Australian almond breeding program

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THE AUSTRALIAN ALMOND BREEDING PROGRAM

AL99008

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The University of Adelaide

Final Report

1 June 2007

AL99008

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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 to help with this aim.

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1. Media Summary

This 2000-2006 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 2000-2006
- development of improved virus detection methods for almond
- establishment of tissue culture techniques for propagation of almond
- in vitro conservation methods to facilitate small-scale storage of germplasm
- investigation of transformation of almond
- fingerprinting and molecular techniques used for almond breeding
- generation of an almond genetic linkage map

Field trials were established in the major inland almond-growing areas of Lindsay Point and Monash to investigate the potential for new almond cultivars to improve market opportunities. Over 29,000 almond seedlings were produced and planted at these test sites in the Riverland. The fruit is being evaluated for export and domestic appeal.

At the time of this report, the primary evaluation trials were established and some cropping and kernel evaluation has begun. The research is expected to identify superior cultivars for each growing area, with self-fertility, improved kernel quality and increased yield.

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2. Technical Summary

Crossing matrices were generated based on the almond ideotype developed. Self-fertility and improved nut quality rank highly in the breeding aims. Controlled pollinations began in 1997 using 11 almond varieties including Nonpareil, Carmel, Ferragnès, LeGrand, self-compatible genotypes, and well-adapted Australian varieties such as Chellaston, Keane's seedling, McKinlay's Magnificent and Johnston's Prolific. Hybridisations are conducted at multiple sites including the Waite Campus (Adelaide), Willunga (Southern Vales) and Loxton (Riverland). Since then the program has produced over 29,000 almond seedlings for evaluation, crossing up to 27 different varieties in one season. A total of 76 different almond cultivars have been used as parents. The seedling progeny have been planted in the Riverland region at Lindsay Point, Victoria and Monash, SA. At the time of this report, the primary evaluation trials were established and some cropping and kernel evaluation has begun. 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. Twenty-four superior selections have been chosen to date to enter the secondary phase of evaluation.

A technique based on the reverse transcriptase-polymerase chain reaction (RT-PCR) has been developed to detect the presence of Prunus necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) simultaneously in almond. This paper presents the results of a 3-year study comparing both enzyme-linked immunosorbent assay (ELISA) and RT-PCR for the detection of PNRSV and PDV using 175 almond leaf samples. Multiplex RT-PCR was found to be more sensitive than ELISA, especially when followed by nested PCR for the detection of PDV. The RT-PCR technique has the added advantage that plant material can be tested at any time throughout the growing season.

Shoot multiplication was successfully achieved from two paper-shell almond cultivars (Nonpareil 15-1 and Ne Plus Ultra) and an almond/peach hybrid rootstock (Titan×Nemaguard) by culturing shoot tips, about 0.7 cm long with 3–5 leaves, on appropriate shoot multiplication media. For Nonpareil 15-1, AP medium with 0.049 μM IBA, 3 μM BAP, 0.058 M sucrose, and 0.7% agar at pH 5.7 was effective, whereas MS medium with 0.049 μM IBA, 5 μM BAP, 0.088 M sucrose, and 0.7% agar at pH 5.7 was suitable for Ne Plus Ultra. For the hybrid Titan×Nemaguard, MS medium supplemented with 10 μM BAP, 0.088 M sucrose, and 0.7% agar provided the best shoot proliferation. Shoots of the rootstock, about 2 cm long, were rooted after one week in the dark and 2 weeks in the light on half strength MS medium supplemented with 2.4 μM IBA, 0.088 M sucrose and 0.7% agar at pH 5.7, with 88.0% rooting efficiency. When almond scions, about 1.5 cm long, were micrografted on rootstock stems and cultured on rooting medium, the survival was 50 and 65% for Nonpareil 15-1 and Ne Plus Ultra, respectively. Rooted rootstocks and rooted micrografted plantlets were successfully acclimatised and transferred to potting mix with 92% survival.

Shoot tips of two almond scion cultivars, Ne Plus Ultra and Nonpareil 15-1, and one almond/peach hybrid rootstock were successfully cryopreserved using a one-step vitrification technique. Three-week-old in vitro cultures were cold-hardened at 4 degrees C on the multiplication medium (Murashige and Skoog for Ne Plus Ultra and the hybrid rootstock; Almehdi and Parfitt for Nonpareil 15-1) for three weeks. Shoot tips, 2-2.5 mm long, were excised and precultured for 1 d at 4 degrees C on the same basal medium, without plant growth regulators, supplemented with 0.7 M sucrose. After the preculture, the shoot tips were incubated in vitrification solution at 25 degrees C for 45 min for the almond scion cultivars and 60 min for the hybrid rootstock, and then stored under liquid nitrogen (LN) for at least 3 d. After rapid thawing at 30 degrees C, the shoot tips were washed with the appropriate liquid basal medium containing 1.0 M sucrose and then cultured on the same basal medium, solidified with agar, but excluding NH_4NO_3 or $(NH_4)(2)SO_4$. Shoot regeneration was usually observed within 2-3 weeks. Survival after LN, recorded as the percentage of shoot tips that produced at least one new shoot four weeks after thawing, was 87.5, 60.0 and

72.5% for Ne Plus Ultra, Nonpareil 15-1 and the hybrid rootstock respectively. The one-step vitrification method is a promising simple technique for cryopreserving almond scion and rootstock shoot tips from in vitro cultures.

A protocol for Agrobacterium-mediated transformation with either kanamycin or mannose selection was developed for leaf explants of the cultivar *Prunus dulcis* cv. Ne Plus Ultra. Regenerating shoots were selected on medium containing 15 μM kanamycin (negative selection), while in the positive selection strategy, shoots were selected on 2.5 g/l mannose supplemented with 15 g/l sucrose. Transformation efficiencies based on PCR analysis of individual putative transformed shoots from independent lines relative to the initial numbers of leaf explants tested were 5.6% for kanamycin/nptII and 6.8% for mannose/pmi selection, respectively. Southern blot analysis on six randomly chosen PCR-positive shoots confirmed the presence of the nptII transgene in each, and five randomly chosen lines identified to contain the pmi transgene by PCR showed positive hybridisation to a pmi DNA probe. The positive (mannose/pmi) and the negative (kanamycin) selection protocols used in this study have greatly improved transformation efficiency in almond, which were confirmed with PCR and Southern blot. This study also demonstrates that in almond the mannose/pmi selection protocol is appropriate and can result in higher transformation efficiencies over that of kanamycin/nptII selection protocols.

Almond cultivars developed in Australia are thought to have descended from 2 breeding lines, 1 from hard-shelled Spanish/Jordan types, and the other from paper-shell Californian types. However, the precise derivation of many individual Australian cultivars is uncertain. Randomly amplified polymorphic DNA (RAPD) was used to estimate the genetic similarities between 50 accessions of almond cultivars derived from Australia, California, Europe and the Middle East, and individual accessions of *Prunus orientalis* (Miller) D. A. Webb and *Prunus webbii* (Spach) Vieh. Amplification products were analysed using the simple matching coefficient and the unweighted pair group method with arithmetic averages to cluster individuals into a dendrogram. Cultivars known to have originated in Europe or the Middle East clustered in a different group from those known to have originated in California confirming the 2 suspected breeding lines. The origin of some common Australian commercial cultivars was inferred by their placement on the dendrogram, and the possible parentage of some Australian selections is discussed.

An integrated genetic linkage map of almond (*Prunus dulcis*) based on RAPD, ISSR, SSR and morphological markers was constructed using a pseudo-testcross mapping format and Joinmap 3.0. A total of 93 individuals from a F_1 full-sib family produced from a cross between 'Nonpareil' and 'Lauranne' were genotyped with 120 molecular markers (60 RAPD, 23 ISSR, 1 SCAR, and 36 SSR) to produce two parental maps. Hybridity of the mapping population was confirmed by DNA fingerprinting and cluster analysis using RAPD and ISSR markers, and SSR inheritance. The two parental maps were aligned using 12 molecular markers (2 RAPD, 4 ISSR, and 5 SSR) that were segregating in both parents (intercross markers). Two distorted markers were observed at the distal region of two linkage groups. A map position was provided for the kernel taste loci, on linkage group B, 49.1 cM from the ISSR marker $(AG)_8$YC-1786. Six linkage groups were obtained for the integrated map with a marker density of 11.5 cM/marker, covering 161.9 cM (Kosambi), representing a 31% coverage of the T x E *Prunus* reference map. This map provides an initial step for producing a SSR saturated integrated genetic linkage map of the almond genome, including morphological markers. The use of both peach and cherry SSRs illustrates the use of interspecific derived primers as a source of polymorphism generation in the *Prunus* genus, further enhancing the collaborative effort to produce a genus wide reference map for application in breeding programs and marker assisted selection.

3. Introduction

The almond (*Prunus dulcis* Mill., syn *Prunus amygdalus* Batsch) is a small deciduous tree, adapted to a Mediterranean climate, which produces an edible nut. It is related to other *Prunus* crops, including peach, plum, apricot and cherry. In 2007 the Australian almond industry is expected to produce in excess of the estimated 23,500 tonnes (kernel), an increase of 46% over the 2006 crop, with an associated farm gate value of $150 million (ABA, 2007). Large increases in production have occurred over the last decade, and production will increase as large areas of young trees come into full bearing. Australian almond acreage has increased five-fold over the past eight years, from approximately 9,000 acres in 1999 to more than 47,000 acres in 2007. Planted orchards are expected to produce annual crops of over 50,000 tonnes (kernel) by 2012 (ABA, 2007).

Commercial almond trees comprise a rootstock to which the scion cultivar is budded. The main commercial scion cultivar is Nonpareil, also known as California Papershell or CPS, which requires pollinator cultivars for production. Rootstocks were traditionally almond seedlings, but most new orchards are planted using seedlings of the Nemaguard peach, which is nematode resistant (Kester and Grasselly, 1987). The best available rootstocks are almond x peach hybrids on shallow soils and Nemaguard on deep soils, but both rootstocks suffer significant limitations under Australian conditions. The majority of Australian almond production is based on cultivars developed many years ago in California, but the Australian industry is still expanding, and many older orchards will require replanting in the near future. There is a need to assess new scion and rootstock cultivars developed overseas, and to fully evaluate local selections, which appear to have potential. It is important to conduct controlled crossing in Australia, in order to develop improved cultivars adapted to local conditions and consumer demands. Rootstock requirements include lime tolerance, nematode resistance, *Phytophthora* tolerance and vigour, whereas scion requirements include yield, kernel quality, self-fertility and resistance to viral, bacterial and fungal diseases.

The almond industry in Australia is currently limited to six commercial cultivars with Nonpareil comprising over 50% of all plantings. The other five are grown primarily for their ability to pollinate Nonpareil. All have significant shortcomings including:

- low quality (kernel & shell)
- disease susceptibility
- physiological disorders
- virus infection
- poor coincidence of flowering times

The industry has been built on the use of old cultivars and rootstocks, which do not perform well under Australian soil and climate conditions. Specific examples of limitations include:

- Productivity of orchards in the North Adelaide plains averages around 60% of the industry standard and only 40% of best practice. Lack of availability of suitable rootstocks for prevailing soil and water conditions is the primary limitation.
- At least 50% of trees are grown for their ability to successfully pollinate the standard cultivar, Nonpareil, and the product quality of these cultivars is inferior to that of Nonpareil. In addition each of these pollinator cultivars has significant limitations including disease susceptibility and/or poor growth habit and/or virus infection and/or timing of flowering does not coincide fully with Nonpareil.
- Early flowering, which leaves the crop vulnerable to frost damage in spring.

The Australian almond industry while having comparatively similar costs of production to other production areas around the world does not have a competitive advantage in product quality or volume. If Australian producers are to remain competitive in the world market place they must have high yielding cultivars, which produce large nuts of high quality.

Virus diseases are a major problem affecting yield or quality of most perennial crops. Those identified in almond are Prunus necrotic ringspot (almond calico), almond mosaic, prune dwarf virus and yellow bud mosaic. They are transmitted by nematodes, pollen or budding, and the correct method of control is the use of virus-free nursery stock. The industry maintains a source area at Monash Vine Improvement Centre in South Australia. It is essential to ensure that all material entering the area is virus-free, and that it remains so. This includes material for existing scion and rootstock cultivars, as well as local selections and imported bud lines for performance assessment. At present, grafting of virus-susceptible Shirofugen trees in field plantings is considered to be the most reliable indexing method. Given that the virus is unevenly distributed throughout the tree, several buds are taken for indexing for a reliable result. Indexing can only be performed in spring and is dependent on successful bud take. Thus the method is slow and costly, and improved methods of both virus detection and virus elimination are required. Prunus necrotic ringspot virus is one of the most severe diseases, and ELISA tests have been developed for detection (Mink, 1992), with more recent efforts concentrated on the development of cDNA probes for detection.

Clonal rootstock propagation is a goal of the industry as this imparts reliability of performance. As with most woody perennials, almonds will establish in tissue culture, and produce multiple shoots, but root initiation is a common problem (Scott et al., 1992). Nevertheless, some cultivars can be successfully propagated via tissue culture, as a result of research aimed specifically at these genotypes. One of the aims of this project is to apply these methods to promising local rootstock selections.

Tissue culture is increasingly used as a method of in vitro conservation. Cultivar and rootstock collections are essential repositories of genetic material for further plantings and for use in selection and breeding programs. In situ collections are expensive to maintain in terms of both land and labour costs, and the prospect of in vitro storage of collections is an attractive alternative. Most methods involve establishment of plant material in culture, followed by treatments, such as high sucrose concentration, which allow the tissue to withstand temperature reduction. The culture is then stored at low temperature until required. These techniques will be adapted for use with the almond cultivars and selections of interest to the industry. In addition to clonal micropropagation and in vitro conservation, tissue culture can also be used as a breeding tool. We propose to use the methodology of genetic manipulation and transformation. Successful transformation techniques have been developed but require refinement.

The specific objectives of this project were:

1. Performance evaluation of local and imported selections and imports of scion and rootstock material of almond.
2. Controlled crossing to produce seedlings for performance evaluation, and selection of new improved cultivars.
3. Development of improved virus detection methods for almond breeding stock and mother plants.
4. Development of virus elimination methods for almond, to ensure freedom from the major virus diseases. (This was abandoned as SARDI pulled out of the project).
5. Investigation of micropropagation for the multiplication and propagation of new improved rootstocks from the selection program.
6. Adoption of in vitro conservation methods to facilitate small-scale storage of germplasm as an alternative to in-ground collections.
7. Investigation of transformation in tissue culture as a method to alter specific traits of almond in otherwise successful cultivars.
8. DNA fingerprinting of almond cultivars, and commencement of genetic mapping.

4. Material and Methods

4.1 Performance evaluation of local and imported selections and imports of scion and rootstock material of almond

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 32 cultivars including several rootstocks from Europe and Israel were released up till April 2004. 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 and these are due for release in the spring of 2007.

Rootstock trial

In 1997/98 the industry identified a range of 8 potential rootstocks not yet evaluated for almonds in Australia. These were GF677, Cadamon, Citation, Hansen 536, Hansen 2168, Atlas, Viking, and Nemasun. Various nurseries have been contacted to provide 30 Nonpareil almond trees budded to each of the eight rootstocks for the field evaluation on a trial block selected on a major property in the Riverland.

Two of the rootstocks involved in this trial, Alnem 88 and H184 peach almond hybrid were identified in the completed rootstock evaluation trial NT96009 as worthy of further evaluation. All eight rootstocks will be evaluated against the current industry standards, Bright's hybrid and Nemaguard peach. As future rootstocks are evaluated they will be evaluated against the same two benchmark controls to allow a continuous evaluation program rather than a series of discrete trials. Rootstocks imported during this study include: from France, GF677 and GF557; from Israel, AI 28-19, AI 27-18, and GF749. The trial is planted at Lindsay Point according to the site plan (Appendix 1).

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 CEBAS-CSIC; Masbovera, Glorieta, Francolí, Felisia, Desmayo Largueta, 155, 12-350, 21-169, 21-323, 21-332, and 22-120 from IRTA. Cultivars imported from Italy include Fascionello and Cristomorto. 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. Local growers have assessed some cultivars in test plantings at Lindsay Point and Paringa and the performance of these cultivars is shown in the results section. 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

The aim is to produce self-fertile almond cultivars with superior kernel quality as well as high productivity and local adaptation that will supply both local and overseas markets. The program focuses on classical breeding via controlled hybridisation, using both Australian and overseas cultivars as breeding stock. The first crosses were made at The University of Adelaide Waite Campus and at Loxton Research Centre in 1997 with Nonpareil, Ferragnès, Mission and LeGrand

pollinated by four Australian varieties, (Keane's seedling, Chellaston, Johnston's Prolific and McKinlay's Magnificent), Carmel, Nonpareil, Ferragnès, LeGrand and self-fertile genotypes. Local varieties were used as these are adapted to our environment and have good qualities such as kernel size and tree habit. Pollen from self-fertile genotypes was imported from INRA, IRTA and CEBAS-CSIC to achieve some self-fertile progeny. All imported pollen was virus tested at The University of Adelaide for PNRSV, PDV and PPV prior to use. Since 1997 to 2006, 79 different cultivars have been used as either female or male parents.

Hybridisations are conducted at multiple sites including The University of Adelaide Waite Campus (Adelaide), Angle Vale (Northern Plains), Willunga (Southern Vales) and Loxton Research Centre (Riverland). 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. In the first year the aim was to produce 1,000 seedlings. In subsequent years the aim was to maximise production of seedlings to 5,000 per year. Pollen was applied using a small paintbrush 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. 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 are planted in the Riverland region at Lindsay Point, Victoria, and at the Riverland Vine Improvement Centre, Monash SA.

Primary evaluation of progeny from breeding program

Primary evaluation is based on nut and kernel characteristics. Nut characters included in the evaluation process are harvest maturity, percent double kernels, shelling percentage, kernel size, kernel weight, shell weight, kernel shape, kernel thickness, kernel taste, kernel colour, testa colour, and testa appearance.

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

Table 4.2.1 Evaluation criteria and selection standards for kernel characteristics.

Character	Records	Selection			Weighted Score[¥]
		High	Medium	Low	
Sweet kernel	Sweet or bitter		sweet	bitter*	1 or 0
Kernel size	Kernels per ounce	≤18/20	20/22 to 23/25	>23/25**	
	or (g)	≥ 1.4	< 1.4 or ≥ 1.1	< 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,	hard, semihard, soft,		stone	1 or 5
	semihard,soft,paper		paper		
Shell seal	Score (1-5)	5	3-4	1-2	1-5
Kernel	Score (1-10)	7-10	5-6	1-4	1-10
appearance****					

[¥] 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 parents used. This was started using a statistical package, ASREML.

Secondary evaluation of progeny from breeding program

It is expected that a small percentage of seedlings will be suitable for secondary evaluation, which will begin in 2007. These will be evaluated in a semi-commercial situation over a range of rootstocks and growing conditions. Secondary evaluation will also include close collaboration of the industry processing and marketing segments to ensure market and consumer acceptability. All evaluation will be against the industry benchmark, Nonpareil. Second stage testing on selected trees

will include assessment of disease tolerance to bacterial spot, tree habit, potential productivity, flowering time, ease of hulling and shelling, harvest time, and propensity for NBF.

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

Development of breeding cage and germplasm collection.
The almond breeding cage at Waite campus contains 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 in November 2004. Two trees per cultivar were budded in spring onto rootstocks. The cultivars include Antoñeta, Fascionello, Francolí, Ferragnès, Ferraduel, Ferralise, Ferrastar, Glorieta, Guara, Mandaline, Marta, Masbovera, Sauret, Steliette and Supernova. Breeding lines from Europe such as R1065, R1049, R1146, 21-332, 22-120,12-350, 21-169, 21-323, and 155 have also been planted. Previous plantings were made in 2001 and included Butte, Padre, Livingston, and Wood Colony from the USA. Virus free accessions of Nonpareil 15-1, Chellaston, Parkinson and Tardy Nonpareil were also planted.

A collection of Australian cultivars was started after an appeal was made to the almond industry for information on the whereabouts of old cultivars (Wirthensohn and Sedgley, 2003). These were subsequently collected, budded onto rootstock, and planted at 'Simarloo' in Lyrup SA. The cultivars collected were Atkinson's hardshell, Baxendale, Bigg's hardshell, Brown Brandis, Brown Nonpareil, Bruce, Chellaston, Federation, Frenzy, Clements, Johnston's Prolific, Keane's seedling, McKinlay's Magnificent, Parkinson's Pride, Pearce, Pethick's Wonder, Somerton, Strout's papershell, and White Brandis. This collection will be invaluable for future reference and testing. All of these early varieties were well adapted to our climatic and environmental conditions. Hence they are important to use as a source of local adaptation in our breeding program. Maintaining a wide range of cultivars is important because they may contain genes useful in the continuing fight against pests and diseases, even those cultivars with overall characteristics that may not be attractive economically. The collection is also important to maintain and preserve our Australian cultivars so they can be used for future reference, and also as part of the history of this important industry, that has played a pivotal role in the economic and cultural development of our nation. All cultivars will be labelled and catalogued and will be available to the industry, researchers, and of course, the Australian almond breeding program.

4.3 Development of improved virus detection methods for almond breeding stock and mother plants

Plant material
One hundred and seventy-five leaf samples were collected randomly from around the canopy of the almond (*Prunus dulcis* (Mill.) D.A. Webb) cultivars Nonpareil (eight trees, 5–67 samples per tree) and Sauret (one tree, five samples) at the Monash bud wood repository (Riverland Vine Improvement Centre, Monash, South Australia) in each of the three seasons from 1999 to 2001. 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. ELISA and RT-PCR were conducted on the same leaf samples.

Detection of PNRSV and PDV by ELISA

Double antibody sandwich ELISA was carried out using the commercially available PNRSV and PDV alkaline phosphatase compound ELISA test kits (Agdia Inc., Elkhart, IN) according to the manufacturer's instructions (Bertozzi et al., 2002).

Isolation of RNA

RNA was isolated from leaves of almond and cherry either by precipitation with lithium chloride (LiCl) or by using a commercially available extraction kit (RNeasy, QIAGEN Inc., CA). For the LiCl method, total RNA was isolated according to the protocol described by Channuntapipat et al., 2001 with some modifications. Approximately 50–100 mg of fresh or frozen leaves were ground to a fine powder in liquid nitrogen and mixed with 1 ml of extraction buffer (0.1 M LiCl, 0.1 M Tris–HCl, pH 8.0, 0.01 M EDTA, pH 8.0, and 1% SDS). Polyvinylpyrollidone (PVP-40) (5%, w/v) and sodium metabisulphite (2%, w/v) were added to the extraction buffer just before use. Eight hundred microlitres of a mixture of Tris-saturated phenol, pH 8.0: chloroform: isoamyl alcohol (25:24:1) were added to the tube and vortexed for 1 min, followed by centrifugation at 14,000 rpm for 15 min at room temperature (RT). The upper aqueous layer was removed and mixed with an equal volume of 4.0 M LiCl. RNA was allowed to precipitate at −20 °C for 4 h, followed by centrifugation at 4 °C for 15 min at 14,000 rpm. The pellet was dissolved in 400 µl of sterile water and re-precipitated in 2 volumes of cold ethanol (−20 °C) in the presence of 40 µL of 3.0 M NaOAc (pH 5.2). RNA was recovered by centrifugation at 14,000 rpm for 15 min at 4 °C. The pellet was washed with cold 70% ethanol by centrifugation at 4000 rpm for 5 min at 4 °C, dissolved in 50 µl of sterile water, and stored at −20 °C (or −80 °C for long-term storage).

RNA was extracted using the RNeasy plant mini kit using the following protocol. Approximately 200 mg of fresh leaf tissue was pulverised in a small plastic bag containing 20 mg of sodium metabisulphite and 2 ml of extraction buffer (RNeasy kit) containing 4.4% (w/v) PVP-40 (Sigma, MO, USA). Alternatively, 200 mg of either fresh or frozen leaf tissue was ground into a fine powder in liquid nitrogen, mixed with 2.0 ml of extraction buffer containing 4.4% (w/v) PVP-40 (Sigma, MO, USA) and 1% (w/v) sodium metabisulphite, and briefly vortexed.

Five hundred microlitres of the homogenate was mixed with 60 µl of 20% (w/v) sarkosyl (*N*-lauroyl-sarcosine, Sigma), and incubated at 70 °C with agitation for 10 min. The contents were then transferred to a QIAshredder mini column and centrifuged at 14,000 rpm for 5 min. The column flowthrough (350 µl) was mixed with 315 µl of 95% ethanol, and the remainder of the protocol was carried out according to the manufacturer's instructions. 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 using the nucleotide sequences of the coat protein published on the GenBank database, National Center for Biotechnology Information (NCBI). The expected amplification products were 722 bp (PDV) and 351 bp (PNSPV) in the first round of RT-PCR (Table 4.3.1).

Table 4.3.1 Primers designed to detect PNRSV and PDV in almond leaves

Virus	Primers	Primer length	Primer sequence	Amplification product size
PNRSV	PNRSVF	19	CTTGAAGG AC CA AC CGAG	
	PNRS VR	19	AT CTGCTA A CG CAG GTAAG	351 bp
PDV	PDVF	22	CCAA TTT A CTT CCAA CTTT CGA	
	PDVR	21	GC A CAA TCAA A TGA TGGA TCA	722 bp
	PDV242F	20	GTATG A TA TCTCGT A CCGAG	
	PDV242R	20	CTG GC TT GTTTCGC TGTGAA	241 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 either 1:100 for almond or 1:500 for cherry. Two microlitres of the diluted first-round PCR products were subjected to nested PCR with primers PDV1F/PDV1R 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) and PDV (722 bp), and the PDV nested PCR product (241 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 and PDV.

4.4 Investigation of micropropagation for the multiplication and propagation of new improved rootstocks from the selection program

Plant materials

In vitro-cultured shoots of an almond/peach hybrid rootstock (*Prunus dulcis* cv. Titan×*P. persica* cv. Nemaguard), called "Bright's hybrid", were obtained from ForBio Research, Brisbane, Australia. Axillary buds on young branches of two almond scions, Ne Plus Ultra and Nonpareil 15-

1, were taken from trees growing at the Waite Campus of the University of Adelaide. Selection 15-1 of Nonpareil was developed by the Washington State Interregional Project as IR number 0015-1.

Young branches of almond scions, with 5–6 axillary buds, were surface sterilised in fresh 7% (w/v) calcium hypochlorite with 0.02% (v/v) Tween 20 for 15 min, and then washed three times in sterile distilled water. Sections with 1–2 buds were excised and cultured on Quoirin and Lepoivre (QL) medium ([Quoirin and Lepoivre, 1977] for 3–4 weeks.

Selection of media

For the almond/peach hybrid rootstock, MS medium was used as previously reported (Channuntapipat et al., 2000). Explants from axillary buds of the two scions, cultured for 3–4 weeks on QL medium, were screened for shoot growth on a number of different media including Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), woody plant medium (WPM) (Lloyd and McCown, 1980), Almehdi and Parfitt (AP) medium (Almehdi and Parfitt, 1986), Tabachnik and Kester (TK) medium (Tabachnik and Kester, 1977), and QL medium. Based on this initial screening, MS medium was chosen for Ne Plus Ultra and AP medium for Nonpareil 15-1.

Stock cultures

Stock cultures of Ne Plus Ultra and the hybrid rootstock were maintained on MS medium supplemented with 4.44 μM BAP, 0.049 μM IBA, 0.088 M sucrose, and 0.7% (w/v) agar. Stock cultures of Nonpareil 15-1 were maintained on AP medium supplemented with 3 μM BAP, 0.049 μM IBA, 0.058 M sucrose, and 0.7% (w/v) agar. The pH of both media was adjusted to 5.7. All cultures were maintained in 250 ml polypropylene containers containing 50 ml of culture media with subculturing every 4–5 weeks.

Effect of BAP and IBA on shoot multiplication of almond scions and the hybrid rootstock

Shoot tips, 0.7 cm long with two or three leaves, were excised from 3-week-old stock cultures and individually transferred to 15 combinations of BAP (0, 1, 5, 10, and 20 μM) and IBA (0, 0.049, and 0.49 μM) on appropriate media. Each treatment was replicated five times. After 4 weeks for almond scions, and 5 weeks for the hybrid rootstock, fresh shoot tips were dissected from each replicate and recultured, in groups of three, on the same medium. Fresh weight, number, and size of multiple shoots were measured 4–5 weeks after this second cycle.

Rooting of the hybrid rootstock

Five-week-old shoots, 2 cm long, were cultured on half strength MS salts with 0.088 M sucrose and 0.7% (w/v) agar at pH 5.7, containing nine different concentrations of IBA (0, 1.4, 2.4, 3.4, 4.9, 7.3, 9.8, 12.3, and 14.7 μM), in the dark at 24±1 °C for 7 days, and then transferred to a 16 h photoperiod for 2 weeks. Shoots were cultured in groups of five in 250 ml polypropylene containers containing 50 ml of culture medium. Each treatment was replicated five times. Callus formation, date of first root emergence (visible to the naked eye), and the number of rooted shoots at 2 weeks were recorded. Root growth was calculated as the increase in length 1 week after rooting commenced, and rooting efficiency as the percentage of shoots producing roots after 4 weeks.

Micrografting of almond scions onto rootstocks

Apical-wedge grafting was used to micrograft 3–7-week-old cultures of almond scions to the hybrid rootstocks in vitro. Stems of rootstocks were cut 1–1.5 cm above the medium and a vertical slit made in the centre of the cut surface to a depth of 3 mm. Scions with similar diameter to the rootstocks were selected from healthy apical shoots with 2–3 nodes (1.5–2.0 cm long). Two slanting cuts about 4–5 mm long were made at the base. The wedge-shaped base of the scion was pushed into the cut in the rootstock, and the junction was either left unwrapped, or wrapped with 3M micropore tape (3M Australia Ltd., Sydney, NSW). The grafted plants were cultured on rooting medium supplemented with 2.4 μM IBA and incubated in the dark for 7 days, then transferred to

the light for 2 weeks. Survival was recorded as the percentage of micrografts with green shoots growing from the scion. Each treatment was replicated 10 times, with one grafted plant per replicate.

In the selection of shoots for micrografting, soft stems were defined as very young stems (about 3-week-old cultures), very light green colour and succulent in appearance, about 0.5–0.7 cm long. Hard stems were defined as mature stems with deep green colour (about 5–7-week-old cultures), 1.5 cm long. Semi-hard stems were intermediate between soft and hard stems with light-green to green colour (about 4-week-old cultures), about 1 cm long.

Culture conditions
All cultures were incubated at 24±1 °C with a 16 h photoperiod of 35–40 μmol m^{-2} s^{-1} provided by cool white fluorescent lights.

Hardening off
Three weeks after shoots or micrografted rootstocks were cultured on rooting medium, the light intensity was increased to 60 μmol m^{-2} s^{-1} for 1 week. Rooted shoots were transferred to pots containing a mixture of Nu-Erth premium potting mix (Nu-Erth Horticultural Supplies, Adelaide, SA), peat moss, and vermiculite in the ratio 3:1:1, covered with transparent plastic bags, and grown under shade cloth (50% light reduction). The plants were watered and ventilated every day by temporarily removing the plastic bags. After 10 days, the plastic bags were punctured to allow greater gas exchange, and after 20 days the tops of the plastic bags were cut open. Controlled released fertiliser, Osmocote® For Pots & Garden Beds (Scotts-Sierra Horticultural Products Co., Marysville, OH) was added and the plants watered daily. The plants were grown under morning sun until they were 40–50 days old, and then exposed to full sunlight.

Statistical analysis
All data were analysed with Duncan's new multiple range test using PlotIT v. 3.20i (Scientific Programming Enterprises, Haslett, MI). For rooting efficiency, a single factor completely randomised design with subsampling was used as described by (Compton, 1994).

4.5 Adoption of in vitro conservation methods to facilitate small-scale storage of germplasm as an alternative to in-ground collections

Plant material
In vitro-cultured shoots of *Prunus dulcis* cvs Ne Plus Ultra and Nonpareil 15-1 and an almond/peach hybrid (*P. dulcis* cv. Titan × *P. persica* cv. Nemaguard) rootstock were used in this study. Stock cultures of Ne Plus Ultra and the hybrid rootstock were maintained on MS medium (Murashige and Skoog, 1962) supplemented with 0.049 μM indole-3-butyric acid (IBA), 4.44 μM 6-benzyl-aminopurine (BAP), 0.088 M sucrose, and 0.7% (w/v) agar (Difco Bitek). Stock cultures of Nonpareil 15-1 were maintained on AP medium (Almehdi and Parfitt, 1986) supplemented with 0.049 μM IBA, 3.1 μM BAP, 0.058 M sucrose, and 0.7% (w/v) agar. The pH of both media was adjusted to 5.7 prior to adding agar and autoclaving at 121°C for 20 min. All cultures were maintained in 250 mL polypropylene pots containing 50 mL of culture media under metal halide lights (40 μmol/m2/s) with a 16 h photoperiod at 25 ± 3°C and subcultured every 4-5 weeks.

Cold-hardening and preculture
Three-week-old shoot cultures were exposed to 4°C for up to 42 days under 15 μmol/m2/s cool white fluorescent lights with a 10 h photoperiod. Shoot tips with 3-5 leaf primordia (2-2.5 mm long, 1-1.5 mm base diameter) were dissected from cold-hardened shoots and precultured on the appropriate medium supplemented with 0.7 M sucrose and 0.7% (w/v) agar for 1 day under the same conditions.

Vitrification
Following preculture, groups of ten shoot tips were transferred to 2 mL cryotubes (Nalgene) containing 1 mL of a vitrification solution (PVS2) (Sakai et al., 1991) and incubated at 25°C for various period of time. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide prepared in either liquid MS for Ne Plus Ultra and the hybrid rootstock, or AP for Nonpareil 15-1, supplemented with 0.4 M sucrose, at pH 5.8. After replacing the PVS2 once during the incubation period, the shoot tips were finally suspended in 0.5 mL of fresh PVS2 and the cryotubes were stored under liquid nitrogen (LN) at –196°C.

Regeneration
Shoot tips were rapidly thawed from LN in a water bath at 30°C, and washed twice with either liquid MS or AP medium as appropriate, supplemented with 1 M sucrose. They were then transferred to either MS medium with 0.008 M sucrose and 0.9% (w/v) agar but excludingNH4NO3 (Kuriyama et al., 1990) for Ne Plus Ultra and the hybrid rootstock, or AP medium with 0.058 M sucrose and 0.9% (w/v) agar but excluding (NH4)2SO4 for Nonpareil 15-1, and cultured with a16-h photoperiod (40 μmol/m2/s) at 25 ± 3°C. Survival was recorded as the percentage of shoot tips that produced at least 1 new shoot 4 weeks after thawing. Shoots were then transferred to either standard MS or AP medium as appropriate, excluding plant growth regulators, for a further 1 or 2 weeks before maintenance as stock cultures as described above.

Statistical analysis
Each treatment was replicated 4 times with 10 shoot tips per replicate, and the results were analysed by Duncan's new multiple range test using PlotIT (Scientific Programming Enterprises, Haslett, MI, US, version 3.20I).

4.6 Investigation of transformation in tissue culture as a method to alter specific traits of almond in otherwise successful cultivars

Plant material
Leaf explants from the *P. dulcis* cv. Ne Plus Ultra were used in the transformation experiments. The explants were taken from clonal shoots propagated *in vitro* using the protocol of Ainsley et al., (2000).

Bacterial Strain and Vectors
Agrobacterium strain EHA 105 (Hood et al., 1993) transformed with the plasmid pBI121mgfp-5-ER (Haseloff et al., 1997) and AGL1 (Lazo et al., 1991) transformed with the plasmid pNOV2819 manA (Syngenta, NC 27709, USA) were used in the transformation of almond leaf explants. The plasmid pBI121mgfp-5-ER has the *npt*II gene under the nopaline synthase (*nos*) promoter, *nos* terminator and the mgfp-5-ER gene under the control of 35S promoter and the *nos* terminator. The plasmid pNOV2819 manA contains the *pmi* gene under the control of CPMS (Cestrium Yellow Leaf Curling Virus Promoter—short version) promoter and *nos* terminator.

Transformation of in vitro leaf explants
The four youngest fully expanded leaves from *in vitro* micropropagated clonal shoots were used for transformation. The explants were pre-cultured for 3 days in liquid MS medium (Murashige and Skoog, 1962) supplemented with IBA (1.96 mg/l) and BAP (2.5 mg/l) at 23±2°C in dark. The pre-cultured explants were dissected transversely across the midrib into 5 mm sections prior to transformation.

Agrobacterium cultures were grown overnight to turbidity (late log phase) in LB medium supplemented with 0.1% (w/v) glucose and appropriate antibiotics (100 µM kanamycin and 25 µg/ml rifampicin for EHA 105 carrying the plasmid pBI121mgfp-5-ER and 50 µg/ml spectinomycin and 25 µg/ml rifampicin for the AGL1 strain carrying the plasmid pNOV2819 manA). The cultures were centrifuged at 4,000 rpm at 18°C for 5 min and adjusted to $OD_{550\ nm}$ of 0.5 with LB medium supplemented with 0.1% (w/v) glucose. Acetosyringone (Sigma) at a final concentration of 100 µM was added to the *Agrobacterium* cultures and incubated at 28°C with shaking for 2 h. The cultures were centrifuged at 4,000 rpm at 18°C for 5 min and the cells diluted to an $OD_{550\ nm}$ of 0.3 with liquid MS medium. The pre-cultured almond leaf explants were co-cultivated with *Agrobacterium* cultures for 1 h on a rotary shaker at 28°C. The explants were blotted on sterile Whatman filter papers and transferred to plates containing RM1 medium (MS supplemented with 1.96 mg/l IBA, 2.5 mg/l BAP, 30 g/l sucrose, 630 µM cefotaxime) and incubated in the dark at 22°C for 3 days. After co-cultivation the leaf explants were washed twice in liquid MS medium with 1 mM cefotaxime for 10 min each. The explants were blotted and transferred to plates with RM1 medium and incubated in the dark at 25±1°C for 3 weeks, placed in dim light for 2 weeks and subsequently transferred to full light.

The tissues transformed with the pBI121mgfp-5-ER construct were subcultured onto RM1 medium every 2 weeks and subjected to four different treatments. Three days or twenty-one days after co-cultivation the tissue was transferred to RM1 medium supplemented with or without kanamycin (15 or 20 µM) for 4–6 weeks. The explants with regenerating shoots from the kanamycin-free medium were transferred to RM2 (MS supplemented with 0.1 mg/l IBA, 1.0 mg/l BAP, 30 g/l sucrose, 630 µM cefotaxime) medium and maintained on this medium until the shoots were 1.5–2 cm long (4 weeks). The shoots were then transferred to RM2 medium supplemented with 15 µM kanamycin (70 days after co-cultivation) and the shoots that survived the selection were rooted and transferred to soil in pots in the glasshouse.

Putatively transformed tissue from the transformation with the pNOV2819 manA construct was transferred to RM3 medium (MS supplemented with 1.96 mg/l IBA, 2.5 mg/l BAP, 15 g/l sucrose, 2.5 g/l mannose, 630 µM cefotaxime) after 3 weeks in culture and maintained on the selection medium for four passages (8 weeks). The shoots were subsequently transferred to RM1 medium and maintained until they were ready to be rooted and transferred to soil in the containment glasshouse.

Leaf antibiotic resistance assay method

Putative transformed leaves from shoots regenerated from the transformations with the pBI121mgfp-5-ER construct were tested for their susceptibility or resistance to the antibiotic kanamycin. A 1 cm piece of leaf from the growing axis was excised and cut into four pieces and placed on plates with RM2 medium supplemented with kanamycin ranging in concentrations from 5 to 15 µM. The plates were incubated for seven days at 25°C under fluorescent light and scored for resistance/sensitivity to kanamycin. The leaf explants that bleached were scored as sensitive while the leaf explants that did not show bleaching were scored as resistant.

Analysis for transgene insertion in almond

Genomic DNA was extracted from the callus and or shoots regenerated from the transformations with the pBI121mgfp-5-ER and pNOV2819 manA constructs using the DNeasy Plant Mini Kit (QIAGEN) as per manufacturer's instructions. The forward and reverse PCR primers used to amplify the *npt*II (KanF: GAGGCTATTCGGCTATGACTG, KanR: ATCGGGAGCGGCGATACCGTA) and the *pmi* gene fragments (PMIF: ACAGCCACTCTCCATTCA, PMIR: GTTTGCCATCACTTCCAG) were designed using the NetPrimer (PREMIER Biosoft International, Palo Alto, CA). All PCR reactions used between 40 and 60 ng of genomic DNA. Aliquots of 5 µl of the PCR products were electrophoresed on a 1.5%

(w/v) agarose gel in 0.5×TBE (Tris Borate EDTA buffer). The gels were stained with ethidium bromide (0.5 µg/ml) and visualised under UV light.

For Southern analysis, 5 µg of genomic DNA from PCR-positive shoots were digested with *Xho*I (transformants containing pBI121mgfp-5-ER) or *Sal*I (transformants containing pNOV2819 manA) to determine the integration of the gene of interest. Following digestion, the DNA fragments were separated on a 1% (w/v) agarose gel and blotted onto Hybond N^+ nylon membrane (Amersham). A 700 bp fragment (for *nptII* gene) or 514 bp fragment (for *pmi* gene) were generated by PCR with labelled with [^{32}P]-dCTP by random priming using the DECAprime II DNA labelling kit (Ambion) and used as probes. Hybridisation was carried out at 65°C in sodium phosphate buffer [0.5 M sodium phosphate, 1 mM EDTA and 7% (w/v) SDS]. The membranes were washed with 2× SSC + 0.1% (w/v) SDS for 15 min, 1× SSC + 0.1% (w/v) SDS for 15 min and 0.1× SSC + 0.1% (w/v) SDS for 5 min at 65°C. The membranes were exposed to the phosphorimager for 3 days.

Rooting of shoots

Using a method very similar to that described by Ainsley et al., (2001b), shoots were rooted by brief treatment with 1 mM IBA (no phloroglucinol). Shoot pre-treatment at 4°C was reduced from 4 weeks to either 1 or 2 weeks; water agar was best solidified using 1.5% agar and, after overnight exposure to IBA, shoots in 1/2 strength MS medium were initially kept in the dark for 2 or 3 days. Shoots with roots were potted in a mixture of Nu-Erth Premium Potting Mix:peat moss:vermiculite (3:1:1) and gradually acclimatised in a glasshouse.

4.7 DNA fingerprinting of almond cultivars, and commencement of genetic mapping

4.7.1 DNA fingerprinting

The aim of this study was to compare the DNA fingerprints of a number of important Australian cultivars, using the RAPD-PCR technique, to establish the breeding lines from which they have developed.

Plant material

Leaves of Australian, Californian, European, and Middle Eastern almond cultivars were obtained from the following sources: the Claremont and Alverstoke orchards at the Waite Institute, Adelaide, South Australia; Loxton Research Centre, Loxton, South Australia; private orchards within South Australia (Angle Vale and Willunga) and Victoria (Lindsay Point); and from trees in Israel and Turkey (Table 4.7.1). Leaves were collected in early spring and stored at –80°Cuntil required for DNA extraction.

DNA extraction

DNA was extracted from leaf tissue using the method reported by Mekuria et al., (1999). Briefly, fresh young leaves were ground to a fine powder in liquid nitrogen and extracted with hot CTAB containing 2-mercaptoethanol and PVP-40T. Contaminating substances were removed with chloroform: isoamyl alcohol and the DNA was precipitated with cold isopropanol and washed in76% ethanol containing 10 mmol/L NH_4Ac until it turned white. The purified DNA was dissolved in TE buffer (10 mmol/L Tris–HCl, 0.1 mmol/L EDTA, pH 8.0) and RNA was removed by incubating the sample with DNase-free RNase A. Additional proteins, including RNase, were precipitated with NH_4Ac, and the DNA was collected by precipitation with ethanol and dissolved in TE buffer. The absorbance of the DNA was measured at 230, 260 and280 nm and the ratios of the absorbances at 260/280 and 260/230 nm were used to determine its purity (Johnson, 1994). DNA samples with absorbance ratios above 1.7 (Sambrook et al., 1989) were used for further analysis and stored at –20°C until needed.

Table 4.7.1 Source of the almond accessions assessed for genetic similarities using RAPD PCR technique

Accession	Source of leaves	Parentage
	Australia	
Baxendale	Waite Claremont Orchard	
Chellaston (Cole)	Waite Claremont Orchard	Selection of Brandis?
H184	Loxton	Peach-almond hybrid
Johnston (Giles)	Waite Claremont Orchard	
Johnston's Prolific	Waite Claremont Orchard	
Keane's Seedling	Angle Vale	Selection of wild hard-shell?
McKinlay's Magnificent	Willunga	
Parkinson 1	Willunga	
Parkinson 2	Waite Claremont Orchard	
Pierce	Willunga	
Pethick Wonder	Angle Vale	Selection of Johnston's Prolific?
Somerton	Loxton	Selection of Johnston's Prolific?
Strout's Papershell	Loxton	
White Brandis 1	Willunga	
White Brandis 2	Willunga	
	France	
Ferraduel	Loxton	Cristomorto × Aï
Ferragnès	Loxton	Cristomorto × Aï
Ferralise	Loxton	Ferragnès × Ferraduel
Ferrastar	Loxton	Cristomorto × Ardechoise
	Iran	
Iranian Seedling 1	Waite Claremont Orchard	
Iranian Seedling 2	Waite Claremont Orchard	
	Israel	
Alnem 88	Loxton	
	Middle East	
Prunus orientalis	Turkey	
Prunus webbii	Waite Alverstoke Orchard	
	USA	
All In One	Waite Claremont Orchard	Peach-almond hybrid
Carmel	Waite Claremont Orchard	Selection of Nonpareil?
Fritz	Waite Claremont Orchard	
Golden State	Angle Vale	
Le Grand	Loxton	
Milo	Waite Alverstoke Orchard	Nonpareil × Selection 24-6?
Mission 1	Waite Claremont Orchard	
Mission 2	Waite Claremont Orchard	
Mission Early	Lindsay Point	
Mission Normal	Lindsay Point	
Ne Plus Ultra	Waite Claremont Orchard	
Nonpareil 15-1	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-4-72	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-5-72	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-6-72	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-7-72	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-8-72	Waite Claremont Orchard	Nonpareil Selection

Nonpareil 3-8-9-72 1	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-9-72 2	Waite Claremont Orchard	Nonpareil Selection
Nonpareil 3-8-11-72	Waite Claremont Orchard	Nonpareil Selection
Nonpareil (Giles)	Waite Claremont Orchard	Nonpareil Selection
Peerless HRU	Waite Claremont Orchard	
Price	Waite Claremont Orchard	
Tardy Nonpareil	Loxton	
Thompson 1	Waite Alverstoke Orchard	
Thompson 2	Waite Claremont Orchard	
Thompson 3	Waite Claremont Orchard	
Thompson 4	Loxton	

PCR amplification and analysis

PCR reactions were performed in a volume of 20 µL containing 40 ng of genomic DNA, 1.5 mmol/L $MgCl_2$, 0.25 µmol/L decamer oligodeoxynucleotide primer (Operon Technologies), 200 µmol/L each of dATP, dCTP, dGTP and dTTP, 1 x *Taq* DNA polymerase buffer (20 mmol/L Tris–HCl, pH 8.4, 50 mmol/L KCl) and 1 unit of *Taq* DNA Polymerase (Life Technologies), overlaid with 2 drops of autoclaved mineral oil (Mekuria et al., 1999). A negative control without genomic DNA was included with each mix. PCR reactions took place in a thermocycler (Programmable Thermal Controller, MJ Research Inc., USA) with the following program: initial denaturation period of 2 min at 95°C, followed by 39 cycles of 1 min at 95°C, 10 s at 50°C, 15 s at 45°C, 20 s at 40°C, 1 min at 35°C, 30 s at 45°C and 1 min 45 s at 72°C, and a final extension step of 5 min at 72°C (Collins and Symons 1993). PCR amplification products were separated by electrophoresis on 1.5% agarose in 1 x TBE buffer (89 mmol/L Tris–HCl, pH 8.3,89 mmol/L boric acid, 5 mmol/L EDTA), and the sizes of the products estimated by running a 100-bp DNA ladder (GeneWorks, Adelaide, South Australia) in one of the wells. Gels were stained with ethidium bromide (0.2 µg/mL) and photographed under UV light with Polaroid film 667. The gel image was also captured by the Tekcap computer program (Version 1.0, Tekram Corporation 1998).

Primer survey

A screening of 20 decamer oligodeoxyribonucleotide primers (Operon Technologies, Alameda, CA, USA) of arbitrary nucleotide sequence was performed in duplicate with the DNA from 5 cultivars selected at random. Six primers that produced clear and reproducible polymorphic bands were selected for DNA fingerprinting: OPA-02, 5′-TGCCGAGCTG-3′; OPA-03, 5′-AGTCAGCCAC-3′; OPA-04, 5′-AATCGGGCTG-3′; OPA-08, 5′-GTGACGTAGG-3′; OPA-10, 5′–GTGATCGCAG-3′; and OPA-12, 5′-TCGGCGATAG-3′.

Analysis of bands

The scanned images of the gels were viewed with a Gel Pro Analyser (Version 3.1, Media Cybernetics, Maryland, USA) and the amplified products were scored as 1 (band present) or 0 (band absent). The Polaroid photographs of the gel assisted in verifying band presence and absence. This information was used to compile a binary data matrix that was analysed using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Version 2.0, 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 common band (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.

4.7.2 Genetic mapping of almond

The objective of this study was to produce an integrated genetic linkage map for a F_1 hybrid population of the cultivars 'Nonpareil' x 'Lauranne', constructed using RAPD, inter-simple sequence repeat (ISSR) SSR and morphological markers.

Plant Material and Morphological Characters

A progeny set of 93 hybrids from a cross between the American self-incompatible cultivar 'Nonpareil' (female parent) and the French self-compatible cultivar 'Lauranne' (male parent) in a pseudo-testcross configuration was selected for linkage map construction. This population is maintained in the field under standard orchard conditions for phenotypic evaluation. For each hybrid two kernels from each of three years were tasted and scored as either sweet or semi-bitter.

DNA Extraction

Total DNA was extracted from frozen (-80°C) leaf tissue according to the method of Lamboy and Alpha (1998). The purified DNA was checked for purity and quantity by spectrophotometry, analysing the A_{260}/A_{230} and A_{260}/A_{280} ratios, and A_{260} respectively (Sambrook et al., 1989).

RAPD, ISSR and SSR Markers

Six RAPD, 13 ISSR and 11 SSR primers were used for polymorphism generation on the 93 F_1 progeny and two parents. PCR amplification was according to Joobeur et al., (2000). PCR products were visualised by polyacrylamide gel electrophoresis with autoradiogram detection for CPACT primers according to Aranzana et al. (2003) PCR products using fluorescent primers were scored using a 377 ABI Prism DNA sequencer and Genotyper software (Applied Biosystems, USA).

Morphological markers

Twelve quantitative traits were evaluated for three years (2001, 2002, and 2003), χ^2 analysis was performed to test for deviation from the expected mendelian ratio. Seven segregating traits were selected including *S*-allele, kernel shape, kernel thickness, kernel taste, double kernels, testa pubescence, and testa colour were scored for the 2003 harvest (third fruiting year) and treated in the same manner as a dominant marker type. Briefly, *S*-alleles were scored using allele specific primers. Each allele was treated independently and entered into the mapping matrix as a single entity. The double character was calculated based on the number of double kernelled fruit in a sample of 20-50 nuts, expressed as a percentage of the total. The segregation class was selected as absent (0%) and present (<0%). Kernel shape and thickness were measured using digital callipers at the widest, thickest and longest points. Kernel shape was determined by the length/width ratio broken into five groups, which were compressed into two classes for linkage analysis, broad (<0.56 cm) and medium/narrow (>0.55 cm). Kernel testa colour was scored visually as a comparison to the two parents and scored as pale or dark. Both kernel taste and testa pubescence (fuzziness) were scored organoleptically and scored on the basis of consistency over three years of data (2001, 2002 and 2003).

Linkage Analysis

The parents and all progeny were scored for presence or absence of bright, reproducible, segregating bands (or peaks for fluorescent detection). All fragment sizes were calculated using GelPro Analyser (version 3.1, Media Cybernetics, USA), segregating bands were identified by the primer code followed by the fragment size. Two data sets were constructed, one for each parent based on the parental origin of the band in accordance to the pseudo-testcross mapping strategy. Chi-squared tests were performed to check for segregation distortion of the markers. The two parental maps were produced using the software Joinmap 3.0 (Van Ooijen and Voorrips, 2001), using the cross-pollination format, at a LOD of 3.0 - 4.0. An integrated map was produced from the corresponding anchor intercross markers common to both parents using the 'combine groups for mapping' function of the program. Map distances in centiMorgans were calculated by converting

recombination frequencies using the Kosambi mapping function (Kosambi, 1944). These data were presented pictorially with the map drawing software MapChart 2.1 (Voorrips, 2002).

5. Results

5.1 Performance evaluation of local and imported selections and imports of scion and rootstock material of almond

Rootstock trial

Most varieties were planted in 1999 with the exception of Atlas, which was planted in 2000. Viking, Alnem 88 and H184 were planted in 2001 after budded trees finally arrived. This was the completion of the planting for the current trial. Harvesting and data collection were expected to commence in 2004. The trees were growing on two soil types: deep mallee sand and shallow sand over lime marl. By 2001 all planted trees were growing well, with the exception of Citation rootstock, which proved more difficult to establish with three trees dying. Preliminary observations indicate that this rootstock seems to be performing erratically under our growing conditions. A wide range of responses was observed between treatments, with some clearly being unsuitable. Citation's performance is less satisfactory and probably non-commercial. One replicate of Citation was removed, as the majority of trees either died or were severely stunted. These were replaced to minimise commercial impact for the property owner. However, we had ongoing problems with sourcing budded trees with only 5 of the 30 Viking trees delivered. One other major problem was the source of some of the rootstocks was unreliable. The ongoing problems of supply meant that to some extent the integrity of the trial was compromised with trees at that time varying in age from zero to three years. Similarly, there were problems with the large disparity between tree sizes supplied between nurseries. These treatments were used for observation purposes only, as valid comparisons between all treatments were not possible. The trial was abandoned due to these reasons as advised by HAL at the time.

Scion trial

Table 5.1.1 shows the characteristics of the new 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.

Most of the new cultivars became available during 2002, either as evaluation trees or as budwood to nurseries. 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.

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

Primary evaluation of progeny from breeding program

Appendix 2 shows a summary of the crosses achieved from 1997 to 2006 and the resulting progeny numbers. Table 5.2.1 shows a summary of almond breeding from 1997 to 2006.

Table 5.2.1 Summary of almond breeding 1997 - 2006

Number of cultivars used	79
Number of different crosses	293
Number of crosses with >200 progeny	23
Number of progeny produced	29,006
Number of selections for further testing	24

To date all progeny produced from 1997 to 2002 have been evaluated on their kernel characteristics. Table 5.2.3 shows the superior selections from these evaluations. Primary evaluations will continue into the new project AL07000.

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 1997-1999 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

Table 5.1.1 Almond varietal characteristics at September 2006.

Variety	***Crop Potential (Calif. Trials)***	***Flowering (Calif)***	***Flowering (Aust)***	***Harvest (Calif)***	***Shell***	***Kernel***	***Market type/Use***	***Disease Susceptibility***		***Comments***
								Bact. spot	A'nose	
Padre	Good 93% Nonpareil	+5 days Nonpareil	+ 7 days?	+26 days Nonpareil	Hard	Medium/ Small	Mission/ Roasting	NA	S	V. upright growth, not fruitful. Butte/Padre combination is the top producer in US.
Livingston	Very Good 109% Nonpareil	+5 days Nonpareil	+ 2 days?	+8 days Nonpareil	Papershell	Medium size, light brown	California/ Blanching	NA	S	Reasonably attractive kernels.
Butte	Very Good 118% Nonpareil	+5 days Nonpareil	+ 4/5 days?	+18 days Nonpareil	Semi Hard	Small/ Medium	Mission/ Roasting	NA	S+	Most productive almond variety in California. V fruitful habit in Aust
Sonora	Good 94% Nonpareil	-3 days Nonpareil	- 3 days?	+7 days Nonpareil	Papershell *Poorly sealed*	Medium/ large, golden, elongate	California/ Blanching	NA	S	Frost sensitive and tendency to alternate bearing. *Appears badly infected with unidentified virus.*
Monterey	Very Good 106% Nonpareil	+2 days Nonpareil (-2 in Aust?)	- 2 days?	+26 days Nonpareil (Less in Aust?)	Soft	Large	California/ Blanching	NA	S+	Large, elongate dark kernels. Up to 20% doubles. Habit does not appear fruitful at 3rd leaf.
Avalon *	Appears Very Good	-3 days Nonpareil		+8 days Nonpareil	Semi Soft	Medium	California/ Blanching	NA	S	Relatively new variety, little acreage to date. Long flowering, overlaps Carmel

Savanna*	Reputedly Good	+14 days Nonpareil		+14 days Nonpareil		Medium/ Large	Nonpareil type	NA	S	Being brought into Australia by commercial nursery
Morley*	Reputedly Good	+14 days Nonpareil		+14 days Nonpareil	Semi-hard	Medium/ Small	Similar to Butte	NA	S	Being brought into Australia by commercial nursery
Sauret #1	Good 93% Nonpareil	+4 days Nonpareil		+5 days Nonpareil	Well sealed paper	Medium	California/ Blanching	NA	S	Under evaluation in Australia (5th leaf). Appears to crop well. Flowering times appear to be getting later as trees mature.
Wood Colony *	Reputedly good.	+2 days Nonpareil	+ 1 day?	+7 days Nonpareil	Semi-soft	Medium size, darkish.		NA	S	Californian Trials indicate shy bearing at 35% Nonpareil. Not fruitful in Aust at 3rd leaf. 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 between California and Australia and 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 5.2.3 Superior selections and their kernel characteristics from 1997, 98, 99, 2000, 01 and 02 progeny

Tree	Cross ID	Cross	*S*-alleles	In-shell wt (g)	%double kernel	% shell hardness	Kernel wt (g)	Kernel taste	Testa colour	Appearance Score/10	%double kernel Score/5	%shell hardness Score/5	Kernel wt Score/5	Kernel taste Score/1	Testa colour Score/5	Shell seal Score/5	Total score /35*
2bT33	97040	Legrand x NP	S_7S_8	2.27	0	62	1.40	sweet	light	8	5	5	4	1	4	-	26
4bT1	97022	NP x Keane	$S_8S_?$	1.98	0	74	1.46	sweet	v light	9	5	5	4	1	5	-	28
1bT31	97001	NP x Lauranne	S_3S_8	4.02	0	39	1.56	sweet	v light	9	5	5	4	1	5	-	28
1bT32	97001	NP x Lauranne	S_7S_f	4.40	11	30	1.31	sweet	light	9	4	5	3	1	4	-	25
8aT48	97018	NP x Carmel	S_7S_5	3.28	0	48	1.56	sweet	light	8	5	5	4	1	4	-	26
10bT35	97011	NP x Carmel	S_5S_8	1.75	4	78	1.37	sweet	light	9	5	5	3	1	4	-	26
R12T17	98028	NP x Lauranne	S_7S_f	3.06	0	42	1.29	sweet	light	9	5	5	3	1	4	5	31
R13T18	98028	NP x Lauranne	S_7S_f	3.93	0	36	1.41	sweet	light	7	5	5	4	1	4	5	30
R30T25	98031	NP x Carmel	S_5S_7	2.18	5	55	1.20	sweet	light	8	5	5	3	1	4	5	30
R33T48	98001	Carmel x Lauranne	S_3S_8	3.49	0	39	1.35	sweet	v.light	8	5	5	3	1	5	5	31
R38T63	98035	NP x Johnston	S_7S_{23}	2.87	5	51	1.47	sweet	light	7	5	5	4	1	4	5	30
R5T19	98027	NP x Somerton	S_1S_8	2.64	0	55	1.46	sweet	light	7	5	5	4	1	4	5	30
R30T45	98031	NP x Carmel	S_5S_8	1.84	4	78	1.43	sweet	light	7	5	5	4	1	4	5	30
R42T106	98042	Price x NP	S_7S_8	1.54	4	80	1.23	sweet	light	9	5	5	3	1	5	3	30
R21T70	99002	Carmel x Johnston	S_8S_{23}	3.87	0	51	1.97	sweet	light	6	5	5	5	1	5	5	31
R23T45	99026	NP x Somerton	S_7S_{23}	2.69	3	60	1.62	sweet	light	8	5	5	4	1	5	5	32
R53T45	99013	LeGrand x Keanes	$S_8S_?$	1.90	0	50	0.96	sweet	light	9	5	5	2	1	5	5	31
R58T27	99012	Johnston x NP	S_8S_{23}	2.13	0	64	1.36	sweet	light	9	5	5	3	1	5	5	32
R61T33	20014	NP x Sauret#1	S_5S_8	1.75	0	71	1.25	sweet	light	8	5	5	3	1	5	4	30
R1T183	01007	Keanes x Antoñeta	S_7	2.17	0	65	1.41	sweet	light	8	5	5	4	1	5	5	32
R2T272	02003	Ferragnès x 1bT32	S_1	4.67	0	30	1.39	sweet	light	9	5	5	3	1	5	5	32
R3T287	02005	Nonpareil x 12-350	S_7	2.12	0	65	1.37	sweet	light	8	5	5	3	1	5	5	31
R9T29	02015	Carmel x 21-323	S_8	1.84	0	67	1.23	sweet	light	7	5	5	3	1	5	5	30
R9T143	02014	Carmel x 12-350	S_8	2.63	5	55	1.45	sweet	light	7	5	5	4	1	5	5	31

* Selections from 1997 were scored out of a total of 30; - shell seal was not scored for 1997 progeny

Secondary evaluation of progeny from breeding program
Eighteen of the 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 spacings 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 3. The remaining selections along with future selections will be planted in upcoming seasons.

5.3 Development of improved virus detection methods for almond breeding stock and mother plants

Both methods of RNA extraction produced identical results from both RT-PCR and nested PCR.

Detection of PNRSV and PDV by ELISA
The sensitivity of ELISA for the detection of PNRSV and PDV was evaluated using 175 almond leaf samples. Nine of the samples gave a positive response for PNRSV in the year 1999, and eight were positive in the year 2000 (Table 5.3.2). Prune dwarf virus was not detected in any of the samples in the years 1999 and 2000 (Table 5.3.2).

Table 5.3.2 Comparison of results obtained by ELISA and RT-PCR in the detection of PNRSV and PDV in almond leaves.

Tree	1999		2000				2001	
	ELISA		ELISA		RT-PCR		RT-PCR	
	PNRSV	PDV	PNRSV	PDV	PNRSV	PDV[a]	PNRSV	PDV[a]
NP04	0 (16)	0 (16)	0 (16)	0 (16)	0 (16)	15 (16)	0 (16)	15 (16)
NP05	0 (13)	0 (13)	0 (13)	0 (13)	0 (13)	12 (13)	0 (13)	12 (13)
NP06	0 (18)	0 (18)	0 (18)	0 (18)	0 (18)	17 (18)	0 (18)	17 (18)
NP07	0 (17)	0 (17)	0 (17)	0 (17)	0 (17)	16 (17)	0 (17)	16 (17)
NP08	0 (17)	0 (17)	0 (17)	0 (17)	0 (17)	17 (17)	0 (17)	17 (17)
NP09	0 (17)	0 (17)	0 (17)	0 (17)	0 (17)	17 (17)	0 (17)	17 (17)
NP10	0 (5)	0 (5)	0 (5)	0 (5)	0 (5)	5 (5)	0 (5)	5 (5)
NP15	4 (67)	0 (67)	3 (67)	0 (67)	4 (67)	67 (67)	4 (67)	67 (67)
Sauret	5 (5)	0 (5)	5 (5)	0 (5)	5 (5)	2 (5)	5 (5)	2 (5)
Total	9 (175)	0 (175)	8 (175)	0 (175)	9 (175)	168 (175)	9 (175)	168 (175)

NP= Nonpareil; Numbers in parentheses indicate the total number of samples tested for each tree; a=Nested PCR for PDV

Detection of PNRSV by RT-PCR
PNRSV was detected in nine out of 175 leaf samples tested using RT-PCR in both the years 2000 and 2001 (Table 5.3.2). Multiplex RT-PCR resulted in the amplification of a 351 bp product 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.

Detection of PDV by RT-PCR and Nested PCR

Total RNA derived from cherry produced an amplification product of 722 bp after the first round of multiplex RT-PCR. No amplification products were detected in any of the almond samples from Australia. However, PDV was detected in an RNA sample extracted from CEBAS1 grown in Spain. The combination of RT-PCR with nested PCR produced an amplification product of 241 bp for cherry, and 168 of the 175 almond samples tested in both 2000 and 2001 (Table 5.3.1 and 5.3.2). The sequence of the amplified product matched the sequence of the virus coat protein of PDV located between the primers.

5.4 Investigation of micropropagation for the multiplication and propagation of new improved rootstocks from the selection program

Effect of BAP and IBA on shoot multiplication of almond scions and the hybrid rootstock

Table 5.4.1 shows the number of shoots per explant within various size ranges for each of the various levels of BAP and IBA. For Nonpareil 15-1, the combination of 5 µM BAP and either 0.049 or 0.49 µM IBA resulted in significantly greater numbers of shoots between 0.5 and 1.0 cm than all other treatments. For shoots longer than 1.0 cm, the largest numbers occurred with either 1 or 5 µM BAP with 0.049 µM IBA, or 5 µM BAP with 0.49 µM IBA. No shoots reached a length of 2 cm (Fig. 5.4.1).

Table 5.4.1 Effect of various concentrations of IBA and BAP on number of healthy shoots of different sizes for Nonpareil 15-1 (AP medium), Ne Plus Ultra (MS medium), and the hybrid rootstock (MS medium)

Concentration (µM)		Number of shoots per explant within specified lengths (cm)[a]							
IBA	BAP	Nonpareil 15-1		Ne Plus Ultra			Hybrid rootstock		
		0.5–1.0	>1.0	0.5–1.0	>1.0	>2.0	0.5–1.0	>1.0	>2.0
0	0	1.0 ^{ab}	0.0 ^{a}	1.0 ^{ab}	0.0 ^{a}	0.0 ^{a}	1.0 ^{a}	0.0 ^{a}	0.0 ^{a}
0	1	4.0 ^{abc}	0.6 ^{a}	4.6 ^{a}	1.6 ^{abc}	1.4 ^{cd}	7.0 ^{ab}	1.0 ^{ab}	0.6 ^{abc}
0	5	9.6 ^{e}	1.2 ^{abc}	35.6 ^{ef}	5.4 ^{de}	0.6 ^{ab}	29.6 ^{ef}	5.0 ^{de}	*1.4* ^{d}
0	10	2.8 ^{abc}	0.6 ^{a}	35.6 ^{ef}	3.4 ^{cd}	0.0 ^{a}	*41.4* ^{g}	*8.0* ^{g}	0.8 ^{bcd}
0	20	1.2 ^{ab}	0.0 ^{a}	24.8 ^{cde}	0.4 ^{ab}	0.0 ^{a}	21.0 ^{cd}	2.8 ^{bcde}	0.0 ^{a}
0.049	0	1.0 ^{a}	0.0 ^{a}	1.0 ^{a}	0.0 ^{a}	0.0 ^{a}	1.0 ^{a}	0.0 ^{a}	0.0 ^{a}
0.049	1	8.8 ^{de}	*3.2* ^{d}	7.2 ^{ab}	2.8 ^{bc}	*2.8* ^{e}	2.2 ^{a}	1.0 ^{ab}	0.0 ^{a}
0.049	5	*17.2* ^{f}	2.6 ^{cd}	*51.6* ^{g}	*11.8* ^{g}	0.8 ^{bc}	19.4 ^{cd}	5.2 ^{ef}	0.2 ^{ab}
0.049	10	5.2 ^{bcd}	0.0 ^{a}	32.4 ^{de}	1.8 ^{abc}	0.2 ^{ab}	24.0 ^{de}	4.2 ^{de}	0.6 ^{cd}
0.049	20	1.6 ^{ab}	0.0 ^{a}	21.8 ^{cd}	1.2 ^{abc}	0.0 ^{a}	14.0 ^{bc}	1.2 ^{abc}	0.2 ^{ab}
0.49	0	1.0 ^{ab}	0.0 ^{a}	1.0 ^{a}	0.0 ^{a}	0.0 ^{a}	1.0 ^{a}	0.0 ^{a}	0.0 ^{a}
0.49	1	6.6 ^{cde}	1.0 ^{abc}	5.4 ^{ab}	1.4 ^{abc}	0.8 ^{bc}	5.2 ^{a}	2.6 ^{abcd}	1.0 ^{abc}
0.49	5	14.6 ^{f}	2.4 ^{bcd}	20.8 ^{c}	6.8 ^{ef}	0.6 ^{ab}	14.4 ^{bc}	3.6 ^{cde}	0.2 ^{ab}
0.49	10	4.0 ^{abc}	0.8 ^{ab}	16.2 ^{bc}	1.2 ^{abc}	0.0 ^{a}	15.0 ^{c}	4.6 ^{de}	0.2 ^{ab}
0.49	20	0.0 ^{a}	0.0 ^{a}	8.4 ^{ab}	0.4 ^{ab}	0.0 ^{a}	13.8 ^{bc}	0.4 ^{ab}	0.0 ^{a}

[a] Each treatment was replicated five times with the one shoot tip per replicate. Data were collected after two culture cycles for almond scion cultivars (4 weeks per cycle) and the hybrid rootstock (5 weeks per cycle).

Values with the same letters within a column are not significantly different at $\alpha = 0.1$ using Duncan's new multiple range test. The most successful levels are given in italics.

A further test compared concentrations of 1, 3, and 5 µM BAP in combination with 0.049 µM IBA (Table 5.4.2). Significantly higher fresh weight and number of leaves occurred with 3 µM BAP. The combination of 3 µM BAP and 0.049 µM IBA was incorporated into all subsequent cultures using Nonpareil 15-1.

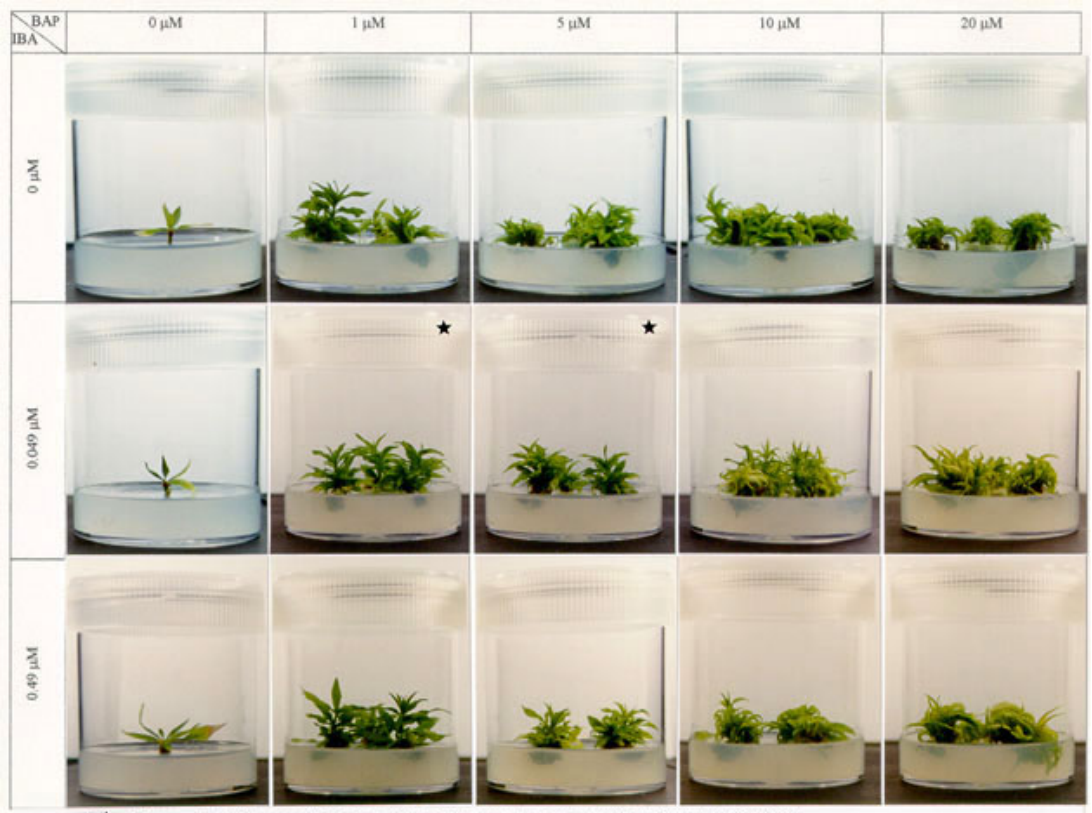

Figure 5.4.1 2nd culture cycle of Nonpareil 15-1 on AP medium supplemented with various combinations of plant growth regulators (BAP and IBA)(4 weeks old)

Table 5.4.2 Effect of three concentrations of BAP (1, 3, and 5 µM) with 0.049 µM IBA in AP medium on growth and multiplication of shoot tips of Nonpareil 15-1

Concentration of BAP (µM)		Fresh weight of shoots (g)[a]		Number of shoots[a]		Length of shoots (cm)[a]	
		Total	Healthy	Total	Healthy	0.5–1.0	>1.0
1		0.74 a	0.74 a	24.2 a	24.2 a	8.8 a	3.2 ab
3		2.20 b	2.20 c	125.8 c	125.8 c	36.0 c	7.8 ab
5		2.45 b	1.09 ab	91.4 ab	55.8 ab	17.2 ab	2.6 a

[a] Each treatment was replicated five times with the one shoot tip per replicate from the first culture cycle. Data were collected after two culture cycles of 4 weeks per cycle. Values with the same letters within a column are not significantly different at $\alpha = 0.1$ using Duncan's new multiple range test.

For Ne Plus Ultra, the combination of 5 µM BAP and 0.049 µM IBA resulted in significantly greater numbers of shoots between 0.5 and 1.0 cm than all other treatments. In contrast to Nonpareil 15-1, several treatments resulted in shoot lengths of 2 cm or greater, the most significant being for a combination of 1 µM BAP and 0.049 µM IBA.

The combination of 5 μM BAP and 0.049 μM IBA was incorporated into all subsequent cultures of Ne Plus Ultra.
For both Ne Plus Ultra and Nonpareil, the fresh weight and shoot number of hyperhydric shoots generally increased as BAP levels were increased up to maximum of about 10 μM, but the effect was not as pronounced as for Nonpareil 15-1 (data not shown). In contrast to the almond scions, the hybrid rootstock produced maximum fresh weight and number of healthy shoots per explant with 10 μM BAP in the absence of IBA (Table 5.4.1). The presence of IBA at either 0.049 or 0.49 μM resulted in a decrease in both fresh weight (data not shown) and number of shoots.

Rooting of the hybrid rootstock
Table 5.4.3 shows the effects of various concentrations of IBA on rooting of the hybrid rootstock. Roots emerged after 10–13 days on medium supplemented with IBA at concentrations between 1.4 and 14.7 μM IBA. IBA significantly increased the number of roots per shoot compared to the control. Concentrations of IBA equal to or greater than 2.4 μM significantly increased rooting efficiency (88.0–96.0%) compared to lower concentrations, but produced increasingly more callus. In the absence of IBA, both late root emergence (22–25 days), and low rooting efficiency (8.0%) were observed. Root growth rate was maximal on media supplemented with IBA between 2.4 and 7.3 μM, but at concentrations greater than this, root growth rate declined. Based on root growth rate, the most appropriate concentration of IBA for rooting of the almond/peach hybrid rootstock was 2.4 μM.

Table 5.4.3 Effect of IBA (μM) on rooting of hybrid rootstock shoots in basal medium consisting of half MS with 3% (w/v) sucrose and 0.7% (w/v) agar at pH 5.7

Concentration of IBA (μM)	Root emergence (days)	Number of roots per shoot[a]	Root growth rate[a,b] (mm per week)	Rooting efficiency[d] (%)	Callus formation[a,c]
0	20-25	0.08 ± 0.00 a	-	8.0 ± 0.0 a	+
1.4	10-12	1.64 ± 0.28 b	5.25 ± 0.22 b	72.0 ± 8.0 b	+
2.4	*10-13*	*2.40 ± 0.30 bc*	*6.05 ± 0.21 c*	*88.0 ± 4.9 c*	++
3.4	10-13	2.68 ± 0.34 bcd	6.00 ± 0.22 c	88.0 ± 4.9 c	++
4.9	10-12	3.04 ± 0.41 cde	5.85 ± 0.17 c	88.0 ± 4.9 c	+++
7.3	10-12	3.48 ± 0.46 cde	5.50 ± 0.15 bc	92.0 ± 4.9 c	+++
9.8	10-12	3.92 ± 0.56 de	3.95 ± 0.20 a	92.0 ± 4.0 c	++++
12.3	10-12	4.24 ± 0.54 e	3.65 ± 0.15 a	96.0 ± 8.0 c	++++
14.7	10-13	3.60 ± 0.55 cde	3.80 ± 0.15 a	96.0 ± 4.0 c	++++

[a] Cultures were incubated in the dark for 7 days. Each treatment was replicated five times with five shoots per replicate. Means ± standard error followed by the same letter within a column are not significantly different at $\alpha = 0.1$ using Duncan's new multiple range test. The most successful levels are given in italics.
[b] Twenty roots from each treatment were marked at the time of emergence and the root lengths were measured 1 week later.
[c] +: no or very small callus; ++: small callus (less than 5 mm diameter); +++: moderate callus (5-10 mm diameter); ++++: large callus (more than 10 mm diameter).
[d] $LSD_{0.05} = 0.13$ analysed by single factor completely randomised design with subsampling.

Micrografting
A comparison of soft, semi-hard, and hard stems of scions that were grafted to hard stems of the hybrid rootstocks, showed that the most successful micrografts (60–70 and 50% for

Ne Plus Ultra and Nonpareil 15-1, respectively) were those where the scion was hard-stemmed (Table 5.5.4). No successful grafts occurred when scions with soft stems were used. One week after transfer from the dark, grafts with soft scion stems turned brown and there was shoot necrosis. Grafts where the scion was semi-hard produced some successful grafts depending on the cultivar (30–40% for Ne Plus Ultra and 0–10% for Nonpareil 15-1).

Table 5.4.4 Survival of plants micrografted by apical-wedge grafting in vitro

Scions grafted on hybrid rootstock	Survival (%)[a]	
	Wrapped grafted area	Unwrapped grafted area
Nonpareil 15-1		
3-week-soft stems (0.5-0.7 cm long)	0	0
4-week-semi-hard stems (1 cm long)	10	0
5-7-week-hard stems (1.5 cm long)	50	50
Ne Plus Ultra		
3-week-soft stems (0.5-0.7 cm long)	0	0
4-week-semi-hard stems (1 cm long)	40	30
5-7-week-hard stems (1.5 cm long)	70	60

[a] The grafted plants were cultured on a rooting medium supplemented with 2.4 μM IBA and incubated in the dark for 7 days, then transferred to the light for 2 weeks. Each treatment was replicated 10 times with one grafted plant per replicate.

Hardening off

A potting mix composed of Nu-Erth premium potting mix, peat moss, and vermiculite was found to be satisfactory for survival of plantlets from in vitro cultures of both rootstocks and micrografted plants. About 92% of in vitro propagated plants were recovered and grown as normal plants.

5.5 Adoption of in vitro conservation methods to facilitate small-scale storage of germplasm as an alternative to in-ground collections

Shoot tips of Ne Plus Ultra were used to determine both the optimum cold-hardening period at 4°C, and the optimum dehydration time in PVS2 at 25°C before storing under LN. For cold- hardening, the highest survival of 87.5% occurred after 21 days, although there was no significant difference between 7 and 21 days. After 28 days, survival was significantly decreased (Fig. 5.5.1).

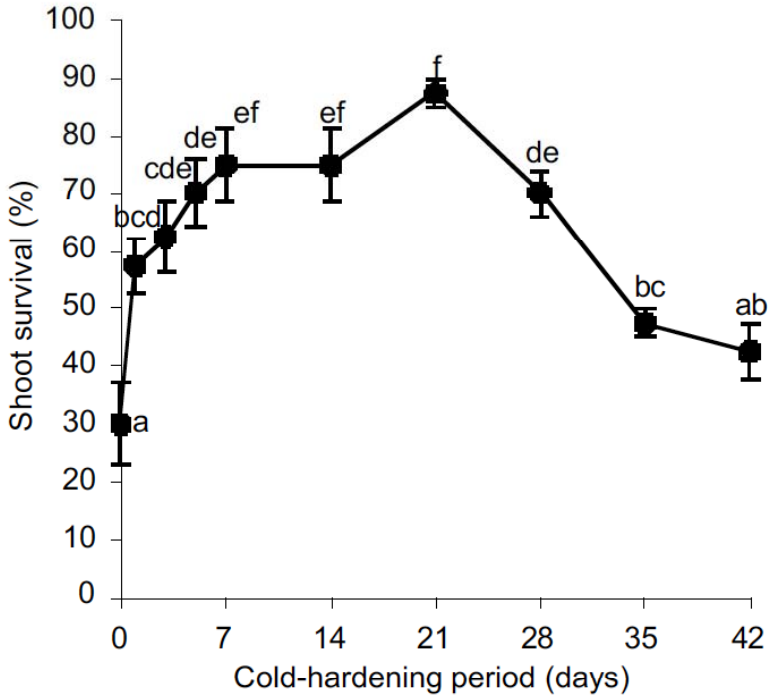

Fig. 5.5.1 Effect of cold-hardening period of shoot cultures at 4°C on shoot survival of Ne Plus Ultra stored in LN by vitrification. Excised shoot tips from cold-hardened shoots were precultured at 4°C for 1 day on MS agar medium supplemented with 0.7 M sucrose. The shoot tips were dehydrated using PVS2 for 45 min before storage under LN. Data were recorded after 3 days of storage in LN. The bars represent mean ± SE. Means with the same letter are not significantly different at the 5% level using Duncan's new multiple range test.

The survival of shoot tips, after removal from LN, increased with the time of incubation in PVS2. The highest survival of 87.5% occurred after 45 min (Fig. 5.5.2). Control shoot tips, treated with PVS2, but not immersed in LN showed survival of between 85-100% (Fig. 5.5.2). Longer periods of incubation in PVS2 decreased the survival of both LN-treated and control shoot tips. For Nonpareil 15-1 and the hybrid rootstock, using the same vitrification procedures as for Ne Plus Ultra, the highest survival was 60.0% at 45 min incubation time in PVS2 and 72.5% at 60 min incubation time in PVS2 respectively (Table 5.5.1). For long-term preservation, up to 180 days, the survival of cryopreserved shoot tips was between 51.7-77.5%. The survival of Ne Plus Ultra was slightly decreased by the time of preservation from 87.5% at 3 days of storage to 76.7% at 180 days of storage. There was no significant decrease in survival after 180 days of storage for Nonpareil 15-1 and the hybrid rootstock (Table 5.5.2).

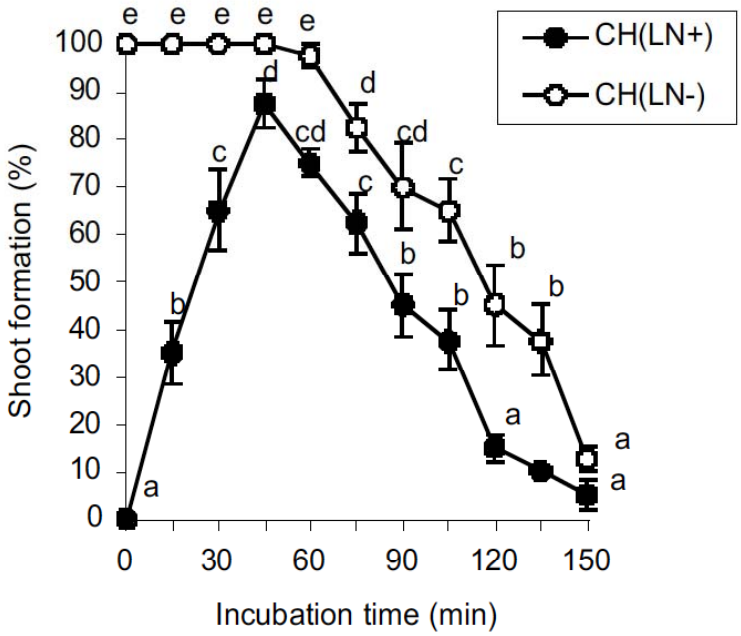

Fig. 5.5.2 Effect of time in PVS2 on shoot survival of Ne Plus Ultra. Shoot cultures were cold- hardened at 4°C for 21 days. Shoot tips were excised, precultured at 4°C for 1 day on MS agar medium supplemented with 0.7 M sucrose, and stored for 3 days ± LN. Values are the mean of four replicates, each with 10 shot tips. The bars represent ± standard error (SE). Means with the same letter on the same graph line are not significantly different at the 5% level using Duncan's new multiple range test. LN+ = stored in LN; LN- = stored at 25°C.

Table 5.5.1 Effect of dehydration time on survival of shoot tips. Shoot tips of 'Nonpareil 15-1' and the hybrid rootstock were cold-hardened at 4°C for 21 days, precultured at 4°C for one day on appropriate agar media (see text) supplemented with 0.7 M sucrose, dehydrated in PVS2 for different periods of time, and subsequently stored in LN for at least 3 days before thawing

Cultivars	Shoot survival (%) ± SE[†] Incubation time in PVS2 (min)			
	30	45	60	75
Nonpareil 15-1	47.5 ± 6.29	60.0 ± 7.07	37.5 ± 4.78	37.5 ± 8.53
Hybrid rootstock	60.0 ± 65.77	65.0 ± 6.45	72.5 ± 7.5	57.5 ± 8.53

[†]Four replicates were used with 10 shoots tips for each replicate. SE = standard error.

Table 5.5.2 Survival of cryopreserved shoot tips. Shoot tips were stored for up to 180 days in LN, and then thawed at 30°C and cultured on appropriate media (see text) without ammonium ions

Period of preservation	Shoot survival (%) ± SE[†]		
	Ne Plus Ultra	Nonpareil 15-1	Hybrid rootstock
3 days	87.5 ± 2.5 b	60.0 ± 3.7 ab	72.5 ± 3.9 a
90 days	78.3 ± 3.7 ab	49.2 ± 7.3 a	82.5 ± 2.2 b
180 days	76.7 ± 3.6 a	51.7 ± 3.4 ab	77.5 ± 3.3 ab

[†]Four replicates were used with 10 shoot tips for each replicate. SE = standard error.
a,b Means followed by the same letter are not significantly different.

Figure 5.5.3 shows the regenerated shoot tips of Nonpareil after cryopreservation micrografted onto hybrid rootstock., and a six-week-old plant after hardening off.

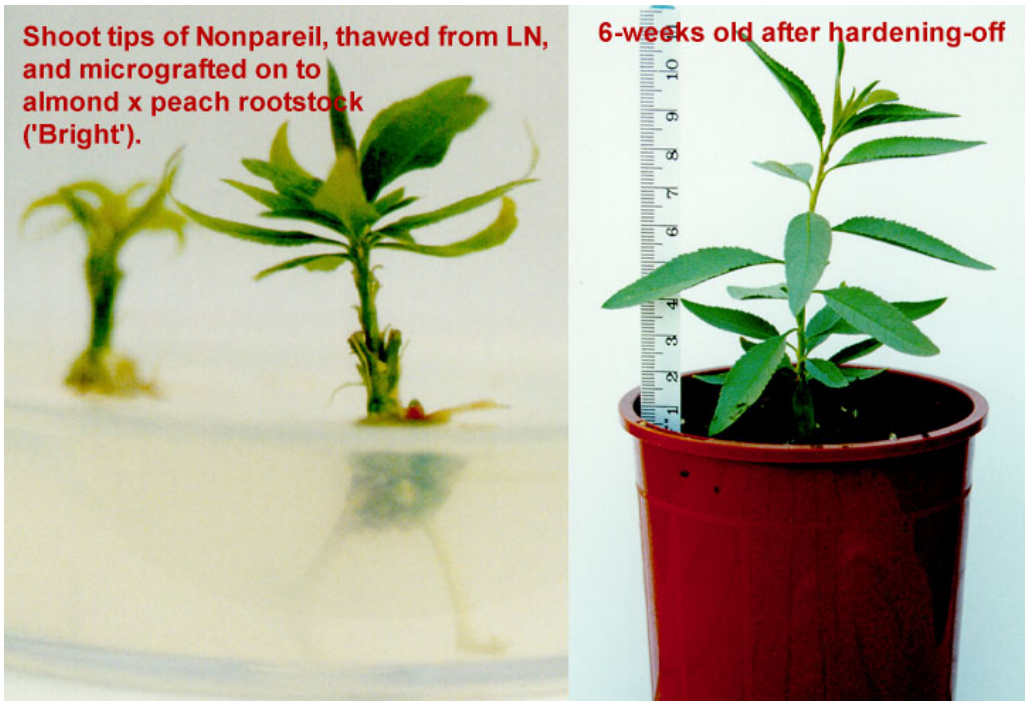

Figure 5.5.3

5.6 Investigation of transformation in tissue culture as a method to alter specific traits of almond in otherwise successful cultivars

The optimum concentration of mannose required for efficient selection against control tissues was determined by observing percent callus growth and regeneration of shoots from inoculated leaf explants on MS medium (Table 5.6.1). As the concentration of mannose in the medium was increased relative to sucrose, the percentage of explants producing callus and regenerating shoots decreased. Mannose concentrations above 2.5 g/l severely impacted upon callus growth and shoot regeneration. Subsequent mannose concentrations in the selection medium were set at 2.5 g/l and accompanied by 15 g/l of sucrose.

Table 5.6.1 Effect of mannose on growth in almond leaf explants

Mannose (g/l)	Sucrose (g/l)	Percent of explants callusing
0	30	80
0.5	15	65
1.0	15	50
2.0	15	35
2.5	15	10
5	15	5
10	10	1
20	0	0

Note. Leaf explants were grown in MS medium supplemented with IBA (1.96 mg/l), BAP (2.5 mg/l) and cefotaxime (630 µM) with indicated amounts of mannose and sucrose.

Transformation of in vitro leaf explants

The leaf explants transformed with *A. tumefaciens* (EHA 105) containing the pBI121mgfp-5-ER construct enlarged and started to produce callus 4–5 days after transformation. Shoot initiation was observed after 2 weeks in culture (Fig. 5.6.1A). Leaf explants were individually subcultured to RM1 medium with cefotaxime every 2 weeks (4–6 weeks in total) to enable the shoots to grow. Each individual callused leaf explant was then placed on medium with kanamycin 3 days after co-cultivation or following initiation of shoot buds 21 days after co-cultivation. No shoot buds were regenerated when placed on medium with kanamycin 3 days after co-cultivation however, a total of 28 shoot buds were regenerated on medium with 15 μM kanamycin while 10 shoot buds were regenerated on medium with 20 μM kanamycin when selection was delayed for 21 days (Table 5.6.2).

Regenerated shoot buds showed low vigour, stunted growth, browning of the tissue and eventually stopped growing in subsequent passages on medium with kanamycin. In comparison, the explants placed on kanamycin-free medium regenerated large numbers of shoots and grew vigorously. Individual shoots from each of the independent lines regenerated on kanamycin-free medium were transferred to RM2 medium containing cefotaxime and maintained on this medium until the shoots were 1.5–2 cm in length. A combined total of 197 shoots from all the surviving transformed lines were regenerated on kanamycin-free medium (Table 5.6.2). If present, multiple shoots per independent line were separated at this stage and placed on RM2 medium with kanamycin (15 μM) for selection of transformed shoots, 70 days after co-cultivation. When transferred to medium with 15 μM kanamycin, 48 shoots across all the lines survived selection (Table 5.6.2). Young leaves from the putatively transformed shoots were also subjected to an in vitro leaf antibiotic resistance assay. The leaves from transformed shoots remained green while those from control shoots bleached showing increasing susceptibility to kanamycin (data not shown).

The putatively transformed explants were placed under UV light to visualise green fluorescent protein (gfp) 6 days, 2 and 6 weeks after co-cultivation. The transformed explants showed green spots under UV light (Fig. 5.6.1C) while the non-transformed explants did not show such fluorescence (Fig. 5.6.1D).

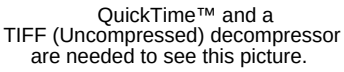

Fig. 5.6.1 **A**–**D** Plate showing regeneration of shoots of almond cultivar Ne plus Ultra transformed with pBI121mgfp-5-ER and pNOV2819 manA constructs. **A** Shoot initiation from tissue transformed with pBI121mgfp-5-ER construct on RM1 [MS + IBA (1.96 mg/l) + BAP (2.5 mg/l)]. **B** Shoot initiation from tissue transformed with pNOV2819 manA construct on RM1 [MS + IBA (1.96 mg/l) + BAP (2.5 mg/l)]. **C** Fluorescence due to *gfp* in the tissue transformed with pBI121mgfp-5-ER construct. **D** No fluorescence was observed in control tissue

The almond leaf explants transformed with *A. tumefaciens* (AGL1) containing the *pmi* gene were placed initially on RM1 medium with cefotaxime for callus initiation and growth. Twenty-one days after transformation, the leaf explants were transferred to RM3 medium containing 2.5 g/l of mannose and proliferating tissue was subcultured onto the same medium every 2 weeks. Regeneration of leaf explants with callus tissue could be observed after 3 weeks in culture (Fig. 5.6.1B). After four passages on selection medium, the individual explants with regenerating shoots were transferred to RM2 medium for further proliferation and growth. In total 70 shoots were regenerated from these explants.

Integration of transgenes

The PCR was used to screen all regenerated shoots from the transformation experiment with the pBI121mgfp-5-ER construct (Table 5.6.2). An expected *npt*II band of 700 bp was amplified in the shoots and four callus lines tested (Fig. 5.6.2A). The control tissue

showed no amplification of the product (Fig. 5.6.2A, lane 18). Overall, 23 independent lines surviving kanamycin selection contained the expected 700 bp PCR-amplified product (Table 5.6.2). A preliminary transformation efficiency of 5.6% was calculated as the percentage of single independent kanamycin-positive lines as confirmed by PCR analysis per total number of initial inoculated leaf explants (Table 5.6.2).

Table 5.6.2 Effect of kanamycin selection on regeneration of almond cultivar Ne Plus Ultra, following transformation with *Agrobacterium* strain EHA 105 carrying the pBI121mgfp-5-ER plasmid

Leaf explants inoculated (A)	Kanamycin (μm)	Selection applied no. of days after co-cultivation	Total shoots regenerated from all lines	Total shoots from all lines surviving after selection	Independent transformed lines PCR positive for Kan gene (B)	Putative transformation efficiency (B/A, %)
321	15	3	0	0	-	-
321	15	21	28	0	-	-
321	20	21	10	0	-	-
414	15	70	197	48	23	5.6

Note. Leaf explants were grown on RMI medium

In the PCR analysis of the shoots transformed with the *pmi* gene, four out of five shoots amplified a 514 bp *pmi* product (Fig.5.6.2B). The control shoot did not amplify any product (Fig. 5.6.2B, lane 7). Overall, of 70 shoots surviving mannose selection, 27 independent lines tested positive for the *pmi* gene. A preliminary transformation efficiency of 6.8% was calculated as the percentage of single independent *pmi* positive lines confirmed by PCR analysis per total number of original inoculated leaf explants.

To confirm the presence and integration of a transgene into the transformants, six PCR-positive transgenic shoots were used to confirm the insertion of the *npt*II gene by Southern blot analysis. In each of the six transformants, the *npt*II probe hybridised to DNA fragments from *Xho*I digested genomic DNA. The restriction enzyme *Xho*I cuts the pBI121mgfp-5-ER plasmid once within the T-DNA. Examination of the *npt*II hybridisation pattern indicated approximately one to three integration sites of the transgene per individual line. Similarly, for the *pmi* transformants, Southern blot analysis was used to confirm the presence of the T-DNA in five PCR-positive lines. The restriction enzyme *Sal*I was used to digest genomic DNA isolated from each sample, which is known to cut the pNOV2819 manA T-DNA once. The *pmi* probe hybridised to *Sal*I digested genomic DNA in each of the transformants tested. The analysis revealed at least one to two integration events of the *pmi* gene into the plant genome. The genomic DNA from the control shoots did not hybridise with any of the probes.

Fig. 5.6.2 **A** PCR analysis of putative transformants (pBI121mgfp-5-ER construct) for the inserted kanamycin gene. *M* = 100 bp marker. *Lanes 1–9*, *13*, *15*, *16 and 17* = putatively transformed shoots. *Lanes 10–12* = putatively transformed callus lines. *Lane 14* = plasmid DNA. *Lane 18* = control. **B** PCR analysis of putative transformants (pNOV2819 manA construct) for *pmi* gene. *Lane M* = 100 bp marker. *Lanes 1–5* = putatively transformed shoots. *Lane 6* = PCR control (no DNA). *Lane 7* = untransformed shoot.

5.7 DNA fingerprinting of almond cultivars, and commencement of genetic mapping

5.7.1 DNA fingerprinting

The 6 decamer primers selected for RAPD-PCR analysis produced a total of 101 bands of which 72 were polymorphic and ranged in size from about 100 bp to greater than 3 kbp. A UV photograph of the PCR products obtained after using primer OPA-04 is shown in Figure 5.7.1.

Figure 5.7.1 DNA fingerprints of 26 accessions produced using primer OPA-04. PCR fragments were separated by gel electrophoresis and visualised under UV light.

The dendrogram (Fig. 5.7.2) shows coefficients of genetic similarity ranging from 0.6 to 1.0 indicating the high level of genetic variation that exists in the almond gene pool. The dendrogram can be divided into 2 distinct clusters. The first cluster (I) mainly consists of cultivars derived from California, and these have less than 72% genetic similarity to those in the second cluster (II) that includes cultivars known to have originated from Europe and the Middle East.

Cultivars within the European and Middle East cluster come from a wider range of geographical locations than the cultivars within the cluster containing the Californian cultivars. The greater degree of genetic diversity shown in Figure 5.7.1 by the European and Middle Eastern cultivars relative to the Californian cultivars would therefore be expected. The accessions of the 2 species, *Prunus orientalis* (Miller) D. A. Webb and *Prunus webbii* (Spach) Vieh, showed genetic similarities of 59 and 61%, respectively, to the commercial *Prunus dulcis* cultivars.

Cultivars with names in parentheses indicate selections propagated by growers who have named a particular mutation or seedling. Therefore it may not be the authentic cultivar for that name. Johnston (Giles) and Johnston's Prolific showed a genetic similarity of 100% indicating that both cultivars are probably the same. No authentic accession of Chellaston was available to compare with Chellaston (Cole).

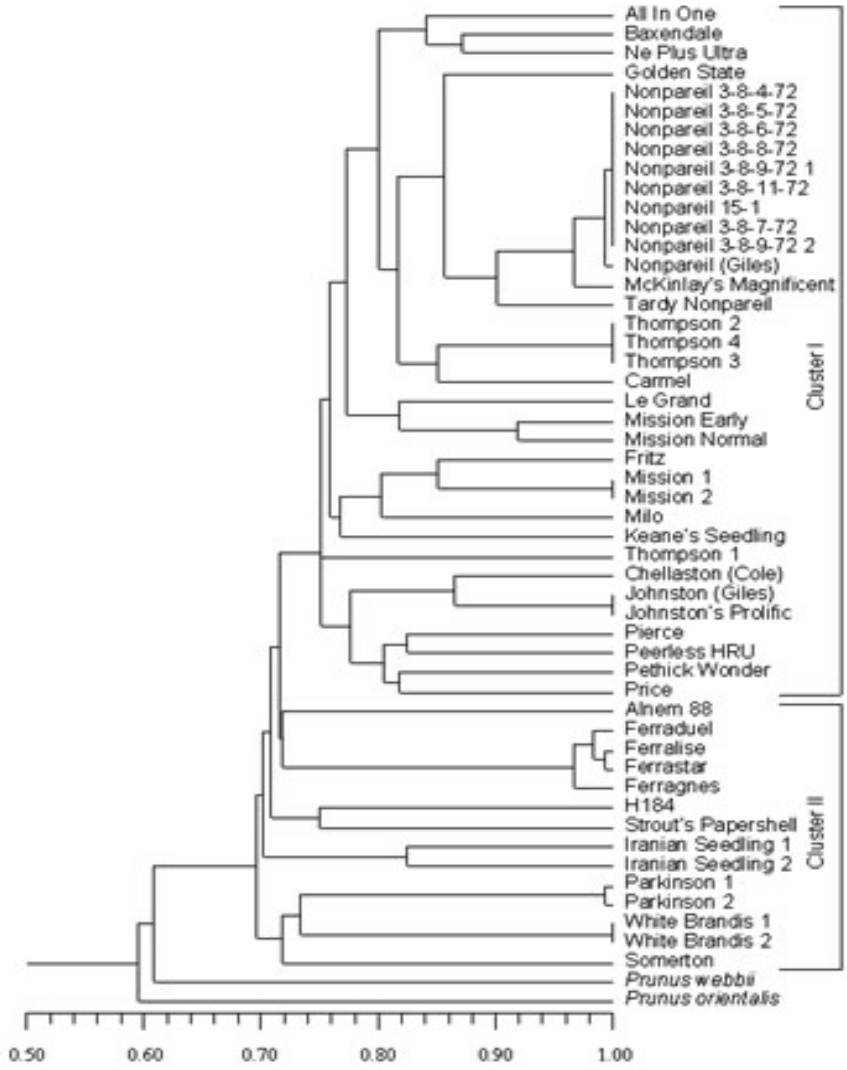

Figure 5.7.2 Dendrogram showing the estimated genetic similarity of 52 accessions (Table 5.7.1) using 6 decamer primers.

5.7.1 Genetic mapping of almond

From the 119 single primers screened, 33 were analysed in the mapping population resulting in 73 segregating markers. From the total 73 segregating markers 19 were inherited from the female parent 'NP', 30 from the male parent 'LA' and 24 inherited from both parents. The segregation ratios were tested for distortion using the internal standard χ^2 test of the mapping program. A total of 19 markers (26%) were distorted from the expected mendelian segregation (α= 0.05%), ranging from 21 – 30% based on parental inheritance. At $\alpha = 0.05$ considering 73 marker loci, around 4 markers were expected to display distortion by chance alone. For the paternally inherited distorted markers, in the majority of cases (78%) the distortion was due to an excess of heterozygotes. No specific correlation was observed for the four distorted markers in the maternal group. There were fewer distorted loci among the SSR marker type for both parental types than for RAPD and ISSR loci. The majority of distorted loci in the male parent were of the ISSR type (56%). Therefore 74% of markers analysed in the progeny segregated according to the expected mendelian inheritance.

For map construction 43 markers from 'NP' and 54 markers from 'LA' were grouped with a LOD score of between 2.0 to 4.0, 77% were mapped with a LOD of 4.0. Two parental maps were constructed according to the DPSTC mapping strategy as described by (Grattapaglia, 1994), resulting in 7 linkage groups for 'NP' (Figure 5.7.3) and 8 for 'LA' (Figure 5.7.4). Linkage groups were composed of between 2 - 11 loci, and between 10.5 – 86.6 cM in length. Average marker density was 9.4 cM/marker for 'NP' and 9.6 cM/marker for 'LA'. On a single linkage group basis, linkage group NP-C showed the highest marker density of 3.5 cM/marker, while NP-B showed the lowest marker density of 24.6 cM/marker. This is likely due to the presence of only two markers on this linkage group. The longest linkage group for both parents was G4 (86.6 and 84.1 cM), which also had the highest number of linked markers (8 and 11). No particular evidence of clustering of the loci was observed, however for six primers $(AC)_8G$, $(AG)_8YT$, $(CA)_8G$, $GCA(CA)_9$, OPC-10, and OPC-11 two different size fragment derived from the same primer were localised on the same linkage group with a distance of 1.9 – 24.9 cM between them . Most of these co-localisations of fragments were observed in the male parent map 'LA'.

Of the 97 markers used for parental map construction, 24 were heterozygous in both parents and allowed the identification of 18 common loci to be used as loci bridges between the maps. Of the 24 markers, 14 were used to produce an integrated parental map, representing about 19% of all markers studied. The integrated map consisted of 6 linkage groups, of 2 – 4 markers, and between 6.0 – 49.1 cM in length (Figure 5.7.5), with a marker density of 1.6 cM/marker.

Of the seven morphological markers selected for mapping, three loci associated with phenotypic characters could be placed on the map. 'NP' has a pale testa, whereas 'LA' has a dark testa, pale versus dark testa segregated 1:1 in the progeny suggesting monogenic inheritance of this trait. Linkage analysis placed this loci at the top of linkage group G4-LA, between the markers PS12e2 and $(AG)_8YC$-1150. Both of these molecular markers are present in the parental maps and have been placed on the integrated map. The loci for taste was loosely linked to the ISSR marker $(AG)_8YC$-1786 and placed at the bottom of B-NP and B-IN, 49.1 cM from the marker. Taste was categorised as sweet in both parents

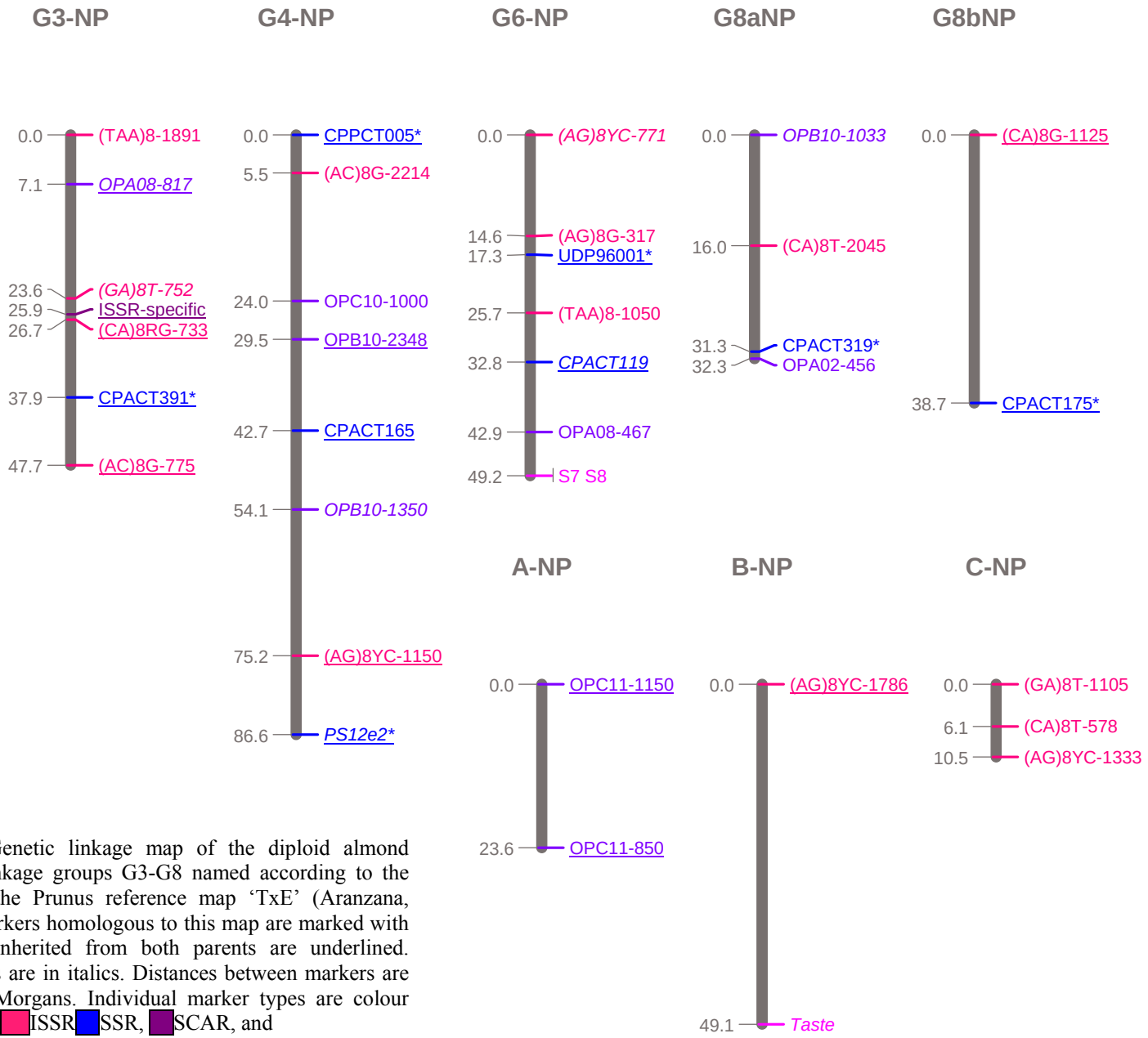

Figure 5.7.3 Genetic linkage map of the diploid almond 'Nonpareil'. Linkage groups G3-G8 named according to the convention of the Prunus reference map 'TxE' (Aranzana, 2003 #281). Markers homologous to this map are marked with a *. Markers inherited from both parents are underlined. Skewed markers are in italics. Distances between markers are given in centi Morgans. Individual marker types are colour coded: RAPD, ISSR, SSR, SCAR, and Morphological

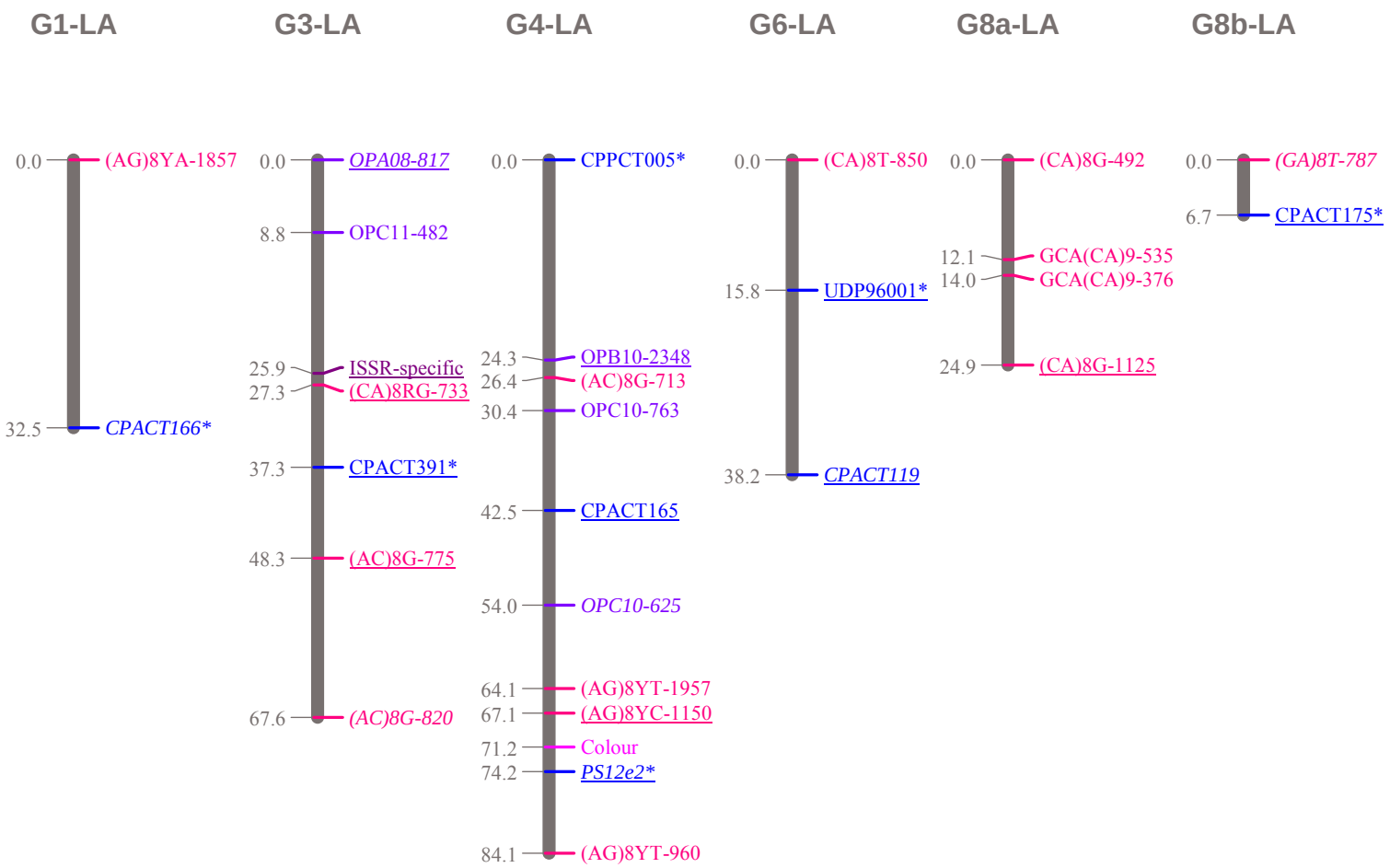

Figure 5.7.4 Genetic linkage map of the diploid almond 'Lauranne'. Linkage groups G1-G8 named according to the convention of the Prunus reference map 'TxE' (Aranzana, 2003 #281). Markers homologous to this map are marked with a *. Markers inherited from both parents are underlined. Skewed markers are in italics. Distances between markers are given in centi Morgans. Individual marker types are colour coded: RAPD, ISSR, SSR, SCAR, and Morphological

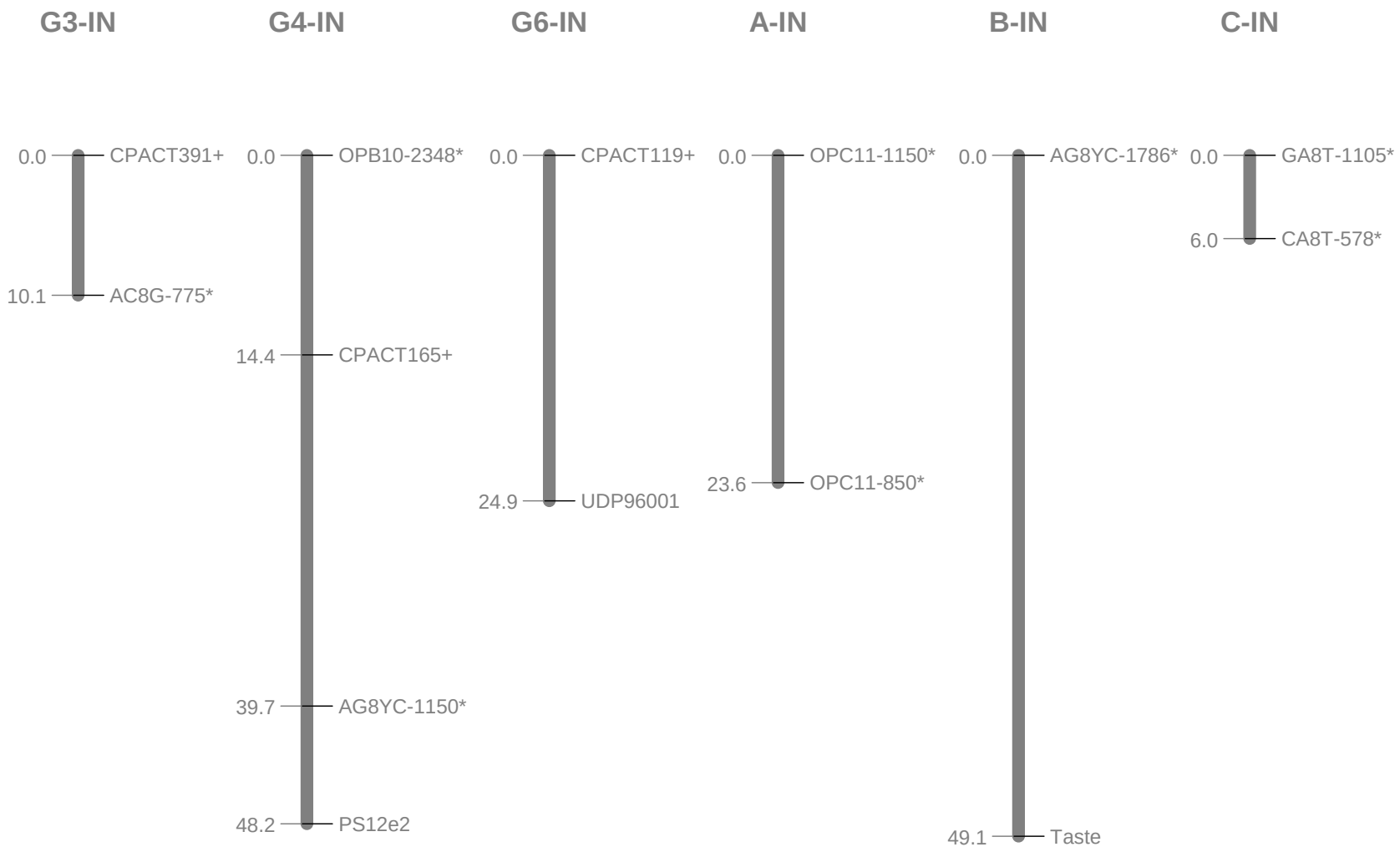

Figure 5.7.5 Integrated genetic linkage map of the diploid almond cross 'Nonpareil' x 'Lauranne'. Linkage groups G3-G6 named according to the convention of the Prunus reference map 'TxE' (Aranzana, 2003 #281). Distances between markers are given in centi Morgans.

and therefore expected to segregate 3:1 for the sweet versus semi-bitter phenotype. It showed distortion from this segregation at $\alpha = 0.05$, indicating it is not likely to be inherited in a simple mendelian fashion, or some other factor associated with scoring this trait distorted the ratio. The loci for S7 and S8 were placed at the distal region of G6-NP, 6.3 cM from the marker OPA08-467 and segregated 1:1 in the population.

6. Discussion

6.1 Performance evaluation of local and imported selections and imports of scion and rootstock material of almond

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 in California. We have different pests and diseases (eg bacterial spot causing major problems for Fritz in Australia) and flowering times can shift, causing pollination problems.

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 are of high quality and consist of both older traditional cultivars as well as some exciting new developments.

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

The almond breeding has been conducted for ten years and has produced over 29,000 trees for evaluation. Selection criteria used in the evaluation process were established in consultation with the industry. The first phase of evaluation 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 trees are spaced at greater distances.

Primary evaluation is completed on progeny from the crosses achieved during 1997 - 2002, 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.

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 will be used and crosses can be designed to use the best with the best.

In conclusion, self-fertility and improved nut quality rank highly in the breeding aims, followed by high productivity and disease resistance. Pollinations will continue but will be targeted at using improved parent combinations using data from the heritability analysis. Evaluations are still underway of the 2002 progeny and assessment of the 2003 progeny will begin in spring 2007. The most promising progeny resulting from the selection process are propagated onto rootstock and planted in 2nd stage trial plots for further assessment.

6.3 Development of improved virus detection methods for almond breeding stock and mother plants

Diagnostic methods used to detect plant pathogens should be reliable, and possess both high specificity and sensitivity. This is of special significance when mother trees are marketed as 'virus tested' in a commercial nursery, as these trees will produce numerous plants via vegetative propagation. Spiegel et al., (1996), Sanchez-Navarro et al., (1998), and Moury et al., (2000) reported that RT-PCR can surpass ELISA in sensitivity for the detection of PNRSV. This work compares the sensitivity of two diagnostic methods, ELISA and RT-PCR, to detect PNRSV and PDV in 175 samples of almond leaves in Australia over a 3-year period, ranging from 1999 to 2001. The ELISA technique detected PNRSV in nine out of the 175 samples of almond leaves tested in 1999, and eight in 2000, whereas with RT-PCR, the same nine samples tested positive in 2000 and 2001. Viral disease intensity can change both temporally and spatially (Thresh, 1983), necessitating assessments to be made throughout the growing season to determine the right time for collection. The detection of PNRSV by ELISA was shown by Bertozzi et al., (2002) to be most sensitive when plant material was collected in spring. Heleguera et al., (2001) reported that samples, collected in summer from chronically infected plants, showed a positive result for PNRSV with IC-RT-nested-PCR and RT-PCR but produced a negative result with ELISA. In the present study, PNRSV could be detected in the leaves of infected almond trees using RT-PCR from early spring to late autumn (data not shown).

ELISA has been used successfully to detect PDV in almond in Europe (Di Terlizzi et al., 1994) and cherry in Australia (Johnstone et al., 1995), but gave negative results with almond trees in Australia (Bertozzi et al., 2002). Multiplex RT-PCR also produced negative results for PDV, but the combination of RT-PCR with nested PCR developed in this study resulted in the detection of PDV in 96% of the 175 Australian samples tested in 2000 and 2001. These results show that nested RT-PCR is more sensitive than ELISA for

the detection of PDV in almonds. Leaves of infected trees from Spain produce a strong positive product after multiplex RT-PCR, suggesting that the titre of PDV may be low in Australian almond trees, and this may be an important factor in the lack of success with ELISA for detecting PDV in Australian almonds. Results with CEBAS1 from Spain also demonstrated that the two viruses could be detected simultaneously by multiplex RT-PCR.

6.4 Investigation of micropropagation for the multiplication and propagation of new improved rootstocks from the selection program

Successful proliferation of almond has been reported for explants derived from seedlings of hard-shelled European cultivars (Antonelli, 1991; Miguel and Oliveira, 1999), and cotyledons of the soft-shelled Nonpareil (Ainsley et al., 2001a). However, previous publications indicate that almond is difficult to micropropagate efficiently from mature tissues. Tabachnik and Kester (1977), collected dormant shoots of Nonpareil, in December/January, and stored these at 3 °C for 1–2 months prior to culture on either MS or a modified Knops medium. These cultures produced between 1 and 10 shoots per explant. Rugini and Verma (1983), produced proliferation and elongation of excised shoot tips of the hard-shelled cultivar, Ferragnès, but only after the explants were cultured on four different media in succession.

Most of the plant growth regulators used for shoot proliferation and elongation of *Prunus* species including almond and rootstocks are based on BAP and auxins (IBA, IAA and NAA). Hammerschlag et al., (1987) found that the best shoot multiplication of peach cultivars was induced when 8.8 μM BAP and 0.05 μM IBA was added to a shoot proliferation medium based on MS. Moreover, Tabachnik and Kester (1977), and Rugini and Verma (1983) reported that high concentrations of BAP promoted shoot proliferation, whereas low concentrations encouraged shoot elongation for almond cultivars. Similar results were obtained in this study for Nonpareil 15-1 using 3 μM BAP for shoot proliferation and 1 μM BAP for shoot elongation, and for Ne Plus Ultra using 5 μM BAP for shoot proliferation and 1 μM BAP for shoot elongation. However, a low concentration of IBA at 0.049 μM was necessary for the almond cultivars in this study to promote longer shoots when compared to the same medium without IBA. For the hybrid rootstock, BAP at 10 μM without BAP was effective for shoot multiplication and IBA was found to inhibit shoot growth. Similar results were obtained from almond–peach hybrid clones using 5 μM BAP in TK medium (Tabachnik and Kester, 1977). In contrast, Lovell and Nemaguard peach rootstocks required IBA at 0.04 μM with BAP at 26.7 μM (Almehdi and Parfitt, 1986).

Hyperhydricity, a common problem in micropropagation, is a physiological condition that manifests as water-soaked or translucent tissue. Hyperhydric plants lack chlorophyll, become brittle, and undergo abnormal growth including short internodes, thick curly leaves, loss of cuticle, and excessive ethylene production (Kyte and Kleyn, 1996). Phan and Hagadus (1988) suggested that high concentrations of cytokinins and low concentration of agar encouraged this condition. For the almond cultivars Nonpareil 15-1 and Ne Plus Ultra used in this study, an increase in BAP up to 20 μM, the highest concentration used, either alone or in combination with IBA, resulted in an increase in hyperhydric shoots. The effect was most pronounced for Nonpareil 15-1. At 3 μM BAP and lower, no symptoms were observed, whereas at 5 μM BAP about 40% of shoots were affected, and this increased to 100% at 10 μM BAP. Leshem et al., (1988) also found that

lowering the cytokinin level could control hyperhydricity. Similarly, this condition was controlled by reducing the level of BAP below 5 μM for Nonpareil 15-1.

The technique of micrografting was used in this study because it was difficult to induce roots on the two almond cultivars, whereas roots were readily induced on the almond/peach hybrid rootstock after 7 days in the dark on medium containing IBA. Martinez-Gomez and Gradziel, 2001 grafted buds of Nonpareil seedlings on different rootstocks with bud survival varying from 30 to 90%, while Ghorbel et al., (1998) micrografted apical buds from in vitro shoots of the almond cultivar Achak onto rootstocks derived from zygotic embryos of the same cultivar with 60–80% success. In the present study, the problem of rooting with the cultivars was overcome by micrografting them to the rootstock, and it was found that an essential condition for the formation of a successful graft union was to have the cambial tissue of rootstock and scion in close contact. This was most readily achieved by the use of a wedge graft when the stem of the scion was the same age and size as that of the rootstock.

These experiments demonstrated that high levels of shoot multiplication could be achieved for Nonpareil 15-1 on AP medium supplemented with 3 μM BAP and 0.049 μM IBA, whilst MS medium with 5 μM BAP and 0.049 μM IBA was suitable for Ne Plus Ultra. BAP at 1 μM resulted in better elongation of almond scion cultivars but the higher concentration resulted in greater multiplication. For the almond/peach hybrid rootstock, MS medium with 10 μM BAP was effective for shoot elongation and proliferation. Maximum rooting of shoots of the hybrid rootstock occurred on half strength MS medium with 2.4 μM IBA when placed in the dark for 2 weeks followed by the light. Five to 7-week-old shoot cultures of scions were micrografted to rootstock stems and rooted grafts were successfully transferred to potting mix.

6.5 Adoption of in vitro conservation methods to facilitate small-scale storage of germplasm as an alternative to in-ground collections

Most woody plant species require cold hardening to improve shoot survival (Niino et al., 1997). This appears to ameliorate any harmful effects of the cryoprotectants and osmotic stress during dehydration (Yamada et al., 1991). For example, the preculture of excised shoot tips at 5°C for 1 day in agar medium supplemented with 0.7 M sucrose was effective for improving survival of cryopreserved shoots in many fruits such as apple, mulberry and cherry (Niino et al., 1992a,b, 1997) and Prunus rootstock (Brison et al., 1995). In the present study, preculturing for 1 day at 4°C was a successful prerequisite to the cryopreservation of two almond cultivars and a hybrid rootstock.

Both the time and temperature of incubation in vitrification solution are important to avoid the damaging effects of crystallisation, chemical toxicity of cryoprotectants, and excess osmotic stress during dehydration (Niino et al., 1992b, 1997). The size of excised shoot tips also seems to be an important factor (Takagi et al., 1997). In the present study, optimum incubation times for the almond cultivars and the hybrid rootstock were 45 and 60 min respectively. Rapid thawing at 25-40°C in a water bath after storage under LN prevents recrystallisation (Sakai et al., 1990) and produces higher survival rates compared to a slow warming (Matsumoto et al., 1995; Takagi et al., 1997). In this study, the plant material was rapidly warmed in a water bath at 30°C.

The medium used for recovery of cryopreserved shoot tips is also very significant, especially the concentration of ammonium ions (Kuriyama et al., 1990). After cryopreserved shoot tips were recultured in the appropriate medium, excluding both ammonium ions and growth regulators, new shoots readily developed that could be successful transferred to a multiplication medium.

Vitrification is a relatively simple method for cryopreserving plant materials that results in a high survival rate of the tissues after their removal from LN compared to other techniques, such as stepwise freezing and simple freezing. The procedure reported here has eliminated the need to vary the composition and concentrations of different reagents. After three days storage in LN, shoot survival was 60.0-87.5% compared to 52-78% after 180 days. Shoot survival for unfrozen control shoot tips was 85-100%. Shoots regenerated from cryopreserved shoot tips did not show any apparent morphological abnormalities, but the possibility of genetic change is being investigated.

6.6 Investigation of transformation in tissue culture as a method to alter specific traits of almond in otherwise successful cultivars

The development of systems for the successful transformation and culturing of almonds are important milestones in the eventual routine genetic modification of the species. Almonds in general display low transformation efficiencies, which have limited the evaluation of different parameters controlling shoot development, flowering and fruit quality. *Agrobacterium* strain EHA105 has previously been an effective agent for genetic transformation of recalcitrant fruit tree species (De Bondt et al., 1994; Mourgues et al., 1996). In this study, in addition to EHA105, we found AGL1 also to be highly effective in transforming almond.

Many plant species including almond show sensitivity to medium supplemented with kanamycin. Alternative selection strategies including ‘delayed selection’ has been successful in obtaining transgenic plants in kanamycin sensitive plants including apple (Yao et al., 1995; Yepes and Aldwinckle, 1994), apricot (Machado et al., 1992) and the almond cultivar Boa Casta (Miguel and Oliveira, 1999). In our study, 15 or 20 μM kanamycin was applied at 3, 21 and 70 days after co-cultivation with *Agrobacterium* in leaf explants transformed with the pBI121mgfp-5-ER construct. Delaying selection by 3 or 21 days after co-cultivation, resulted in the formation of a small number of buds, which developed into small shoots that remained stunted and did not grow further. Kanamycin-free induction medium was subsequently used which resulted in the formation of increased numbers of putatively transformed cell clusters or shoot initials. These shoots were allowed to grow and were subsequently screened on kanamycin selection medium 70 days after transformation. A total of 48 of the 197 shoots from independent lines continued to grow in the presence of kanamycin from which 23 independent lines gave positive results using the PCR to screen for T-DNA insertion. We were able to calculate a preliminary transformation efficiency of 5.6% based on the positive results from the PCR screens relative to the initial number of independent leaf explants used at the beginning of the experiment. This result was encouraging and was further supported by Southern blot analysis on six randomly chosen kanamycin-resistant PCR-positive shoots, which showed integration of the TDNA into the genomes of each of the almond shoots.

There is growing public concern about the widespread use of antibiotic selection in plant transformation and its perceived risk associated with both human consumption or lateral transfer to other plants or organisms. In recent years, there have been new developments in alternative positive selection systems such as the use of the *pmi* gene from *E. coli*. The classical procedure in choosing a selection agent involves identifying concentrations, which limit non-transgenic plants regenerating. In our study, we found 2.5 g/l mannose supplemented with 15 g/l of sucrose to be an effective agent to select and regenerate transgenic almond plants using the *pmi* system. Combining mannose with sucrose has also previously been shown to give positive results in other *pmi* transformed plants including sugar beet, maize, rice and cassava (Joersbo et al., 1998; Lucca et al., 2001; Negrotto et al., 2000; Zhang and Puonti-Kaerlas, 2000). In the control callus and shoots, mannose selection reduced overall tissue vigour and tissues developed a distinctive brown colour. Interestingly, mannose selection eliminated tissue necrosis (in both transformed and control tissues), which is commonly observed with kanamycin selection in almond (Lindsey and Gallois, 1990). However, 5 g/l severely curtailed shoot development in the transgenic shoots. Overall, final transformation efficiencies using both mannose and sucrose were calculated to be 6.8% an improvement over the parallel kanamycin-based selection protocol reported in this and other studies (Miguel and Oliveira, 1999). Our results obtained using mannose selection are encouraging and show much promise for the continued use of the mannose/*pmi* transformation and selection method in almond. Transformed shoots quantified by both PCR and Southern blot analysis for the presence of the T-DNA were rooted in culture and transferred to soil. In less than 18 months, the transgenic almond plants had flowered. This is a significant improvement on the 5–7 years normally required for plants to flower after propagation via traditional breeding methods. It appears that in vitro culture may have overcome the extended vegetative phase common in almond plants raised from seed using traditional breeding methods and offers a promising faster route for further molecular based genetic discovery and manipulation in almond.

In conclusion, this study demonstrated that the *P. dulcis* cv. Ne Plus Ultra can be efficiently transformed and cultured to soil using an initial tissue culture selection strategy based on the use of *npt*II/kanamycin resistance or more favourably through the activity of the *pmi* gene as a selectable marker with mannose as a selective agent. Previous to this work, success in almond transformation has been limited to in vitro seedlings and the generation of a single transformed line from *P. dulcis* cv. Boa Casta (Miguel and Oliveira 1999). As also observed in this study, almonds were found to be sensitive to kanamycin and this sensitivity is most likely the source of the reduced transformation efficiencies observed in previous studies (Ainsley et al., 2001a; Archilleti et al., 1995; Miguel and Oliveira, 1999). The switch to the positive mannose/*pmi* section system has enhanced the number of transformed shoots and renders the use of kanamycin markers in almond transformation redundant. It is reasonable to suggest that positive selection may be the preferred option in subsequent almond transformation experiments.

6.7 DNA fingerprinting of almond cultivars, and commencement of genetic mapping

6.7.1 DNA fingerprinting

According to Mekuria et al. (1999), the genetic distance required to distinguish 2 related accessions as different cultivars still remains unclear, and they suggested that accessions of olives showing at least 20% genetic difference from each other may be regarded as

distinct cultivars. However, Ferraduel, Ferralise, Ferrastar, and Ferragnès are regarded as distinct cultivars of almond and show a maximum genetic distance of 4%. Therefore, it is likely that the Australian cultivars Somerton, White Brandis, Parkinson, Strout's Papershell and H184 are distinct cultivars. The low similarity values and placement of these cultivars on the dendrogram suggests that they are distinct from all others and are more likely to be derived from Europe or the Middle East than from California. This is possibly true for Somerton which was derived from a seedling originating from wild almond trees at Somerton, a suburb of Adelaide (F. Keane pers. comm.). The wild trees were probably planted by settlers and could therefore be of European origin.

White Brandis is believed to be derived from Spanish/Jordan types (Quinn, 1941; F. Keane pers. comm.) but because Parkinson shows morphological characteristics similar to those of Johnston's Prolific, it is believed to be a bud mutation of the latter (F. Keane pers. comm.). However, the accessions of Parkinson and Johnston's Prolific show only a 70% genetic similarity, so inferring the derivation of Parkinson based on morphology may lead to an erroneous interpretation of its derivation.

The French cultivars Ferraduel, Ferralise, Ferrastar and Ferragnès showed a large genetic distance from all other cultivars. However, the genetic distance between the 4 cultivars was less than 4%, confirming their shared parentage. Ferraduel, Ferragnès, and Ferralise share common S-alleles and are therefore cross-incompatible (Yamashita et al., 1987; Socias i Company and Felipe, 1994).

Cluster I, consisting mainly of Californian cultivars, formed clusters of increasing genetic similarity. Chellaston (Cole) was expected to show a strong genetic similarity to White Brandis because Quinn (1941) suggested that it is likely to be a seedling of the Brandis strain. Instead, it clustered with an 86.5% similarity to Johnston (Giles) and Johnston's Prolific, the latter believed to be derived from the Jordan strain (Quinn, 1941). These 3 cultivars showed a similarity of about 78% to Pierce and Peerless HRU, which clustered at 82.5%, and Pethick Wonder and Price, which clustered at 82%. Pethick Wonder originated in Angle Vale and is believed to be a bud mutation of Johnston's Prolific (F. Keane pers. comm.). Although the genetic distance of 22% does not support this derivation, it can be concluded that Johnston's Prolific and Chellaston have more resemblance to the Californian cultivars, Peerless and Price, than to the suspected European Jordan cultivar.

The next main cluster showed that Fritz and the 2 accessions of Mission were genetically similar at 85%. Milo had a genetic distance of 20% from these cultivars, and Keane's Seedling was 3% more distant from Milo. This suggests that Milo and Keane's Seedling are distinct cultivars from each other and from Mission and Fritz. This is consistent because Keane's Seedling originated from wild hard-shell varieties on the Sturt Creek (F. Keane pers. comm.) and is therefore likely to be distantly related to all other cultivars. Milo is assumed to be a misspelled version of the Californian Nonpareil ´Selection 24-6 (Nonpareil ´ Eureka) pedigree, Milow (Kester, 1994). However, because Milo showed little similarity to Nonpareil it is probably a distinct cultivar from Milow, although it showed 80% similarity to the Mission cultivars, supporting the isozyme study of Hauagge et al. (1987b) which found that Milow had 80% alleles in common with both Nonpareil and Mission. Comparison of Milo with an authentic Milow would be necessary to confirm these results.

Hauagge et al., (1987b) found that Fritz had several possible origins based on the inheritance of 5 isozyme systems. These were Mission and Nonpareil, Mission and Peerless, or Mission and Drake. Since Fritz clustered with Mission, any of these origins is supported. The cluster containing 2 other cultivars known as Mission, with one showing earlier flowering than the other, showed only 76% similarity to the 2 accessions of Mission that clustered in the previous group. This finding suggests that Mission Early and Mission Normal are not cultivars of Mission.

The 2 peach–almond hybrids, Le Grand and All In One, showed about 77% similarity. All in One showed 84.5% similarity to Baxendale and Ne Plus Ultra which clustered at 88%. This strongly suggests that Baxendale and All in One are derivatives of Ne Plus Ultra.

Carmel has been reported as originating from a bud mutation of Nonpareil (Bartolozzi et al., 1998). In the present study an 18% genetic difference was shown between Nonpareil and Carmel confirming the conclusions of Hauagge et al., (1987a) and Vezvaei et al., (1994) that Carmel could not have originated this way. Thompson clustered with Carmel at 85.5%. Bartolozzi et al., (1998) suggested that Carmel had the same parentage as Thompson, and this is supported by the close similarity shown between the cultivars in this cluster.

The 2 accessions of Thompson used in this study were sourced from different orchards (Claremont and Alverstoke) at the Waite Campus of the University of Adelaide. Because the DNA fingerprints of these 2 accessions were different, additional DNA samples were obtained from 2 other trees labelled as Thompson, one from the Claremont orchard and the other from the Loxton Research Centre, Loxton, South Australia. Comparison of the DNA fingerprints from the 4 samples showed that there was a large genetic distance between the accession from the Alverstoke orchard and the 3 other accessions that clustered at 100%. One possible reason for this variation is that the different accession was a grower's selection, as occurred in the case of Milo and Chellaston (Cole). A second possibility is that mislabelling occurred in the Alverstoke orchard, and a third is that a separate introduction of the cultivar was made from a different region, as has been reported for some garlic accessions brought into Australia (Bradley et al., 1996).

Quinn (1941) treated Golden State and American I.X.L. as separate cultivars, whereas Gathercole (1989) stated that American I.X.L. was wrongly named as Golden State in the early 1970s. Analysis of additional accessions of the 2 cultivars would clarify this uncertainty. The clustering of Golden State with Nonpareil may explain the incompatibility found between these 2 cultivars (Gathercole, 1989). Californian records indicate that Nonpareil, Ne Plus Ultra, I.X.L., and Mission may be siblings of each other since they were selections from the same Languedoc seed (Bartolozzi et al., 1998). The close proximity of the first 3 cultivars to each other on the dendrogram consequently supports this relationship. However, Mission appears to be too genetically distant to belong in this group.

Tardy Nonpareil is reported to bloom 7–10 days after Nonpareil and produce lower yields (Kester, 1994). Bartolozzi et al. (1998), reported no difference between the RAPD patterns of Nonpareil and Tardy Nonpareil, whereas in the present study, the 2 cultivars showed a genetic distance of 9%. Considering the small genetic distance shown by the 4 French cultivars, it is possible that Tardy Nonpareil is derived from a seedling of Nonpareil rather than a somatic mutation.

Vezvaei et al. (1994), concluded that Nonpareil contributed to selections that gave rise to Australian cultivars, unlike Mission which shows isozyme loci not detected in any Australian cultivars. Only one Australian cultivar, McKinlay's Magnificent, shows a close genetic similarity to Nonpareil to be regarded as a selection. This probably accounts for the cross-incompatibility of the 2 cultivars (T. Bertozzi pers.comm.). Nonpareil (Giles) differed from the other selections of Nonpareil by less than 1%, and no genetic differences were found between the 9 selections of Nonpareil that represent the largest and tightest cluster on the dendrogram.

6.7.2 Genetic mapping of almond

A total of 73 polymorphic markers were used to construct two low to moderate density genetic linkage maps for almond using a F_1 full-sib hybrid population, derived from a cross between the cultivars 'Nonpareil' and 'Lauranne'. This population was used to construct these genetic linkage maps as the parents were assumed to have a high level of heterozygosity between them, leading to a high frequency of gene recombination in the progeny a prerequisite of the DPSTC strategy (Grattapaglia and Sederoff, 1994). The allelic transmission from parent to offspring via recombination during meiosis enabled the relative position of genetic loci in relation to each other to be inferred. Using this strategy seven and eight separate linkage groups were constructed for the female and male parent respectively, putatively corresponding to the haploid chromosome number of almond (n = 8). Markers that were in an intercross segregation (heterozygous in both parents) were used to merge the two parental maps into a preliminary consensus map for this cross, consisting of six linkage groups.

The discrepancy between the number of linkage groups expected to coalesce and the number observed for the female parent and the integrated map is most likely due to the high number of unincorporated markers and the low number of homologous markers for the integrated map. The inclusion of more markers of the testcross co-dominant type would be expected to alleviate this situation and increase the number of linkage groups to the anticipated number. The incorporation of more markers would also be expected to merge single markers so far unlinked to the major linkage groups that may presently be in regions of very low marker frequency. (Debener, 1999) proposed increasing the mapping population size as a means of introgressing unlinked single and linked marker pairs into the main linkage groups. Analysis of genetic maps produced for other *Prunus* species, indicates that the population size used for this study is unlikely to be the reason for the discrepancy in linkage group number in comparison to the haploid chromosome number. The population size of this study is toward the higher end of those used in other studies, and is higher than the 'TxE' population used as the reference map for *Prunus* (Aranzana et al., 2003; Joobeur et al., 1998). Therefore increasing the marker number and using more informative marker types such as RFLP and SSR would be more beneficial for further map construction.

No obvious clustering was observed in either the parental or integrated maps, which may be masked by the low marker density and may be observed if more markers were introduced into the map. The dispersal of distorted loci did not appear to be random, rather they tended to be localized toward the distal ends of the linkage groups. Eleven groups, 5 in the female, and 6 in the male had distorted markers present. (Maliepaard, 1998) observed markers that showed skewed segregation patterns usually could not be mapped. In this study 80% of skewed markers in 'NP' and 53% in 'LA' were mapped, indicating

this is not the case in almond. The percentage of skewed loci is comparable to levels observed in rose, 11-23% (Debener, 1999), 18% in oak (Barreneche, 1998), 11.3% in apricot (Lambert, 2003), and 10% in another almond map (Joobeur, 2000). However the number of skewed loci was approximately half the levels of distorted markers obtained in intraspecific peach x almond mapping programs, 43% (Aranzana, 2003), 46% (Joobeur, 1998) and 37% (Foolad, 1995). Segregation distortion was observed in approximately one third of markers mapped in a peach x *Prunus davidiana* cross, and although several mechanisms were proposed to account for this, one mechanism could not account for the total number of distorted loci observed (Foulongne, 2003). In intraspecific hybrids, segregation distortion is proposed to be a consequence of hybrid breakdown (Foolad, 1995). Several reasons have been proposed for segregation distortion observed in tree species including genetic load, segregation with a pollen lethal gene or SI locus (Ballester, 1998; Gebhardt, 1991), statistical bias or genotyping and scoring errors. Several authors have discounted distorted markers from the first round of map construction and observed including these markers in a later round made no significant difference to gene order or linkage map order (Conner, 1997; Debener, 1999; Kuang, 1999; la Rosa, 2003).

The localisation of three morphological traits on the map is the first step toward using this map as a tool for marker assisted selection. Both testa colour and the sweet/semi-bitter taste character have not been mapped prior to this study. The gene for self-incompatibility (SI) has been mapped to the end region of the linkage group G6 from a 'Ferragnès' x 'Tuono' cross (Ballester, 1998). (Foulongne, 2003) also proposed the location of a SI locus in the same region for a *P. persica* x *P. davidiana* cross, on the end of LG6. This is the same location as *S7* and *S8* were localised to in this study, suggesting a strong homology to morphological trait locations, between maps obtained from a variety of crosses. It would be interesting to determine if this were a genus wide phenomenon, and markers for self-incompatibility have been identified in both apricot (Badenes, 2000) and cherry (Wiersma, 2001), which could be placed on these species maps developed by (Lambert, 2003) and (Wang, 1998). In this study morphological traits were selected for analysis on the basis of their importance for release as a commercial cultivar.

The integration of the parental maps was accomplished using heterozygous and co-dominant markers, which also allowed the identification of homologous linkage groups between the parents. No change in marker order was observed between the parents and the integrated maps although a change in the distances between markers was observed. This was expected, since although the values were relatively close, in the integrated map the combined recombination frequency estimate is the average over the male and female meioses. The integrated map is therefore a statistical compromise between the maternal and paternal recombination values rather than a biological reality. However, the development of integrated maps are the ideal situation for mapping programs, since identifying conserved loci in related species and using them as reference points it is possible to transfer linkage information. SSRs are transportable across *Prunus* as shown by a number of studies (Aranzana, 2003; Martínez-Gómez, 2003; Mnejja, 2004; Lambert, 2003). Peach and cherry SSRs have also been used for construction of genetic maps in pear (Yamamoto, 2002), indicating cross-genus transfer is a possible for these markers in the Rosaceae. Among the ten SSRs used in this study six were developed from almond, three from peach, and one from cherry. These markers can be regarded as the preferential marker type for mapping and particularly for the development of genera specific consensus maps. Seven of these SSRs are anchor loci to the 'TxE' map (Aranzana, 2003) (P. Arús, unpublished). Aranzana (2003) has proposed a set of SSRs based on the 'TxE'

map which could be used as a 'genotyping set' for this genera. Two SSRs used in this study are part of this set.

The ultimate application of this map is to improve almond-breeding efficiency through the use of molecular marker technologies. MAS is the most common objective of genetic map construction, which can be used for accelerated backcrossing by tracking the segregation and inheritance of traits through a number of backcrosses (Foulongne, 2003). Or as is the case in most woody perennials for the direct selection of desirable traits in breeding programs. It is desirable that the selected marker is closely linked to the trait of interest to ensure linkage is not lost through successive breeding cycles. Therefore marker saturation is important to reduce linkage disequilibria and diminish the effectiveness of selection. The incorporation of more molecular markers to integrate the remaining five unlinked morphological traits is the next step for improving this map for further use in MAS. In the future when additional markers are added to the map to improve genome coverage, marker saturation, and accuracy in some regions, gene pyramiding can be employed. Combining screening for a number of traits makes MAS more cost effective since the majority of costs are associated with sampling and DNA extraction. This map provides another step towards this goal.

7. Technology Transfer

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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:

10 November 2000, Australian Almond Industry Seminar, Adelaide,

15-16 November 2001, Australian Almond Industry Seminar, Renmark

1 November 2002, Australian Almond Industry Seminar, Renmark

25 September 2003, Australian Almond Industry Seminar, Adelaide

11-12 November 2004, Australian Almond Industry Seminar, Renmark

10-11 November 2005, Australian Almond Industry Seminar, Berri

2-3 November 2006, Australian Almond Industry Seminar, Berri

8. Recommendations

We strongly recommend the continuation of the breeding program to achieve the initial aim of providing the industry with new cultivars. Currently the program is at a point where new superior trees require further secondary evaluation before release to the almond industry.

We recommend the continued use of molecular markers as part of the breeding program as they allow faster analysis of *S*-alleles and for use in virus detection.

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

- Maintain the crossing program with a more targeted approach until 2009
- Evaluate all superior selections in second stage trials
- Evaluate all imported cultivars for kernel characteristics
- Develop more molecular markers for use in the breeding program
- Estimated breeding values and heritabilities will be determined
- Develop methods for analysing water use efficiency in almond seedlings
- Determine fatty acid profiles in almond progeny

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THE AUSTRALIAN ALMOND BREEDING PROGRAM

AL99008

Dr Michelle Wirthensohn and Prof. Margaret Sedgley

The University of Adelaide

Final Report Appendices

1 June 2007

Appendix 1. Rootstock trial filed plan at Lindsay Point

Rootstock Trial #2 Site Plan

Row	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Tree															
1	B	Carmel	B	Peerless	B	Carmel	B	Carmel	B	Peerless	B	Carmel	B	Carmel	B
2	B	"	B	"	B	"	B	"	B	"	B	"	B	"	B
3	B	"	B	"	B	"	B	"	B	"	B	"	B	"	B
4	B	"	B	"	B	"	B	"	B	"	B	"	B	"	B
5	H	"	N	"	7	"	H	"	B	"	B	"	B	"	B
6	10	"	4	"	7	"	9	"	B	"	3	"	B	"	B
7	10	"	4	"	7	"	9	"	B	"	3	"	B	"	B
8	10	"	4	"	7	"	9	"	B	"	3	"	B	"	B
9	H	"	4	"	7	"	H	"	B	"	3	"	B	"	B
10	N	"	1	"	N	"	6	"	B	"	B	"	B	"	B
11	5	"	1	"	4	"	6	"	B	"	B	"	B	"	B
12	5-died	"	1	"	4	"	6	"	B	"	B	"	B	"	B
13	5-died	"	1	"	4	"	6	"	B	"	B	"	B	"	B
14	N	"	1	"	4	"	6	"	B	"	B	"	B	"	B
15	6	"	6	"	1	"	N	"	B	"	B	"	B	"	B
16	6	"	6	"	1	"	N	"	B	"	B	"	B	"	B
17	6	"	6	"	1	"	5	"	B	"	B	"	B	"	B
18	6	"	6	"	1	"	5	"	B	"	B	"	B	"	B
19	6	"	6	"	1	"	N	"	B	"	B	"	B	"	3
20	7	"	H	"	H	"	B	"	3	"	B	"	B	"	3
21	7	"	10	"	H	"	B	"	3	"	B	"	3	"	3
22	7	"	10	"	10	"	B	"	3	"	B	"	3	"	3
23	7	"	10	"	10	"	B	"	3	"	B	"	3	"	B
24	7	"	H	"	H	"	B	"	B	"	B	"	3	"	B
25	H	"	7	"	N	"	B	"	B	"	B	"	B	"	B
26	9	"	7	"	5	"	B	"	B	"	B	"	B	"	B
27	9	"	7	"	5	"	B	"	B	"	B	"	B	"	B
28	9	"	7	"	5	"	B	"	B	"	B	"	B	"	B
29	H	"	7	"	N	"	B	"	B	"	B	"	B	"	3
30	1	"	N	"	H	"	B	"	3	"	B	"	B	"	3
31	1	"	4	"	H	"	B	"	3	"	B	"	B	"	3
32	1	"	4	"	9	"	B	"	3	"	B	"	B	"	3
33	1	"	4	"	9	"	B	"	3	"	B	"	B	"	B
34	1	"	4	"	H	"	B	"	B	"	B	"	B	"	B
35	B	"	B	"	B	"	B	"	B	"	B	"	B	"	B
36	B	"	B	"	B	"	B	"	B	"	B	"	B	"	B
37	B	"	B	"	B	"	B	"	B	"	B	"	B	"	B
38	B	Carmel	B	Peerless	B	Carmel	B	Carmel	B	Peerless	B	Carmel	B	Carmel	B

Rootstock Trial #2 Site Plan

16	17	18	19	20	21	22	23	24	LEGEND
									Trees planted in 1999:
Peerless	B	**Carmel**	B	**Carmel**	B	**Peerless**	B	**Carmel**	1 = GF677
"	B	"	B	"	B	"	B	"	4 = CADAMON
"	B	"	B	"	B	"	B	"	5 = CITATION
"	B	"	B	"	B	"	B	"	6 = BRIGHT'S HYBRID ex Forbio
"	H	"	4	"	N	"	6	"	7 = NEMAGUARD
"	10	"	4	"	N	"	6	"	9 = HANSEN 536
"	10	"	4	"	5	"	6	"	10 = HANSEN 2168
"	10	"	4	"	5	"	6	"	B = Buffer tree (seed hybrid)
"	H	"	4	"	N	"	6	"	H = Hybrid (buffers to replace shortages within treatments)
"	7	"	6	"	N	"	H	"	N = Nemaguard (buffers to replace shortages within treatments).
"	7	"	6	"	4	"	H	"	
"	7	"	6	"	4	"	9	"	**Trees planted in 2000:**
"	7	"	6	"	4	"	9	"	3 = ATLAS
"	7	"	6	"	4	"	H	"	
"	1	"	1	"	N	"	N	"	**Unavailable for planting:**
"	1	"	1	"	N	"	4	"	2 = VIKING
"	1	"	1	"	5-died	"	4	"	8 = NEMASUN
"	1	"	1	"	5	"	4	"	
"	1	"	1	"	N	"	4	"	
"	H	"	B	"	7	"	B	"	
"	9	"	10	"	7	"	B	"	
"	9	"	10	"	7	"	B	"	
"	9	"	B	"	7	"	B	"	
"	H	"	B	"	7	"	B	"	
"	6	"	7	"	H	"	B	"	
"	6	"	7	"	10	"	B	"	
"	6	"	7	"	10	"	B	"	
"	6	"	7	"	H	"	B	"	
"	6	"	7	"	H	"	B	"	
"	N	"	B	"	1	"	B	"	
"	5	"	B	"	1	"	B	"	
"	5	"	9	"	1	"	B	"	
"	5	"	9	"	1	"	B	"	
"	N	"	B	"	1	"	B	"	
"	B	"	B	"	B	"	B	"	
"	B	"	B	"	B	"	B	"	
"	B	"	B	"	B	"	B	"	
Peerless	B	**Carmel**	B	**Carmel**	B	**Peerless**	B	**Carmel**	

Appendix 2. Crosses achieved and progeny numbers from 1997-2006

Female	Male	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	Total
11aT44	Nonpareil								63			**63**
1aT26	self						9	10				**19**
1aT26	Somerton						21	17				**38**
1aT30	self						15	108				**123**
1aT30	Somerton						12	79				**91**
1aT4	self						2					**2**
1aT4	Somerton						10	23				**33**
1aT40	self						1	1				**2**
1aT40	Somerton						10	7				**17**
1aT9	Somerton						4					**4**
1bT31	Nonpareil								70			**70**
1bT31	self						2	23				**25**
1bT31	Somerton						35	16				**51**
1bT32	Nonpareil								67			**67**
1bT32	self						22	26				**48**
1bT32	Somerton						5	22				**27**
1bT42	self						4	6				**10**
1bT42	Somerton						32	16				**48**
1bT47	self						15	9				**24**
1bT47	Somerton						14	8				**22**
1bT51	self						2	6				**8**
1bT51	Somerton						3					**3**
2bT33	Nonpareil								9			**9**
4bT1	1bT32								43			**43**
4bT1	Nonpareil								10			**10**
8aT48	Nonpareil								9			**9**
8aT48	1bT31								19			**19**
Carmel	155										251	**251**
Carmel	1bT32						558			139		**697**
Carmel	21-323						90				173	**263**
Carmel	2bT33							39		85		**124**
Carmel	4bT1							67				**67**
Carmel	8aT48								65	87		**152**
Carmel	A-22							125				**125**
Carmel	Antoñeta $S_fS_?$										213	**213**
Carmel	Desmayo Largueta							32	49			**81**
Carmel	Genco									142		**142**
Carmel	Glorieta										23	**23**
Carmel	Guara S_1S_f							8				**8**
Carmel	Johnston's Prolific			140								**140**
Carmel	Lauranne		43					110				**153**
Carmel	Mandaline										181	**181**
Carmel	Marta $S_?S_f$							7				**7**
Carmel	McKinlay's Magnificent			5								**5**
Carmel	Nonpareil								86			**86**
Carmel	P. webbii		53									**53**
Carmel	Pearce			4								**4**
Carmel	R23T45										326	**326**
Carmel	R30T25									136		**136**
Carmel	R30T45							12		14		**26**
Carmel	R33T48										157	**157**
Carmel	R38T63									28		**28**
Carmel	R53T45										122	**122**
Carmel	Somerton			23								**23**
Carmel	Steliette									88		**88**

Carmel	Supernova							201		**201**
Carmel	12-350					171				**171**
Carmel	3aT31			9						**9**
Chellaston	1aT26					219	83			**302**
Chellaston	1aT30						125			**125**
Chellaston	1bT30					106				**106**
Chellaston	1bT31						57		163	**220**
Chellaston	1bT32								39	**39**
Chellaston	1bT47					112				**112**
Chellaston	21-169					76				**76**
Chellaston	21-323					14				**14**
Chellaston	Antoñeta						48			**48**
Chellaston	Carmel								219	**219**
Chellaston	Desmayo Largueta						27			**27**
Chellaston	Marcona				24		17			**41**
Chellaston	Marta				17					**17**
Chellaston	Nonpareil								187	**187**
Chellaston	Parkinson's Pride								384	**384**
Chellaston	Primorskiy					33		114		**147**
Chellaston	R30T25								128	**128**
Chellaston	R33T48								120	**120**
Chellaston	Steliette								167	**167**
Chellaston	Supernova							155		**155**
Chellaston	Tuono							133		**133**
Chellaston	12-350					66				**66**
Chellaston	Lauranne				28			16	108	**152**
Ferraduel	Johnston's Prolific		17							**17**
Ferraduel	Keane's Seedling		43							**43**
Ferraduel	Nonpareil			40						**40**
Ferraduel	Parkinson's Pride		62							**62**
Ferraduel	Somerton			141						**141**
Ferraduel	1bT51					194				**194**
Ferraduel	Chellaston		94							**94**
Ferragnès	1aT30*									**0**
Ferragnès	1bT31					66				**66**
Ferragnès	1bT32					23				**23**
Ferragnès	Carmel	3								**3**
Ferragnès	Chellaston	18								**18**
Ferragnès	Johnston's Prolific	4								**4**
Ferragnès	Keane's Seedling	50	3							**53**
Ferragnès	Le Grand	16								**16**
Ferragnès	McKinlay's Magnificent	12	14							**26**
Ferragnès	Nonpareil		13	62						**75**
Ferragnès	Parkinson's Pride		3							**3**
Ferragnes	1aT26/1aT30*				31					**31**
Ferragnès	12-350					31				**31**
Ferragnès	P. webbii			11						**11**
Ferragnès	Somerton		2							**2**
Ferralise	Carmel		1							**1**
Ferralise	Somerton			35						**35**
Ferralise	Tardy Nonpareil		29							**29**
Ferrastar	Nonpareil			99						**99**
Ferrastar	Somerton			96						**96**
Frenzy	Ferragnès			23						**23**
Johnston's Prolific	12-350					198				**198**
Johnston's Prolific	21-169					82				**82**
Johnston's	21-323					96				**96**

Prolific Johnston's										
Prolific Johnston's	8aT48								71	**71**
Prolific Johnston's	A-22					81				**81**
Prolific Johnston's	Carmel		6						198	**204**
Prolific Johnston's	Chellaston	61								**61**
Prolific Johnston's	Cristomorto				132					**132**
Prolific Johnston's	Desmayo Largueta					138				**138**
Prolific Johnston's	Felisia S?Sf					50				**50**
Prolific Johnston's	Ferraduel								50	**50**
Prolific Johnston's	Guara					160				**160**
Prolific Johnston's	Iranian 2	1	11							**12**
Prolific Johnston's	Keane's Seedling	1								**1**
Prolific Johnston's	Lauranne	26	75		189					**290**
Prolific Johnston's	McKinlay's Magnificent	10								**10**
Prolific Johnston's	Ne Plus Ultra	25	46							**71**
Prolific Johnston's	Nonpareil		153							**153**
Prolific Johnston's	OP	234								**234**
Prolific Johnston's	Parkinson's Pride	9							82	**91**
Prolific Johnston's	Pearce	6								**6**
Prolific Johnston's	Primorskiy						25			**25**
Prolific Johnston's	R21T70								84	**84**
Prolific Johnston's	R23T45								59	**59**
Prolific Johnston's	R33T48								106	**106**
Prolific Johnston's	Somerton	42								**42**
Prolific Johnston's	Steliette								225	**225**
Prolific Johnston's	Supernova						94			**94**
Prolific Johnston's	Thompson		8							**8**
Prolific Johnston's	Tuono						29			**29**
Keanes	12-350				118					**118**
Keanes	1bT31					106				**106**
Keanes	21-169				48					**48**
Keanes	21-323				89					**89**
Keanes	Antoñeta			24						**24**
Keanes	Cristomorto				155					**155**
Keanes	Felisia					112				**112**
Keanes	Glorieta						65			**65**
Keanes	Keane's					2				**2**
Keanes	Lauranne			35			86			**121**
Keanes	Marcona			19						**19**
Keanes	R30T45					75				**75**
Keanes	Steliette						137			**137**
Keanes	Supernova						58			**58**
Lauranne	1bT32							7		**7**

Lauranne	8aT48								7		**7**
Lauranne	Carmel								20		**20**
Lauranne	Falsa Barese								15		**15**
Lauranne	Nonpareil							22	15		**37**
Lauranne	OP								76		**76**
Lauranne	R12T17								18		**18**
Lauranne	R13T18								40		**40**
LeGrand	Keane's Seedling			121							**121**
LeGrand	Le Grand	3									**3**
LeGrand	Nonpareil	122									**122**
LeGrand	OP		10								**10**
LeGrand	Somerton			66							**66**
McKinlay's Magnificent	McKinlay's Magnificent		2								**2**
McKinlay's Magnificent	Iranian 2		15								**15**
McKinlay's Magnificent	Keane's Seedling		13								**13**
McKinlay's Magnificent	Parkinson's Pride		37								**37**
Mission	Chellaston	16	21		9						**46**
Mission	Carmel	11	17								**28**
Mission	Ferragnès	4	107		9						**120**
Mission	Iranian 2		12								**12**
Mission	Johnston's Prolific	21	16	94							**131**
Mission	Keane's Seedling	54	41								**95**
Mission	Le Grand	15									**15**
Mission	McKinlay's Magnificent	4	3								**7**
Mission	Nonpareil	12	138	153	14						**317**
Mission	P. webbii			80							**80**
Mission	Parkinson's Pride		18								**18**
Mission	Pearce		12	189							**201**
Mission	Somerton			161							**161**
NePlus Ultra	McKinlay's Magnificent			2							**2**
NePlus Ultra	Pearce			105							**105**
NePlus Ultra	Somerton			75							**75**
Nonpareil	12-350						489				**489**
Nonpareil	1aT26					35					**35**
Nonpareil	1bT30					67	153				**220**
Nonpareil	1bT31								101		**101**
Nonpareil	1bT32						449				**449**
Nonpareil	1bT51						9				**9**
Nonpareil	21-169						34				**34**
Nonpareil	21-323						142				**142**
Nonpareil	2bT33								72		**72**
Nonpareil	3aT31				2						**2**
Nonpareil	3bT45				7						**7**
Nonpareil	4bT1							124	24		**148**
Nonpareil	8aT48								110		**110**
Nonpareil	A-22							256			**256**
Nonpareil	Antoñeta					3					**3**
Nonpareil	Carmel	105	87								**192**
Nonpareil	Chellaston	214	80	44							**338**
Nonpareil	Cristomorto						46				**46**
Nonpareil	Desmayo Largueta							95			**95**
Nonpareil	Falsa Barese								193		**193**
Nonpareil	Felisia							151			**151**
Nonpareil	Ferraduel									142	**142**

Nonpareil	Genco							127		**127**
Nonpareil	Glorieta						50		267	**317**
Nonpareil	Guara					43				**43**
Nonpareil	Iranian 2		4	37						**41**
Nonpareil	Johnston's Prolific	126	140	149	9					**424**
Nonpareil	Keane's Seedling	207	65	25						**297**
Nonpareil	Lauranne	182	156			74		101		**513**
Nonpareil	Le Grand		54							**54**
Nonpareil	Mandaline								314	**314**
Nonpareil	Marcona					12				**12**
Nonpareil	Marta					17			167	**184**
Nonpareil	McKinlay's Magnificent	18		172						**190**
Nonpareil	Mission 126/1	12								**12**
Nonpareil	ModAlnem 6			79						**79**
Nonpareil	Moncayo					27				**27**
Nonpareil	Ne Plus Ultra		33							**33**
Nonpareil	OP		332							**332**
Nonpareil	P. webbii	1	151							**152**
Nonpareil	Parkinson's Pride		90	56	48					**194**
Nonpareil	Peach r11 t4			4						**4**
Nonpareil	Pearce		24	86						**110**
Nonpareil	Peerless			61						**61**
Nonpareil	R12T17							181		**181**
Nonpareil	R13T18							82		**82**
Nonpareil	R14T17							100		**100**
Nonpareil	R21T70							76		**76**
Nonpareil	R23T45							30		**30**
Nonpareil	R30T25							165		**165**
Nonpareil	R30T45					128		64	186	**378**
Nonpareil	R33T48								231	**231**
Nonpareil	R38T63							31		**31**
Nonpareil	R5T19					60		151		**211**
Nonpareil	Sauret#1				22					**22**
Nonpareil	Somerton		79	136	5					**220**
Nonpareil	Steliette						45	114	175	**334**
Nonpareil	Tuono						46			**46**
P. webbii	OP		40							**40**
Parkinson	Ne Plus Ultra			10						**10**
Parkinson	Nonpareil			228						**228**
Parkinson	Carmel			20						**20**
Parkinson	Lauranne			84						**84**
Parkinson	Thompson			104						**104**
Pearce	Nonpareil			169						**169**
Pearce	Lauranne			29						**29**
Pearce	Ne Plus Ultra			28						**28**
Pearce	Thompson			14						**14**
Price	Iranian 2		2							**2**
Price	McKinlay's Magnificent		27							**27**
Price	Nonpareil		82							**82**
Price	Parkinson's Pride		42							**42**
Price	Somerton			201						**201**
Price	Johnston's Prolific		3		14					**17**
Price	Keane's Seedling		61	141						**202**
R12T17	Nonpareil						17			**17**
R12T28	Nonpareil						24			**24**
R29T105	Nonpareil						8			**8**
R30T25	Nonpareil						40			**40**
R33T48	Lauranne						10			**10**

Somerton	12-350										116	**116**
Somerton	1bT31							83			255	**338**
Somerton	1bT32										265	**265**
Somerton	21-169										57	**57**
Somerton	A-22							155	56			**211**
Somerton	Antoñeta					49						**49**
Somerton	Carmel			70							175	**245**
Somerton	Desmayo Largueta							42	169			**211**
Somerton	Felisia							103				**103**
Somerton	Ferragnès			20								**20**
Somerton	Guara							203				**203**
Somerton	Marcona							45				**45**
Somerton	Marta					23						**23**
Somerton	Ne Plus Ultra			86								**86**
Somerton	Nonpareil			151							286	**437**
Somerton	Pearce		2									**2**
Somerton	R30T25										17	**17**
Somerton	R33T48										334	**334**
Somerton	Steliette										305	**305**
Somerton	Supernova								59			**59**
Somerton	Thompson			105								**105**
Somerton	Tuono								75			**75**
Somerton	1aT26							198				**198**
Somerton	Lauranne					41						**41**
Thompson	Keane's Seedling			1								**1**
Thompson	Somerton			17								**17**
Thompson	Strout			2								**2**
	Total trees	**1230**	**2808**	**4353**	**148**	**396**	**4507**	**3573**	**2224**	**2639**	**7128**	**29006**

Appendix 3. Planting plan for 2nd stage evaluations Lindsay Point

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

Filter unit **N**

Nemaguard rootstock