using the programme NTSYSpc (Numerical Taxonomy and Multivariate Analysis System, Version 2.2, Exeter Software, New York). The program is based on an algorithm, devised by Sokal and Sneath (1963), which calculates genetic similarity by matching individuals that share or lack a marker data (Bartolozzi et al., 1998). The SAHN procedure of NTSYS-pc used the unweighted pair group method with arithmetic averages (UPGMA) to cluster individuals, according to the simple matching coefficient, into a dendrogram (Stiles et al., 1993; Bradley et al., 1996; Bartolozzi et al., 1998). The dendrogram was based on the pairwise comparison of the binary matrix of all cultivars being considered to show the percentage of genetic similarity between cultivars.

Table 4.5.1. Source of the almond accessions assessed for genetic similarities using SSR technique.

Cultivar	**No. accessions**	**Origin**	**Cultivar**	**No. accessions**	**Origin**
Tom Strout	1	Australia	Monarto 1	1	Australia
Somerton	2	Australia	Monarto 2	2	Australia
Brown Nonpareil	1	Australia	Monarto 3	1	Australia
Strout's papershell	1	Australia	Monarto 4	1	Australia
Johnston's	1	Australia	Marion St	1	Australia
Pethick Wonder	1	Australia	Parkinson	1	Australia
Bigg's hardshell	2	Australia	Atkinson's hardshell	1	Australia
White Brandis	1	Australia	Nonpareil	1	USA
Keane's	2	Australia	Carmel	1	USA
Pearce	2	Australia	Ferragnès	1	France
Frenzy	1	Australia	Lauranne	1	France
Bruce	1	Australia			

Table 4.5.2. SSR primers used for fingerprinting analysis, number and size of alleles detected and linkage group covered.

Primer Name	Reference	No. alleles	Min. size (bp)	Max. size (bp)	Linkage group
BPPCT001	Dirlewanger et al., 2002	15	127	178	G2
BPPCT007	Dirlewanger et al., 2002	11	126	159	G3
BPPCT025	Dirlewanger et al., 2002	12	155	191	G6
CPDCT025	Mnejja et al., 2005	11	161	201	G3
CPDCT045	Mnejja et al., 2005	17	134	187	G4
CPPCT006	Aranzana et al., 2002	9	179	211	G8
CPPCT022	Aranzana et al., 2002	9	221	295	G7
CPPCT044	Aranzana et al., 2002	10	160	190	G2
CPSCT012	Mnejja et al., 2004	11	145	181	G6
CPSCT018	Mnejja et al., 2004	10	158	183	G8
CPSCT021	Mnejja et al., 2004	9	134	159	G2
EPPCU9168	Howad et al., 2005	11	172	192	G5
PMS40	Cantini et al., 2001	9	90	133	G4

4.5.b Genetic mapping of almond

The objective of this study was to enhance the original integrated genetic linkage map of the F_1 hybrid population of the cultivars 'Nonpareil' x 'Lauranne' (Gregory et al., 2005), constructed using RAPD, inter-simple sequence repeat (ISSR) SSR and morphological markers.

Mapping population and DNA extraction

An almond pseudo-testcross population with 93 progeny, derived from the cross between the American cultivar 'Nonpareil' as maternal parent and the French cultivar 'Lauranne' as pollen donor, was used as the mapping population (NxL) (Wu et al., 2009). The population was planted in a commercial orchard at Lindsay Point - Victoria, Australia (34°15'S – 141°00'E) with a fertile and well drained soil and an average of 223 mm annual rainfall. Standard orchard management including fertilisation, irrigation and pruning were applied. Agronomic traits segregating in the population include: in-shell weight, kernel weight, percentage double kernels, shell hardness, kernel shape, kernel thickness, kernel length, kernel width, testa colour, testa pubescence, kernel taste, bloom time and self-incompatibility. Total genomic DNA was extracted from fresh young leaves using the protocol of Lamboy and Alpha, (1998). DNA quantity and quality was measured spectrophotometrically by Nanodrop ND-1000® (Thermo Scientific, USA).

Molecular markers

A total of 241 SSR initially reported in different *Prunus* species were screened for polymorphism in the parents and selected progeny (Table 4.5.3). The designation of the markers, the original species from which the markers were developed, and the reference information are listed in Table 4.5.3. The PCR was performed in a total volume of 20 µl containing 1 × PCR reaction buffer (Bioline, Sydney, Australia), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 40 ng of template DNA and 1 unit of *Taq* polymerase (Bioline, Sydney, Australia). Amplification was achieved through the following cycles: first denaturation at 95°C for 5 min, 34 cycles of denaturation at 95°C for 30 seconds, annealing at appropriate temperatures (mostly based on the information provided in the cited literature, and available on request from authors) for 30 seconds, and extension at 72°C for 30 seconds, and a final extension at 72°C for 7 min. Electrophoresis was performed on 8% (w/v) polyacrylamide gel, or automated capillary gel on the ABI PRISM 3730 DNA Analyzer (Applied Biosystems) to visualise PCR products. Markers with good reproducibility and clearly decipherable loci were chosen for construction of linkage maps.

Thirteen SNPs used in the present study have been described previously (Wu et al., 2009 & 2010). Primers were designed using Primer 3 (Rozen and Skaletsky, 2000) for high resolution melting (HRM) analysis and the amplicons were designed at a length between 60-100 bp when possible. PCR amplifications were performed in a total volume of 10µL on a Rotor-Gene 6500 realtime PCR Thermocycler (Corbett Research, Sydney, Australia). The reaction mixture contained 40 ng almond genomic DNA, 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 300nM forward and reverse primers, 1.5 µM Syto® 9 (Invitrogen, Sydney, Australia), and 0.5 U Biotaq DNA polymerase (Bioline, Sydney, Australia). The amplification was achieved by a touchdown PCR protocol: first denaturation at 95°C for 2 min, then 50 cycles of denaturation at 95°C for 5 sec, annealing and extension for 10 sec at 62°C for the first cycle and thereafter at 0.5°C decrease each for 10 cycles, and a final extension at 72°C for 2 min. HRM was performed as follows: pre-melt at the first appropriate temperature for 90 sec, and melt at a ramp of 10°C in an appropriate temperature range at 0.1°C increments every 2 seconds. The fluorescence data were acquired at the end of each annealing step during PCR cycles and each of the HRM steps with automatic gain optimisation. High resolution melting curve analysis was performed using the HRM module of Rotor-Gene 6500 series software (Corbett Research, Sydney, Australia). The melting data were normalised by adjusting start and end fluorescence signals respectively of all samples analysed to the same levels. Genotypes of the individuals were scored automatically by the software and verified visually.

ISSR and RAPD markers used in this study were described previously (Gregory, 2004; Gregory et al., 2005). In brief, six RAPD primers and 13 ISSR primers were used to genotype the population for map construction. For RAPD analysis PCR was performed in a total volume of 20 μL on a PCT-100 thermocycler (MJ Research Inc., USA). The reaction mix contained 40 ng of DNA, 1x PCR buffer, 3 mM $MgCl_2$, 0.25 μM primer, 200 μM of dNTPs, 1.1 U Taq polymerase and 1x cresol red dye (2% sucrose, 0.1 mM cresol red sodium salt). The amplification was conducted according to following protocol: first denaturation at 95°C for 2 min, then 45 cycles of denaturation at 95°C for 30 sec, annealing at 48^o for 45 sec and extension at 72°C for 1 min, and a final extension at 72^{o}C for 10 min. For ISSR analysis, PCR was performed in a total volume of 20 μL on a PCT-100 thermocycler (MJ Research Inc., USA). The reaction mix contained 50 ng of DNA, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.5 μM primer, 200 μM of dNTPs, 1.0 U Taq polymerase and 1x cresol red dye. The amplification was conducted using the same protocol as for RAPD or the following protocol: first denaturation at 94°C for 7 min, then 41 cycles of denaturation at 93°C for 1 min, annealing at 55^o for 1 min and extension at 72°C for 2 min, and a final extension at 72^{o}C for 8 min. PCR products of RAPD and ISSR were visualised on 1.5% or 1.75% (w/v) agarose gel respectively. The assay of self-incompatible genes *S3*, *S7*, and *S8*, and self-fertile gene *Sf* was performed using a previously described protocol (Channuntapipat et al., 2001).

Map construction

Linkage maps were constructed using JoinMap® 3 software (Van Ooijen and Voorrips, 2001). Two different mapping methods were applied and the resulting maps were compared and analysed to assess their synteny with the *Prunus* TxE reference map. The first method constructed two parental maps separately, which was followed by the production of an integrated map. As this approach involved two map construction steps (Grattapaglia and Sederoff, 1994; Maliepaard et al., 1998; Wu et al., 2004) we denoted it as 'Two-step method' and the map or linkage groups labelled as "II". This included preparing two separate parental data sets as described elsewhere for a pseudo-testcross population (Grattapaglia and Sederoff, 1994). Both sets of genotyping data were loaded into JoinMap® 3 and two parental maps were constructed individually. Chi-square analysis was performed for goodness of fit to the expected Mendelian segregation ratio for each marker and skewed markers were identified using a threshold of $P < 0.05$. Framework linkage groups were created by omitting the skewed markers from the data for all the linkage groups except linkage group 7 (G7), due to the high degree of skewed markers in this group (see Results section). These framework groups were used as fixed orders for the individual final map construction that included all skewed markers. Linkage groups were established at a LOD score of > 5 and recombination fraction < 0.40. The Kosambi mapping function was used for the calculation of map distances (Kosambi, 1994). Two parental maps (as frameworks or final maps) were integrated using the "Combine Groups for Map Integration" function of Joinmap® 3 to produce the combined maps (framework or final maps). This method uses mean recombination frequencies and combined LOD scores for mapping calculations. The second method constructed a map by using all the markers heterozygous in both or either of the parental trees as one set of data (Hanley et al., 2002 & 2007; Clarke et al., 2009). As this method involved using all markers in a single map construction, it was denoted as the 'One-step method' and the map or linkage groups labelled as "I". The map was constructed strictly following the steps to construct a single parental map in the 'Two-step method'. In brief an initial analysis was performed by excluding skewed markers except those in G7, and the resulting framework map was used as fixed order for final map construction that included all the markers. The parameters used were exactly the same as 'Two-step method'. Markers in common between our maps and the *Prunus* TxE reference map were used to identify corresponding linkage groups. The resulting maps were graphically presented and their alignment was performed using Mapchart 2.2 (Voorrips, 2002).

Sequence blast and localisation in Prunus genome
Sequences of the SNP-anchored genes were blasted against peach genome v1.0 scaffolds (http://www.rosaceae.org/peach/genome), and the resulting homolog sequences were located in the scaffolds (corresponding to the linkage groups of Prunus genetic maps) using the GBrowse function.

Table 4.5.3. List of identifiers and numbers of the SSR markers tested, segregated and mapped in this study.

Identifier	Number of markers tested	Number of markers segregated	Number of markers mapped	Species of origin	References
AMPA	1	0	0	Apricot	Decroocq et al., 2003
BPPCT	29	17	16	Peach	Dirlewanger et al., 2002
CPDCT	25	15	13	Almond	Mnejja et al., 2005
CPPCT	25	13	12	Peach	Aranzana et al., 2002
CPSCT	23	13	10	Japanese plum	Mnejja et al., 2004
EMPA	3	1	1	Sweet cherry	Clarke and Tobutt, 2003
EMPaS	4	0	0	Sweet cherry	Vaughan and Russell, 2004
EPDCU	14	6	5	Almond	Mnejja et al., 2010
MA	1	1	1	Peach	Yamamoto et al., 2002
Pac	2	0	0	Apricot	Decroocq et al., 2003
PaCITA	1	1	1	Apricot	Lopes et al., 2002
PceGA	3	1	1	Sour cherry	Downey and Iezzoni, 2000
Pchcms	3	1	1	Peach	Sosinski et al., 2000
Pchgms	8	3	3	Peach	Sosinski et al., 2000; Wang et al., 2002
PMS	3	0	0	Peach	Cantini et al., 2001
PS	4	2	2	Sour Cherry	Joobeur et al., 2000
UCD-CH	8	2	1	Sweet cherry	Struss et al., 2003
UDA	41	15	14	Almond	Testolin et al., 2004
UDAp	28	9	9	Apricot	Messina et al., 2004
UDP	15	8	6	Peach	Cipriani et al., 1999
Total	**241**	**108**	**96**		

4.6 Estimation of water use efficiency of breeding stock

Increased crop water use efficiency is a high priority for any irrigated crop in Australia. This can be achieved in a number of ways including increasing harvest index, increasing efficiency of irrigation water use, or increasing transpiration ratio. Various agronomic techniques can also influence the total crop water use efficiency, ie planting densities etc. Harvest index and transpiration ratio are the two plant-based parameters that are mainly determined by gene x environment. Transpiration ratio can be screened across a population of seedlings by determining the extent to which the heavier isotope of carbon (C^{13}) is discriminated against during gas exchange in the leaf and photosynthesis. This parameter is measured relative to standards and expressed as a delta ratio. The values are generally proportional to transpiration ratio of the particular genotype (eg for grapevines Gibberd et al., 2001). This technique has been used in other breeding programs, for example the successful breeding of a more water use efficient bread wheat (Drysdale) by CSIRO (Condon et al., 2004).

Five different breeding lines of almond (Carmel x Tarraco, Johnston x Lauranne, Nonpareil x Tarraco, Nonpareil x Lauranne and Nonpareil x Vayro), were selected with 4 replicates for each cross. Each tree was grown in a 30 cm pot containing coco peat mix (2/3 peat, 1/3 sand) plus slow release fertiliser. Pots were maintained in a greenhouse set at 26^{o}C with a 12 hour day/night light regime.

From 11/07/2011 to 09/08/2011, each week one replicate of each cross was moved to a growth chamber for exposure to a constant environment. The temperature in the growth chamber was 22^{o}C and the light regime was set at 12 hours light/dark. After one week, assimilation rate (*A)*, evaporation rate (*E)* and stomatal conductance (g_s) of three leaves from each plant were measured using a Li-COR Biosciences portable photosynthesis system (Model LI-6400; LI-COR, Lincoln, Nebraska, USA). It is important to note that the leaf chamber was equipped with an extra light source in order to measure gas exchange under light saturated conditions. The photosynthetic photon flux density (PPFD) was set at 1500 μmol photons m^{-2} s^{-1} which is more than is required to achieve light-saturation in almonds (Egea et al., 2012). The external CO_2 concentration was set at 400 μmol mol^{-1}, temperature was 22˚C and air flow rate was 350 mmol s^{-1}. The relative humidity was kept nearly constant throughout the experiment (50-55%).

For each plant, three upper undamaged, fully expanded and healthy leaves were selected for measurement. All measurements were performed on the same branch and every measured leaf was tagged with a unique number. After that the tagged branch was separated from the plant for measuring the leaf specific hydraulic conductance of a shoot using the Hydraulic Conductance Flow Meter (HCFM, Dynamax, Houston, Texas, USA). The branch was cut in water to avoid the entrance of air bubbles into the veins and vessels (Figure 4.6.1). The transient method was used to measure flow rate as a function of pressure as outlined in (Vandeleur, 2007) to give the conductance (*k*).

Leaf area of all the separated leaves was measured with an AM300 Portable Leaf Area Meter and the leaves were dried in the oven. The measured conductance was normalised by dividing by total leaf area to give (L_{shoot}) ($kg.s^{-1}.MPa^{-1}.cm^{-2}$). Intrinsic water use efficiency (WUE_{intr}) was calculated as A/g_s.

Carbon isotope measurement

At the end of the experiment leaf samples were taken from each tree from the 5th node and used for C^{13} discrimination (Δ) measurement. Leaf samples were dried at a temperature of 61˚C for 3 days. After grinding and weighing into 3-4 mg capsules, the isotope composition (δ) was measured using a mass spectrometer. The carbon isotope discrimination Δ was calculated as the following equation (Hubick et al., 1986):

$\Delta = (\delta_a - \delta_p)/(1 + \delta_p)$

δ_a= carbon isotope composition in atmosphere (-7.6×10^{-3})

δ_p = carbon isotope composition in plant tissue

HCFM methodology

The *k* was measured with a Hydraulic Conductance Flow Meter (HCFM, Dynamax, Houston, Texas, USA). First, the HCFM was tightly attached to an almond branch (Figure 4.6.1). A suitably chosen rubber for fitting the branch in the compression fitting (CF), reduced the probability of leakage. The nitrogen gas flow was regulated by a needle valve (NV) connected to a captive air tank (CAT). This tank, contained degassed water and air, and was either pressurised or depressurised by regulating the NV. The pressurisation rate increased linearly. There were two series of the 8-way manifolds comprised of inlet and outlet manifolds. These two manifolds were connected to pressure transducer 1 (PT1) and pressure transducer 2 (PT2). They were also connected to each other by 6 capillary tubes which varied in diameter. The flow rate was calibrated by passing water through the different capillary tubes between the two transducers. Also there was a dual channel A/D circuit which was used to regulate the pressure transducers (Vandeleur, 2007).

To study the non-stomatal limitations of photosynthesis, three cultivars of almond trees (Nonpareil, Carmel and Masbovera) were selected for measuring g_s at field capacity. This experiment was started on 7 June 2012 and ended on 15 June 2012. Normally in the field, these trees would be dormant at this time of year, however in the glasshouse the trees remain evergreen under these conditions. Four replicates of each cultivar were grown in the same conditions as the previous almond crosses in the glasshouse. The soil surface was covered by white polyethylene beads to minimize water evaporation from the soil. Pots were adequately watered, were left to drain for 8 hours to reach constant weight. In the next step the weight of pots, representing field capacity, was recorded. Every second day thereafter, pots were re-weighed and watered to field capacity. During one week the g_s of leaves was recorded daily by a leaf porometer (Decagon, model SC). The data were analysed by ANOVA using Statistical Analysis Software (SAS Institute Inc. 2004, SAS/STAT, 9.1).

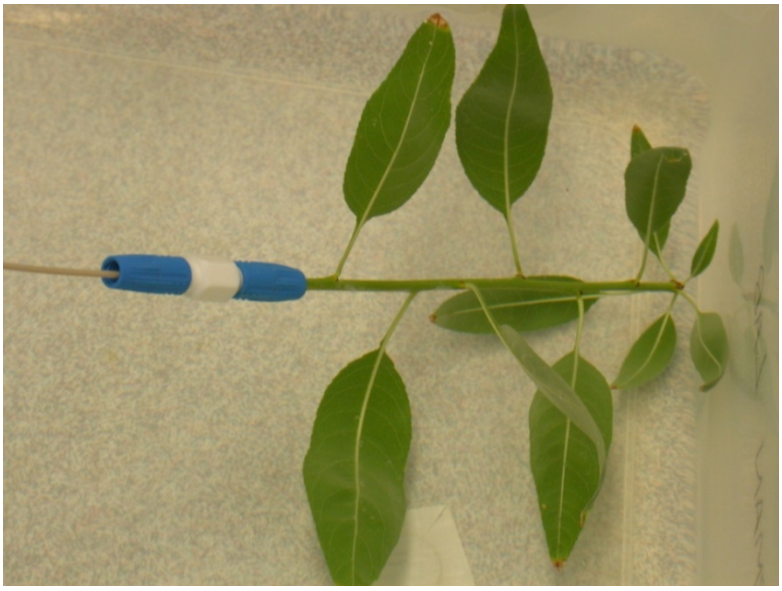


Figure 4.6.1. After cutting under water, the branch is tightly attached to the tube connected to the HCFM.

5. Results

5.1 Performance evaluation of local and imported selections and rootstocks

5.1.a Rootstock trial

At the time of writing, the rootstock trial was still to be planted. The results will be reported in the Final report for Project AL11012.

5.1b Scion trial

Table 5.1.1 shows the characteristics of the cultivars imported from California. The table contains the best information currently available for these cultivars and includes information in regard to existing cultivars for comparison. This is the second season of evaluations.

Table 5.1.2 shows the characteristics of the new cultivars imported from Spain. Phenology observations began in the first week of August through to mid October. Flowering was heavy for most cultivars, especially Tarraco which had a very long period of visible pink buds in 2011 but this did not transfer into high yields in 2012. Generally full bloom was one week later for most cultivars compared to Nonpareil, although Marcona was a week earlier. A number of cultivars are self-fertile, namely; Vayro, Constantí and Francolí. All are hard-shelled with relatively thin hulls that peel away easily at harvest, which is an ideal trait for in-field hull removal. 2012 was the first cropping year with no individual yields recorded (harvested as mixed nuts). 2013 yield results are shown in Table 5.1.2 (4^{th} leaf).

Most of the cultivars are freely available, as is the current situation, but a few are patented. These patented cultivars are also available but require growers to sign a “Non-propagation Agreement” and pay a royalty to the cultivar owners in California.

Table 5.1.1. Californian almond varietal characteristics at April 2013.

Variety	***Yield (kg/ha)***	***Flowering time***	***Harvest date***	***Shell***	***Kernel***	***Crackout***	***Market type/Use***	***Disease Susceptibility***		***Comments***
								Bact. spot	Hull Rot	
Padre	4082	+ 5 days Nonpareil	+20 days Nonpareil	Hard	Small, 0.99 g	24%	Mission/ Roasting	T	S	V. upright growth, not fruitful. Butte/Padre combination is the top producer in US.
Livingston	4030	+ 6 days Nonpareil	+18 days Nonpareil	Papershell	Small, 1.19 g	34%	California/ Blanching	NA	T	Reasonably attractive kernels.
Butte	3553	+ 4 days Nonpareil	+20 days Nonpareil	Semi Hard	Small, 1.08 g	27%	Mission/ Roasting	S+	T	Most productive almond variety in California. Very fruitful habit in Aust.
Sonora	4262	- 6 days Nonpareil	+10 days Nonpareil	Papershell *Poorly sealed*	Medium, 1.24 g	31%	California/ Blanching	NA	S	Frost sensitive and tendency to alternate bearing.
Monterey	3689	- 5 days Nonpareil	+22 days Nonpareil	Soft	Large, 1.57 g	31%	California/ Blanching	NA	T	Large, elongate dark kernels. Up to 20% doubles.
Avalon *	2860	- 3 days Nonpareil	+20 days Nonpareil	Semi Soft	Medium, 1.24 g	28%	California/ Blanching	NA	T	Relatively new variety, little acreage to date. Long flowering, overlaps Carmel
Wood Colony *	3561	+ 2 days Nonpareil	+20 days Nonpareil	Semi-soft	Medium, 1.22 g	34%		NA	T	Californian Trials indicate shy bearing at 35% Nonpareil. Tasteless kernel.

* Patented variety T: Tolerant S: Susceptible NA: Information not available to date

Please note: This information is collated from a number of sources. There will be variation within regions in Australia, therefore flowering and harvest times and comments regarding disease susceptibility and cropping potential are to be used as a guide only.

Table 5.1.2. Spanish almond varietal characteristics at April 2013.

Variety	***Yield (kg/ha)***	***Flowering time***	***Harvest date***	***Shell***	***Kernel***	***Crackout***	***Disease Susceptibility*** Bact. spot	***Comments***
R1065	543	+ 7 days Nonpareil	NA	Hard	Large, 1.5 g	15%	T	Reasonably attractive kernels.
Tarraco*	1437	+ 11 days Nonpareil	NA	Hard	Large, 1.48 g	21%	S	Self-incompatible, medium vigour, early bearing.
Vayro*	809	+ 9 days Nonpareil	NA	Hard	Medium, 1.37 g	21%	T	Self fertile, good Autogamous level. Vigorous.
Glorieta	1278	+ 6 days Nonpareil	NA	Hard	Medium, 1.31 g	19%	T	Strong vigour, drought tolerant.
21-332	381	+ 14 days Nonpareil	NA	Soft	Large, 1.46 g	17%	S	Unattractive, semibitter kernels,
Constantí*	1161	+ 7 days Nonpareil	NA	Hard	Small, 1.02 g	18%	T	Self fertile. Many windfalls. Vigorous. Drought tolerant.
Francolí	1512	+ 9 days Nonpareil	NA	Hard	Small, 1.11 g	21%	S	Self-fertile. Vigorous.
Marcona	1794	- 7 days Nonpareil	NA	Stone hard	Small, 1.07 g	20%	T	Many windfalls.
Masbovera	1343	+ 6 days Nonpareil	NA	Hard	Small, 1.19 g	19%	T	Strong vigour. Many windfalls. Drought tolerant.

* Patented variety T: Tolerant S: Susceptible NA: Information not available to date

Please note: This information is collated from a number of sources. There will be variation within regions in Australia, therefore flowering and harvest times and comments regarding disease susceptibility and cropping potential are to be used as a guide only.

5.2 Controlled crossing to produce seedlings for performance evaluation, and selection of new improved cultivars

Primary evaluation of progeny from breeding program
Table 5.2.1 shows a summary of the crosses achieved from 2008 to 2010 and the resulting progeny numbers.

Table 5.2.1. Crosses achieved and progeny numbers for 2008 – 2010.

Year crossed	Female	Male	# Progeny produced
2008	Johnston's Prolific	UA-2	102
	Johnston's Prolific	21-323	114
	Johnston's Prolific	22-120	74
	Johnston's Prolific	4bT1	55
	Johnston's Prolific	Somerton	105
	Nonpareil	21-169	139
	Nonpareil	4bT1	189
	Nonpareil	Chellaston	77
	Nonpareil	Johnston's Prolific	101
	Nonpareil	R1065	120
	Nonpareil	R1146	84
	Nonpareil	Somerton	155
2009	21-169	4bT1	26
	21-169	Nonpareil	149
	Chellaston	R30T45	161
	Chellaston	Lauranne	195
	Ferraduel	Nonpareil	52
	Ferraduel	Chellaston	98
	Ferrastar	Chellaston	39
	Johnston's Prolific	4bT1	169
	Nonpareil	21-169	89
	Nonpareil	22-120	165
	Nonpareil	Chellaston	39
	Nonpareil	Ferraduel	27
	Nonpareil	Lauranne	90
2010	12-350	R42T106	27
	UA-3	12-350	238
	UA-3	21-323	161
	UA-3	UA-5	32
	Antoñeta	Nonpareil	215
	Carmel	UA-2	73
	Chellaston	UA-1	51
	Nonpareil	12-350	26
	Nonpareil	21-323	65

	Nonpareil	UA-5	47
	Nonpareil	White	79
	UA-1	Nonpareil	111
	Somerton	UA-5	154

To date all progeny produced from 1997 to 2005 have been evaluated on their kernel characteristics. The project is currently evaluating progeny from 2006 and 2007. Primary evaluations will continue into the new project AL13000.

The large amount of data generated from the evaluations was used to calculate heritability estimates and estimated breeding values of the parents used. To date, only data generated from the 1997-2003 progeny has been analysed. The heritability estimates of some of the traits measured are shown in Table 5.2.2.

Table 5.2.2 Heritability estimates of some almond traits.

	Appearance	Colour	Hardness	Kernel wt	% Doubles	Shell wt
Heritability	0.29	0.38	0.90	0.48	0.20	0.89

Secondary evaluation of progeny from breeding program

Eighteen superior selections from the primary evaluation were grafted onto Nemaguard rootstock along with comparator and reference cultivars (five of each selection and cultivars). These were planted at Lindsay Point in August 2006 at commercial spacing of 5 x 7 m under drip irrigation. Comparator and reference cultivars used were Ferragnès, Ne Plus Ultra, Aï, Mission, Marcona, Peerless, Nonpareil, Tardy Nonpareil, Monterey, Somerton, and Guara. The planting plan is shown in Appendix 1. This trial was first harvested in 2009 (3rd leaf) and every subsequent year. All measurements required for PBR have been taken on the most productive lines.

Each year at flowering, one branch from each tree replicate of the selections, was bagged to exclude insects, in order to measure the level of autogamy; the ability to self-pollinate. There was marked variation between years and selections in percentage self set. Inclement weather at flowering time in the 2011 may have impacted on that year's result.

A second trial was planted in 2010, also at Lindsay Point. This trial contains six superior selections grafted onto GF677 rootstock along with comparator cultivars Guara, Sonora, Somerton, Chellaston, Nonpareil and Mission. The planting plan is shown in Appendix 2. The first harvest from this trial was in 2013 (3rd leaf). Two selections have out performed Nonpareil based on yield and kernel size, UA-8, UA-9 and UA-10. Several selections have also produced kernels much larger than Nonpareil, however with lower yields.

Flowering phenology was measured on all parent trees and other accessions planted at the Waite campus. Flowering times were also recorded each year on the selections and comparators in the secondary trials. Flowering phenology is shown in Appendix 3.

Assessment of Bacterial spot tolerance

Water-soaked areas on the leaves caused by MilliQ water soon disappeared (Figure 5.2.1), tiny yellowish marks caused by the syringe occurred at a few sites. Water-soaked spots developed 9-14 days after inoculation on the 4-5mm diameter areas infiltrated with almond strain inoculum suspension. The symptom was discrete and dark necrotic areas (Figure 5.2.2). During the inoculation, inoculum suspension was frequently spilled at random, but the symptoms developed only at infiltrated area.

Table 5.2.7 shows the percentage of infected sites and the diameter of water-soaked spots. There was a significant difference between the percentages of infected sites indicated by analysis of variance, and also significant difference in diameter of bacterial spot legions between cultivars was observed. Padre showed the least number of infected sites but not significantly less than three of the selections, UA-3, UA-2 and UA-1. The diameter of water-soaked legions on leaves of Padre, UA-2 and UA-1 was significantly lower than that of Fritz and Butte.

Figure 5.2.1. Control leaf - detached leaf of Nonpareil inoculated with water

Figure 5.2.2. Detached leaf of Butte inoculated with *X. campestris* pv *pruni* showing symptom of dark necrotic legions.

Table 5.2.7. Detached leaf response to *Xanthomonas campestris* pv. *pruni* at 28°C under dark incubation condition.

Cultivars	Sites inoculated on leaves	Percentage of infected sites¥	Average diameter of necrotic legions (mm)¥
Fritz	90	60.0^{ab}	9.51^{a}
Nonpareil	90	40.0^{b}	4.10^{c}
Butte	90	75.6^{a}	5.21^{b}
Padre	90	1.1^{d}	0.56^{e}
UA-3	90	12.2^{cd}	2.94^{d}
UA-2	90	12.2^{cd}	1.02^{e}
UA-5	90	65.6^{a}	4.27^{c}
UA-1	90	11.1^{cd}	1.01^{e}
UA-4	90	34.4^{bc}	2.86^{d}

¥Numbers in columns followed by the same letter were not significantly different according to ANOVA at $P<0.001$.

5.3 Virus detection in breeding stocks, selections and budwood repository and any required virus elimination

Detection of PNRSV and PDV by RT-PCR

From 2008 to 2011, PNRSV was detected in 57 out of 1,334 leaf samples, and PDV was detected in only 14 samples tested using RT-PCR (Table 5.3.1). Multiplex RT-PCR resulted in the amplification of a 351 bp product for PNRSV and the combination of RT-PCR with nested PCR produced an amplification product of 241 bp for PDV in some Australian almond samples and a sample extracted from CEBAS1, a Spanish cultivar. The sequence of the amplified product matched the sequence of the virus coat protein of PNRSV located between the primers.

Trees that tested positive at Monash budwood repository were removed from the site and any parent trees that tested positive were not used again. No trees underwent thermo therapy.

Detection of ApMV and ACLSV by RT-PCR

All of the samples tested negative for ApMV and ACLSV.

Table 5.3.1. Results of virus testing of Monash samples, breeding parents and selections.

Year	No. trees tested	No. trees positive for PNRSV	No. trees positive for PDV
2008	408	2	14
2009	402	20	0
2010	441	27	0
2011	43	3	0
2012	40	5	0

5.4 Analysis of free fatty acid profiles in almond selections and breeding stock

Biochemical testing first began in 2009 on all the selections in the 1st secondary trial plus comparators. The fatty acid profile was determined from the extracted lipids from each sample. Table 5.4.1 shows the profile from 2009. Numbers in red indicate the value of the selection is greater than Nonpareil. Eleven of the selections have higher levels of oleic acid (18:1), six selections have higher levels of linoleic acid (18:2) and two have higher lipid levels. NPU had the highest linoleic acid level at 30.78% and Somerton had the highest lipid content at 49.98%.

Table 5.4.2 shows the profiles from 2010 sampling. Again eleven selections showed higher levels of oleic acid, six selections have higher levels of linoleic acid and nine have higher lipid levels than Nonpareil. For that year R30T45 had the highest linoleic acid level at 22.94% and 4bT1 had the highest lipid content at 57.95%. Carmel had the highest linoleic acid level at 29.83%.

Table 5.4.3 shows the results from 2012 of a regional trial comparing cultivars and seven of the better producing selections, from Willunga area, Adelaide plains, Riverland and Sunraysia growing districts. Unsaturated fatty acids (USFA) consisted of oleic acid (58.53 – 70.77% of total lipids), linoleic acid (18.9 – 29.9% of total lipids), palmitoleic acid (0.20 – 0.53% of total lipids) and vaccenic acid (0.8 – 1.5% of total lipids) which made up more than 90% of the total lipids. Saturated fatty acids (SFA) including palmitic acid (5.9 – 7.47% of total lipids), stearic acid (1.0 – 2.43% of total lipids), arachidic acid (0.07 – 0.10% of total lipids) and myristic acid (0.02 – 0.05% of total lipids, data not shown) accounted for less 10% of the total lipids. This result shows USFA oleic acid and linoleic acid as the dominant fatty acids, followed by SFA palmitic acid and stearic acid, as well as other minor USFA and SFA. Riverland grown selection UA-4 had the highest oleic acid level (70.77%), Willunga grown Nonpareil had the highest linoleic acid level at 29.93% and the highest lipid level at 63.5%.

Table 5.4.1. Fatty acid profile from 2009 sampling of seventeen selections and comparators from the 1st secondary trial.

Variety	C16:0 %	C16:1 %	C18:0 %	C18:1 %	C18:2 %	C20:0 %	C18:1/ C18:2	SFA[z] %	UFA[z] %	MUFA[z] %	PUFA[z] %	MUFA/PUFA	oil %
10bT35	7.45	0.54	1.42	65.09	25.01	0.07	2.60	9.06	90.94	65.76	25.08	2.62	45.39
2bT33	7.32	0.40	1.69	63.73	26.50	0.08	2.41	9.18	90.82	64.24	26.49	2.43	42.44
4bT1	6.86	0.42	1.49	69.90	20.98	0.06	3.33	8.43	91.57	70.56	21.01	3.36	43.76
R30T25	6.76	0.40	1.29	65.90	25.19	0.07	2.62	8.26	91.74	66.52	25.22	2.64	49.19
R30T45	7.53	0.50	1.76	59.58	29.91	0.08	1.99	9.54	90.46	60.28	30.07	2.00	47.78
R33T48	6.45	0.40	1.40	69.58	21.72	0.07	3.20	8.05	91.95	70.21	21.74	3.23	46.93
R38T63	6.80	0.61	1.40	71.09	19.64	0.11	3.62	8.37	91.63	71.96	19.67	3.66	48.44
R42T106	7.36	0.39	1.33	67.70	22.82	0.09	2.97	8.89	91.11	68.27	22.85	2.99	45.83
R58T27	6.73	0.33	1.40	71.29	19.89	0.07	3.58	8.25	91.75	71.84	19.92	3.61	47.30
R61T33	6.41	0.45	1.15	70.77	20.78	0.06	3.41	7.73	92.27	71.46	20.80	3.43	46.64
Guara	6.05	0.24	2.47	71.12	19.86	0.11	3.58	8.69	91.31	71.42	19.88	3.59	42.22
Lauranne	7.03	0.54	1.69	69.11	21.27	0.12	3.25	8.91	91.09	69.81	21.29	3.28	49.24
Marcona	6.15	0.40	1.64	69.61	21.84	0.09	3.19	7.89	92.11	70.24	21.87	3.21	46.73
Monterey	6.59	0.29	1.37	67.55	23.81	0.09	2.84	8.13	91.87	68.03	23.77	2.86	49.05
Nonpareil	**6.59**	**0.46**	**1.64**	**68.68**	**22.23**	**0.08**	**3.09**	**8.38**	**91.62**	**69.30**	**22.23**	**3.12**	**48.31**
NPU	6.70	0.31	1.45	60.35	30.78	0.08	1.96	8.28	91.72	60.90	30.77	1.98	44.33
Somerton	5.89	0.35	2.14	72.29	18.76	0.12	3.85	8.30	91.70	72.90	18.80	3.88	49.98

[z] SFA: Saturated Fatty Acids; UFA: Unsaturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids

Table 5.4.2. Fatty acid profile from 2010 sampling of seventeen selections and comparators from the 1st secondary trial.

Variety	C16:0 %	C16:1 %	C18:0 %	C18:1 %	C18:2 %	C20:0 %	C18:1/ C18:2	SFA[z] %	UFA[z] %	MUFA[z] %	PUFA[z] %	MUFA/PUFA	oil %
10bT35	7.01	0.54	1.37	68.34	19.38	0.06	3.53	8.44	91.56	68.9	19.38	3.56	53.33
2bT33	6.84	0.61	1.22	70.67	17.61	0.05	4.01	8.11	91.89	71.3	17.61	4.05	50.59
4bT1	6.98	0.46	1.37	72.28	16.31	0.05	4.43	8.40	91.60	72.75	16.31	4.46	57.95
R3Tt25	7.33	0.58	1.25	66.55	21.44	0.05	3.10	8.63	91.37	67.15	21.44	3.13	55.6
R30T45	7.66	0.63	1.43	64.79	22.94	0.05	2.82	9.14	90.86	65.42	22.95	2.85	56
R33T48	6.82	0.52	1.44	66.65	21.76	0.05	3.06	8.31	91.69	67.18	21.76	3.09	55.02
R38T63	6.69	0.73	1.25	73.47	14.69	0.05	5.00	7.99	92.01	74.22	14.69	5.05	51.65
R42T106	6.93	0.46	1.4	70.16	18.21	0.05	3.85	8.38	91.62	70.64	18.21	3.88	53.46
R58T27	6.82	0.53	1.68	71.91	16.18	0.06	4.44	8.56	91.44	72.45	16.19	4.47	53.47
R61T33	6.38	0.52	1.12	73.87	14.94	0.05	4.94	7.55	92.45	74.41	14.94	4.98	55.55
Aï	6.35	0.43	1.77	68.69	19.4	0.07	3.54	8.19	91.81	69.13	19.41	3.56	53.53
Ferragnès	5.63	0.38	1.9	74.73	14.34	0.07	5.21	7.60	92.40	75.13	14.35	5.24	56.21
Guara	6.61	0.35	2.01	69.12	18.68	0.1	3.70	8.72	91.28	69.49	18.69	3.72	51.12
Marcona	6.57	0.56	1.65	68.32	19.69	0.06	3.47	8.28	91.72	68.88	19.69	3.50	53.36
Mission	6.62	0.38	1.54	66.86	21.24	0.06	3.15	8.22	91.78	67.26	21.24	3.17	52.25
Monterey	6.22	0.42	1.55	71.97	16.91	0.06	4.26	7.83	92.17	72.41	16.91	4.28	53.44
Nonpareil	**6.99**	**0.6**	**1.46**	**69.75**	**18.32**	**0.06**	**3.81**	**8.51**	**91.49**	**70.36**	**18.32**	**3.84**	**53.58**
NPU	7	0.47	1.41	65.84	22.66	0.06	2.91	8.47	91.53	66.32	22.66	2.93	53.64
Peerless	7.03	0.49	1.31	67.58	20.32	0.06	3.33	8.40	91.60	68.09	20.32	3.35	53.12
Somerton	5.55	0.51	2.34	75.87	12.97	0.07	5.85	7.96	92.04	76.4	12.98	5.89	56.32
TNP	6.24	0.45	1.43	74.2	14.28	0.05	5.20	7.72	92.28	74.66	14.28	5.23	57.24

[z] SFA: Saturated Fatty Acids; UFA: Unsaturated Fatty Acids; MUFA: Monounsaturated Fatty Acids; PUFA: Polyunsaturated Fatty Acids

Table 5.4.3. Fatty acid composition from 2012 sampling of seven superior selections compared with cultivars from various regions.

Genotype	Site	palmitic (C16:0)	palmitoleic (C16:1n-7)	stearic (C18:0)	arachidic (C20:0)	vaccenic (C18:1n-7)	oleic (C18:1n-9)	linoleic (C18:2n-6)	Ratio Oleic/Linoleic	Oil %
Nonpareil	Riverland	**6.7**	**0.43**	**1.27**	**0.07**	**1.3**	**63.6**	**24.8**	**2.57**	**56.5**
	ADL plains	6.87	0.5	1.43	0.1	1.3	64.23	23.87	2.69	59.98
	Willunga	6.77	0.4	1.4	0.1	1.2	58.53	29.93	1.96	63.5
	Sunraysia	6.93	0.5	1.07	0.1	1.5	63.77	24.4	2.61	57.66
Johnston	ADL plains	6.67	0.53	1.8	0.1	1.27	66.93	21	3.19	58.22
	Willunga	6.87	0.4	2.0	0.1	1.1	62.97	25	2.52	60.75
Somerton	Riverland	5.9	0.4	1.7	0.1	1.2	70.13	18.9	3.71	59.42
	Willunga	6.07	0.4	2.43	0.1	1	66.97	21.4	3.13	60.45
Chellaston	ADL plains	6.8	0.43	1.97	0.1	1.2	66.6	21.27	3.13	58.33
Peerless	Riverland	6.9	0.4	1.07	0.1	1.3	61.13	**27.30**	2.24	54.53
Price		6.23	0.3	1.27	0.1	1.2	63.20	**25.93**	2.44	53.09
Carmel		6.8	0.4	1.17	0.07	1.2	58.6	**29.83**	1.96	53.66
Guara		5.9	0.2	2.4	0.1	0.8	**66.43**	22.33	2.97	**60.41**

ADL, Adelaide plains

5.5 Adoption of molecular techniques to facilitate the breeding program

5.5.a DNA fingerprinting

The *S*-allele primers produced mostly two bands in each cultivar, however some cultivars produced only one band (Table 5.5.1). In general most cultivars could be placed into an existing incompatibility group. No Australian cultivars contained the self compatible *Sf* allele.

Amplification of the 13 SSR loci was successful in all the almond genotypes included in this study. The SSR markers showed variation in the number of alleles identified, varying from 9 alleles for primers CPPCT006, CPPCT022, CPSCT021 and PMS40, to 17 alleles for primer CPDCT045 (Table 4.5.2). All primers produced a maximum of two bands per genotype in accordance with the diploid level of almond. Genotypes showing a single band were considered homozygous for that particular locus. All linkage groups were covered except for linkage group 1. A dendrogram was constructed from the SSR marker data and is shown in Figure 5.5.1. In this preliminary work 28 accessions were analysed and these clustered into one large group and four smaller groups.

Table 5.5.1. *S* genotypes deduced from amplification products of some Australian and imported cultivars.

Cultivar	**No. of accessions**	**Origin**	**First intron bands**		**Second intron bands**		***S*-alleles**	**Possible parents/ancestors**
Tom Strout	1	Australia	371	419	1000	1200	$S_{13/19}S_{22}$	Kapareil/Jordan
Somerton	2	Australia	-	375	685	742	S_1S_{23}	NPU/Jordan
Brown Nonpareil	1	Australia	878	346	745	1750	S_1S_7	NPU/Nonpareil
Strout's papershell	1	Australia	433	325	1217	833	$S_{22}S_{25}$	Jordan/?
Johnston's	1	Australia	379	321	710	813	$S_{23}S_{25}$	Jordan/?
Pethick Wonder	1	Australia	371	381	700	1317	S_{23}/S_{27}	Jordan/?
Bigg's hardshell	2	Australia	554	1015	358	1143	S_6S_{14}	Peerless/Jordanolo
White Brandis	1	Australia	346	367	558	692	S_6S_{23}	Peerless/Jordan
Keane's	2	Australia	358	381	1711	1333	S_7S_{27}	Nonpareil/?
Pearce	2	Australia	360	384	2133	685	S_8S_{23}	Nonpareil/Jordan
Frenzy	1	Australia	-	405	283	2233	S_5S_8	Carmel/Nonpareil
Bruce	1	Australia	366	410	667	1250	$S_{22}S_{23}$	Jordan/?
Monarto 1	1	Australia	-	432	745	1250	S_1S_{22}	NPU/Jordan
Monarto 2	2	Australia	325	354	825	2250	S_8S_{25}	Nonpareil/?
Monarto 3	1	Australia	370	400	1107	1329	$S_{13/19}S_{27}$	Kapareil/?

Monarto 4	1	Australia	-	375	685	720	S_1S_{23}	NPU/Jordan
Marion St	1	Australia	414	594	736	1240	S_1S_{22}	NPU/Jordan
Parkinson	1	Australia	389	427	692	1260	$S_{22}S_{23}$	Jordan/?
Atkinson's hardshell	1	Australia	381	1129	988	1400	$S_{14}S_{27}$	Jordanolo/?
Nonpareil	1	USA	350	400	1733	2200	S_7S_8	?
Carmel	1	USA	350	400	280	2314	S_5S_8	Nonpareil/?
Ferragnès	1	France	333	594/ 891	727	906	S_1S_3	?
Lauranne	1	France	333	413	853	905	S_3S_f	Ferragnès x Tuono

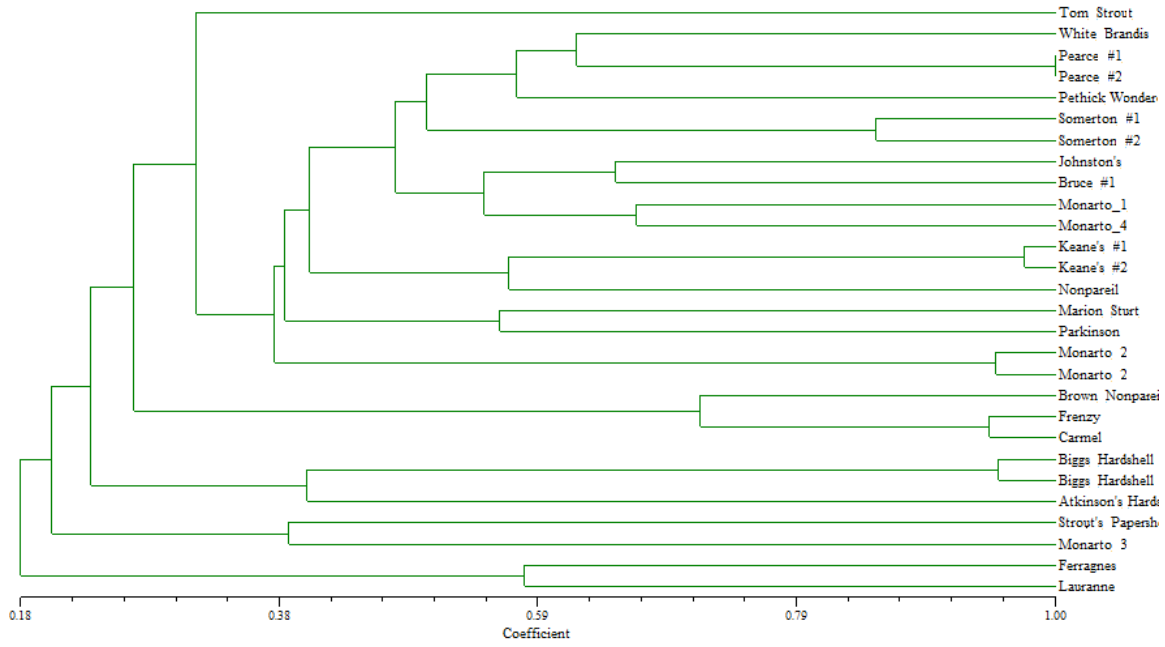


Figure 5.5.1. UPGMA dendrogram constructed from SSR marker data showing genetic relationships.

5.5.b Genetic mapping of almond

Marker polymorphism

Altogether, 179 markers showed polymorphism in the population under analysis. Of these, 92 (51.4%) were heterozygous in both parents, 34 with 4 alleles, 37 with 3 alleles, and 21 with 2 alleles; 40 (22.3%) were heterozygous only in Nonpareil; and 47 (26.3%) were heterozygous only in Lauranne. SSR markers BPPCT009, CPDCT020, CPSCT039 and UDAp-479 demonstrated multi-locus amplifications, each with two loci, across the population. The mapping results (see

following description) indicated, however, that the loci of BPPCT009 were located closely in the same linkage group (at a distance of 6.0cM).

Segregation of the markers

Of 179 markers analysed, 147 (82.1%) segregated in the expected ratio following the Mendelian segregation model, and 32 (17.9%) showed skewed segregations ($P < 0.05$). Among those, 19/113 (16.8%) SSR, 6/34 (17.6%) ISSR, 5/14 (35.7%) RAPD, and 2/14 (14.3%) SNP markers segregated in skewed ratios in the population. Following grouping of the markers in the mapping process, 50% (10/20) of the markers grouped in G7 had skewed segregation ratios, which was extremely high compared to the average skewed marker percentage. Interestingly, both skewed SNP markers were grouped in G7. To avoid using biased framework map as the fixed order for final map construction, all the skewed markers in G7 were included in the first step of mapping, and second step mapping for this group was omitted.

A plot of genotype frequency χ^2 values of the loci in the G7 versus their map positions is shown in Fig. 5.5.2. A main peak was identified in the area between markers MA020 and OPC07-1688. While a few markers with low χ^2 values were present in the adjacent areas of the peak, a trend that the χ^2 values declined gradually towards two ends of the linkage group was clearly illustrated.

Linkage maps constructed using One-step and Two-step methods

A final map of NxL containing 158 markers (94 SSRs, 35 ISSRs, 18 SNPs/INDELS, and 11 RAPDs) and covering 602.7 cM of the genome was constructed using the One-step method of JoinMap® (Table 5.5.2). The average marker distance of the map was 4.0 cM, and 28 skewed markers were mapped in the genome of the population. Individually, G1 was the longest group covering 108.2 cM of the genome with 22 markers mapped, and G5 the shortest group covering 54.8 cM with 17 markers mapped. The average marker distance varied from 2.4 (G6) to 5.7 (G8) cM. As indicated previously, G7 mapped the highest number of segregation ratio skewed markers (10), whereas G5 had no skewed markers in the group.

Table 5.5.2. The number of loci, linkage group size, average marker distance, and number of markers segregated in skewed ratios of the maps constructed using One-step and Two-step methods.

	One-step				Two-step			
Group	Number of loci	Size (cM)	Average marker distance (cM)	Number of skewed markers	Number of loci	Size (cM)	Average marker distance (cM)	Number of skewed markers
G1	22	108.2	5.2	3	24	113.0	4.9	3
G2	17	67.3	4.2	2	17	72.5	4.5	2
G3	21	69.4	3.5	5	21	72.3	3.6	6
G4	21	83.6	4.2	3	21	89.4	4.5	3
G5	17	54.8	3.4	0	17	54.4	3.4	0
G6	28	65.0	2.4	2	28	72.8	2.7	2
G7	18	80.5	4.7	10	18	81.6	4.8	10
G8	14	73.9	5.7	3	15	76.5	5.5	3
Total/ average	**158**	**602.7**	**4.0**	**28**	**161**	**628.5**	**4.1**	**29**

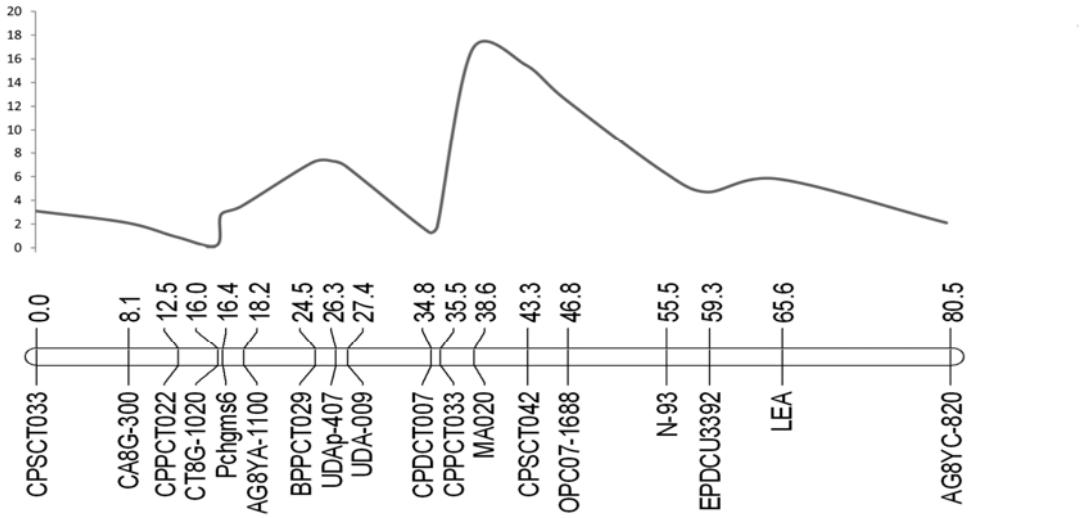


Figure 5.5.2. A plot of genotype frequency χ^2 values of the loci in the G7 versus their map positions resulting from the One-step mapping method.
A main peak was shown in the area between markers MA020 and OPC07-1688. The map of G7 is the x-axis shown in the genetic distance of the loci in centimorgans (cM), and the genotype frequency χ^2 values is the y-axis.

Using the Two-step method, the final map constructed contained 161 markers (96 SSRs, 35 ISSRs, 18 SNPs/INDELS, and 12 RAPDs), and covered 632.5 cM of the genome (Table 5.5.2). The average marker distance of the map was 4.1 cM and 29 skewed markers were mapped in the genome of the population. Similar to the One-step map, G1 was the longest group covering 113 cM of the genome with 24 markers mapped, and G5 the shortest group covering 54.4 cM with 17 markers mapped. The average marker distance varied from 2.7 (G6) to 5.5 (G8). As with the One-step method, G7 mapped the highest number of skewed markers (10) whereas G5 had no skewed markers mapped.

For most of the markers, these two methods produced consistent mapping results as shown in Fig 5.5.3. The linkage groups G5 and G7 produced by the One-step and Two-step methods were completely collinear, and no rearrangement of the loci was observed. Some markers had unstable positions on the other linkage groups when the two maps were compared. Although the divergence in positions were not substantial when most of the markers were subjected to the two different mapping methods, seven markers showed shifts larger than 10 cM, i.e., CT8G-743 and UDAp-479A in G1, UDA-008 in G2 , UDA-014 and CPDCT008 in G3, AG8YT-960 in G4, AG8YC-771 in G6 and AG8YA-763 and OPA08-1175 in G8 with shifts of: 48 cM, 17.7 cM, 43.4 cM, 14.7cM, 12.3 cM, 61.8 cM, and 29.5 cM respectively. Among these, CT8G-743, CPDCT008, AG8YC-771 and AG8YA-763 showed significantly skewed segregation ratios. The segment from marker UDAp-479A to marker CPPCT029 of G1 (in map I) and from marker CA8T-2045 to marker UDP96-019 of G8 (in map I) was inverted between the two maps.

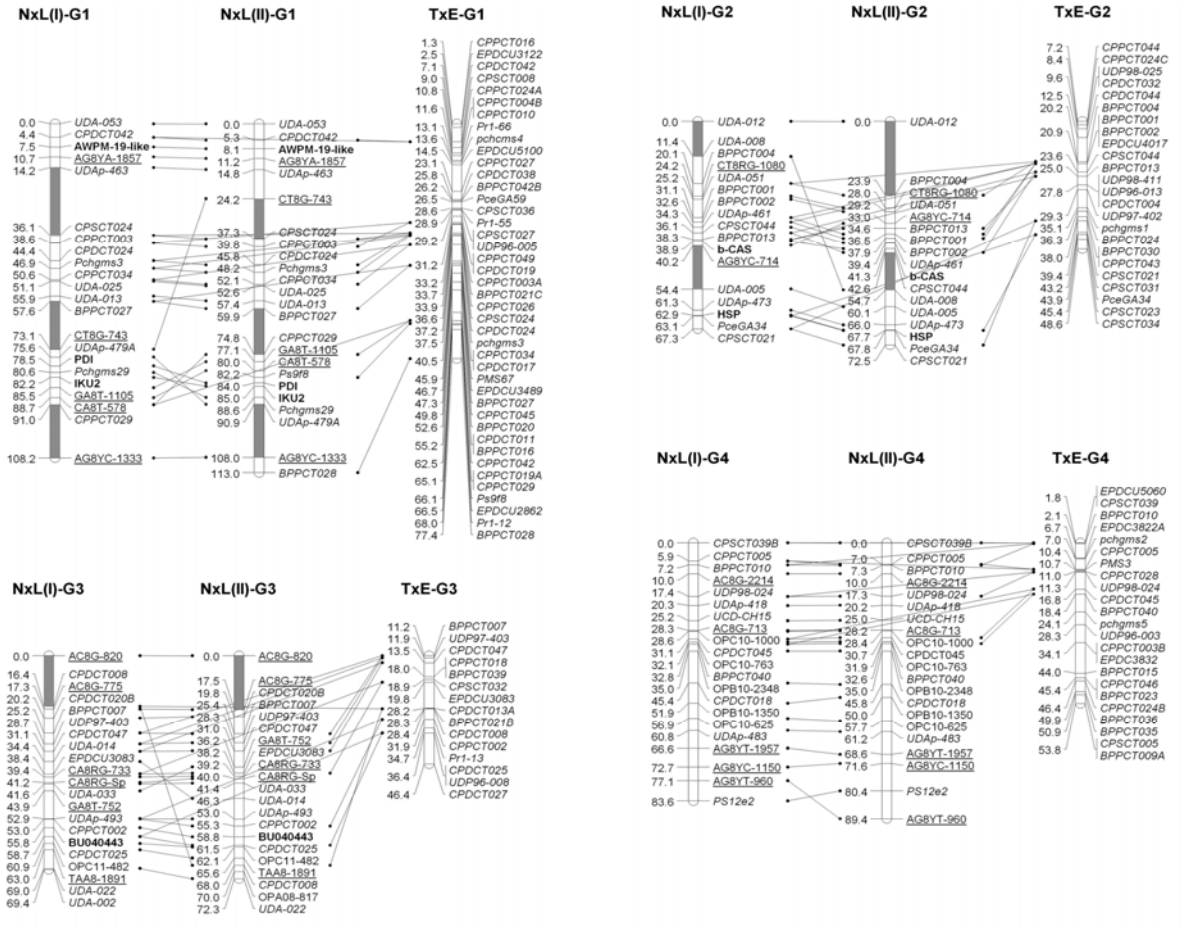
Among the markers mapped in the NxL population, the ISSRs were developed in our initial analysis, and therefore were newly mapped markers in the *Prunus* genome. The SSRs were sourced from published studies, and the majority of the markers were mapped in the same linkage groups as previously reported. However, a few SSR markers were mapped for the first time: CPDCT018 on G4, CPDCT006 on G6, and CPDCT007 on G7. UDA-012 was mapped to G8 of TxE Bin map (Howad et al., 2005), while in this study, it was mapped to G2. CPDCT020 has been mapped to G8 of TxE reference map (Dirlewanger et al., 2004) and “Contender” x “Fla.92-2C” peach map (Fan et al., 2010) as a single locus marker. This marker, however, amplified two loci in the NxL population, and they were located on G3 as well as G8. UDAp-479 also amplified at two loci, which were mapped to G1 and G5 in our study. A recent report identified four loci in the apricot population “Z506-07” (Z) x “Currot” (C) and all four loci were assigned to G8 (Campoy et al., 2010). Two loci of BPPCT009 were mapped to G4 of peach “Ferjalou Jalousia” x “Fantasia” (Dirlewanger et al., 2006) and TxE reference maps (Dirlewanger et al., 2004) for locus A, and G7 of “Ferjalou Jalousia” x “Fantasia” map (Dirlewanger et al., 2006) and G6 of TxE reference map (Dirlewanger et al., 2004) for locus B . In contrast, two loci of BPPCT009 were mapped to G6 of our mapping population in an interval of 8.5 cM.

In the maps constructed by both methods, gaps bigger than 10 cM were observed (segments in grey shown in Fig. 5.5.3). Those included one gap on G3, G5, G7 and G8, two gaps on G2, and three gaps on G1 in the One-step map, and one gap on G3, and G7, two gaps on G2 and G5, and three gaps on G1 and G8 in the Two-step map. The Two-step map had more gaps (12) of > 10cM than the One-step map (9). The biggest gap (23.9 cM) was between the markers UDA-012 and BPPCT004 on G2 of the Two-step map.

Synteny of the NxL and TxE maps and between the almond and peach genomes
The almond NxL and *Prunus* TxE reference genetic maps were compared using common SSR markers to visualise the syntenic regions. As shown in Fig.5.5.3, a high degree of macro-synteny between NxL and TxE was evident across the whole genome. For example, the linkage groups G1,

G4 and G6 did not show any order conflict between the NxL and TxE maps. Despite the high degree of macro-synteny, rearrangements of markers in small sections occurred in the rest of the linkage groups. Furthermore, a few markers showed inconsistency of position over larger distances between the NxL and TxE maps. For instance, the marker CPSCT033 mapped to the top of G5 in NxL maps (I and II), while it was located in the middle segment of TxE 28.4 cM away. The marker CPDCT008 was mapped to the upper part (NxL map I) or the lower part (NxL map II) of G3 but to the lower middle part in TxE. With reference to the TxE map, the NxL map coverage of the genome varied with linkage groups. G1 and G6 covered the whole length of the corresponding groups of TxE; G2, G3, and G7 covered most of their corresponding groups with one end or both ends having no common markers with TxE but covering equivalent lengths; G4 and G5 had fewer markers in common with the TxE map but comparison with maps in the GDR database (http://www.rosaceae.org) indicated full coverage of G4; and G8 alignment showed that at least the bottom part of approximately 10 cM was not mapped in NxL. Hence, this NxL map can be regarded as moderately saturated.

The sequences of the SNP-anchored genes were compared using Blastn with the peach genome v1.0 database, and homologous sequences were located in the scaffolds of the peach genome. As the scaffolds correspond to each of the linkage groups of *Prunus* maps, the relative positions of the genes can be identified in the genome. The results showed that the locations of the majority of the genes mapped in the NxL population agreed with the positions of their homologous sequences in the corresponding peach genome scaffolds (Fig. 5.5.4). AWPM-19-like, however, was located near the top of G 1 rather than in the lower middle part of the group, where the homologous sequence was identified in the peach genome scaffold_1. In G6, the segment involving MT2, dhn3 and AP2 showed inversion compared to the peach genome despite spanning only a small fragment with genetic distance 5.9 cM or DNA length 2.6 Mbp.



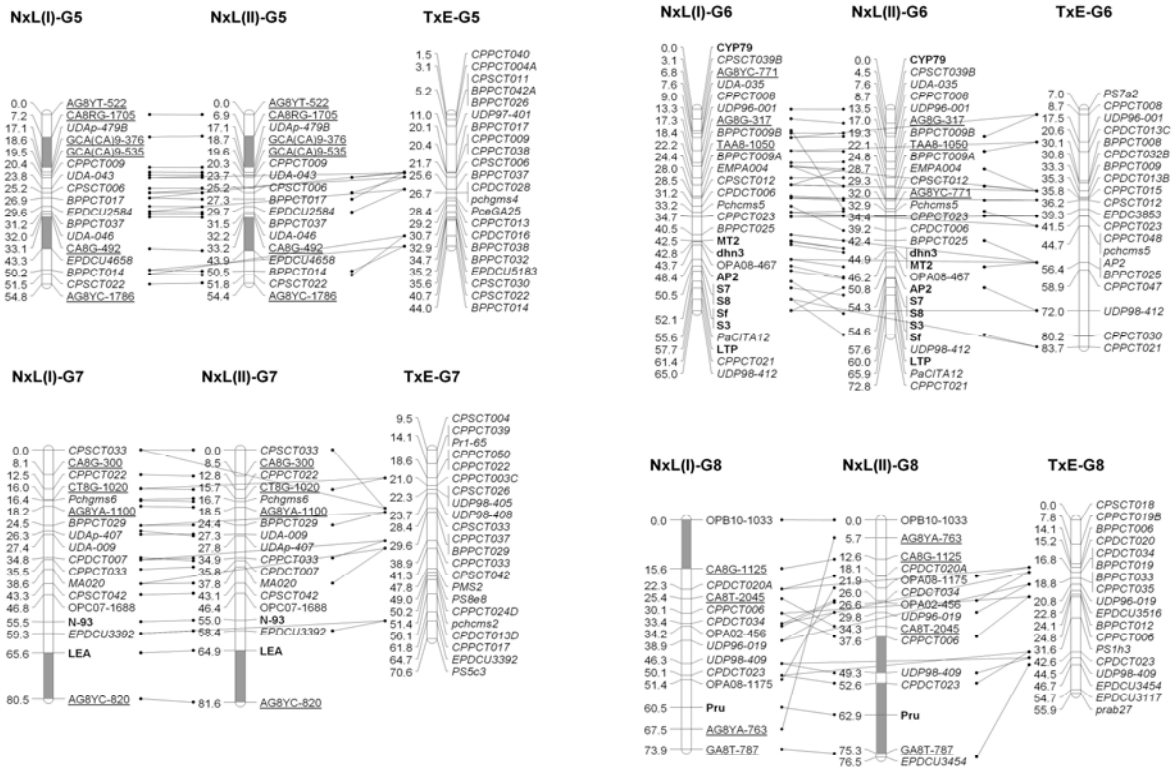


Figure 5.5.3. The alignment of the linkage groups between maps of the cross 'Nonpareil' x 'Lauranne' (NxL) F1 population constructed using One-step and Two-step methods, and with the *Prunus* TxE reference map that include only SSR markers.
The maps of NxL population were constructed using the JoinMap® 3, and the maps were viewed and aligned by the MapChart 2.2. The SSR markers are shown in *italics*, the SNP-anchored genes and S-loci are in bold, ISSR markers are underlined, and the RAPD markers are in plain font. The genetic distance of the loci are shown in centimorgans (cM) and the gaps between two adjacent markers > 10 cM are highlighted in grey segments on the linkage group bars.

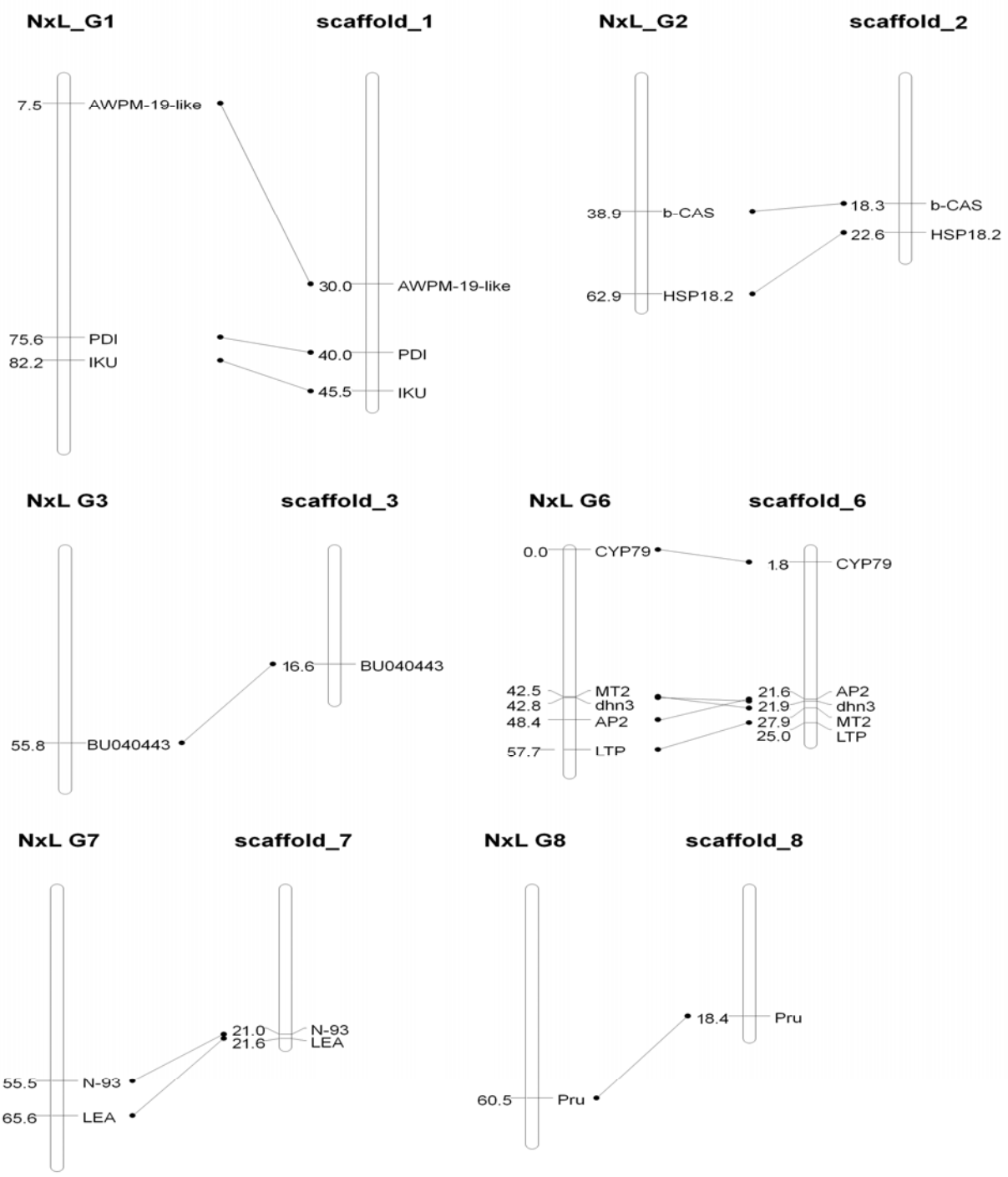
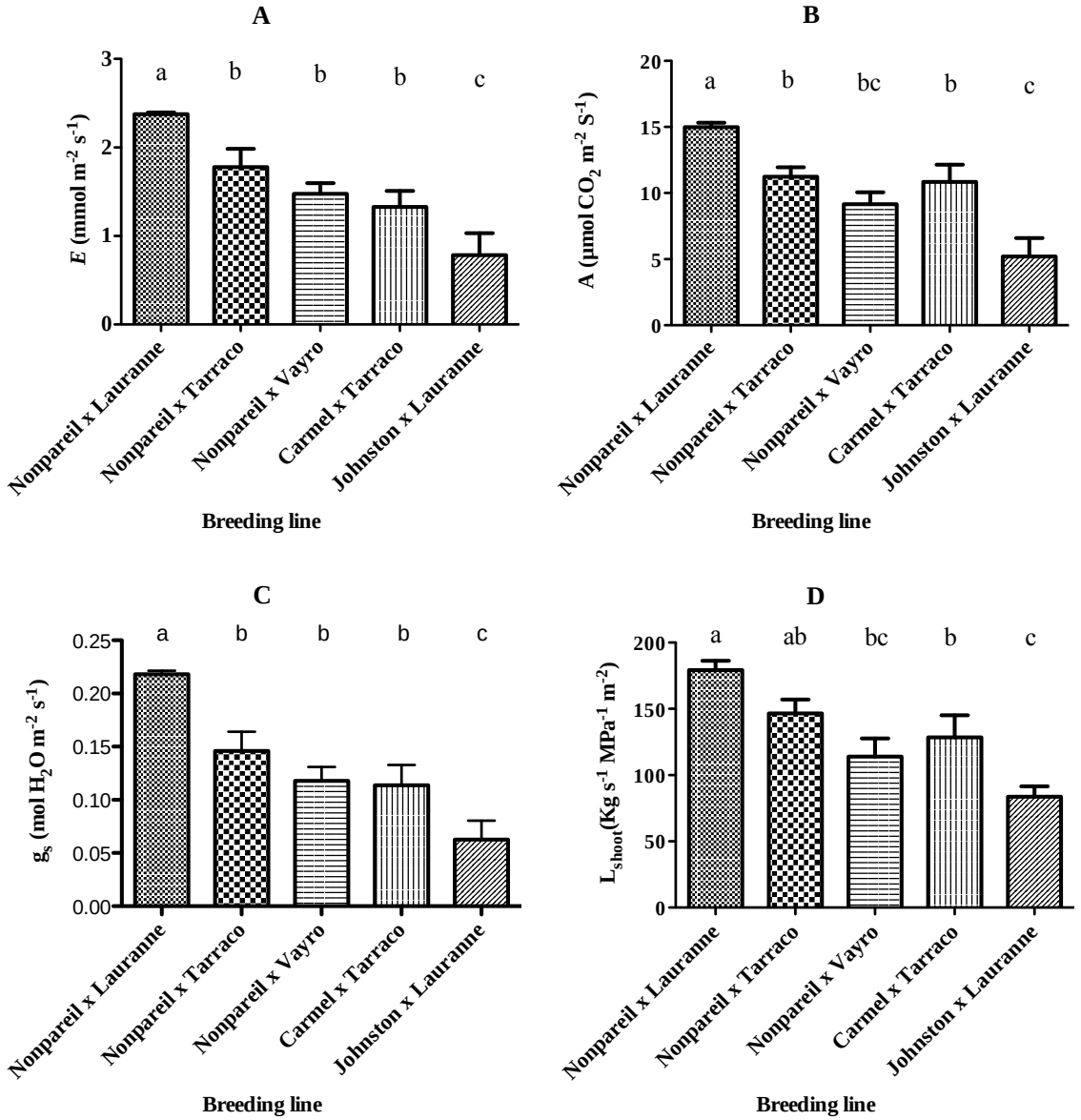


Figure 5.5.4. Comparison of the positions of SNP-anchored genes mapped in the NxL population (map I) with the positions of their homologous sequences in the scaffolds of peach genome v1.0. The genetic distance in the NxL map is shown in centimorgans (cM), and the positions of the homologous sequences in the scaffolds of peach genome v1.0 are shown in mega basepairs (M). The scaffold bars represent the whole length of the corresponding chromosomal DNA. The locations of all the genes except AWPM-19-like mapped in the NxL population agreed with the positions of their homologous sequences in corresponding peach genome scaffolds. AWPM-19-like gene positioned near the top of NxL G1, but in the lower part of the peach genome scaffold_1. An inversion is present in the segment including genes MT2, dhn3, and AP2.

5.6 Estimation of water use efficiency of breeding stock

There were significant differences in transpiration *E*, carbon assimilation *A*, stomatal conductance g_s and *k* normalized to leaf area L_{shoot}. For all the mentioned parameters the observed differences were most significant between Johnston x Lauranne and Nonpareil x Lauranne (Figures 5.6.1 A, B, C and D). The differences between Johnston x Lauranne, Nonpareil x Vayro and Nonpareil x Tarraco were not always significant. Differences in intrinsic water use efficiency (WUE_{intr}) were only significant between Carmel x Tarraco and Nonpareil x Lauranne. In fact, Carmel x Tarraco had the highest WUE_{intr} compared to other varieties (Figure 5.6.1 E) and the lowest C^{13} discrimination value (Figure 5.6.1 F).
There were significant differences ($P < 0.05$) in stomatal conductance between Nonpareil, Carmel and Masbovera measured at field capacity. The lowest values of g_s were observed in Masbovera which was 5.9 times lower than Carmel (Table 5.6.1). Although, g_s of Nonpareil was approximately 4.8 times greater than that of Masbovera, its g_s was still ($P < 0.05$) much lower than that of Carmel.



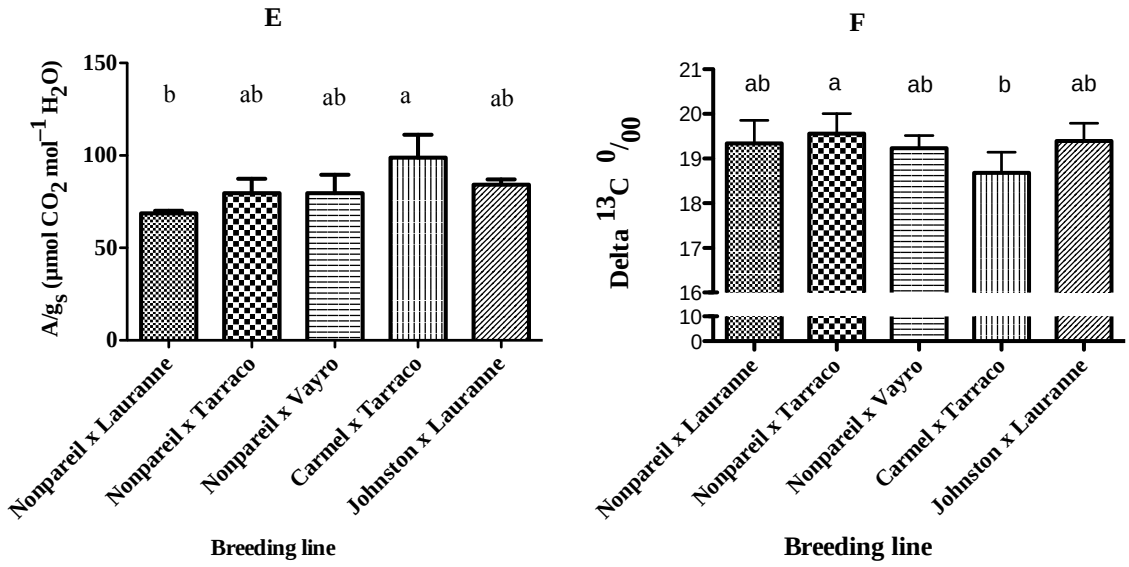


Figure 5.6.1. The genotypic variation in (A), transpiration rate *E;* (B), assimilation rate *A;* (C), stomatal conductance $g_{s,;}$ (D), leaf hydraulic conductivity normalized to leaf area $L_{shoot;}$ (E), intrinsic water use efficiency WUE_{intr}; (F), carbon isotope discrimination delta $^{13}C_{,}$ for 5 genotypes of almond. Each column represents the average of 4 replicates. Different letters demonstrate statistical differences according to the Duncan's test ($P < 0.05$).

Table 5.6.1. The variation between stomatal conductance (g_s) for three varieties of almond. Values for g_s are means (n = 28). Numbers within a column followed by different letters are significantly different ($P < 0.05$).

Variety	g_s (mol H_2O $m^{-2}S^{-1}$)
Carmel	1.42^a
Nonpareil	1.16^b
Masbovera	0.24^c

6. Discussion

6.1 Performance evaluation of local and imported selections and rootstocks

The importation of a wide range of new cultivars from around the world is aimed at finding the best commercial mix of cultivars for our conditions. This is important for the almond industry's development. Initially these importations have come from California and these cultivars are now available to the industry to plant commercially for evaluation after the long process of quarantine and propagation for budwood trees.

Experience has taught us that the cultivars do not always perform here as they do overseas. We have different pests and diseases (eg bacterial spot causing major problems for Fritz in Australia) and flowering times can shift, causing pollination problems. For example testing in Australia has shown that the Californian cultivar Butte is very sensitive to bacterial spot but Padre is quite tolerant.

There is increasing grower interest in cultivars from areas other than California which have commercial potential. These cultivars have high market acceptance in European and Mediterranean countries and in many cases also have some production advantages. These cultivars produce well and are of high quality and consist of both older traditional cultivars as well as some exciting new developments. In the future Australian marketers will decide if these new cultivars can fit into their market niche.

6.2 Controlled crossing to produce seedlings for performance evaluation, and selection of new improved cultivars

The almond breeding has been conducted for fifteen years and has produced over 34,000 trees for evaluation. Selection criteria used in the evaluation process were established in consultation with the industry. The first phase of evaluation on the seedling progeny, deals with fruit and kernel characteristics only, as tree characteristics such as architecture, vigour, yield and disease tolerance are more easily studied during stage 2 trials where the selected trees are propagated onto rootstocks and grown at greater distances apart.

Primary evaluation is completed on progeny from the crosses achieved during 1997 - 2005, with the remaining progeny to be evaluated in the near future. As expected the progeny exhibit a wide range of values for all measured characters. This is essential for the success of the program and is the result of the incorporation of imported almond cultivars into the breeding program thus widening the gene pool.

In the Australian almond industry, Nonpareil is the main cultivar in use because of its high kernel quality, although, as is the case with most other cultivars, it is self-incompatible and relies on cultivars of a different incompatibility group for cross-pollination. Gametophytic self-incompatibility occurs in the family *Rosaceae*, which includes a number of ornamental and fruit tree crops such as *Malus*, *Prunus*, *Pyrus*, *Rosa*, and *Rubus*. Self-fertility has been assigned to the allele S_f and breeding for this trait is a major focus of the Australian almond breeding program. Self-compatibility is tested on selected progeny using molecular markers developed at Waite. In particular the primer for S_f is used to screen progeny from self-incompatible x self-fertile crosses to identify self-fertile seedlings.

The heritability of a trait is a measure of the proportion of the trait variation which is due to the genes involved. An estimated breeding value of an individual tree is the value of its genes to its progeny. Thus using the data generated from this breeding program, a more directed breeding approach can be used and crosses can be designed to use the best with the best.

Pollinations ceased in 2010, but may continue if the need arises, and would use improved parent combinations using data from the heritability analysis. Evaluations are still underway on the 2006/2007 progeny and assessment of the 2008 progeny will begin in spring 2013. The most promising progeny resulting from the selection process are propagated onto rootstock and planted in 2nd stage trial plots for further assessment. To date five selections have been chosen to go into commercial tertiary trials, based on their productivity, flowering time and self-fertility. One hundred trees of each will be planted at three sites across the Riverland and Sunraysia areas. Three of the selections carry the self-fertile gene and the results from the bagging of these trees to exclude pollinators shows a moderate to high self fruit set which is promising. 2013 was the first year of harvest for the second of the secondary trials and two selections are showing promise with yields greater than Nonpareil. Further harvests will be required to establish their long-term productivity.

Assessment of Bacterial spot tolerance
Tests on detached leaves kept under controlled environmental conditions demonstrated differential tolerance to *Xanthomonas campestris* pv. *pruni* on four almond cultivars and five selections from the breeding program. There was defined development of bacterial spots on the leaves inoculated with the almond strain. Difference in symptom expression of *Xanthomonas campestris* pv. *pruni* suggest that some almond cultivars are immune. The percentage of infected sites showed large variation among the cultivars, and significant difference was detected on diameter of bacterial spots. This may indicate that although some of the almond cultivars were highly susceptible, the severity of symptoms on leaves is different. Bacterial spot development on leaves of Nonpareil, Padre, UA-2 and UA-1 was slower than that of Fritz and Butte. Nonpareil, Padre, UA-2 and UA-1 may be partially resistant to the almond strain of *Xanthomonas campestris* pv. *pruni*.
For the upcoming project all selections will be tested for tolerance to bacterial spot and rated against Nonpareil and Butte/Fritz.

6.3 Virus detection in breeding stocks, selections and budwood repository and any required virus elimination

It is important for an industry such as the almond industry, which relies heavily on propagated material, that the mother stock for budwood is as clean as possible and tested annually for viruses. It is also important that the mother trees are minimally exposed to vectors which may carry viruses such as sap-sucking insects. As part of the breeding program, virus testing was undertaken to identify clean parent stock to minimise contamination of progeny. Monash budwood trees were also tested annually on a rotation basis of each tree tested every two years. Prunus necrotic ringspot and Prune dwarf virus are endemic within Australia, so it wasn't surprising that PNRSV appeared every year at Monash. PDV however is harder to detect at ELISA level but can be picked up at PCR level when using a nested protocol. This in fact tells us that the amount of PDV within a tree is at a very low level and generally symptoms are not visible. When suspected positive trees were tested at Knoxfield using woody indexing, no trees were found to be positive. Thus the nested protocol for PDV may be too sensitive and unnecessary.
The following project will continue to test selections but the bulk of Monash testing will be done by Knoxfield DPI Victoria.

6.4 Analysis of free fatty acid profiles in almond selections and breeding stock

The health benefits of almonds have been largely attributed to the high proportions of unsaturated fatty acids (USFA) present in almond lipids (Spiller et al., 1998; Hyson et al., 2002; Rajaram et al., 2010). Plant-based oils, for example olive oil, typically contain an abundance of mono-unsaturated fatty acids (MUFA), such as oleic acid, whereas almond lipids contain polyunsaturated fatty acids (PUFA), such as linoleic acid and Vitamin E. Linoleic acid, an omega-6 fatty acid, is an essential fatty acids for humans and is involved in children's growth and the prevention of cardiovascular disease. However, high levels of linoleic acid have been considered a marker of almond spoilage, since linoleic acid's double bonds are susceptible to oxidation, thus, lower levels of linoleic acid are advocated (Kodad and Socias i Company, 2008). High levels of oleic acid and low linoleic acid are often associated with prolonged shelf-life of almonds.

In this study USFA oleic acid and linoleic acid are the dominant fatty acids, followed by SFA palmitic acid and stearic acid, as well as other minor USFA and SFA, which is consistent with other regional studies such as in Turkey, California, Spain, Iran, Italy, France, Greece, Morocco and Syria (Kodad and Socias i Company, 2008; Askin et al., 2007; Moayedi et al., 2011; Kodad et al., 2010). Most almonds have a similar fatty acid profile, although the proportions are changeable between variety and region, that is except for some studies which found margaric (C17:0), margaroleic (C17:1), gadoleic (C20:1), behenic (C22:0) and lignoceric (C24:0) acids (Piscopo et al., 2010). So far, there are no other studies showing similar findings.

The variation in lipid content among all the tested samples was high and varied across years. The lowest lipid content was observed in Guara almonds grown in the Riverland in 2009, while Nonpareil almonds from Willunga contained the highest lipid levels in 2012. In general all almond samples had lower levels of lipids in 2009. The 2008/2009 growing season in the Riverland was quite dry (normal) while the 2009/2010 and the 2011/2012 growing seasons were wet. This may have influenced lipid accumulation during kernel development. Environment had a significant effect on lipid content in some cultivars and not others. Both Somerton and Johnston showed no difference between sites. On the other hand, genotype showed more influence on almond lipid content. In the Riverland, lipid content varied significantly between genotypes ($P < 0.05$). In all years one or more selections from the breeding program had higher lipid content than Nonpareil grown in the same site. This is a good outcome for the industry as these selections will be more nutritious than Nonpareil. This result will add to the knowledge from previous studies (Kodad et al, 2011; Vezvaei and Jackson, 1995) which reported the lipid content of Australian almond cultivars ranged from 35 – 61 g/100 g and update the data for research and industry to refer to.

Our regional study demonstrated that linoleic acid is significantly affected by both environment and genotype. Willunga grown almonds have higher linoleic acid than other sites. Willunga Nonpareil was greater than Riverland, Sunraysia and ADL plains Nonpareil by 17.1%, 18.5% and 20.3%, respectively. Willunga Somerton was greater than Riverland Somerton by 11.7% and Willunga Johnston higher than ADL plains Johnston by 16%. The impact of rainfall on Australia grown almonds could be probably related to cloud conditions, rather than water supply, as all orchards in Australian regions are fully irrigated. In general, Willunga has lower solar exposure and higher rainfall than the other three sites. Such microclimate conditions could have an impact on almond fruit development and lead to higher linoleic acid content. The effect of genotype on linoleic acid was highly significant at three sites, Nonpareil was relatively higher than the local varieties Johnston, Somerton and Chellaston, but significantly lower than Carmel, Peerless and Price. Linoleic acid is an essential fatty acid for humans, thus these selections are more nutritious than Nonpareil in that respect.

6.5 Adoption of molecular techniques to facilitate the breeding program

6.5.a DNA fingerprinting

Prior to this study only the *S*-alleles of Pearce and Somerton were fully known. *S*-allele identification has shown that several imported almond cultivars have at some stage provided parental material for the named Australian cultivars. The source of S_{25} and S_{27} may be from the early imported cultivars which haven't had their *S*-alleles identified. These could be from early importations from Europe such as Jordan Almond.

A dendrogram of the 28 almond cultivars was drawn based on the UPGMA cluster analysis, thus allowing the genotypes to be classified into one main group and four smaller groups of very different size. The large grouping consisted of mostly Australian cultivars but included the American 'Nonpareil'. 'Keane's' clustered the closest to 'Nonpareil' and also shares one *S* allele, *S7*. In one of the smaller groupings 'Carmel' clustered with 'Frenzy' and 'Brown Nonpareil'. 'Frenzy' also shares identical *S* alleles with 'Carmel', which may suggest it is a sport of 'Carmel' or a misnamed cultivar. The second and third smaller clusters contained only Australian cultivars while the fourth smaller cluster contained 'Ferragnès' and its offspring, 'Lauranne'. The SSR analysis has shown the co-ancestry of many Australian cultivars. Future work will include many more European, Californian and Australian cultivars. To facilitate this, a germplasm collection has been started containing 48 accessions of Australian material.

It was shown that the SSRs developed in other *Prunus* species such as peach, sweet cherry or Japanese plum can be effectively used for evolutionary and fingerprinting studies in almond, confirming the high level of synteny within the *Prunus* species (Aranzana et al., 2003; Arús et al., 2006; Mnejja et al., 2010).

The study has demonstrated the utility of the molecular markers for genetic fingerprinting in almond and their high level of genetic variation explained by its condition of self-incompatibility. The SSRs used in this study will be used in future for maintaining the integrity of our germplasm collection, and can also be used as part of the PBR process as a means of identifying each accession.

6.5.b Genetic mapping of almond

In this study, we constructed almond linkage maps of an Australia population derived from the cross between the American cultivar 'Nonpareil' as maternal parent and the French cultivar 'Lauranne' as pollen donor (NxL). Two maps were constructed using One-step and Two-step methods, with total lengths of 602.7 cM and 628.5 cM respectively. 158 molecular markers were positioned on the one-step and 163 markers were positioned on the two-step map. The resulting maps showed high colinearity with the *Prunus* TxE reference map (Howad et al., 2005; Dirlewanger et al., 2006).

Individual parental maps were constructed and then integrated to produce a consensus map of the population by estimation of the average recombination frequency of the loci in the two parents, as has been used for other pseudo-test cross mapping populations in many tree species (Grattapaglia and Sederoff, 1994; Maliepaard et al., 1998; Wu et al., 2004). In this study, we denoted this as the Two-step method. Since the release of JoinMap® version 3 (Van Ooijen and Voorrips, 2001), the construction of an integrated map of the population can be undertaken by loading all the genotyping data of the population, bypassing individual parental map construction in a one-step strategy. Genetic maps constructed in this way have been published in recent years (Hanley et al., 2002; Hanley et al., 2007; Clarke et al., 2009). During initial mapping analysis, we tried both the One-step and Two-step methods, and variations of marker distances and positions were identified. As the comparison studies had not been reported, we applied both methods in our NxL almond

population to investigate whether different methods can produce significantly different maps. Based on our study, these two integration methods did not result in substantial differences for all the linkage groups, and only a small proportion of markers showed positional instability between the two maps. According to segregation analysis, the majority of the markers (6/9) which showed position shifts > 10 cM between two maps were distorted or adjacent to distorted markers in the map, indicating inaccurate estimation of their recombination frequencies with linked markers in the groups. Therefore, it can be suggested that different mapping methods may not be a main factor contributing to the unstable positioning of the markers, but rather that the nature or quality of the markers can cause such instabilities. On the other hand, fewer large gaps and the slightly higher marker density generated in the One-step map may indicate that One-step mapping is an appropriate method to construct an integrated map of a pseudo-test cross population such as in the almond and other tree plants. Therefore, in this study, the One-step map was used to represent the NxL genome for other analyses (Figs. 5.5.2 and 5.5.4).

A few genetic linkage maps produced using intraspecific crosses of almond have been reported since 1994 in the crosses of 'Ferragnes' x 'Tuono' (FxT) (Arús et al., 1994; Joobeur et al., 2000; Viruel et al., 1995), 'Felisia' x 'Bertina' (FxB) (Ballester et al., 2001; Ballester, 1998), 'R1000' x 'Desmayo Largueta' (RxD) (Sánchez-Pérez et al., 2007), and 'Nonpareil x Lauranne' (NxL) (Gregory et al., 2005; Wu et al., 2009). While FxT and FxB maps consisted mostly of RFLP and RAPD markers, the RxD map contains 56 SSR markers with less density across the genome. SSRs are the most favoured marker type used for many applications in plant genetics including genetic mapping because of easy transferability between intraspecific populations and across closely related species, and a high number of alleles per locus that provides greater information content (Gupta and Varshney, 2000; Wünsch, 2009, Mnejja et al., 2005; Mnejja et al., 2010). Therefore, a saturated map containing additional SSRs is warranted in almond intraspecific crosses. The NxL map initially started with RAPDs, ISSRs and the small numbers of SSRs available, and a sparse integrated map was produced (Gregory et al., 2005). Using high resolution melting curve analysis, Wu et al. (2008) mapped 12 SNP-anchored genes on six linkage groups with the addition of more SSRs in the map (Wu et al., 2009). In the present study, we have constructed a molecular linkage map in the almond intraspecific cross NxL with SSRs, SNP, RAPD, and ISSR markers. In comparison with the *Prunus* TxE reference map and other maps reported in *Prunus* (data not shown), linkage groups of the NxL map almost covered the whole length of the almond linkage groups with the exception of G8 which requires further extension on the bottom end of the group. With reasonable coverage of the genome by SSRs and SNPs, the map can be readily used in the Australian almond breeding program (Wirthensohn and Sedgley, 2002) and contribute to international almond genome research.

The clustering of distorted loci in G7 suggests a possible association of lethal or deleterious genes with this part of the linkage group and a more in depth study to investigate this possibility is warranted. In a peach F2 mapping population, no linkage of markers could be established for G7 (Blenda et al., 2007) possibly due to the complexity of marker segregation. Half of the mapped markers segregated in a skewed ratio at the significant level of $P < 0.05$, and most of the skewed markers clustered on the lower part of the linkage group where a peak of genotype frequency χ^2 appeared in the area around marker MA020. This finding indicates that the area may harbour one or more lethal or deleterious genes. Although some genes or traits related to biotic or abiotic stress have been mapped to this group such as the nematode resistance trait MA (Lalli et al., 2005) and the DHN gene involved in freezing and drought tolerance (Bliss et al., 2002), those genes were probably not the cause of the distorted segregations as these occur in a different region of G7. It would be interesting to search for lethal gene alleles in the region around MA020. The recent release of peach genome v1.0 provides a good opportunity for conducting such investigations.

With the release of a >7 fold coverage peach genome in April 2010 (v1.0), with 27,852 genes predicted (http://www.rosaceae.org/peach/genome), genomic exploration in *Prunus* and more widely in the family Rosaceae and perhaps other tree plants will undoubtedly accelerate. Indeed, as the closest relative of peach, genetic and genomic studies in almond will benefit significantly from the publication of the peach genome sequences. For almond researchers and breeders to fully utilise the sequence information becoming available for peach, well-assessed almond populations and genetic maps are required to associate important agronomic traits of the species with predicted genes in peach. Thus, genetic maps such as NxL will be valuable locally and internationally for almond genetic research and breeding programs.

6.6 Estimation of water use efficiency of breeding stock

Improving WUE is an effective solution for increasing food productivity under water limited conditions. According to previous studies, two basic solutions are suggested for improving WUE; first is improving water management by adopting effective irrigation methods. The second is breeding new varieties and genotypes which are more water-use efficient (Condon et al., 2004, Parry and Lea, 2009). Regarding the second mentioned solution for improving WUE, significant differences in WUE_{intr} were revealed between Carmel x Tarraco and Nonpareil x Lauranne. Since all the measurements were taken under well watered and light saturated conditions, the differences were probably related to non-stomatal limitations of *A* (Condon et al., 2004).
Consistent with our results, Wong et al (1979) confirmed the strong correlation between *A* and g_s under different environmental conditions (Figure 5.6.1 C). They reported that the limiting factors for carbon assimilation could, independently from each other, have similar effects on g_s. In other words, stomatal closure can be controlled by the photosynthetic capacity of the mesophyll cells. Naturally, stomata tend to maintain the internal concentration of CO_2 (C_i) at a constant level. As a result, any changes in *A* can be closely correlated with g_s (Wong et al., 1979). According to literature, drought-tolerant plants tend to have lower steady state g_s rates and close their stomata more gradually than sensitive or moderately tolerant plants, thus the low g_s reading of Masbovera and the cross Johnston x Lauranne may indicate drought tolerance compared to other cultivars and the other families. Cowan and Farquhar (1977) suggested that stomatal behaviour follows the optimality theory for gas exchange regulations. According to this theory, assimilating the maximum levels of carbon per unit of water transpired is considered as the optimal control of gas exchange (Cowan, 1977, Cowan and Farquhar, 1977). The differences in stomatal conductance (Figure 5.6.1 A) may also come from the differences in the distribution of stomata.
Previous work has found a negative correlation between carbon isotope discrimination and crop water use index, ie the lower the discrimination value the higher the WUI. In this study the Carmel x Tarraco breeding line showed the lowest carbon discrimination value and the highest water use efficiency of the five breeding lines studied. This information will be helpful in planning future crosses with these parents and breeding lines.

7. Technology Transfer

7.1 Conferences

Franks, T.K., Wirthensohn, M.G., Guerin, J.R., Kaiser, B.N., Wu, S., Gibson, J., Hunt, P., Yadollahi, A., Sedgley, M. and Ford, C.M. Bitterness in almond. Poster presented at the Horticulture for the Future Conference in Lorne, Victoria, 18 – 22nd September 2011.

Wirthensohn, M.G., Fernández i Martí, A. and Rahemi, M. Identification of self-incompatibility genotypes and DNA fingerprinting of some Australian almond cultivars. V International Symposium

on Pistachios and Almonds, Turkey 6 – 10 October 2009.

Wirthensohn, M.G., Chin, W.L., Franks, T.K., Baldock, G., Ford, C.M. and Sedgley, M. Investigation of flavour compounds from sweet, semi-bitter and bitter almond kernels. XIV Meeting of GREMPA, Greece, 30 March – 4 April 2008.

8 – 10th October 2012, 14th Australian Almond Conference, Barossa Valley
26 – 28th October 2011, 13th Australian Almond Conference, Victor Harbor
27 – 29th October 2010, 12th Australian Almond Conference, Mildura
28 – 30th October 2009, 11th Australian Almond Conference, Barossa Valley
29 – 31st October 2008, 10th Australian Almond Conference, Barossa Valley

7.2 Reports/ Publications

Fernández i Martí, A., Wirthensohn, M., Alonso, J.M., Socias I Company, R. and Hrmova, M. (2012). Molecular modeling of S-RNases involved in almond self-incompatibility. Frontiers in Plant Science **3,** 1-4.

Zhu, Y., Wilkinson, K. and Wirthensohn, M. (2012). Abundance of α-Tocopherol (Vitamin E) identified in almond kernels. Australian Nutgrower **26**(3), 11-13.

Wirthensohn, M.G., Fernández i Martí, A. and Rahemi, M. (2011). Identification of self-incompatibility genotypes and DNA fingerprinting of some Australian almond cultivars. Acta Horticulturae **912,** 561-566.

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Wirthensohn, M.G., Chin, W.L., Franks, T.K., Baldock, G., Ford, C.M. and Sedgley, M. (2010). Investigation of flavour compounds from sweet, semi-bitter and bitter almond kernels. Options Méditerranéennes Série A, **94**, 117-122.

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Yadollahi, A., Arzani, K., Franks, T., Wirthensohn, M., Kaiser, B., Guerin, J.R.,Sedgley, M., Ford, C. and Tavassolian, I. (2009). Cyanogenic biosynthesis study of bitter and sweet Almond (Prunus dulcis). 6th National Biotechnology Congress of Iran, Tehran- Iran.

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Wirthensohn, M. (2009). Report on the 5th International Symposium on Pistachios and Almonds and visit to CEBAS-CSIC, Spain. Australian Nutgrower **23**(4), 26-27.

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Yadollahi A., Franks, T., Wirthensohn, M., Kaiser, B., Sedgley, M. and Ford, C. (2008). Bitterness mechanism of Almond (Prunus dulcis). 15th National & 3rd International Conference of Biology, 19-21 August 2008, Tehran, Iran.

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Wirthensohn, M.G., Chin, W.L., Franks, T.K., Baldock, G., Ford, C.M. and Sedgley, M. (2008). Characterising the flavour phenotypes of almond (Prunus dulcis Mill.) kernels. Journal of Horticultural Science and Biotechnology **83**, 462-468.

7.3 Field days/Meetings with growers

Research findings were communicated to the Almond Board of Australia's annual seminar and field days on the following dates:

Field days

27th September 2010, Field day at Lindsay Point with John Slaughter from Burchell Nursery, California.

PIC/Strategic Meetings

Almond Plant Improvement Committee meeting in Adelaide on 28th August 2012.
A meeting with Michelle Wirthensohn, Ben Brown, Brett Rosenzweig and Andrew Lacey occurred in Berri on the 12th September and in Adelaide on 22nd October 2012 and a fourth meeting with Michelle Wirthensohn, Ben Brown and Ross Sinner in Adelaide on 5th November 2012 to discuss the new Proposal.
Almond Plant Improvement Committee meetings in Berri on 17th January 2012
Joint meeting with the Production and Processing committees in Renmark on 10th May 2012.
Advanced Production Systems Workshop in Adelaide on 17th August 2011.

Commercialisation meeting at Waite on the 28th January 2011
Almond Plant Improvement Committee meeting in Berri on 17th January 2011.
Almond Plant Improvement Committee meeting at Berri on 28th January 2010 and on 11th August 2010.
Almond Industry R&D Strategic Planning workshop, Mildura. 10th & 11th May 2010
Almond Plant Improvement Committee meeting at Berri on 28th January 2010.
Almond Plant Improvement Committee meeting at Berri on 27th May 2009 and a follow up telephone conference was held on 18th June.
Almond Plant Improvement Committee meeting at Waite on August 7th and 18th December 2008.

Media
Michelle Wirthensohn appeared on Channel 10's Scope TV, May 8th 2010, a children's science show, and talked about almond characteristics and breeding.
Michelle Wirthensohn was interviewed on ABC Radio Country hour by Tim Marshall regarding self-fertile almonds. Monday 10th May 2010.
Michelle Wirthensohn was interviewed live over the phone on Rural Live TV, regarding the almond breeding program. Monday 17th May 2010.
Michelle Wirthensohn appeared on ABC Landline on Sunday 28th April 2013, for a story on the almond industry.
Michelle Wirthensohn was interviewed on ABC Radio 891 and 1062 Riverland, on Tuesday 21st May 2013, for a story on the new HAL funding for Project AL13000. Also interviewed live on Radio National Bush Telegraph program on Tuesday 21st May 2013.
Michelle Wirthensohn was interviewed on Radio Adelaide about the almond breeding program, which was aired on Sunday 26th May 2013.

7.4 Web page
An information web page detailing the progress of this research is being developed as a resource for almond growers, and can be linked to the ABA web page.

8. Recommendations

The current program has yet to evaluate approximately 8,000 progeny. It has been recommended to us that early elimination of trees for undesirable traits will help speed up the primary evaluation stage. The program has identified five superior breeding lines so far, which out perform Nonpareil in yield by up to 15%. We recommend further large scale trials of these five lines at different sites across Australia.

We propose to investigate the following issues during the next five years:

- Maintain the primary evaluation program with appropriate culling at an early stage until all progeny are assessed.
- Evaluate all selections in second stage trials.
- Initiate tertiary trials for superior selections.
- Commercialise the most promising selections.
- Supply growers and industry with informative fact sheets on each commercially released selection.

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Appendices for HAL Project AL08000

Appendix 1. Planting plan for the first 2nd stage evaluation site, Lindsay Point Victoria

7 x 5m grid

				Row 1	Row 2	Row 3	Row 4	Row 5
	***S*-alleles**	**Flowering time**	Tree					
2bT33	S_7S_8	early mid	1	Nonpareil				
4bT1	$S_8S_?$	early mid	2	Peerless	4bT1	R61T33		
1bT31	S_3S_8	late	3	Peerless	4bT1	R61T33	Nonpareil	
1bT32	S_7S_f	mid	4	Peerless	4bT1	R61T33	Nonpareil	
8aT48	S_5S_7	early	5	Peerless	4bT1	R61T33	Nonpareil	
10bT35	S_5S_8		6	Peerless	4bT1	R61T33	Nonpareil	
R12T17	S_7S_f	mid late	7	R30T45	Monterey	1bT32	R38T63	
R13T18	S_7S_f	mid late	8	R30T45	Monterey	1bT32	R38T63	
R30T25	S_5S_7	mid	9	R30T45	Monterey	1bT32	R38T63	
R33T48	S_3S_8	late	10	R30T45	Monterey	1bT32	R38T63	
R38T63	S_7S_{23}	early mid	11	R30T45	Monterey	1bT32	R38T63	
R5T19	S_1S_8	mid	12	Mission	R42T106	10bT35	Marcona	
R30T45	S_5S_8	mid	13	Mission	R42T106	10bT35	Marcona	
R21T70	S_8S_{23}	mid late	14	Mission	R42T106	10bT35	Marcona	
R23T45	S_7S_{23}	early	15	Mission	R42T106	10bT35	Marcona	
R42T106	S_7S_8		16	Mission	R42T106	10bT35	Marcona	
R53T45	$S_8S_?$		17	R53T45	Guara	1bT31	2bT33	
R61T33	S_5S_8		18	R53T45	Guara	1bT31	2bT33	
R58T27	S_8S_{23}		19	R53T45	Guara	1bT31	2bT33	
Ferragnès	S_1S_3	v late	20	R53T45	Guara	1bT31	2bT33	
Ne Plus Ultra	S_1S_7	early	21	R53T45	Guara	1bT31	2bT33	
Ai	S_3S_4	late	22	Ne Plus Ultra	R13T18	R12T17	R30T25	
Mission	S_1S_5	late	23	Ne Plus Ultra	R13T18	R12T17	R30T25	
Marcona	$S_{11}S_{12}$	mid	24	Ne Plus Ultra	R13T18	R12T17	R30T25	
Peerless	S_1S_6	early mid	25	Ne Plus Ultra	R13T18	R12T17	R30T25	
Nonpareil	S_7S_8	mid	26	Ne Plus Ultra	R13T18	R12T17	R30T25	
Tardy Nonpareil	S_7S_8	ex late	27	R33T48	Ferragnès	Aï	8aT48	TNP
Monterey	S_1S_8	mid late	28	R33T48	Ferragnès	Aï	8aT48	TNP
Somerton	S_1S_{23}	early	29	R33T48	Ferragnès	Aï	8aT48	TNP
Guara	S_1S_f	late	30	R33T48	Ferragnès	Aï	8aT48	TNP
			31	R33T48	Ferragnès	Aï	8aT48	TNP
			32	Nonpareil	R58T27	R23T45	Somerton	R21T70
			33	Nonpareil	R58T27	R23T45	Somerton	R21T70
			34	Nonpareil	R58T27	R23T45	Somerton	R21T70
			35	Nonpareil	R58T27	R23T45	Somerton	R21T70
			36	Nonpareil	R58T27	R23T45	Somerton	R21T70
				7m	7m	7m	7m	

Planted
17/8/2006

Nemaguard rootstock

Filter unit

N

Appendix 2. Planting plan for the second 2nd stage evaluation site, Lindsay Point Victoria

ROW	Nonpareil	Pollinator	Nonpareil	Pollinator	Nonpareil	Pollinator
Tree						
1	NP	B	NP	B	NP	B
2	NP	B	NP	B	NP	B
3	NP	B	NP	B	NP	B
4	NP	Guara	NP	R7T50	NP	R20T42
5	NP	Guara	NP	R7T50	NP	R20T42
6	NP	Guara	NP	R7T50	NP	R20T42
7	NP	Guara	NP	R7T50	NP	R20T42
8	NP	Guara	NP	R7T50	NP	R20T42
9	NP	Guara	NP	R7T50	NP	R20T42
10	NP	Guara	NP	R7T50	NP	R20T42
11	NP	Guara	NP	R7T50	NP	R20T42
12	NP	Guara	NP	R7T50	NP	R20T42
13	NP	R10T142	NP	R7T50	NP	R20T42
14	NP	R10T142	NP	Somerton	NP	Nonpareil
15	NP	R10T142	NP	Somerton	NP	Nonpareil
16	NP	R10T142	NP	Somerton	NP	Nonpareil
17	NP	R10T142	NP	Somerton	NP	Nonpareil
18	NP	R10T142	NP	Somerton	NP	Nonpareil
19	NP	R10T142	NP	Somerton	NP	Nonpareil
20	NP	R10T142	NP	R15T85	NP	Nonpareil
21	NP	R10T142	NP	R15T85	NP	Nonpareil
22	NP	R10T142	NP	R15T85	NP	Nonpareil
23	NP	Sonora	NP	R15T85	NP	Nonpareil
24	NP	Sonora	NP	R15T85	NP	R14T100
25	NP	Sonora	NP	R15T85	NP	R14T100
26	NP	Sonora	NP	R15T85	NP	R14T100
27	NP	Sonora	NP	R15T85	NP	R14T100
28	NP	Sonora	NP	R15T85	NP	R14T100
29	NP	Sonora	NP	Chellaston	NP	R14T100
30	NP	Sonora	NP	Chellaston	NP	R14T100
31	NP	Sonora	NP	Chellaston	NP	R14T100
32	NP	R21T70	NP	Chellaston	NP	R14T100
33	NP	R21T70	NP	Chellaston	NP	Mission
34	NP	R21T70	NP	Chellaston	NP	Mission
35	NP	R21T70	NP	B	NP	Mission
36	NP	R21T70	NP	B	NP	Mission
37	NP	R21T70	NP	B	NP	Mission
38	NP	R21T70	NP	B	NP	Mission
39	NP	B	NP	B	NP	Mission
40	NP	B	NP	B	NP	Mission
41	NP	B	NP	B	NP	B
42	NP	B	NP	B	NP	B

N

Row #	18	17	16	15	14	13

GF677 Hybrid rootstock	Planted 10th August	2010

Appendix 3. Phenology of flowering of 65 almond accessions at Waite Campus 2010. Red boxes denote full bloom.