National citrus scion breeding program

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Project Number: CT00012

CT00012

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HAL PROJECT NUMBER *CT00012*

THE NATIONAL CITRUS SCION BREEDING PROGRAM

(July 2000 – June 2004)

STEPHEN SYKES *et al.*

CSIRO PLANT INDUSTRY
QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES & FISHERIES

HAL PROJECT NUMBER *CT00012*

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Statement of purpose:

The purpose of this document is to formally to report the progress made by the research conducted from July 2000 until June 2004 which formed HAL project CT00012, the National Citrus Scion Breeding Program.

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Media summary

The National Citrus Scion Breeding program is a long-term program that has been supported by the Australian Citrus Industry since 1991 through a series of discreet one-to-four year projects funded by Horticulture Australia Limited and the research providers CSIRO Plant Industry and DPI&F Queensland. Since 1996 the program has been funded as a fully coordinated project with research focused in four main areas of activity, namely conventional diploid hybridisation (CSIRO Plant Industry, Merbein), the production of triploids for seedlessness (DPI&F Queensland, Bundaberg), mutation breeding (Merbein and Bundaberg) and gene technology (CSIRO Plant Industry, Adelaide).

The development of new scion varieties through breeding, selection and introduction is a high priority for the Australian Citrus Industry. The National Citrus Scion Breeding Program is focused to address industry priorities for new fresh fruit varieties. Major characteristics targeted are seedlessness, easy peel, flavour and size, internal and external quality, and agronomic characteristics such as ease-of-harvest, amongst others. The breeding program aims to produce new varieties adapted to Australia's varied regional conditions and the research has been designed to provide marketing, processing and production advantages to the Australian Citrus Industry.

Key outcomes of the program will be the adoption of innovative new varieties that will address the needs of key industry identified market windows of opportunity resulting in increased profitability for Australian citrus growers. Key windows of opportunity identified during project CT00012 have been for early and late maturing, seedless, sweet, easy-to-peel varieties primarily for export.

General conclusions from the project are that the program is producing results that will have application to industry in the form of new varieties, as well as results that are having immediate application to the breeding program itself. In terms of results for industry, data from second stage evaluation trials being conducted on grower-based regional test-plots are indicating that a nomination for release of two new seedless easy-peel varieties will most likely occur in late 2005. Results impacting on the program itself include data on the inheritance of key traits and improved breeding and screening methods. Increased numbers of triploid hybrids produced during CT00012 is an important project output that will impact on future directions taken at Bundaberg.

With regard to future R&D, evaluation of new variants and selections in replicated plantings are key components of the project. In addition, as selections show promise in regional test-plots, it will be important to progress the most promising to larger scale evaluation blocks of 0.5-1.0 ha so that sizable volumes of fruit will be available for test marketing. Market-testing will be important for when new varieties are released. These larger trials can be initiated prior to commercial release so that fruit will be available to test markets while commercial plantings are established and before large quantities of commercially-grown products are available. Other areas for future R&D will be to resurrect the biotechnology component of the project, which was suspended during the course of CT00012. This may be achieved through exploring and developing mutually beneficial overseas collaborations. With the success shown in irradiating parthenocarpic Kara mandarin to give two seedless lines, further consideration should be given to entering high quality parthenocarpic hybrids that are capable of setting seedless fruits in the absence of pollination into a mutation program as well. Such hybrids, due to self-compatibility, are normally seedy in an open-pollination situation. Irradiation could result in eliminating seeds in these hybrids giving them potential for progression to regional evaluation and ultimately variety release.

Industry is involved with the project by evaluating selections from the program under testing agreement so that regional adaptability can be assessed. Practical application of the research to industry will occur when new varieties are released from the program.

Technical summary

Conventional diploid hybridisation component

Conventional citrus breeding through hybridisation with diploid parents is a long term proposition requiring a clear and dedicated commitment by both industry and R&D agencies. The strategic hybridisation program has been in progress at Merbein since 1984 and has received support from industry via HAL funding since 1991. During this period the genetic foundations of the program have been built so that crosses are made to accommodate industry requirements for new varieties. These requirements are documented in the breeding plan, which was prepared during CT00012.

The hybridisation program is essentially a pipeline approach for the delivery of new varieties. Activities are now high for all phases of the pipeline with an anticipated release notice for some selections in late 2005. As a result, a commercialisation strategy is being developed in consultation with the reference committee. In preparation for release, source trees of the promising selections are being indexed by AusCitrus to assess their health status. Once their health status is known, budwood multiplication will commence.

Further evidence demonstrating the value of Imperial mandarin as a parent to transmit autonomic parthenocarpy was obtained during CT00012. Satsuma was also shown to transmit seedless traits, but difficulties in using it as a parent mean that progeny sizes can be restrictively small. Viable pollen, however, was obtained and used to generate larger progenies using satsuma as a male parent crossed to monoembryonic females. Data from one such progeny have reinforced conclusions made from other progenies concerning the inheritance of traits affecting the seedless phenotype.

Information concerning the inheritance of seedless characteristics in the breeding populations can now be used to predict better the outputs from new crosses aimed at incorporating the seedless phenotype with other desirable fruit quality traits.

Promising new hybrids have been identified with potential and entered into second phase evaluation trials. Agreements for regional testing of promising selections were entered into with grower cooperators. Fruits have been harvested from these trials and positive reactions were received from grower groups during fruit displays and tastings.

New crosses were performed to further examine the inheritance of seedless characteristics in the breeding population at Merbein and to address market windows of opportunity with product specifications as outlined in the breeding plan. Crosses were designed to maximise full- and half-sib family relationships enabling investigation of the inheritance of other key characteristics.

Once a series of early x early crosses has been completed, the hybridisation program at Merbein will be at a stage whereby the need for further crosses in the foreseeable future will have been met. The hybridisation program in terms of new variability will have addressed the aims and objectives with regard to product specifications in the breeding plan. The hybridisation program at Merbein will then be in a phase where most activities will centre on evaluation and commercialisation of potential new varieties. Future crosses in this component of the breeding program will have specific research aims to investigate key characteristics aimed at improving efficiency for breeding

research into the future. There is, however, scope to increase mutation breeding activities with new parthenocarpic hybrids that produce high quality fruits, which are seedy due to pollination.

Triploidy component

The triploidy breeding program has been successful in developing large numbers of hybrids, which are now well established in the field. More than 3200 hybrids, mostly from crosses between diploid seed parents and tetraploid pollen parents, are field-established and another 2400 hybrids are in the nursery ready for field-planting in early 2005. There is no information available as to the ploidy levels of these hybrids, or how the proportion of triploids to tetraploids varies between families. However it is hoped that a significant number of them are triploids and will produce seedless fruit of sufficient quality for further evaluation.

New autotetraploid and allotetraploid pollen sources became available during the course of the project and were immediately incorporated into the hybridisation work. These included some high quality mandarin genotypes, and their availability has enabled a reduction in the utilisation of tetraploid sweet orange pollen (which was used extensively, particularly in the early years of the crossing program).

A major obstacle associated with poor seed formation and contamination of tissue-cultured embryos was overcome during the project. The problem was extremely severe in 2004 with the result that very few fruit were suitable for embryo rescue. It was discovered that the cause of these problems was Hemiptera insects that were stinging the young fruit and in so doing injecting a yeast (*Nematospora coryli*) that grew within the fruit. This yeast inhibited seed formation and caused contamination when embryos were placed on culture media. The problem was recognised many years ago, but its cause was unknown and efforts to alleviate the symptoms had failed. By improved control of the relevant Hemiptera insects it is anticipated that significantly improved embryo-rescue rates will be achieved in coming seasons.

Experiments were established to reduce sucker growth on hybrids. These showed that NAA was not particularly effective in reducing sucker growth, and may even be phytotoxic. It was discovered that the white acrylic paint, used as a carrier for the NAA, was more effective in reducing sucker growth. Simply painting the trunks of hybrids with white paint significantly reduced sucker production and had no detrimental affect on hybrid growth.

Pollen from two triploid cultivars was shown to have the potential to induce seed production in a range of monoembryonic mandarin genotypes. Much of this seed was viable and the resulting hybrids will be field planted and fruited. The ploidy level of these hybrids has not been determined though they show leaf and stem distortion consistent with complex ploidy. Poor fruit set on supposedly parthenocarpic genotypes reinforces previous observations at Bundaberg that this characteristic may be strongly influenced by environment.

Mutation breeding

At Merbein, the seedless or low-seeded characteristics of 2 budlines (258.2 and 258.4) derived from a Kara mandarin bud that was exposed to 60gy gamma irradiation were confirmed. The stability of this trait in these budlines after a generation of vegetative propagation was demonstrated, but needs to be confirmed by further experiments. The two seedless (or low-seeded) budlines appeared to arise as chimeric mutations which affected both female and male fertility. Further experiments will confirm this. The results have justified the approach taken and have demonstrated the value of using induced mutagenesis to affect seediness in a known parthenocarpic variety. This supports a proposal to irradiate hybrids from the diploid program that are highly parthenocarpic with superior

fruit quality and appropriate fruit maturity, but are capable of self-pollination and thus seedy in an open-pollination situation. Irradiation to reduce or eliminate seeds in such hybrids would improve their chances for adoption.

At Bundaberg, sixteen putative low-seeded mutants from eight different cultivars have been propagated for further evaluation. These will be assessed to ensure they remain low-seeded and that other characteristics, particularly fruit size, have not been adversely affected. The frequency of low-seeded mutations varied between the nine cultivars subjected to irradiation. Four putative mutants were selected from 41 trees of Afourer, whereas no low-seeded mutations were detected from 80 trees of Kinnow.

Gene technology

A report was prepared and submitted to HAL in May 2003 when a decision was made by HAL to suspend further funding for the gene technology research. The report summarised the work carried out at CSIRO Plant Industry's Adelaide Laboratory from July 1992-June 2003. This research pioneered molecular breeding of citrus in Australia where none existed previously. It established procedures to regenerate transgenic citrus (West Indian lime) plants and examine the utility of the introduced genes for citrus crop improvement. It successfully identified, produced and tested genes that can prevent seed formation or reduce seed size and number in citrus fruit. Proof of concept was successfully obtained in West Indian Lime for all but one gene, which requires further tailoring. Transformation and regeneration of mandarins and mandarin hybrids was not successful. The report provided a clear statement of where the work was at when funding ceased, the utility of the genes, their ownership, current bottlenecks in citrus molecular improvement and routes to market for varieties developed in the future utilization of these genes.

Introduction

With fresh fruit, as for all other horticultural produce, change is ever present and producers and markets can no longer rest assured that traditionally favoured varieties, or indeed existing crops, will continue to command premium prices. It is important that those trading in fresh citrus are continually innovative like others in the horticultural sector. Innovation should be effective at all stages in the market chain from planting materials through to packaging and presentation to the consumer. The use of genetic improvement techniques, whether conventional or bio-technological, offers great opportunity for the generation of novelty. New varieties and types of seedless citrus with novel colour, size, taste, texture and other quality characteristics that address market requirements, or perhaps even alter market perceptions, provide innovation through genetic improvement that will maintain or improve market share and thus command premium prices.

The development of new scion cultivars through breeding, selection and introduction is a high priority for the Australian Citrus Industry. Project CT00012 continued the breeding research that forms part of a nationally coordinated citrus improvement program. This national program involves varietal improvement projects covering breeding, evaluation and repository maintenance. The breeding component is primarily focused to address industry priorities for fruits consumed as fresh products. Major characteristics targeted are seedlessness, easy peel, flavour and size, internal and external quality, and agronomic characteristics such as ease-of-harvest, amongst others. The breeding program aims to produce new scion varieties adapted to Australia's varied regional conditions and the research has been designed to provide marketing, processing and production advantages to the Australian Citrus Industry.

During CT00012 traditional breeding approaches have been pursued in the environments in which the varieties will be grown. These approaches have been coordinated with newer biotechnological methodologies conducted in a laboratory that has international recognition for this field of research. Each line of research in CT00012, and indeed in previous projects conducted under the umbrella of the National Citrus Scion Breeding Program, has had specific, short- and long-term goals and thus has been designed to be flexible in response to changing industry and market requirements. Innovation is important for competitiveness in the global market and new varieties need to be developed which grow well in Australia and ship well to provide the industry with an export edge. The research in the project has been tailored for market needs and an important aspect of this research has been focused on producing seedless varieties and breeding lines. The breeding program is designed to generate outstanding new varieties which can be tested in the market place where their novel features can capture consumer interest and thus gain the industry a unique competitive advantage.

By coordinating traditional breeding methods such as hybridisation and mutation breeding, with newer genetic engineering technologies, the research team has ensured that the best approach is adopted. In this way each targeted aim can be achieved within the overall framework of producing improved, locally adapted citrus scion varieties for the Australian citrus industry. Before the project commenced the research team put together its application to HAL knowing that an expert team had reviewed the research in November 1995 and again in February 1999. The outcome of these reviews was that the program employs an appropriate range and balance of methodologies to achieve its goals. Thus at the start of CT00012, the project covered the following four distinct areas within an overall coordinated breeding project:

1. Conventional hybridisation (Merbein)
2 Triploidy breeding (Bundaberg)
3. Mutation breeding (Merbein and Bundaberg)

4. Genetic transformation (Adelaide)

In this way, project CT00012 continued the research of CT96014 (1996-2000), which was a coordinated project, and before that projects CT111, CT206, CT225, CT315, CT319 & CT522, and so built on the successes of previous citrus scion breeding projects supported by HAL.

As a coordinated project, the components of CT00012 have complimented and not duplicated the research effort and contributed collectively to the overall goal of innovative and improved Australian varieties that address market requirements leading to expanded market opportunities.

CT00012 was originally funded for the period July 2000 - to - June 2003. The project was reviewed in February 2003 by Dr. Luis Navarro, of IVIA, Spain. A new project application was also submitted to HAL to continue the National Citrus Scion Breeding Program beyond July 2003. As a result of the review and other deliberations, the Industry Advisory Committee and HAL decided to suspend support for the gene technology research and extend CT00012 for a further 12 months by supporting only the hybridisation (diploid and triploid) and the mutation research. As a consequence of suspending the gene technology research, Dr. Anna Koltunow submitted a final report covering this component of CT00012 to HAL during 2003. Dr. Koltunow's report on the gene technology component of CT00012 has been included as an appendix to this report.

This final report documents progress in the other components of the research undertaken from June 2000 until July 2004 for project CT00012.

3. Diploid hybridisation program (CSIRO Plant Industry, Merbein)

3.1 Introduction

The conventional hybridisation program at CSIRO Plant Industry Merbein is based on crossing diploid parents to yield hybrid progenies, which are evaluated for key characteristics. The data generated are used to identify promising hybrids for:

- entry into second phase replicated evaluation plantings, and
- use as parents in future breeding, thus building on the genetic foundations of the program.

The data are also used to study the inheritance of key traits to develop breeding and selection strategies. As such, the program is dynamic, can be responsive to changing industry priorities, and takes the form of a pipeline approach for the delivery of outputs to achieve the overall industry outcome of successful new scion varieties.

Citrus breeding research at Merbein commenced in the 1960s when CSIRO's citrus germplasm arboretum was established. However, it was not until 1991, when industry supported the research through matching HAL funding, that breeding for new scion varieties received a much higher profile. Before 1991, industry had assisted with in-kind support for testing new selections and with funds from the Citrus Management Company (now Murray Valley Citrus Board) for purchasing isozyme analytical equipment. This equipment was used in HAL project CT111 (1991-92) to identify new seedless Satsuma mandarin hybrids, and since in other projects to identify zygotic from nucellar seedlings where female parents have been polyembryonic.

In breeding new Australian varieties, the hybridisation program at Merbein has sought to provide industry with new material for testing and at the same time build on the genetic foundations underpinning the program. In this way, the direction taken by the research can respond to current as well as future industry priorities for new varieties without the need to adopt a hit-or-miss approach in making new crosses.

This section outlines the progress made in the hybridisation and associated research at Merbein since July 2000. Only summaries of the data are reported here for the sake of brevity. Large data sets have been generated and are used for making key decisions in the program. Progress was also documented in 6-monthly milestone reports submitted to HAL during the course of the project and are available for further information.

3.2 Crossing program

A significant outcome from the research conducted over the last decade at Merbein has been to generate new parent material specifically for use in breeding new Australian varieties. This research has recombined and fixed characteristics deemed essential in easier-to-use parents for the development of new varieties to address current, and more importantly, future market requirements.

Historically, both in Australia and overseas, breeding new citrus varieties by hybridisation has involved pair crosses between common knowledge varieties, often repeating the same cross year after year without learning much about the characteristics targeted in the program. In conducting a strategic hybridisation program to develop new parents, the research at Merbein has made a

departure from this approach. Resources are being expended to understand the way that key fruit characteristics are inherited. In this way, breeding and selection strategies are smarter. To achieve this, the evaluation of progenies has to be extensive and detailed. Progenies are screened extensively for the characteristics that contribute to the complex seedless trait. Pollination experiments are conducted to assess the effects of different pollen sources on fruit set and seediness, and observations made on pollen fertility and other flower characteristics. As a result we know more about the genetic control of the characteristics that lead to seedlessness under Australian conditions so we can plan breeding activities with greater certainty of outputs.

Now seedless genes are firmly established in our breeding parents, the hybridisation program has progressed to place greater emphasis on other quality traits such as fruit size, sugar and acid levels, and rind characteristics. These, along with maturity season, are mostly quantitative traits influenced by the actions of many genes. Crosses conducted in CT96014 were aimed at using parents identified in CT319 that would transmit seedless characteristics. The progenies from these crosses as described later were planted out during CT00012.

New crosses made during CT00012 were aimed at varieties required by industry as identified at a series of grower fora held during 1999 and were primarily designed to yield late and early maturing varieties. The following describes the crosses made during CT00012.

3.2.1 Crosses aimed at new late maturing varieties

Consistent market place messages for citrus fruits are that:

- Seedless fruits are preferred in some markets and demanded in others.
- Small fruit size, seediness and peeling-difficulty reduce market acceptance.
- Fruits must be **widely available (seasonality)**, sweet and juicy.
- Convenience (ease of peeling and not messy) will become increasingly more important.
- The demand for sweet, pigmented grapefruits will continue to increase.

The diploid hybridisation program at Merbein has sought to take on board these messages in developing new parent material and thus new hybrids.

These messages, along with the issues identified for new varieties from a series of industry fora held during June 1999, have been taken on board by the breeding plan developed during CT00012 for the overall breeding program. In developing product specifications for new varieties, it was concluded that new seedless, late maturing varieties are needed in Australia to fill a recognised export market window of opportunity, namely fruit for the period August through to October. Thus, a series of crosses was started with one controlled cross-pollination in 1999 during CT96014 and continued through until spring 2002.

Female parents used were hybrids between Silverhill satsuma and a range of sweet oranges that were shown to possess key traits that will give seedless phenotypes, namely autonomic parthenocarpy and pollen sterility. Male parents were a range of late-maturing hybrids that are parthenocarpic and of high fruit quality, and late-maturing named varieties. The details of the successful crosses are as follows:

Table 3.1. Crosses made from 1999 until 2002 aimed at late maturing seedless easy peel varieties.

Family	Years in which cross was made	Family	Years in which cross was made
88-02-07 x Fortune	2001	88-02-30 x 2916	2001
88-02-07 x 2751	2001	88-02-30 x Encore	2001, ‘02
		88-02-30 x Murcott	2001
88-02-12 x 2916	2000, ‘01	88-02-30 x 2952	2000, ‘01
88-02-12 x 2360	2000, ‘01	88-02-30 x VAL 9	2000, ‘01
88-02-12 x 2127	2000		
88-02-12 x 2751	2000, ‘01	88-05-40 x 2916	2001
88-02-12 x Fortune	2000, ‘01	88-05-40 x 2952	2001
88-02-12 x Encore	2000, ‘01	88-05-40 x 2360	2001
88-02-12 x Murcott	2000, ‘01	88-05-40 x 2751	2001
88-02-12 x 2952	2001	88-05-40 x Fortune	2001
88-02-12 x Val 9	2001		
		88-02-44 x 2916	2000
88-02-14 x 2916	2001	88-02-44 x 2751	2000, ‘01
88-02-14 x Encore	2001	88-02-44 x 2127	2000
88-02-14 x Murcott	2001	88-02-44 x Fortune	2000, ‘01
88-02-14 x Fortune	2001	88-02-44 x Encore	2000, ‘01
88-02-14 x 2751	2001	88-02-44 x Murcott	2001
		88-02-44 x VAL 9	2001
88-02-18 x 2751	2001	88-02-44 x 2360	2001
88-02-18 x Murcott	2001	88-02-44 x 2952	2001
88-02-18 x 2916	2001	88-02-44 x 2916	2001
88-02-18 x Fortune	2001		
		88-02-57 x Murcott	2001
88-02-21 x 2916	2000, ‘01 ‘02	88-02-57 x 2916	2001
88-02-21 x 2360	2000, ‘01	88-02-57 x 2751	2001
88-02-21 x Fortune	1999, ’00, ‘01 ‘02	88-02-57 x 2360	2001
88-02-21 x Murcott	2001	88-02-57 x 2952	2001
88-02-21 x Val 9	2001	88-02-57 x Fortune	2001
88-02-21 x Encore	2001		
88-02-21 x 2952	2001	88-03-08 x Fortune	2001, ‘02
88-02-21 x 2751	2001	88-03-08 x 2751	2001, ‘02
		88-03-08 x Encore	2001
88-02-21(b) x Fortune	2001	88-03-08 x 2360	2002
88-02-21 (b) x 2916	2001	88-03-08 x 2952	2002
		88-03-08 x Murcott	2002
88-02-30 x 2360	2001, ‘02		
88-02-30 x 2751	2001	88-03-10 x Fortune	2001, ‘02
88-02-30 x Fortune	2000, ‘01’ ‘02	88-03-10 x 2916	2001
88-03-10 x Encore	2002		
88-03-10 x 2360	2002	88-05-24 x VAL 9	2001
		88-05-24 x 2916	2001
88-04-11 x 2916	2000, ‘01	88-05-24 x 2952	2001

88-04-11 x Fortune	2001	88-05-24 x Murcott	2001
88-04-11 x 2751	2001		
88-04-11 x Murcott	2001	88-05-40 x Murcott	2001
		88-05-40 x 2916	2001
88-05-08 x 2916	2001, '02		
88-05-08 x 2952	2001	88-05-53 x 2751	2001, '02
88-05-08 x 2751	2001, '02	88-05-53 x VAL 9	2001, '02
88-05-08 x VAL 9	2001	88-05-53 x 2916	2001, '02
88-05-08 x Fortune	2001, '02	88-05-53 x Fortune	2001, '02
88-05-08 x Encore	2002	88-05-53 x 2952	2002
88-05-08 x 2360	2002	88-05-53 x Encore	2002
88-05-08 x 2127	2002	88-05-53 x 2360	2002
88-05-08 x Murcott	2002	88-05-53 x Murcott	2002
Female parents were: 88-02-07, -12, -14, -21, -21(b), -30, -40, -44, -57 (Silverhill satsuma x Joppa) 88-03-08, -10 (Silverhill satsuma x Red Siletta) 88-04-11 (Silverhill satsuma x White Siletta) 88-05-08, -24, -40, -53 (Silverhill satsuma x Hamlin)		Male parents were: 2127, 2916, 2360, 2751, 2952 (imperial mandarin x Ellendale tangor) Named varieties: Fortune, Encore, Murcott and Valencia orange (Val 9)	

Progeny from crosses made in 1999 and 2000 were planted in the breeding field during spring 2003. Progeny from 2001 and 2002 crosses will be planted during spring 2004 and autumn 2005 respectively.

3.2.2 Crosses aimed at new early maturing varieties

The breeding plan also highlights the export market window of opportunity that exists for very early maturing fruits. The feasibility for breeding improved very-early maturing hybrids was demonstrated by a hybrid between Clementine and Imperial mandarin, which was first reported in milestone report no. 5 (31/08/02). Hybrid 91-03-04 reaches full maturity in early April at Merbein and, as described later, has been fast-tracked into second phase evaluation. As this hybrid was selected from only a small family of ten hybrids, and also since other hybrids in the family were shown to have seedless traits and good fruit quality, a priority was to repeat the cross and also generate other new families with Clementine, Imperial and their hybrids that produce early maturing fruits. Three series of crosses were designed and controlled pollinations commenced during 2003 with the aim of continuing until all combinations were completed. The crosses were as follows:

Series 1. The aim here was to repeat the Clementine Marisol x Imperial cross that resulted in the selection of 91-03-04 for further evaluation. In addition to repeating this cross, other Clementine varieties were included and reciprocal crosses were conducted as follows:

Table 3.2. Crosses made between Clementine varieties and Imperial mandarin in spring 2003. The seeds were sown in September 2004. Further crosses will be conducted in 2004 and 2005.

Female	Male	No. pollinations	No. seeds harvested in 2004*
Marisol	Imperial	23	72
Fina	Imperial	20	0
De Nules	Imperial	20	0
Oroval	Imperial	20	23
Clem. – Old clone	Imperial	20	35
Imperial	Marisol	20	5
Imperial	Fina	20	77
Imperial	De Nules	20	7
Imperial	Oroval	20	0
Imperial	Clem. – Old clone	21	0

* Unfortunately a severe storm occurred at Merbein on December 4, 2003 and many fruits were lost from the trees that were used as parents. This resulted in fewer seeds being collected than anticipated.

The seeds collected from the fruits were sown in September 2004.

Series 2. The second of this series of crosses was made using Fischer navel orange as the female parent. Fischer is an early maturing navel and the aim was to produce new early maturing tangor varieties. Being polyembryonic, seeds from the crosses will be germinated *in-vitro* to maximise the chances for hybrid rescue. As a consequence, the plan was also to rescue as many nucellar seedlings of Fischer to compliment crosses aimed at providing a population of Fischer nucellar seedlings from which to select nucellar mutants (this component of the program is described in section 3.2.3). Table 3.3 summarises the crosses made with Fischer navel aimed at early maturing tangor varieties.

Table 3.3. Controlled cross-pollinations made with Fischer navel in spring 2003 aimed at producing new early maturing tangor hybrids.

Female	Male	No. pollinations conducted in Spring 2003	No. fruits harvested in 2004
Fischer navel	Imperial	20	5
Fischer navel	Marisol	20	2
Fischer navel	Fina	20	1
Fischer navel	De Nules	20	2
Fischer navel	Oroval	20	0
Fischer navel	Clem. - Old clone	20	5

* Unfortunately a severe storm occurred at Merbein on December 4, 2003 and many fruits were lost from the Fischer navel tree used as a parent. This resulted in fewer fruits being collected than anticipated. At this stage the fruits are in cold storage until an opportunity arises to extract the seeds and germinate them *in-vitro*.

Series 3. The third series of crosses aimed at early maturing seedless easy-peels involved hybrids bred at Merbein and which have been identified as potential parents based on seedless traits, fruit maturity and quality, particularly high juice sugar concentrations. The crossing plan designed is presented in Table 3.4 along with the number of pollinations conducted in spring 2003 and the number of seeds extracted from the fruits harvested in 2004.

Table 3.4. Controlled cross-pollinations made between parents bred at Merbein and selected to generate new early-maturing, seedless, easy-peel hybrids. Crosses were made during spring 2003 and seeds collected in June 2004. Seeds have been sown.

Female parent	Male parent					
	23-09		23-26		29-57	
	No. pollinations	No. seeds	No. pollinations	No. seeds	No. pollinations	No. seeds
87-03-09	1	1				
87-03-21			5	5	4	0
87-03-23						
88-22-13						
88-22-30	3	0	21	31	23	0
88-22-41	1	1	2	3	1	0
91-03-01						
91-03-04						
91-03-07			20	2	20	3
91-03-10			20	0	13	10
92-01-02	20	6	18	37	15	44
92-01-07	20	0	20	44	20	42
92-01-24	20	5	20	30	25	104
92-01-31	20	0	20	31	20	0
93-05-05						
93-05-09	6	0	20	6	20	9
93-05-10						

Female parents were:
87-03-09, -21 & -23 (Clementine old clone x Sunset mandarin)
88-22-13, -30 & 41 (Clementine old clone x Valencia orange)
91-03-01, -04, -07 & -10 (Clementine Marisol x Imperial mandarin)
92-01-02, -07, -24 & -31 (Clementine Fina x Silverhill satsuma)
(3-05-05, -09 & -10 (Imperial mandarin x Clementine old clone)

Male parents were:
23-09, 23-26 & 29-57 (Imperial mandarin x Ellendale tangor)

Not all combinations in series 3 were made in 2003 and of the crosses attempted, some resulted only in seedless fruits. Others yielded fruits with low seed numbers. Further controlled pollinations are planned in 2004-to-2006 to complete this series of crosses. The seeds that were extracted were sown in seed flats during September 2004.

3.2.3 Crosses aimed at generating early maturing nucellar mutants of navel orange

A nucellar seedling selection program was initiated in response to concerns primarily from growers in the MIA for better early maturing navel oranges. Selection of nucellar seedlings, which can be variable due to localised mutation, has been successful for the improvement of satsuma mandarins in Japan.

Fischer navel has been the target of this research as it was shown in 2002-03 that it produces more seeds than other varieties when deliberately cross-pollinated with *Poncirus trifoliata* pollen (Table 3.5).

Table 3.5. Results from a series of exploratory cross-pollinations made in 2002 with a range of navel orange varieties to identify which may be suitable for use as seed parents to maximise seed and thus seedling recovery.
Poncirus trifoliata pollen was used so that hybrid seedling emergence could be assessed rapidly. The trifoliate leaf characteristic of *P. trifoliata* is dominant and allows a quick separation of zygotic and nucellar seedlings.

Female parent	Male parent	Number of pollinations.	No. fruit	No. seeds
Bellamy navel	*P. trifoliata*	20	5	4
Lowes Late navel	*P. trifoliata*	20	1	0
Lanes late navel	*P. trifoliata*	20	0	-
Biggs Early navel	*P. trifoliata*	20	0	-
Navelina	*P. trifoliata*	20	0	-
Navelate	*P. trifoliata*	20	0	-
Newhall	*P. trifoliata*	20	2	0
Fischer	*P. trifoliata*	20	7	28
Atwood	*P. trifoliata*	20	3	2
Washington (Houghton)	*P. trifoliata*	20	0	-
Leng navel	*P. trifoliata*	20	2	2
Washington (Coomealla)	*P. trifoliata*	20	0	-

Clearly some navel selections yielded more fruits and seeds than others, even though these data are limited as the numbers of pollinations were quite low. Fischer yielded more seeds than other varieties and it was decided this variety should be used as a female to transmit navel orange characteristics into new parents for the breeding program. Of particular interest would be seedless traits, and in particular male sterility, which is thought to be due to a different mechanism to that displayed by Satsuma. Fischer is a popular early selection in California (Saunt 2000), indicating its potential also to transmit early fruit maturity, which has been identified as a priority in the breeding plan.

In 2003 Fischer was cross-pollinated with early maturing mandarin varieties (see section 3.2.2) and also with *Poncirus trifoliata* pollen. A total of 115 flowers of Fischer were emasculated and cross-pollinated with pollen from *Poncirus trifoliata*. As with some other crosses, however, the Fischer tree used as the female parent was in the path of a severe storm on December 4, 2003 and many developing fruits were lost. Twelve fruits were harvested from the controlled pollinations in June 2004 and have been placed in cool store until the seeds can be extracted and germinated *in-vitro* to maximise embryo recovery. Hybrids will be identified and rogued and the nucellar seedlings will be rowed out at high density and fruit evaluated once flowering occurs.

3.2.4 Conclusions

Once the series of early x early crosses have been completed, the hybridisation program at Merbein will be at a stage whereby the need for further crosses in the foreseeable future will have been met. The hybridisation program in terms of new variability will have addressed the aims and objectives with regard to product specifications in the breeding plan. The hybridisation program at Merbein will then be in a phase where most activities will centre on evaluation and commercialisation of potential new varieties. It is anticipated that the first nomination for release of a new variety will be in late 2005 and a commercialisation strategy is being developed in consultation with the Citrus Scion Breeding Reference committee. Any new crosses anticipated for this component of the

breeding program after the early x early series has been completed will most likely have specific research aims, for example to investigate the inheritance of key characteristics such as early flowering. As such, future crosses will really be aimed at improving efficiency of breeding research into the future.

3.3 First phase evaluation of hybrids

After controlled crosses have been made and hybrids rowed out in the breeding fields, the hybridisation program at Merbein is divided essentially into two phases. Phase one is where single hybrid trees, planted at high density on their own roots, are screened and evaluated for a range of key characteristics. From the results generated in phase 1, promising hybrids may be selected for further testing in phase two or for use as parents in new crosses. Phase two involves testing replicated trees in regional test plots on growers' properties and in comparative trials on CSIRO property, which also serve as DUS trials for PBR purposes. From the results obtained during phase two evaluation, decisions will be made concerning the commercialisation of any hybrid considered to have potential for release to industry for production.

The methods used and the data collected during phase 1 evaluation have been described in previous final reports for projects within the national citrus scion breeding program and will not be described again here. This section of the report aims to report on key developments with regard to first phase evaluation of the various progeny groups that have been listed before. The updated list of progenies and activities is presented here as Table 3.6.

3.3.1 Screening for seedless traits

Methods used to screen hybrids for characteristics that contribute to the seedless phenotype were as developed and used in previous projects. In brief, treatments were:

- One or two flowering limbs were encased in a mesh (2mm) bag to exclude pollinating insects.
- Flowers were emasculated and left un-pollinated.
- Flowers were emasculated and self-pollinated.
- Flowers were left to open-pollinate.

Floral characteristics were also recorded, with particular attention paid to pollen fertility.

3.3.1.1 Results for progeny groups 2, 3, 4, and 5

Table 3.7 provides the final results with regard to segregation for autonomic parthenocarpy in the families included in these progeny groups.

With the exception of family 88-21 (Clementine x Murcott), autonomic parthenocarpy was observed in all families in these progeny groups. Data collected at Merbein and reported elsewhere have demonstrated that Murcott tangor will only set fruit if pollinated and seeds develop. Clementine mandarin is a stimulative parthenocarpic variety in that fruit set is triggered by pollination and seedless fruits form if the pollen is non-functional as in self pollen which is incompatible. The absence of parthenocarpic fruit development in this family suggests that the hybrids had not inherited an ability for this characteristic from either parent.

Table 3.6. Summarised timetable for activities leading to variety release for the strategic hybridisation and mutation components (CSIRO Merbein) of the National Citrus Scion Breeding Program (HRDC projects CT111, CT319, CT96014 and CT00012). The hybridisation program is seen as an ongoing exercise whereby new selections can be fed back into the program as new parents to address industry and market requirements for new varieties.

ACTIVITY	FEEDBACKS	PROGENY GROUP (Hybrids or mutants)*												
		#1 hybrids	#2 hybrids	#3 hybrids	#4 hybrids	#5 hybrids	#6 hybrids	#7 hybrids	#8 mutants	#9 hybrids	#10 hybrids	#11 hybrids	#12 hybrids	#13 nucellar mutants
BREEDING • Hybridisation • Mutation	⇦ ⇦ ⇖ ⇧	1984 *84-01*	1987 *87-03 → -05*	1988 *88-01 → -11*	1988 *88-12 → -19*	1988 *88-21 → -23*	1991 *91-01 → 92-01*	1993 *93-01 → -05*	1994 *mutat-ions*	1994 *94-01*	1996 → 98 *96-01 → 98-133*	1999 → 2002 *99-01 →*	2003 → 2006 *03-01 →*	2003 → 2005
FIRST PHASE SCREENING AND EVALUATION (single trees)	⇧ ⇧ ⇧	1993-‘97	1998-2002	1995-2001	1997-2001	1997-2001	2000-2004	2001-2005	1996-2001	1999-2006	2004 onwards	2008 onwards	2008 onwards	2008 onwards
SELECTIONS	⇗ ⇨ ⇨ ⇧	1996-‘97	Anticip-ated 2000-02	1999 onwards (parents 1994+)	Anticip-ated 2000-02	Anticip-ated 2000-02	Anticip-ated 2005	Anticip-ated 2006	Anticip-ated 2001-2	Anticip-ated 2006	Anticip-ated 2008	Anticip-ated 2008	Anticip-ated 2008	Anticip-ated 2008
SECOND PHASE EVALUATION (replicated trees) • Grower trials • Comparative trial	⇧ ⇧ ⇧	Trials planted 1999-2000	Entry to trials anticipat ed in 2002-3	Some propag-ated in 1999 for entry to trial in 2000	Entry to trials anticipat ed in 2002-3	Entry to trials anticipat ed in 2002-3	Entry to trials anticipat ed in 2006-7	Entry to trials anticipat ed in 2007-8	Entry to trials anticipat ed in 2002	Entry to trials anticipat ed in 2007	Entry to trials anticipat ed in 2008	Entry to trials anticipat ed in 2008	Entry to trials anticipat ed in 2008	Entry to trials anticipat ed in 2008
ADVANCED SELECTIONS	⇧ ⇗	Anticip-ated from 2005	Anticip-ated after 2008	Anticip-ated after 2007	Anticip-ated after 2008	Anticip-ated after 2008	Anticip-ated after 2011	Anticip-ated after 2012	Anticip-ated after 2006	Anticip-ated after 2008	Anticip-ated after 2012	Anticip-ated after 2012	Anticip-ated after 2012	Anticip-ated after 2012
VARIETY RELEASE	⇨ ⇨	Anticip-ated from 2005	Anticip-ated after 2008	Anticip-ated after 2007	Anticip-ated after 2008	Anticip-ated after 2008	Anticip-ated after 2011	Anticip-ated after 2012	Anticip-ated after 2006	Anticip-ated after 2008	Anticip-ated after 2012	Anticip-ated after 2012	Anticip-ated after 2012	Anticip-ated after 2012

* For brief explanations see following sheet.

Table 3.6 contd.

Briefly the progeny groups referred to in table 3.6 are as follows:

1. The original cross (Imperial mandarin x Ellendale tangor) made in 1984 that started the hybridisation program at Merbein.

2. Crosses made involving Sunset mandarin as well as the old clone of Clementine mandarin to introduce self-incompatibility and fruit quality genes into the program.

3. Crosses made with Silverhill satsuma mandarin aimed at new tangors and tangelos. These crosses were made to introduce seedless genes from satsuma into the breeding program. (CT111)

4. Crosses made with Imperial mandarin aimed at parthenocarpic tangors and tangelos.

5. Crosses made with the old clone of Clementine aimed at self-incompatible, parthenocarpic tangors.

6. Crosses made with new introductions of Clementine to yield self-incompatible, parthenocarpic mandarins and tangors.

7. Some repeat crosses involving Imperial, Clementine old clone and pummelos made for experimental purposes to investigate the inheritance of parthenocarpy and self-incompatibility.

8. Mutations generated to produce pollen sterile variants of Imperial, Kara and Ellendale, all of which have been shown to be parthenocarpic.

9. One cross involving two Satsuma hybrids that had short juvenile periods. The hybrid parents were a tangor and a tangelo and the aim was to maintain a short juvenile period and combine their seedless genes in new hybrids for use as parents.

10. Large crossing program conducted in CT96014 using parents with seedless genes generated from the program. Crosses aimed at easy-peel, seedless mandarin, tangor and tangelo types that mature over a wide range of seasons. Crossing program was designed to yield information on the inheritance of key fruit quality characteristics by capitalising on full- and half-sib family relationships. In addition crosses aimed at generating new triploids were made using pollen of tetraploid selections from QDPI with monoembryonic diploid selections containing genes for seedlessness, in particular parthenocarpic ability.

11. Crosses using large-fruited, monoembryonic tangor hybrids from the program that possess seedless genes aimed at generating new dual purpose (fresh fruit and juicing types) sweet orange varieties and late maturing mandarins. These crosses were started in response to individual industry requests and the outcome of a series of grower fora held in 1999 (Started in CT96014 and to be continued in CT0012).

12. New crosses commenced in 2003 aimed specifically at early maturing seedless easy-peels. This series of crosses was stimulated by results obtained for a relatively low number of exploratory hybrids produced in the early 90s between early mandarin varieties. Transgressive segregation for early maturity suggests that very early maturing varieties can be bred. One hybrid bred at Merbein matures in late March, which is far earlier than either of its parents, with a juice Brix of 12 and a ratio of 12:1 and full colour development. The main problem facing this hybrid is size and this is where further breeding is warranted. This and other hybrids from these earlier crosses are seedless.

    The breeding plan supports the development of earlier varieties as there are market opportunities and gaps in supply. Growers have supported the development of good early seedless easy-peel varieties. A comment received from Dr. Alan Legge, formerly of Mack Multiples, which is a large UK importer of fresh fruits, during a visit to Merbein in April 2003 was that with nothing comparable being available at the time, a good seedless mandarin maturing in March would warrant air freighting to the UK for marketing as a premium fruit.

    This crossing program commenced in October 2003 and is planned to continue until 2006 to obtain family sizes to warrant rigorous selection and genetic analysis. Parents used are Clementine and Imperial mandarins as well as hybrids with desired characteristics bred at Merbein.

13. A nucellar seedling selection program has been initiated in response to concerns from primarily the MIA for better early maturing navel oranges. Selection of nucellar seedlings, which can be variable due to localised mutation, has been successful for the improvement of satsuma mandarins. Initially Fischer navel has been the target of this research as it was shown in 2002-03 that it produces more seeds than other varieties when deliberately cross-pollinated with *Poncirus trifoliata* pollen. Fischer, a selection from California, is also an early maturing navel. In 2003 Fischer has been cross-pollinated with early maturing mandarin varieties and also with *Poncirus trifoliata* pollen. Resulting seeds are germinated in-vitro to maximise embryo recovery. Hybrids will be identified and rogued; those hybrids with mandarin parents will be retained while those with trifoliata orange parents will be discarded. Nucellar seedlings will be rowed out at high density and fruit evaluated.

All other families segregated for autonomic parthenocarpy. The family sizes for the Satsuma hybrids were not large and the ratios observed cannot be used to form a hypothesis concerning the genetic control of this characteristic. The segregation ratios in the larger families, however, could all be fitted to a model suggesting that autonomic parthenocarpic fruit development is under the control of more than one gene. The data presented here reinforces the discussion presented in the final report for CT96014 and supports the data presented by Vardi *et al* (2000), who proposed that three complimentary dominant genes are responsible for the expression of parthenocarpy in *Citrus*.

In breeding for seedlessness, the program has aimed to combine parthenocarpic fruit development with pollen sterility or self-incompatibility. As also reported in the final report for CT96014, pollen sterility occurred sporadically amongst hybrid families with no clear segregation patterns that would indicate simple gene action. As discussed in previous reports, these results support other evidence (Ueno, 1986 and Yamamoto *et al*., 1992a and b) demonstrating that crosses made between pollen viable parents may yield a small proportion of pollen sterile offspring. This was not the case, however, for families from the satsuma crosses made in 1988 (Table 3.8). Segregation for pollen sterility in these families indicated the action of a single gene, although family size was small.

Table 3.7. Segregation for autonomic parthenocarpic fruit development for a range of families in progeny groups 2, 3, 4, and 5.

Cross	Parents	Parthenocarpic: Not parthenocarpic.	Suggested ratio	Sig χ^2
87-03	Clementine x Sunset	2 : 19	1 : 7	P = 0.2 – 0.5
88-02	Silverhill x Joppa	7 : 1		
88-03	Silverhill x Red Siletta	2 : 0		
88-04	Silverhill x White Siletta	0 : 1		
88-05	Silverhill x Hamlin	2 : 2		
88-07	Silverhill x Pummelo CS26	3 : 6		
88-08	Silverhill x Pummelo CS27	4 : 0		
88-09	Silverhill x Pummelo CS28	5 : 0		
88-11	Silverhill x ?	1 : 1		
88-12	Imperial x Red Siletta	6 : 5	1 : 1	P = 0.5 – 0.8
88-13	Imperial x Hamlin	4 : 11	1 : 3	P = 0.9 – 0.95
88-14	Imperial x Joppa	3 : 13	1 : 3	P = 0.5 – 0.7
88-15	Imperial x Pummelo CS26	5 : 7	1 : 1	P = 0.5 – 0.7
88-16	Imperial x Pummelo CS27	3 : 6	1 : 2	
88-17	Imperial x Pummelo CS28	11 : 4	3 : 1	P = 0.8 – 0.9
88-18	Imperial x White Siletta	5 : 10	1 : 2	
88-19	Imperial x Med. Sweet	8 : 7	1 : 1	P = 0.7 – 0.8
88-21	Clementine x Murcott	0 : 19	0 : 1	
88-22	Clementine x Valencia	4 : 42	1 : 7	P = 0.3 – 0.5
88-23	Clementine x Joppa	5 : 18	1 : 3	P = 0.7 – 0.8

Table 3.8. Segregation for pollen sterility/fertility in Silverhill satsuma crosses.

Family	Pollen sterile	Pollen fertile
Satsuma x Red Siletta	1	1
Satsuma x Hamlin	2	2
Satsuma x Joppa	4	4
Satsuma x White Siletta	0	1
Satsuma x Pummelo CS26	5	4
Satsuma x Pummelo CS27	3	1
Satsuma x Pummelo CS28	4	1

3.3.1.2 Results for progeny groups 6, 7, and 9

The families in these groups are still being screened for traits associated with the seedless phenotype and the data are still incomplete and thus not presented here. However, the results for one family are, with the exception of two hybrids, complete and are presented herein. Family 92-01 (Clementine Fina x Miho satsuma) segregated 1:1 for autonomic parthenocarpic fruit development (actual data being 28 parthenocarpic to 22 non-parthenocarpic hybrids, $\chi^2 = 0.72$ NS), suggesting that a single gene effect was responsible for the expression of this trait. If the expression of parthenocarpy is under the control of three complimentary dominant genes, then this result when compared to the result when Clementine was crossed with Murcott (family 88-21, see table 3.7), suggests that Clementine is homozygous recessive for one of the genes and that Satsuma is heterozygous for this gene and most likely homozygous dominant for the other two complimentary genes. Thus, in the cross between Clementine x Satsuma, and with the three complimentary genes represented by P_1,P_2 and P_3, the 1:1 segregation ratio would be caused by -- -- p_3p_3 x $P_1P_1P_2P_2P_3p_3$.

Pollen fertility also segregated within this family with a ratio of 1:1 for male fertile to sterile hybrids (actual data being 29 sterile to 21 male fertile hybrids; $\chi^2 = 1.28$ NS) suggesting a single gene for pollen sterility from Satsuma. As Miho satsuma was the male parent in this cross, these data indicate that male sterility in satsuma is nuclear and not cytoplasmic.

3.3.1.3 Conclusions

Further evidence, supporting existing data, was obtained from new hybrids demonstrating the value of Imperial mandarin as a parent to transmit autonomic parthenocarpy. Data collection for satsuma hybrids was also completed and satsuma was shown to be a good parent for transmitting seedless traits. In CT00012, a larger satsuma family was available for which Miho wase satsuma had been used as the male parent. New data from this family have supported earlier results for this genotype in breeding for seedlessness.

During August 2001, container-grown trees of Imperial mandarin and Miho wase satsuma flowered in the glasshouse at Merbein. Two Miho trees produced flowers with viable pollen similar to when it was used to pollinate Fina clementine to give family 92-01. As outlined here and in other reports, Imperial and Satsuma have proved valuable parents for transmitting autonomic parthenocarpy and other traits that contribute to the seedless phenotype. Normally any cross involving a satsuma parent is difficult to make due to Satsuma's pollen sterility, ovule/embryo abortion and high degree of polyembryony. Imperial is monoembryonic and thus a more suitable female parent than a low-seeded polyembryonic parent for generating larger progenies. Thus, when satsuma pollen was available, it was fortuitous that an Imperial tree was also flowering and the cross could be made (Table 3.9). Based on data collected to date indicating that the expression of autonomic parthenocarpy is controlled by the action of three dominant complimentary genes, 75% of the progeny between Imperial and Miho should be parthenocarpic.

There were three open-pollinated fruits on the Miho trees and none on the Imperial trees. The Miho fruits were seedless, as were 40 open-pollinated fruits sampled from a Miho tree in the arboretum at Merbein. The seeds were germinated and hybrid trees propagated, which will be described later in this chapter.

Table 3.9. Details of crosses made between Miho wase satsuma and Imperial mandarin under glasshouse conditions during August 2001. Fruits were harvested on March 25, 2002						
Female	Male	Date	No. flowers pollinated	No. fruit	No. seeds	Mean seeds per fruit
Imperial	Miho wase	8/8/01	4	1	11	11
Imperial	Miho wase	9/8/01	4	2	24	12.0 ± 1.4
Imperial	Miho wase	13/8/01	11	9	145	16.1 ± 6.2
Imperial	Miho wase	15/3/01	3	2	21	10.5 ± 3.5

During CT00012, further information on the inheritance of seedless characteristics in the breeding populations was obtained and can now be used to predict better the outcomes of new crosses aimed at incorporating the seedless phenotype with other desirable fruit quality traits.

3.3.2 First phase evaluation of hybrids for fruit quality and other key characteristics

During the project, first phase evaluation was completed for progeny groups 2, 3, 4 and 5 and as a result selections were made for entry into second phase evaluation. Hybrids in other progeny groups either flowered for the first time and were subjected to monthly sequential harvests or had flowered before and were thus harvested annually according to predicted fruit maturity based on previous seasons' data. Again large data sets have been generated, but are not presented here. For the sake of brevity only data for hybrids selected for entry to the next stage of evaluation are presented.

During the course of CT00012 a decision was made to fast-track promising material earlier during the first phase evaluation process. It was agreed that any hybrid exhibiting outstanding qualities during its first year of fruiting would be propagated to seedling rootstocks and topworked to establish orchard trees before the usual full set of data over four seasons had been collected. Once propagated, evaluation in the first phase would continue so that supporting data could be collected while second phase trees are establishing and growing on CSIRO land such that evaluation in the second phase would start earlier and so reduce the time taken to arrive at a possible release. It was agreed that if subsequent data from first phase evaluation do not support the selection of a candidate, however, it could be removed from the second phase evaluation process.

During CT00012, 4 hybrids were selected from progeny group 6 for fast-tracking to phase 2 evaluation. The following described the features of hybrids selected during CT00012 for entry to second phase evaluation.

3.3.2.1 Hybrids selected on the basis of at least four seasons' fruit quality data.

Thirteen hybrids from groups 2, 4 and 5 were selected for further evaluation as replicated trees (Table 3.10). Six of these hybrids possess the potential for seedless fruits and a further two have yielded fruits with low seed numbers. The others, as the data collected so far suggests, will be seedy, although open-pollinated seed numbers may be less than given in Table 3.10 when they are grown in an environment with reduced pressure from cross-pollination. The pressure for cross-pollination is perhaps at its greatest under the conditions of the breeding field where there are many pollen fertile hybrids growing at high density. Fruit quality (Table 3.10) of the non-parthenocarpic hybrids weighed in their favour for inclusion in the next stage of evaluation. All selections yield fruit with high juice sugar concentrations, a characteristic that is becoming increasingly important in

export markets, especially Asian, and gives the fruit a rich taste. Maturity season for the selected hybrids was wide ranging from April through to August/September and similar in range for the current group of Imperial x Ellendale hybrids being evaluated in regional test plots.

Table 3.10. Hybrids selected from progeny groups 2, 4 and 5 for entry into phase two evaluation. Details concerning parentage, if fruit will develop parthenocarpically and fruit maturity are presented.

Progeny group	Hybrid	Parents	Parthenocarpic	Fruit maturity season
2	87-03-01	Clementine mandarin x Sunset	Yes	June
	87-03-05	Clementine mandarin x Sunset		May/June
	87-03-06	Clementine mandarin x Sunset		June
	87-03-12	Clementine mandarin x Sunset	Yes	April
	87-03-14	Clementine mandarin x Sunset		June
	87-03-26	Clementine mandarin x Sunset		May
3	88-13-23	Imperial mandarin x Hamlin	Yes	June/July
	88-14-03	Imperial mandarin x Joppa	Low seeded	Aug/Sept
	88-14-18	Imperial mandarin x Joppa	Low seeded	August
	88-18-02	Imperial mandarin x White Siletta	Yes	July/August
	88-18-18	Imperial mandarin x White Siletta	Yes	July/August
5	88-21-18	Clementine (old clone) x Murcott	Yes	August
	88-22-55	Clementine (old clone) x Valencia	Low seeded	June

Table 3.11. A summary of the key characteristics for the 13 hybrids selected for entry into second phase evaluation trials. The data were collected from individual hybrids on their own roots growing at high density over a four-year period. The metrical data are presented as ranges over three years of full tree harvests and were collected from the three most common grade sizes harvested each year.

Hybrid	Rind Colour	Shape	Easy peel	Rind texture	Diameter (range mm)	Fruit weight (range g)	Rind thick. (range mm)
87-03-01	10.3-11.6	Oblate	Yes	Pebbled	44-53	49-73	3.0-3.2
87-03-05	10.5-11.0	Oblate	Yes	Smooth/ Pebbled	54-63	66-93	3.0
87-03-06	8.0-10.7	Oblate	Yes	Smooth/ Pebbled	45-52	54-75	2.2-3.2
87-03-12	7.6-7.8	Oblate	Yes	Pebbled	58-67	98-128	3.0
87-03-14	7.8-8.5	Oblate	No	Pebbled	53-61	79-111	2.7-2.8
87-03-26	10.2-10.5	Oblate	Yes	Pebbled	57-65	80-114	3.0-3.3
88-13-23	8.0	Oblate	Yes	Pebbled	57-66	79-112	3.2-3.3
88-14-03	9.0-9.8	Oblate	No	Smooth/ grainy	44-50	50-69	3.0
88-14-18	9.0-10.0	Oblate	No	Smooth /pebbled	59-68	109-138	3.2-3.5
88-18-02	8.7-9.2	Pyriform	Yes	Smooth/ Pebbled	57-69	84-123	4.3-5.7
88-18-18	8.7 – 9.0	Oblate	Yes	Smooth /pebbled	58-66	77-109	3.7-4.0
88-21-18	9.1	Oblate	Yes	Pebbled	63	118	3.1
88-22-55	9.5	Oblate	Yes	Grainy	54	89	2.8

Rind colour has been scored using a rating system developed by Yamazaki and Suzuki (1980). Using this system the higher the number the redder the rind – Imperial mandarin generally receives a score of 7.0.

Table 3.11 contd.			
Hybrid	% juice (range extractable on weight basis)	Open-pollinated Seed no. (range)	Juice sugar concentrations (range °Brix)
87-03-01	35-37	0.4-0.7	15.2-16.4
87-03-05	33-39	7-21	12.4-13.8
87-03-06	36-45	11-14	12.0-15.9
87-03-12	21-33	9-11	12.8-14.0
87-03-14	31-33	10-13	13.8-14.0
87-03-26	35-38	7-18	11.9-12.8
88-13-23	12-18	0.5-1.3	11.4-12.7
88-14-03	40-42	4-5	12.1-12.7
88-14-18	33-35	7.6-10.5	11.1-11.9
88-18-02	26-34	2-13	11.9-13.9
88-18-18	28-33	11-16	13.8-14.6
88-21-18	40	12.8	14.8
88-22-55	38	13.4	16.8

The selected hybrids from progeny groups 2, 4 and 5 have been propagated to seedling rootstocks (Carrizo citrange, Cleopatra mandarin and Symons sweet orange) and established on CSIRO land. The selections have also been topworked to established trees at CSIRO.

3.3.2.2 Hybrids selected from progeny group 6 for fast-tracking to stage two evaluation

Data for two Fina Clementine x Silverhill satsuma hybrids (92-01-05 and –23) indicated that they are autonomic and stimulative parthenocarps in that seedless fruits developed when flowers were either self- or unpollinated. Their fruit quality after only two seasons (only one of which involved a full tree harvest) suggested that they should be fast-tracked to second stage testing. Another hybrid from the same cross (92-01-31) was also shown to be parthenocarpic and pollen sterile. This hybrid stood out in that rind maturity varied according to the seed content of the fruits. Rind colour break occurred sooner in fruits that developed without seeds, which could be distinguished on the tree. This characteristic has a potential benefit for harvesting seedless fruits and so it was decided to include it in second phase evaluation for further investigation. Fruit quality of 92-01-31 fruits also justified its propagation for the next phase of evaluation.

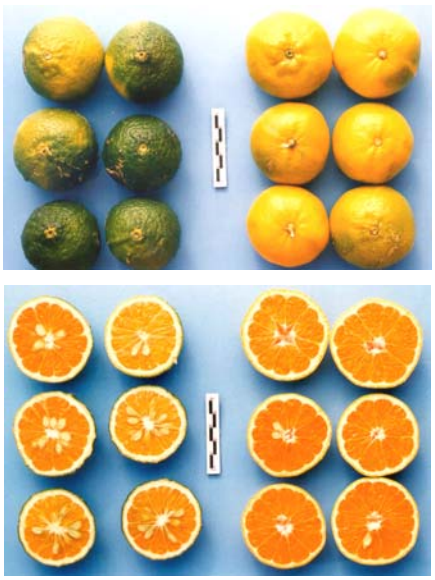
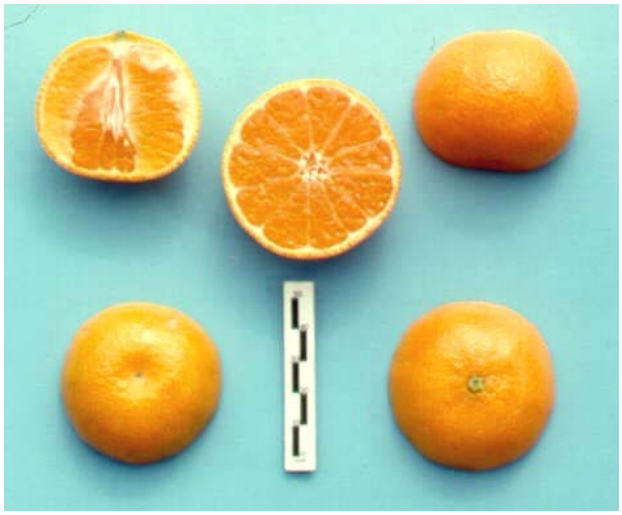


Figure 3.1. Variation in colour break of fruits from hybrids 92-01-31 in relation to seediness. The upper image is of the fruits before they were cut as shown in the lower image. The presence of seeds not only delayed colour break but were also associated with a coarser and thicker rind. The variation in rind colour break could be used as an indicator in the orchard for seedless or low seeded fruits.

The other hybrid that has been propagated for fast-tracking is a one between Marisol clementine x Imperial mandarin (Fig. 3.2) and has been discussed earlier in relation to the crossing program aimed at early maturing fruits. This hybrid was observed in the breeding field as having fruits that started to break colour towards the end of March, which is very early at Merbein. Fruits harvested at the end of March had excellent juice quality characteristics with a good flavour. The hybrid is also parthenocarpic and self pollen was non-functional. Its potential to be seedless and its very early maturity led to its selection for fast-tracking.



Date:	**26/3/03**	**3/4/03**	**10/4/03**
Weight	51.46 ± 6.57g	61.4 ± 10.7	52.5 ± 10.1
Brix	10.78 ± 0.29	11.47 ± 0.45	11.55 ± 0.58
Acid	0.94 ± 0.08	0.87 ± 0.09	0.84 ± 0.03
Brix:Acid	11.6 ± 0.9	13.3 ± 1.2	13.78 ± 0.63

Seed number 0-9 (open-pollinated); seedless if pollination is prevented. Self pollen poor.

Fig. 3.2. Fruits and fruit quality data for hybrid 91-03-04, which has been fast-tracked into stage 2 evaluation based on its earliness and potential for being seedless. This hybrid has generated interest from a major importer of citrus into the United Kingdom because of its early maturity, which means it could fill a key market opportunity due to the lack of good quality seedless easy-peels available at

this time of the year.

3.3.3 Progenies produced during CT96014

Hybrids generated during CT96014 were planted in the breeding field during spring 2000. The hybrids were from a range of crosses made between CSIRO-bred hybrids that were shown to possess the range of characteristics that contribute to the seedless phenotype. The design of the planting was based on a series of randomised blocks with family groups randomised within blocks. Some hybrids within these families commenced flowering during 2003 and fruits have been harvested and assessed during winter 2004. These progenies will feature in the first phase evaluation plans during the next 3-4 years at Merbein.

3.3.4 Transgenic West Indian lime at Merbein

Transgenic citrus trees were transported from the Adelaide laboratory during July 2000 and again in February 2001, and have been held within a secure facility at Merbein to prevent pollen flow either to or from them. Trees have been maintained according to guidelines agreed to by the Merbein laboratory Institutional Biosafety Committee and ratified by first the Genetic Manipulation Advisory Committee (GMAC) and subsequently the Office of the Gene Technology Regulator (OGTR).

Although the trees have been maintained in an insect proof facility, which has been proven experimentally to prevent pollen flow from the trees, when these trees have flowered, flower buds have been enclosed in glassine bags before they have opened. These bags serve two purposes. First, they further reduce the chances of pollen escape from the trees and, second, they retain all flowers that abscise from the plant facilitating their collection and appropriate disposal.

Experiments conducted to prove that the facility prevented pollen flow in either direction are not reported here except to say they involved parthenocarpic pollen sterile hybrids selected from the breeding program and that they investigated the chances for insect-mediated pollen transfer and for wind blown pollen transfer.

Flowering of the transgenic citrus housed at Merbein was monitored during the course of CT00012 and different pollination treatments were applied as deemed appropriate. The results from these experiments were communicated to Dr. Koltunow in Adelaide who collated the information along with data generated in Adelaide. Pollen sterile variants were observed amongst the trees at Merbein.

The trees at Merbein have been maintained by codes without knowledge concerning which specific gene or genes have been inserted. In this way, experiments were conducted and observations made without bias. Thus, results have been communicated directly to the Adelaide team for interpretation.

Observations at flowering of transformed West Indian Lime trees held in the PC2 insect-proof facility at Merbein confirmed that the pollen sterile transformants are indeed pollen sterile. Thus, on the basis of there being no need to be concerned about pollen flow from transformed citrus into commercial orchards, these trees could be grown outside of an insect-proof facility. The pollen sterile transformants, however, have not been shown to be parthenocarpic and require pollination to set fruits, which are then seedy. Seedy fruits do represent a risk for transgene transfer into commercial orchards, which would need to be considered should a recommendation be made for the release of these trees for trial under restricted but otherwise normal orchard conditions.

The results from transgenic trees at both Merbein and Adelaide have provided proof of concept for the approach used to yield pollen sterile citrus. However, and as the funds for the transformation component of the breeding program have been withdrawn by HAL, further work with these West Indian lime transformants has been suspended with no recommendation at this stage that they be released for restricted field-based evaluation.

A strictly monitored audit trail has been documented for these transgenic trees following guidelines developed by the Merbein site Institutional Biosafety Committee, which were ratified by the OGTR.

3.3.5 Imperial mandarin x Miho wase satsuma

This progeny was described earlier in section 3.2.1.3. The usual practice with new progenies at Merbein has been to row out the hybrid seedlings in the breeding field on CSIRO property at high density on their own roots along with nucellar seedlings of their parents or, if the parents are monoembryonic, grafted trees of the parents. This allows comparison with parent genotypes. The hybrids from this cross between Imperial and Miho, however, are being treated differently.

A recommendation made by Luis Navarro after his review of the breeding program in February 2003 was that the approach used in Spain of budding new hybrids to citrange rootstock be investigated as an attempt to shorten the juvenile period of young seedlings. As a result it was decided to use this progeny from Imperial x Miho to see if the juvenile period of the hybrids could be shortened. Each hybrid seedling has been budded to each of three rootstocks under glasshouse conditions. The rootstocks are Carrizo citrange, Symons sweet orange and Cleopatra mandarin and these will be planted out along with the own-rooted hybrids in the breeding field at CSIRO during autumn 2005.

3.3.6 Inheritance data

At the end of CT00012 it was decided that the evaluation of progeny groups 2, 3, 4 and 5 would be viewed as completed even though a small number of hybrids have never flowered or fruited. The data sets for the hybrids are currently being updated and will be used to investigate the inheritance of key characteristics that have been recorded during their evaluation as own-rooted hybrids grown at high density in the breeding fields. While the data will be analysed fully over the next 12 months, early analyses have given some information on the inheritance of some selected traits.

Significant differences ($P<0.001$) between full-sib families have been shown for seedling juvenility, measured as years until first flowering for each hybrid within a family. From data analyses, intra-class correlation coefficients have ranged from 0.20 to 0.28. The intra-class correlation coefficient for full-sib families gives an estimate of broad sense heritability (Falconer, 1960) such that $t>½h^2$. This indicates that seedling juvenility is heritable and it should be feasible to select parents that will transmit a shorter juvenile period to their progeny. For some of the crosses made in project CT96014, which used CSIRO-bred hybrids to increase the frequency of seedless offspring, a number of parthenocarpic, pollen sterile hybrids that had flowered after 4 and 5 years were used as parents. The shorter juvenile period of these parents may be transmitted to their progeny. The progenies generated between 1996 and 2000 have now commenced flowering and flowering times are being recorded.

Between and within family variation in fruit maturity based on forecast and actual harvest times have also been analysed for some seasons. Again significant differences have been shown between full-sib families and intra-class correlation coefficients ranging from 0.35 to 0.87 indicate that fruit

maturity is heritable. This suggests that early x early and late x late crosses will generate mostly early and late maturing hybrids respectively. Similarly, regressions of family on mid-parent means have been significant and in one case the slope of the line was 1.4 ± 0.2 indicating that parent selection on phenotype will give a high proportion of hybrids with the desired fruit maturity season.

Other preliminary analyses investigating the variation for juice sugar as °Brix and mean fruit weight in some seasons indicate that these traits have reasonably high heritabilities, again suggesting that parent selection based on phenotype is feasible. Further data analyses will be conducted over the next 12 months to investigate genetic parameters affecting the inheritance of key fruit quality traits.

3.4 Second phase evaluation of hybrids

3.4.1 Trees of selections made from progeny group 1 and budded to seedling rootstocks distributed to grower-co-operators for phase 2 evaluation and selection.

Selections from progeny group 1 were established during spring 2000 for second phase evaluation by grower cooperators in the main citrus growing regions of South Australia (the Riverland), Victoria (Sunraysia), Queensland (Central Burnett) and NSW (Sunraysia and the MIA). Trees were established in all regions except the MIA by the end of 2000. A grower cooperator was identified in the MIA during 2001 and the hybrids established there as both topworked trees and young trees propagated on seedling rootstocks. The trees in the MIA were planted and topworked during November 2001.

Fig 3.3 Trees of selections from progeny group 1 that had been entered into phase two evaluation one year after being established on a cooperating grower's property in NSW.
The left hand image shows a Valencia tree topworked with a hybrid selection 12 months after stump grafting showing development of the unions. The image on the right shows the test plot of selections that were planted as trees propagated on seedling stocks (the scale is 1 metre).

In addition to the trees in the major regions, a trial has also been established in cooperation with the NT DPI&F. A comparative trial of the ten selections with six comparator varieties grafted to three rootstocks was also established on the CSIRO farm. This trial, which also includes ten other selections that warranted further observation before release to grower cooperators, was planted in spring 2000.

Growth of trees on grower-cooperator properties was for the most part excellent and the first trees flowered and held fruit in 2002-03 (Fig. 3.4). Fruit yields were not sufficiently large enough in 2003 to warrant grower demonstrations of the fruit, although fruits from the original trees were available for viewing and tasting at the New Varieties Day held June 19, 2003 at Renmark (see Fig. 3.5).

Figure 3.4 The first seedless and low-seeded fruits harvested from a grower-based trial of selected hybrids during 2003.

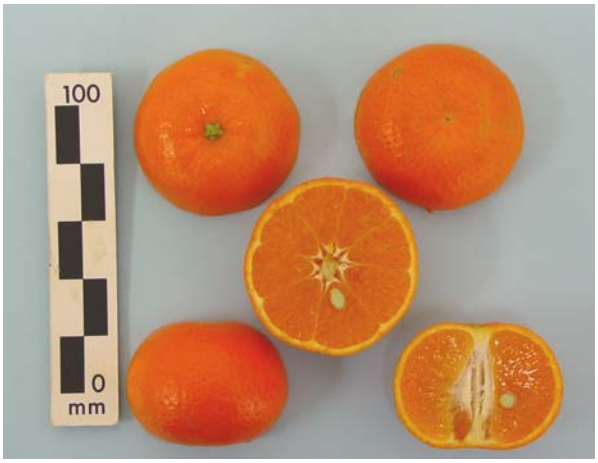
Figure 3.5 Citrus growers inspecting fruits of selections from the hybridisation program at the New Varieties Day held June 19, 2003 at Renmark, SA (photograph courtesy of Kevin Lacey).

Unfortunately, the grower who established the test plot in Queensland decided to withdraw from the regional testing network and the trees were removed, delaying any harvest of fruits from the selections in the Central Burnett region. A new planting was established with another grower during spring 2003, but because of these changes to the location of the test plots, fruits for display to Queensland growers will be delayed.

Trees in the Riverland of SA and the Sunraysia region of NW Victoria and SW NSW produced good yields on some properties in 2004. Fruits were harvested and displayed to growers in the Sunraysia region during late June through to September 2004. The data are not reported here but will be presented to the reference committee as part of the new project. The fruits of selected varieties (fig 3.6) presented to growers at a varieties field walk at DPI NSW Dareton were well received.

Hybrid 2336

Hybrid 2127



Hybrid 2350

Imperial mandarin

Fig. 3.6 Three hybrid selections being evaluated in regional test plots compared with Imperial mandarin from the same site. The fruits were harvested during late June 2004 at which stage Imperial was mature. Selection 2336 was scheduled for harvest during early July, 2350 during August and 2127 during September.

At this stage, it is anticipated that a formal notice of release will occur during late 2005 for some of the selections in the regional test plots. It is anticipated that yields on trees in test plots in the southern regions will be sufficient to conduct further grower demonstrations during 2005.

3.4.2 Propagation and establishment of trees of selections made from progeny groups 2, 4, 5 and 6.

Trees of hybrids 87-03-01, 87-03-05, 87-03-06, 87-03-12, 87-03-14, 87-03-26 (progeny group 2 hybrids), 88-13-23, 88-14-03, 88-14-18, 88-18-02, 88-18-18 (progeny group 4 hybrids), 88-21-18, 88-22-55 (progeny group 5 hybrids), 91-03-04, 92-01-05, 92-01-23 and 92-01-31 (progeny group 6 hybrids) have been propagated by budding to three rootstocks, namely Cleopatra mandarin, Symons sweet orange and Carrizo citrange. These trees were planted in spring 2003.

The same hybrids have been topworked to established trees. Trees were prepared during winter 2003 by pruning away one side to allow new shoots to grow for budding into. Four trees of each scion in each of 2 rows were topworked by inserting buds of the scion into the new shoots during spring/summer 2003. Each group of four trees was assigned randomly within rows.

Growth of the topworked trees has been good (Fig 3.7) and it is anticipated that fruits may be harvested from these trees during 2006 or 2007.

3.5 Summary

The hybridisation program is essentially a pipeline approach for the delivery of new varieties. The program is now at the stage where activities are high for all stages in the pipeline with an anticipated release notice for some selections in late 2005. As a result, a commercialisation strategy is being developed in consultation with the reference committee. In preparation for release, the source trees for the ten selections from progeny group 1 currently being evaluated in regional test-plots are being indexed by AusCitrus to assess their health status. Once their health status is known, an agreement will be entered into between AusCitrus and CSIRO for budwood multiplication. The results of the indexing should be available in early 2005.

Conventional citrus breeding through hybridisation with diploid parents is a long term proposition requiring a clear and dedicated commitment by both industry and R&D agencies. This is not peculiar to citrus but is true for most long-lived woody perennial fruit and nut tree crops. The strategic hybridisation program aimed at new scion varieties has been in progress at Merbein since 1984 and has received support from industry via HAL funding since 1991. During this period the genetic foundations of the program have been built to a stage where crosses are now being made to accommodate industry requirements for new varieties as documented in the breeding plan, which was prepared during CT00012. Hybrids have been selected from the program for entry into second phase testing by grower cooperators and it is anticipated that a new variety may be nominated for release from within these towards the end of 2005. In summary, highlights from project CT00012 have been:

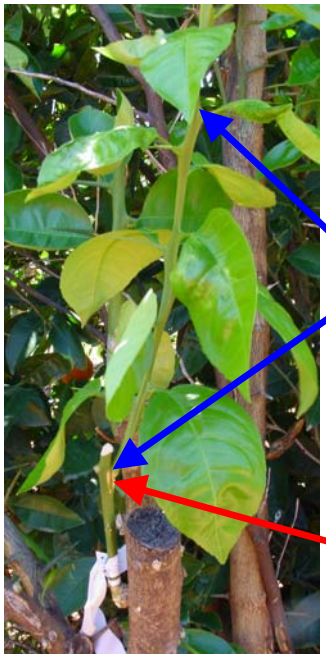
- Further evidence demonstrated the value of Imperial mandarin as a parent to transmit autonomic parthenocarpy.
- Satsuma was also shown to transmit seedless traits, but difficulties in using it as a parent mean that progeny sizes can be restrictively small. Viable pollen, however, has been obtained at Merbein and used to generate larger progenies using satsuma as a male parent crossed to

monoembryonic females. Data from one such progeny have reinforced conclusions made from other progenies concerning the inheritance of traits affecting the seedless phenotype.

Tree prepared for topworking after pruning in winter 2003

Growth of trees following pruning and budding with new scion wood

Close up of shoots, which had grown since winter pruning, after budding in November/December 2003.



Growth of shoot by February 18, 2004 from bud inserted in spring.

Fig 3.7. Trees topworked with hybrids selected from progeny groups 2, 4, 5 & 6. The figure shows the sequence of events from July 2003 through to February 2004.

- Information concerning the inheritance of seedless characteristics in the breeding populations can now be used to predict better the outputs from new crosses aimed at incorporating the seedless phenotype with other desirable fruit quality traits.
- Promising new hybrids have been identified and entered into second phase evaluation trials.
- Agreements for regional testing of promising selections were entered into with grower cooperators. Fruits have been harvested from these trials and positive reactions were received

from grower groups during fruit viewing and tasting. A commercialisation strategy for the release of new varieties is being developed in consultation with the reference committee.

- New crosses were performed to further examine the inheritance of seedless characteristics in the breeding population at Merbein and to address market windows of opportunity with product specifications as outlined in the breeding plan that was documented during the project. Crosses were designed to maximise full- and half-sib family relationships to investigate the inheritance of other key characteristics as well.

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4. Triploidy (DPI&F, Bundaberg)

4.1 Introduction

Triploid breeding has been the principle project activity conducted by the Department of Primary Industries and Fisheries, at Bundaberg Research Station, Queensland. The aim is to generate genotypes with high fruit quality that are seedless on account of their triploid chromosome number. While many triploids have now been generated and established in field plantings, only a small number of these had fruited by the end of the project period and none look suitable for commercial production. However it has been demonstrated that hybrids from the program are seedless (or near seedless) even under intense pollination pressure, and that the trees are productive. During the project period important new parents were added to the breeding program, improvements made to laboratory and field management techniques, and some long-running technical problems solved.

(**NB** Tables referred to in this chapter, but not embedded in the text are to be found in Appendix 2)

4.2 Crossing program

4.2.1 Materials and methods

Pollination and embryo rescue techniques were detailed in the previous report. The nature and number of pollinations/embryo rescues/field-plantings that occurred in each of the project years are detailed in the extensive tables that follow.

4.2.2 Results and discussion

Tables 4.1a-g show the number of pollinations performed and resulting fruit set, for each parental combination in each pollinating season 1998 to 2004. These varied from season to season because of the availability of pollen parents, the priority attached to particular parental combinations and how this changes with time, and the effects of climatic condition on the length of the flowering season and amount of fruit set. The largest number (4338) of pollinations was performed in 2000 and the lowest number (2417) in 1999. Average fruit set was reasonable constant at around 25%, but lower in 2001 (~15%) and 2003 (~12%). Clementines (eg. DeNules, Arrufatina, Corsica) tended to give the best fruit set rates (often around 40%), while seed parents like Hickson were consistently low (~5%). More than 100 parental combinations were attempted in each of 2000, 2001, and 2002, with the largest number of tetraploid parents (19) utilised in 2002.

Tables 4.2a-f show the numbers of seeds that were produced from each family in each year of the project. In the early years (1998 and 1999) all seeds (whether plump or small) were embryo-rescued. However it was realised that the plump seeds were capable of germinating without the assistance of tissue-culture techniques, so in 2000 some of the plump seeds were simply sown in the nursery. This also reduced the loss of plants that normally occurs when plantlets are transferred from tissue-culture tubes to the nursery. From 2001 onwards all plump seeds were simply peeled and sown, whereas flat seeds were submitted to embryo-rescue. The number of seeds sown and seeds rescued are shown for each family in each season. For the later years of the crossing program the numbers of seeds ‘Sown’ and ‘Rescued’ directly reflect whether the seeds were plump or flat (near-microscopic).

Tables 4.3a-e indicate the numbers of field-planted trees that have been produced in the program. For more recent years there are well-established nursery trees awaiting field-planting so these have

also been included in this set of tables (where indicated). These tables also divide the numbers of field-planted trees into those that were derived from normal seeds (sown) and those from flat seeds (embryo-rescued). The program has averaged more than 1000 field-planted hybrids per year with around 20% of these plants being derived from near-microscopic embryos, and the remainder from plump seeds that were simply sown. While the emphasis has been on easy-peel production, it can be seen that significant populations of hybrids with pummelo and sweet orange parentage have also been established in the field. The number of field-planted trees resulting from embryo rescue of small embryos has increased steadily over recent years, partly reflecting improvements in technique and efficiency. The recent discovery for the cause of poor fruit set, seed formation and culture contamination, which have affected the program for many years (particularly in 1998, 2001 and 2004), and the development of procedures to overcome these problems, should see a future increase in numbers of field-planted trees derived from small embryos.

Tables 4.4a-e show the percentage of pollinations that have resulted in field-planted trees (or well established nursery trees awaiting field planting). These tables reveal very large differences between parental combinations in terms of the number of pollinations that need to be performed in order to obtain a field-grown hybrid. In 2001 for example, each Arrufatina pollination resulted in around 1.2 field planted hybrid trees. Conversely in this same year, only around 2% of Hickson pollinations resulted in a field-grown tree (~50 pollinations to produce a single tree). In terms of tetraploid pollen sources, 4X Murcott has given low tree numbers relative to the numbers of pollinations performed (particularly in 2000 and 2001) while 4X Joppa often produced amongst the best results (~40% of pollinations giving a field-grown tree). These figures contrast sharply with rates normally achieved from diploid X diploid hybridisation where each pollination often results in more than 5 field-grown hybrids.

4.3 Incorporation of new parents to the crossing program

4.3.1 Materials and methods

New genotypes were added to the hybridisation program during the course of the experiment. The utilisation of these new parents is detailed in Tables 4.1a-g.

4.3.2 Results and discussion

In previous years, the triploid breeding efforts have been hampered by the limited range of tetraploid genotypes available for crossing. DPI&F recognised this problem some years ago and made bulk-plantings of seed from polyembryonic varieties that displayed desirable characteristics. When these seedlings emerged they were visually screened for tetraploid characteristics and any potential tetraploid seedlings potted-on and checked for ploidy level. Fourteen new autotetraploids were developed in this way, and were top-worked to hasten the commencement of flowering. Some of these top-worked autotetraploids flowered for the first time in 2001 and were immediately incorporated into that seasons crossing program. This process has continued in subsequent years with new autotetraploids used as parents as soon as they produce any flowers. Nine of the 14 new autotetraploids have now been incorporated in hybrids.

Similarly, some of the hybrids from previous crossing were known to be tetraploid (allotetraploid) and pollen from these has also been incorporated into the crossing program (commencing in 2001). They have been utilised to a lesser extent than the new autotetraploids because their phenotype is unknown, and likely to be inferior to other parents being used. However, as more of these

allotetraploids produce fruit and are assessed it should be possible to identify individuals with superior fruit qualities and make use of them as parents. Of most interest will be high quality allotetraploids that are monoembryonic since this opens the possibility of utilising them as seed parents as well as pollen parents. Pollen from *Poncirus trifoliata* (syn. *Citrus trifoliata*) has been applied to many of the allotetraploids as they start fruiting in order to determine their capacity to produce hybrid seed. Visual inspection of seeds from some of these allotetraploids proved a little unreliable in determining whether they were mono or polyembryonic, so the utilisation of *P.trifoliata* pollen will continue as new allotetraploids commence flowering.

4.4 Improving embryo rescue techniques

4.4.1 Material and methods

Modifications continue to be made to the embryo-rescue procedures used in the breeding program. Primarily these are aimed at increasing the survival rate of embryos, but also involve reducing the workload and improving the efficiency of the process. The modifications arise from new information gained from scientific literature, visitors to the research station, or from related problems that offer prospects for improvement.

4.4.2 Results and discussion

The two major improvements to embryo rescue techniques made during the course of this project were the result of information gained from Prof. Luis Navarro during his visit to the research station in February 2003, and from the independent discovery of the cause of contamination in culture tubes which has long been a problem at Bundaberg.

Navarro suggested changing from a single-stage recovery culture, to a two-stage process in which embryos are initially germinated in petri dishes before being transferred to large culture tubes. This enabled the use of two different culture media, one suited to germination and the other to growth. Under the previous system, embryos were placed in small tubes (30mL, with ~10mL media) where they remained until being transferred to the nursery. Because some embryos failed to germinate, a lot of effort and cabinet space was taken-up with unproductive tubes. Where the embryo did germinate, the resulting plant was restricted in the size that it could achieve before being transferred to the nursery. Under the new system, many embryos could be placed on a single petri dish and only those that germinated were transferred to tubes. These tubes were of similar volume (35mL) but twice the height enabling twice as much media to be used (~25mL) while still allowing plantlets to grow to be larger and stronger before being transferred to the nursery. The new system is also better suited to the facilities available at Bundaberg, because less culture cabinet space is required.

There has always been some level of contamination in the embryo rescue cultures, which caused complete failure of germination or poor growth. A range of chemicals had been used in these contaminated tubes to kill the pathogen(s) but none had been successful. This problem was discussed in some detail in the Final Report for the previous project, where it was shown that some seed-parent trees had far higher levels of contamination than others. As a result the decision was made not to include certain seed parents in the crossing program. However this did not completely solve the contamination problem, and in the 2004 season contamination levels were extremely high. In view of the persistent nature of the problem, despite the high standard of sterile technique being practiced, a pathology colleague agreed to look at the contamination with a view to finding a chemical that might be used in the media to contain the pathogen(s) growth. There was some

difficulty in identifying the pathogen, until it was recognised as a yeast genus that had never been previously found in Australia (Shivas et al. *in press*). Eventually it transpired that this yeast was transmitted into the developing fruit on the mouth parts of a group (Pentatomidae) of bugs (Hemiptera). This group of insects included the common Spined Citrus Bug (*Biprorulus bibax*). Consequently the strategy for reducing contamination in the tissue culture media is to manage the Pentatomid insects in the field to prevent contamination of the fruit in the first place. Insect numbers are being carefully monitored from flowering through to fruit picking. This should enable us to use any of the germplasm collection in the breeding program (instead of excluding certain trees because of high contamination levels). The explanation for the seed-parent trees that gave high contamination levels is that they were more attractive to the insect, or more susceptible to yeast development when stung by the insect.

4.5 Ploidy determination

4.5.1 Materials and methods

Ploidy determination remains a major obstacle in the breeding program. Despite the strong recommendation of the Navarro Review to purchase a flow cytometer for the laboratory at Bundaberg, this has not eventuated due to unavailability of funds. A morphological technique was developed based on hybrids of known ploidy levels. The leaf morphology of these hybrids was measured and then a logistics regression model used to match these characteristics to the known ploidy level. Leaves were scanned on a flat-bed scanner then processed through the image software SigmaScan. Stomata were also removed from these leaves by epidermal stripping and measured under a microscope. The resulting variables were analysed using logistics regression with ploidy level as the independent variable and the resulting model was then used to predict the ploidy level of new hybrids.

In a separate experiment, attempts were made to stain and count chromosomes in pollen mother cells. Standard cytology techniques were employed.

4.5.2 Results and discussion

Leaf morphological features have been used to segregate hybrids into putative triploids and tetraploids, and they are planted in different areas in the field based on this determination. A total of 14 morphological characteristics were assessed in order to find those that were strongly linked with ploidy level. The need for replication, position at which the sample leaf was taken, and age of plant were also examined.

The position at which the sample leaf was taken from the plant did not significantly affect the morphological characters measured. The only exception to this was stomatal numbers, which tended to be greater on higher leaves at the top of the tree. Within-leaf variability in morphological characters was not influenced by the position of the leaf on the tree. It was therefore decided to sample a leaf from near the top of the tree, and to only make one set of measurements from each leaf.

The variables that proved most useful in predicting ploidy level included: stomata size, number and percentage cover of leaf; and leaf thickness, density and diameter.

It remains to be seen how effective this morphological model is in predicting ploidy level in hybrids. This will not be known until many of these hybrids have fruited, and even then there may be some uncertainty as the presence of seeds may not simply be because the plant is tetraploid (it could also be a diploid that slipped into the program). Of the few hybrids that have fruited so far, indications are that the use of morphological characters to predict ploidy has not been particularly successful. The use of the technique was suspended following the Navarro visit when there were strong indications that a flow cytometer would be supplied to the program.

During the project period attempts were also made to stain pollen mother cells and count chromosomes. This is a well established technique sometimes used in other crops. The technique met with moderate success but was hampered by the usual problems of chromosome counting in *Citrus*, principally the small size of the chromosomes. It was difficult to determine with any certainty whether the cells that were observed were triploid or tetraploid. The technique was as slow and cumbersome as the conventional root-tip squashes used previously, and seems to offer no advantage to the citrus breeding program.

At the end of this current project period, it seems clear that there is no substitute for flow cytometry in terms of rapidly screening large numbers of hybrids. Currently, all hybrids are being field planted without any knowledge of their ploidy level, and this situation seems likely to continue for the foreseeable future.

4.6 A trellis system for hybrids

4.6.1 Materials and methods

The current system used in the ploidy manipulations program involves investment of significant resources in individual hybrids early in their life (via embryo rescue). Consequently each plant that is produced in this way needs to be well established before it is transferred to the field, in order to reduce field-losses. Hybrids, that survive embryo rescue and transfer from tissue-culture tubes to the nursery, are grown as a supported single stem. Side-shoots are removed at regular intervals to promote top-growth. Once these nursery plants have reached about 1.2m they are transferred to the field.

In the field, trees are planted onto a high (~0.5m) mounded bed. A 2m high single vertical trellis is installed above the bed, and plants are attached to the wires of this trellis at the time of planting. After field-planting, hybrids are kept free of side-shoots below 0.5m but allowed to grow without any other pruning. Hybrids are planted at spacing of either 0.375 or 0.5m (depending on putative ploidy level) in a single row, giving a plant density of 6675 or 5000 hybrids per hectare.

4.6.2 Results and discussion

The trellis system now in use for the triploid breeding program evolved almost by accident. It was the result of a delay in field planting which caused excessively tall nursery plants that would have required cutting-back at planting in order to prevent them snapping under the strong winds regularly experienced at Bundaberg. Attaching these tall plants to a single upright trellis meant that all the nursery growth could be retained and that the plants could be established at high density.
The use of a high raised bed was designed to make planting easier (using large numbers of plants from large nursery bags) and to reduce any drainage problems in a low-lying area of the research

station originally used for planting. However the growth rate on these raised beds has been so impressive that it is now standard practice.

Plate 4.6
Trellis grown hybrids 2 years after planting.

The system has created considerable interest from overseas breeders interested in developing an efficient method for rapid growth of field-grown hybrids.

4.7 Reducing sucker growth on field-planted hybrids

4.7.1 Materials and methods

Hybrids can sucker profusely when field-planted. This creates problems with maintenance, including the capacity to apply knock-down herbicides to the beds containing the progeny rows. It also aggravated the overcrowding problem brought about by the high density planting system being utilised. Manual removal of these side-shoots is labour intensive and expensive. Alternative methods of preventing side-shoot development on the lower 0.5m section of the tree trunk were investigated.

Prior to 1998, NAA had been used extensively in the conventional breeding program at Bundaberg to reduce side-shoot development in progeny blocks. This procedure was stopped because better residual herbicides reduced the need to apply knock-down herbicides, the small citrus trees quickly branched and filled the high density area, and there was some thought that the NAA was having a deleterious affect on plant growth. The situation in the triploid program was somewhat different because hybrids were planted out when they were larger, and it was desirable not to have new suckers emerging from low on these trees.

Two experiments were conducted to test the efficacy and phytotoxicity of NAA used to reduce sucker production.

Expt 1: Three treatments (11,500ppm NAA made up in white vinyl paint; 11,500ppm NAA made up in water plus Shirwet (13ppm); water plus Shirwet (13ppm)). Treatments were applied to nursery-growing hybrids (range of parental combinations) in individual 5L pots. Each of the 3 treatments was replicated 10 times. Measurements of tree height, diameter and side-shoots were

made at monthly intervals for 5 months after treatment application. Trees averaged 1.1m high at the time of treatment application.

Expt 2: This experiment was based on initial results from Expt 1, and was conducted using hybrids that had recently been field planted. It consisted of 6 treatments:
Treatment 1: 11,500ppm NAA made up in white vinyl paint
Treatment 2: 23,000ppm NAA made up in white vinyl paint
Treatment 3: 11,500ppm NAA + 13ppm Shirwet made up in water
Treatment 4: 23,000ppm NAA + 13ppm Shirwet made up in water
Treatment 5: 13ppm Shirwet made up in water
Treatment 6: 13ppm Shirwet made up in white vinyl paint
These treatments were applied to hybrids that had recently been field planted. The 6 treatments were applied to 10 trees each of 2 different parental combinations (4Xpummelo x Murcott; Wilking x 4XEmperor). Tree height, diameter and sucker production was assessed 1 month, 6 months and 12 months after treatment application. After 12 months, suckers were removed and their height and biomass determined.

4.7.2 Results and discussion

Expt 1: There were no significant differences between treatments in terms of tree height and trunk circumference at any of the 4 measurement dates. However when results were expressed as changes in height and circumference significant differences emerged (Table 4.7a) Trees treated with NAA in paint (and to a lesser extent with NAA in water) had a lower growth rate (in terms of increasing tree height) than trees that had been treated with water. Conversely trees treated with NAA in paint had a greater rate of trunk increase than the other 2 treatments. Trees treated with NAA in water produced more shoots than either NAA in paint or water alone.

Table 4.7a Effect of NAA treatment on the height and diameter increase, and side-shoot production, of citrus seedlings, Nursery, BRS.

Treatment	Ht. Increase (mm)	Diam. Increase (mm)	Side-shoot production
(1) NAA in paint	59	1.72	0
(2) NAA in water	114	0.62	6
(3) Water alone	245	0.65	2.3
LSD (0.05)	132	0.98	2.9

These results suggested that the reduction in shoot production was more a function of the carrier used for the NAA rather than the NAA itself. It also suggested the possibility of some growth reduction from the use of NAA, as well as a stimulatory effect of paint on trunk growth. These issues were investigated further in the second experiment.

Expt 2: As previously, there was no difference between treatments in tree height or diameter at any measurement date. Similarly there was no difference between treatments in the change in tree height between measurement dates. However there were significant treatment effects on the rate of increase in trunk diameter. Paint alone (6), produced the greatest growth rate (in terms of trunk expansion), while those treatments with high rates of NAA (2 & 4) had the slowest trunk expansion rates (Table 4.7b).

Table 4.7b Effect of NAA on the growth of citrus seedlings at 1 month and 12 months after treatment application, New Trellis block, BRS.					
Treatment	Diam. Increase (mm)	1st month shoot production	Final shoot production	Av. Shoot length/plant (mm)	Av. Shoot biomass/plant (g)
1) NAA in paint	17.7	0	0.15	22.6	16
2) 2X NAA in paint	12.6	0	0.29	21.6	26
3) NAA in water	19.2	0.05	2.30	65.6	138
4) 2X NAA in water	14.8	0.10	1.25	71.2	115
5) Water alone	15.6	1.25	2.85	94.9	200
6) Paint alone	21.7	1.10	0.55	41.9	39
LSD (0.05)	5.6	0.85	1.03	41.4	85

Shoot production was influenced by treatments, but the effect changed over time. Within a month of treatment application, treatments containing NAA (1 to 4) produced less shoots than straight water (5) or paint (6). However this effect quickly changed, and by the end of the experiment it was the treatments containing paint (1, 2 and 6) that had the least side-shoots. The average length of side-shoots and their biomass was also least in the treatments containing paint.

The results suggest that the principle cause of the reduction in side-shoot production was the paint rather than the NAA, and that NAA had only a small and short-lived effect that was little different from water by the end of the experiment. There is also some indication that the high rate of NAA was phytotoxic, causing reduced trunk growth rates.

The two families used in the experiment performed differently, but the effect of the 6 treatments was similar. The 4Xpummelo x Murcott hybrids had higher growth rates (both in terms of height increase (136mm vs 70mm) and trunk expansion (19mm vs 15mm)) but produced far less side-shoots (0.4 vs 2.1) and of greatly reduced biomass (32g vs 146g) compared with the Wilking x 4XMurcott hybrids.

Results for the two experiments were similar. They suggest that NAA is not particularly effective (or long-term) in reducing side-shoot production on young citrus hybrids. Simply applying paint is far more effective (and there seems to be some stimulatory effect on trunk growth rates). Results also suggest the possibility of some phytotoxicity from NAA. We had started using NAA in paint as a routine treatment in the nursery at Bundaberg, but ceased this when we observed slow growth and stunting on treated trees. More work needs to be done on this if it is to resume as a nursery treatment, and care needs to be taken if treating small plants (such as those used in Expt 1.).

From a practical standpoint, NAA will not be used in the breeding program at Bundaberg but instead the trunks will simply be treated with white paint where sucker-production is a problem.

4.8 Utilisation of triploid pollen

4.8.1 Materials and methods

Pollen was collected from 2 known triploids in August/September 2001, and applied to 16 diploid cultivars to determine effects on seed production and the viability of this seed. The 2 triploids used as pollen parents were derived from open-pollinated Ellendale mandarin, and had been confirmed

by both root-tip chromosome counting and flow cytometry to be triploids. Both had consistently produced seedless (or near-seedless) fruit for the previous 4 seasons. The 16 cultivars used as seed parents were all mandarins, and had been extensively used (and confirmed as producing only hybrid seed) in previous seasons. Fifteen pollinations (except where shown) were applied to each of the 16 cultivars. These were then monitored for percentage fruit set, and whether the set fruit contained plump and/or flat seed. Seeds were sown, and the number of plants surviving for more than 2 years counted.

4.8.2 Results and discussion

Table 4.8a shows the fruit set from each of the pollen sources on the 16 seed parents. The seediness of this fruit is also shown.

Table 4.8a Effect of two triploid pollens on fruit set and seed formation in 16 monoembryonic diploid seed parents, BRS.

	'EL3' triploid pollen				'EL5' triploid pollen			
	% set	Plump seeds per fruit	Flat seeds per fruit	Nursery trees	% set	Plump seeds per fruit	Flat seeds per fruit	Nursery trees
Arrufatina	0				0			
Aust.Clem	7	3	1	1	0			
Corsica 1	0				0			
Corsica 2	20	7.5	13	7	0			
Daisy	13	9	5	15	13	14.5	1.5	22
DeNules	0				20	4.3	5.7	10
Ellendale	27	3.8	3.5	12	0			
Encore	7	6	3	4	7	16	19	12
Fallglo	-				0			
Fortune	0				7	3	2	3
Hickson	0				0			
IM111	20	13.7	2.3	24	0			
Imperial	5	26	7	22	0			
Monarch	53	8.5	7.1	15	13	11.5	1.5	24
Umatilla	14	14	9	11	80	7.8	0.3	27
Wilking	7	2	5	0	0			

It can be seen that fruit set was achieved on more than half of the parental combinations, and 6 of the combinations gave at least 20% fruit set. However the average fruit set for the whole experiment was only 8.7% of pollinations. Fruit produced from triploid pollen were seedy, containing a mixture of normal plump seeds as well as flat seeds. For example, with Daisy the fruit averaged 12 normal seeds and 3 flat seeds per fruit.

It is also important to note that of the 38 fruit produced in this experiment, only one fruit (a Monarch X EL3) was seedless, and many crosses failed to set any fruit. This is despite the use of a number of parents (eg. Imperial, Arrufatina, Corsica, de Nules, Ellendale) that are reported to have parthenocarpic ability. However this result is consistent with our experience at Bundaberg, and the work of Wallace and Lee (1999) and Wallace (2004), in suggesting a strong environmental influence on the expression of parthenocarpy under subtropical conditions.

Many of the seeds were viable and have been successfully established as nursery trees, with at least one tree from all but one of the families that set fruit (15). These 209 trees will be field planted in early 2005. While the seeds germinated and have grown reasonably well, many of the resulting plants have abnormal/distorted leaf and stem development, as might be expected from plants with unusual chromosome numbers. Unfortunately it has not been possible as yet to determine the ploidy level of these hybrids. It also remains to be seen whether they are capable of flowering and setting fruit (or producing viable pollen).

These results have demonstrated the capacity of triploid pollen to cause seed set in a range of mandarin cultivars, and this needs to be kept in mind when promoting new triploid varieties. Furthermore, the results demonstrate limitations on the expression of parthenocarpy in this environment and the high fertility of the Citrus genus.

4.9 Selections fruiting on the top-worked block

4.9.1 Materials and methods

The first hybrids generated in the triploid breeding program at Bundaberg were top-worked onto an established block of Murcott. Top-working commenced in August 1998 and a total of 76 hybrids were established in this manner (see previous report for detail).

Fruiting hybrids were assessed in the field.

4.9.2 Results and discussion

The first hybrids produced fruit in 2002, four years after top-working. In this season seven triploids and two tetraploids produced sufficient fruit to conduct a preliminary assessment. In the 2003 season 16 triploids and 36 tetraploids fruited and were assessed (Table 4.9a & 4.9b).

This material has clearly demonstrated the potential of the triploid program to produce hybrids which are seedless even under intense pollination pressure, that are of good fruit size, and on fruitful trees. Larger progenies (such as have now been established on the trellis system) are required in order to combine these desirable characteristics of triploids with suitable fruit quality for commercial production.

In terms of efficiency in breeding, it is noteworthy that hybrids in the top-worked block did not commence fruiting any earlier than we have seen seedling fruit under the trellis system. The former is far more labour intensive and expensive to maintain, and appears to offer no advantage over growing seedlings on their own roots. Based on experience with other citrus breeding projects at Bundaberg it is anticipated that around 90% of hybrids will be culled soon after they commence fruiting (because of poor fruit quality) so there seems little value in investing significant resources in hybrids before they have fruited.

4.10 Selections fruiting on the trellis.

4.10.1 Materials and methods

Hybrids generated in the ploidy breeding program were planted onto a trellis system (see section 4.6) commencing in March 2001. A small number of these trees fruited for the first time in 2004 and were assessed. The fruit were assessed in the field by two people and each tree was described in terms of its fruit characteristics.

4.10.2 Results and discussion

Twenty one trees produced at least one piece of fruit and were assessed. This represents 1.6 % of the 1312 trees planted on 'Trellis 1". Some of the characteristics of these hybrids are shown in Table 4.10a.

None of this material satisfies commercial requirements and will not be propagated further. However it will be retained and re-assessed in 2005, particularly considering the fruit quality problems caused by Nematospora in the 2004 season (see section 4.4). Although numbers assessed in each family are extremely small, some trends can be seen in this limited data. For example there is a clear tendency for the tetraploid orange parents to produce hybrids with orange-like characteristics such as poor external/internal colour and rounded fruit shape. The orange-like taste was also transmitted in two of the hybrids. The program has generated many hybrids using tetraploid orange parents and it is hope that some of these hybrids may display the desirable characteristics of oranges with some improvement, and thus become potentially useful for orange growers. Many of these hybrids should fruit over the next few years.

Another aspect that the 2004 data reveals is the inadequacy of the morphology-based ploidy determination method. This technique was used on a trial basis for two seasons because no other efficient technique was available. It can be seen that 3 of the hybrids which were predicted to be triploids actually produced >10 seeds/fruit suggesting that they are not triploids. The morphology-based technique is no longer being used at Bundaberg although we will continue to monitor the seed number of hybrids that were assessed using this technique. Although the acquisition of a flow cytometer was recommended by the Navarro review, it was not supported in the new project and so the ploidy breeding program remains severely hampered by the absence of an efficient means of determining the ploidy level of hybrids produced.

Two of the hybrids were considered worthy of further assessment, though they are by no means outstanding at this stage. Both of these are hybrids with tetraploid Murcott. This has been an important pollen source in the triploid breeding program (see Tables 4.1a-g) and its use has continued despite problems caused by late flowering and poor pollen production from this genotype. It is hoped that some of the future fruiting hybrids produced from this pollen source will display some of the desirable characteristics of Murcott that have made it the most important commercial mandarin variety for exports.

4.11 References

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5. Mutation breeding (Bundaberg and Merbein)

5.1 Introduction

Induced mutation in *Citrus* and in particular irradiation of buds has been shown to be effective in producing low-seeded mutants as well as affecting other fruit quality characteristics of existing varieties. Research conducted during projects CT315, CT319 and CT614 investigated irradiation as a means of altering current commercial varieties with regard to seediness. This research continued as part of this project CT00012.

5.2 Irradiation research at CSIRO Merbein

5.2.1 Introduction

Induced mutation has been used successfully to generate new seedless variants of citrus (eg Hearn, 1986). The aim of the research in this component of the project was originally to investigate whether floral phenotype could be altered through induced mutagenesis to capitalise on the parthenocarpic nature of three candidate varieties.

Ellendale tangor (unpublished data), Imperial (Sykes and Possingham, 1992) and Kara (Sykes *et al.*, 1994, see Fig 5.1) mandarins will set seedless fruits in the absence of pollination. The original aim of the work was to generate and thus investigate if pollen sterile variants would produce seedless fruits when grown in isolation from other sources of viable pollen. Seedless variants of lemons and Minneola tangelo were recovered following gamma irradiation of buds (Spiegal-Roy and Vardi, 1989) and in the case of Eureka and Villafranca lemons, the variants were pollen sterile.

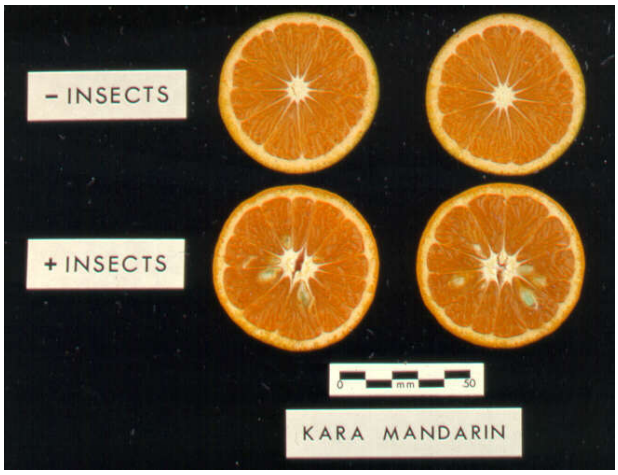

Fig 5.1 The effect of preventing pollination of Kara mandarin on seediness of fruits. Pollination was prevented by excluding insects during flowering.

In project CT319, buds and small trees were treated either with gamma irradiation or short-wave (254nm) UV light. The final report for CT 319 provides further details. This research continued in CT614 when trees propagated from gamma irradiated buds or UV-treated rooted cuttings were assessed for seedlessness and fruit quality. The main finding regarding this component of the research in CT614 was that two M2 trees derived from buds cut from an M1 tree grown from a Kara bud treated with 60gy gamma irradiation had very low mean seed numbers in their fruits (2.1 ± 1.5 and 1.4 ± 1.5 respectively compared to 18.8 ± 7.4 and 20.1 ± 5.9 for two other M2 trees

growing next to them). The research continued in CT00012 focused on these two M2 lines and progress is outlined in this section of the report.

5.2.2 Materials and methods

The following summarises the methods and results obtained used up until the start of CT00012:

1. Buds of Kara were irradiated in 1994 (CT319).
2. Rootstocks were budded with the irradiated buds and allowed to grow to give M1 generation trees.
3. Buds from nodes 3-12 of M1 generation trees were used to propagate M2 generation trees (see Fig 5.2).

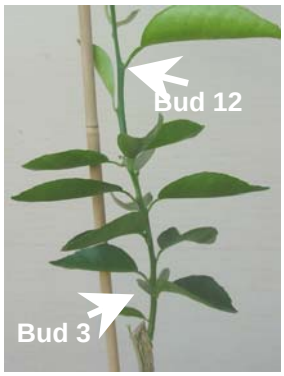

Fig 5.2. Single shoot from an irradiated bud grafted to a rootstock (M1) demonstrating the positions from which buds were taken to propagate the next vegetative generation (M2) trees.

4. M1 and M2 generation trees were rowed out in the orchard and open-pollinated fruits screened for seeds.
5. Two M2 generation trees (258.2 and 258.4) produced seedless fruits (CT614). These trees were propagated using buds from an M1 tree (258) propagated from a bud treated with 60gy gamma irradiation.
6. The parent M1 tree 258 and a control tree grown from a non-irradiated bud produced seedy fruits under the same orchard conditions.
7. Other M2 trees (eg 258.7) grown from M1 tree 258 buds also produced seedy fruits.

The following details the research conducted with these Kara mandarin budlines in CT00012:

Verification of observations made prior to season 1999-2000

Although these data were presented in the final report for CT614, they are presented again here as they were obtained in September 2000, which was at the start of CT00012. Two trees that were propagated from buds 2 and 4 from M1 tree 258, which developed from a bud treated with 60gy gamma irradiation, were harvested in September 2000. Fruits were also harvested from two control Kara mandarin trees, grafted on Carrizo and Sweet orange rootstocks respectively, the M1 tree grown from the original irradiated bud, and another daughter tree from M1 258 namely 258.7. Fruits were weighed, percentage juice determined, juice sugar, acid and sugar:acid ratio determined, and seed numbers recorded.

Fruits harvested in 2001

In October 2001, all fruits were harvested from the seedless or low-seeded M2 lines 258.2 and 258.4 along with fruits from 258.7, which was another daughter tree from the M1 mother tree 258. Fruits were also harvested from M1 tree 258 and from two other M2 daughter trees derived from

other gamma irradiated buds of Kara mandarin. These two latter trees were selected because they were growing alongside trees 258.2 and 258.4 and thus subjected to similar pollination pressures. As other commitments with pollination experiments and controlled crosses were high at this time, fruits were simply weighed and seeds extracted and numbers recorded.

Propagation of M3 generation daughter trees in 2001

When fruits of 258.2 and 258.4 were harvested in October 2001, a short piece of shoot including several buds immediately behind the fruit was also collected, labelled with the fruit number and stored at 4°C. Once fruits had been analysed, buds from shoots that had borne seedless fruits were used to propagate M3 generation daughter trees. These trees were budded to Symons sweet orange rootstock seedlings under glasshouse conditions, where they were maintained until large enough to be re-potted into larger containers (12l) and relocated to a shadehouse at ambient temperatures.

Pollination experiments in 2001

A series of pollination treatments were applied to the two seedless M2 trees along with a control Kara, the M1 mother tree and another M2 tree, namely 258.7, which had produced only seedy fruits.

The treatments applied were:

1. Emasculation with no pollen applied to the stigma
2. Emasculation with self pollen applied to the stigma
3. Emasculation with Valencia orange pollen applied to the stigma
4. Open-pollination

Fruits from these treatments were harvested prior to normal maturity when seed and undeveloped ovule numbers were recorded.

Pollination experiments in 2002 and 2003 with control, M1 and M2 trees

Pollination experiments involving bagging, self pollination, and emasculation accompanied either by zero pollination or controlled cross-pollination with Valencia pollen were conducted again during 2002 and 2003. Fruits were harvested during October 2003 and 2004 respectively and analysed as before.

Pollination experiments with M3 daughter trees

In spring 2003, a number of the M3 trees, which were still in large pots under shadehouse conditions, had flowered and these were subjected to a range of pollination treatments as follows:

- Flowers were emasculated and selfed
- Flowers were emasculated and cross-pollinated either with Valencia or *Poncirus trifoliata* pollen
- Flowers were left untreated for open-pollination.

Fruits were harvested from these trees during June 2004 and seed numbers recorded.

5.2.3 Results and discussion

Verification of observations made prior to season 1999-2000

Table 5.1 summarises the data for fruits collected from Kara mandarin trees that had been propagated from non-irradiated buds (controls), from a bud exposed to 60gy gamma irradiation, and M2 daughter trees propagated from the M1 tree.

Table 5.1. Fruit quality data for three M2-generation Kara mandarin trees, the M1 mother tree and two control trees. The M1 and M2 trees were propagated to Symons sweet orange rootstocks. The control trees were propagated to Carrizo citrange and Symons sweet orange rootstocks. Fruits were harvested on September 18, 2000 and data are means ± sd for 12 fruits.

Tree	Mean fruit weight (g)	% juice	Juice Brix	Juice acid	Brix:acid	Mean seed number	Range in seed numbers
Kara/citrange (control)	159.7 ± 19.5	48.4	11.3	1.54	7.3	23.1 ± 6.8	14 – 33
Kara/SWO (control)	69.1 ± 12.8	42.2	12.0	2.67	4.5	15.5 ± 2.3	11 – 22
258 (M1)	178.2 ± 21.1	45.7	11.0	1.10	10.0	19.2 ± 4.4	13 – 28
258.2 (M2)	128.4 ± 19.4	54.4	11.0	1.59	6.9	1.1 ± 1.1	0 – 3
258.4 (M2)	120.8 ± 19.2	48.3	12.8	1.24	10.3	1.5 ± 1.0	0 – 4
258.7 (M2)	141.9 ± 26.0	48.2	12.8	1.79	7.2	17.7 ± 6.8	7 – 30

Fruits harvested in 2001

Seedless fruits were again harvested in 2001 from two M2 generation trees propagated from gamma irradiated Kara mandarin (Fig. 5.3) and the data are presented in Table 6.2. Seed numbers ranged from 0-2 and 0-3 with mean seed number per fruit of 0.8 ± 1.0 and 0.5 ± 0.7 for 258.2 and 258.4 respectively compared to 4 – 22 and 12.9 ± 5.8 for the M1 generation tree from which these trees were propagated. Seed numbers for another M2 generation tree propagated from the same M1 tree ranged from 8 – 24 with a mean of 15.8 ± 7.9. This suggested that the mutation giving rise to the two trees yielding seedless fruits was present in a chimeric sector. To ensure stability of these seedless or low-seeded budlines, M3 generation trees were propagated from buds immediately proximal to seedless fruits.

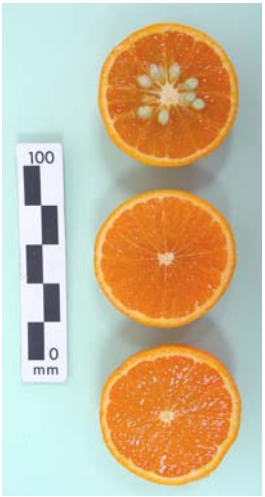

Fig. 5.3. Fruits from three lines of Kara mandarin. The topmost seedy fruit is from a normal Kara tree. The mid and bottom most seedless fruits are from two lines (258.2 and 258.4) derived from gamma irradiated buds.

Table 5.2. Seed characteristics for all open-pollinated fruits harvested in 2001 from Kara mandarin trees propagated from buds or budlines with a history of gamma irradiation.

Tree 258 (M1) was propagated using a bud that was exposed to 60gy gamma irradiation and trees 258.2, 258.4 and 258.7 were daughter trees of 258. Trees 225.6 and 251.10 were M2 generation trees propagated from other M1 trees that had been propagated using buds exposed to 40gy and 60gy gamma irradiation respectively. These trees were growing alongside the 258 M2 trees and subjected to similar open-pollination pressures.

Tree	Mean seed no.	Range in seed numbers	% of fruit seedless
258 (M1)	12.9 ± 5.8	4 – 22	0
258.2 (M2)	0.8 ± 1.0	0 – 3	51.5
258.4 (M2)	0.5 ± 0.7	0 – 2	61.3
258.7 (M2)	15.8 ± 7.9	8 – 24	0
225.6 (M2)	6.7 ± 3.4	2 – 16	0
251.10 (M2)	19.1 ± 8.7	5 – 39	0

Propagation of M3 generation daughter trees in 2001

Thirty one M3 generation trees were propagated successfully and were retained under shadehouse conditions. Details of the trees are presented in Table 5.3. The growth of the trees indicated that as pot-grown plants that they would carry their first fruits in 2003-04 when the stability of the budlines with regard to seediness could be investigated. It was decided to maintain these trees under shadehouse conditions until data concerning flowering could be obtained. By doing this, there would be some certainty that they had maintained their seedless character before being planted out for evaluation of other fruit quality characteristics and yield potential.

Table 5.3. Number of seeds in individual fruits harvested from Kara mandarin trees 258.2 and 258.4 in 2001 and the success of propagating M3 trees from buds taken from immediately behind seedless fruits.

258-2			**258-4**		
Fruit number from behind which buds were retained	Number of seeds in fruit	M3 tree propagated, code and success*	Fruit number from behind which buds were retained	Number of seeds in fruit	M3 tree propagated, code and success*
1	1		**1**	**0**	**258-4-1 ✓**
2	**0**	**258-2-2 X**	2	1	
3	**0**	**258-2-3 X**	**3**	**0**	**258-4-3 ✓**
4	1		**4**	**0**	**258-4-4 ✓**
5	2		5	2	
6	1		**6**	**0**	**258-4-6 ✓**
7	1		**7**	**0**	**258-4-7 ✓**
8	**0**	**258-2-8 ✓**	**8**	**0**	**258-4-8 ✓**

Table 5.3 contd.

258-2			258-4		
Fruit number from behind which buds were retained	Number of seeds in fruit	M3 tree propagated, code and success*	Fruit number from behind which buds were retained	Number of seeds in fruit	M3 tree propagated, code and success*
9	2		**9**	**0**	**258-4-9 ✓**
10	**0**	**258-2-10 ✓**	**10**	**0**	**258-4-10 ✓**
11	3		**11**	**0**	**258-4-11 X**
12	**0**	**258-2-12 ✓**	12	1	
13	**0**	**258-2-13 ✓**	**13**	**0**	**258-4-13 ✓**
14	**0**	**258-2-14 ✓**	**14**	**0**	**258-4-14 ✓**
15	**0**	**258-2-15 ✓**	**15**	**0**	**258-4-15 ✓**
16	**0**	**258-2-16 ✓**	16	1	
17	**0**	**258-2-17 ✓**	17	1	
18	1		**18**	**0**	**258-4-18 ✓**
19	**0**	**258-2-19 ✓**	19	1	
20	1		20	2	
21	2		**21**	**0**	**258-4-21 X**
22	**0**	**258-2-22 ✓**	**22**	**0**	**258-4-22 ✓**
23	1		**23**	**0**	**258-4-23 X**
24	3		**24**	**0**	**258-4-24 ✓**
25	3		25	1	
26	1		26	1	
27	1		27	2	
28	2		**28**	**0**	**258-4-28 ✓**
29	**0**	**258-2-29 ✓**	29	1	
30	**0**	**258-2-30 ✓**	**30**	**0**	**258-4-30 ✓**
31	**0**	**258-2-31 ✓**	31	1	
32	**0**	**258-2-32 ✓**			
33	**0**	**258-2-33 ✓**			

* ✓ or **X** after the code assigned to an M3 tree indicates if propagation was successful.

Pollination experiments in 2001

Summarised data from the pollination experiments conducted in 2001 and harvested in 2002 are presented in Table 6.4. All trees produced seedless fruits when flowers were emasculated and then left un-pollinated. This was expected as Kara has been shown to be capable of parthenocarpic fruit development, which was one of the main reasons why Kara was selected as a candidate for mutation breeding originally in project CT319.

Only trees 258.2 and 258.4 produced seedless or very low-seeded fruits when their flowers were challenged by pollen be it by controlled- or open-pollination (Fig 5.4). This contrasted with the control, M1 and the other M2 trees, which all yielded seedy fruits when pollinated with Valencia pollen or were left to be open-pollinated, although seed numbers were higher for fruits from open-

pollination. Self pollination of the control trees resulted in high seed numbers, which contrasted with the trees derived from the bud that was exposed to gamma irradiation. The M1 tree (258) only yielded one fruit after self-pollination and this only contained a solitary seed.

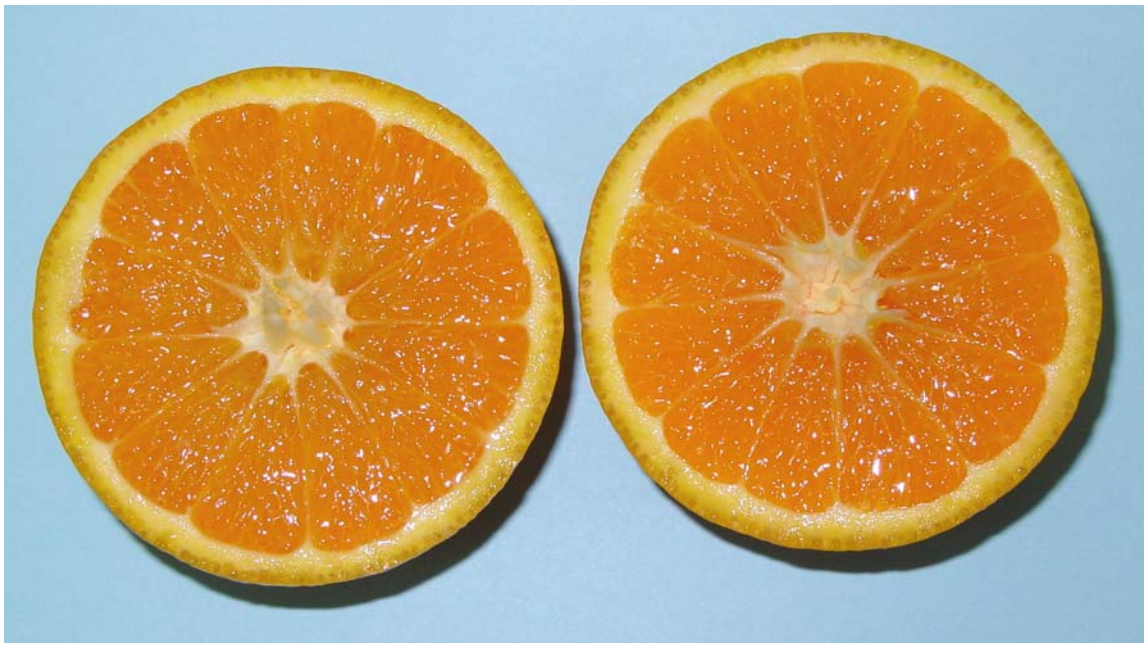

Fig 5.4. Seedless fruits from a line of Kara mandarin derived from a gamma-irradiated bud. These fruits developed after their flowers were cross-pollinated with Valencia orange pollen.

The variation in mean number of undeveloped ovules in fruits was interesting and there were differences between the two M2 seedless trees and the other trees investigated. In un-pollinated trees, only the M1 tree had a large number of undeveloped ovules. Where fruits developed following pollination, only the M2 trees 258.2 and 258.4 had low numbers of undeveloped ovules. These data suggest that a mutation has occurred affecting female fertility in budlines derived from the original irradiated bud, but this has only been shown up in two and not all of the M2 trees. The absence of undeveloped ovules in the un-pollinated fruit of the control tree compared to the 11 in the un-pollinated M1 tree is difficult to understand, although only one fruit resulted in each case. The results for the M2 trees, however, indicate that the three trees have maintained their ability for parthenocarpic fruit from the original Kara budline but only trees 258.2 and 258.4 have been altered with regard to ovule development or survival. Their reduced female fertility would appear to have arisen as a sectoral mutation and for this reason the propagation of M3 generation trees was important to ascertain the stability of this altered characteristic.

Kara mandarin is a hybrid between Owari satsuma and King mandarin (Saunt, 1990). Satsuma is a seedless due to parthenocarpy, pollen sterility, ovule and embryo abortion (Iwamasa, 1966). King mandarin is seedy and the author is unaware of any reports that it displays parthenocarpy. Kara fruits may be seedy or seedless and our experiments have shown it is parthenocarpic. Presumably Kara has inherited its parthenocarpic ability from its satsuma parent but not pollen sterility or ovule abortion. In M2 trees 258.2 and 258.4 it is possible that a mutation is expressed that has affected female fertility and caused a reversion to the satsuma genotype with regard to ovule and/or embryo abortion.

Table 5.4. Mean fruit weight, % rind, seed number and undeveloped ovule number for Kara mandarin trees propagated from non-irradiated (control) and gamma irradiated buds. Tree 258 was propagated using a bud that was exposed to 60gy gamma irradiation and trees 258.2, 258.4 and 258.7 were daughter trees of 258.

Treatment	Tree	Mean fruit weight	Mean % rind	Mean seed number	Mean number of undeveloped ovules
No pollen	Control [a]	74.4	13.5	0	0
	258 (M1) [a]	46.4	13.5	0	11
	258.2 (M2)	76.2 ± 20.2	13.8 ± 1.8	0	0.2 ± 0.5
	258.4 (M2)	79.9 ± 14.3	14.8 ± 1.0	0	1.4 ± 1.7
	258.7 (M2)	66.3 ± 8.1	13.2 ± 0.4	0	0.3 ± 0.6
x self pollen	*Control*	*52.1 ± 11.8*	*15.3 ± 0.6*	*18.5 ± 9.2*	*8.0 ± 8.5*
	258 (M1) [a]	*51.0*	*13.7*	*1*	*9*
	258.2 (M2)	*82.4 ± 19.6*	*13.0 ± 1.4*	*0*	*0*
	258.4 (M2)	*67.1 ± 33.9*	*14.9 ± 1.1*	*0*	*1.5 ± 1.7*
	258.7 (M2) [b]	-	-	-	-
x Valencia	Control	84.0 ± 1.2	13.1 ± 6.5	4.5 ± 6.4	25.5 ± 7.8
	258 (M1)	50.7 ± 18.7	14.6 ± 1.4	2.3 ± 2.5	6.3 ± 1.5
	258.2 (M2)	75.3 ± 22.0	13.3 ± 2.3	0.2 ± 0.4	1.4 ±1.4
	258.4 (M2)	79.7 ± 15.1	14.2 ± 2.3	0.8 ± 1.1	2.2 ± 1.8
	258.7 (M2)	93.0 ± 24.3	12.7 ± 1.1	3.8 ± 2.5	6.8 ± 3.9
Open-pollinated	Control	101.4 ± 6.6	9.8 ± 0.7	8.6 ± 6.8	8.7 ± 3.0
	258 (M1)	78.1 ± 9.9	14.3 ± 2.2	20.0 ± 3.0	7.7 ± 2.6
	258.2 (M2)	90.9 ± 11.2	13.2 ± 1.7	0	1.2 ± 0.8
	258.4 (M2)	84.0 ± 16.6	12.7 ± 0.7	0.3 ± 0.5	0.7 ± 0.8
	258.7 (M2)	87.0 ± 5.2	12.0 ± 0.7	12.0 ± 8.4	6.2 ± 2.1

[a] only one fruit harvested
[b] fruits absent

Pollination experiments in 2002 and 2003 with control, M1 and M2 trees

Experiments with the same treatments as used in 2001 were conducted in 2002 and 2003. The results of the 2003 experiments are yet to be collected and analysed with fruits to be harvested in October 2004. The results from the experiment conducted in 2002 were similar to and supported the data obtained in 2001.

Pollination experiments with M3 daughter trees

Not all of the M3 trees maintained under shadehouse conditions flowered during spring 2003 (see Table 5.5). Those that did flower were challenged with four sources of pollen, namely self-, open- or cross-pollination with Valencia orange and *Poncirus trifoliata* pollen. The number of flowers pollinated per tree per pollen source varied due to differing numbers of flowers between trees. Nevertheless, a high proportion of deliberately pollinated flowers had developed fruits by December. Unfortunately, however, the trees were hit by a severe storm at Merbein on December 4 and most of the developing fruits were lost. The fruits that remained on the trees after the storm were enclosed in mesh bags and allowed to develop until colour break occurred when they were harvested. The results are presented in Table 5.5.

Table 5.5. Effects of different pollination treatments on seed numbers in M3 generation daughter trees propagated from two budlines originating from a single Kara mandarin bud that was exposed to 60gy gamma irradiation.

It should be noted that the trees were severely damaged during a storm on December 4, 2003, after which only a small number of the developing fruitlets remained on the trees. Consequently the data presented here have been pooled for each tree-treatment combination.

Budline	Pollination treatment		Number of fruits harvested	Range in seed numbers between fruits	Mean seed number per fruit
	Pollen	No. of flowers pollinated [c]			
258.2 [a]	Valencia (10) [b]	105	1		1
	Poncirus (4)	21	1		1
	Self (6)	32	1		0
	O/P (3)	?	4	0 - 2	0.5 ± 1.0
258.4 [a]	Valencia (9)	76	8	0 - 2	0.75 ± 0.71
	Poncirus (2)	11	1		0
	Self (4)	16	1		0
	O/P (10)	?	17	0 - 1	0.21 ± 0.43

[a] Of the M3 trees that were propagated (15 and 16 for the 258.2 and 258.4 budlines respectively, see table 5.3), 11 and 12 M3 daughter trees of the 258.2 and 258.4 budlines respectively flowered in 2003.

[b] The numbers in parentheses are the number of M3 trees that received the pollination treatment.

[c] The number of pollination treatments per tree varied and have been pooled to give the total per budline.

Although fruit numbers were low, the number of seeds per fruit for the different pollination treatments indicated that the seedless or low-seeded nature of the M2 trees from which buds were taken to propagate the M3 trees was stable over at least one generation of vegetative propagation. This result was encouraging but requires further data to confirm the stability of these two budlines with regard to seed development. These trees will now be planted out in the research orchard where further data concerning seed development will obtained along with information on their yield potential and other fruit quality characteristics.

When the M3 trees flowered, it was noticed that pollen production was very low and was different from normal Kara mandarin. The anthers of opened flowers were pale yellow and produced little pollen resembling satsuma mandarin and pollen sterile hybrids that have been bred in the diploid hybridisation program (fig. 5.5). Self pollen was not functional in that only seedless fruits resulted, although only two fruits from self-pollination remained after the storm. This may have been caused by low pollen fertility or ovule abortion. Other activities during the flowering season prevented further work with the M3 trees, but pollen development and function will need to be investigated further in the M3 trees.

Fig 5.5. A flower from a M3 Kara tree showing pale yellow anthers and only scant pollen production. Future research will determine if the pollen produced by M3 trees is functional or if the irradiation treatment has also resulted in pollen sterility.

5.2.4 Conclusions

- The seedless or low-seeded characteristics were confirmed for 2 budlines (258.2 and 258.4) derived from a Kara mandarin bud that was exposed to 60gy gamma irradiation.
- The stability of this trait in these budlines after a generation of vegetative propagation was demonstrated, but needs to be confirmed by further experiments.
- The two seedless (or low-seeded) budlines appeared to arise as chimeric mutations which affected both female and male fertility. Further experiments will confirm this.
- The results have justified the approach taken and have demonstrated the value of using induced mutagenesis to affect seediness in a known parthenocarpic variety. This supports a proposal to irradiate hybrids from the diploid program that are highly parthenocarpic with superior fruit quality and appropriate fruit maturity, but are capable of self-pollination and thus seedy in an open-pollination situation. Irradiation to reduce or eliminate seeds in such hybrids would improve their chances for adoption.

Kara mandarin is not a major variety, but is grown to a limited extent in the Murray Valley, especially the Riverland of SA. Seediness is one of its limitations and a rough, coarse rind is another. It is a late maturing variety that stores well, suggesting that it could fulfil one of the aims of the breeding plan, namely a late maturing variety for export. A seedless variant of Kara would help towards this goal and a finer rind is one fruit characteristic observed for the budlines developed at Merbein. Fruits from these budlines also store well and have been held at 3°C for up to 6 months with little noticeable sensory deterioration. The aim now is to observe and evaluate the M3 trees propagated during CT00012 under orchard conditions to ensure the low-seeded or seedless trait is maintained and also investigate further fruit yield, quality and storage capacities. It is interesting that there has been interest in these budlines from overseas on more than one occasion.

5.2.5 References

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5.3 Mutation breeding research at DPI&F, Bundaberg

The Queensland citrus industry is currently commercialising two new varieties derived from the irradiation of Murcott budwood. These will be available to southern growers in the near future. The success in developing improvements to Murcott using this technique prompted the inclusion of a small irradiation component in the breeding project conducted at Bundaberg.

5.3.1 Materials and methods

The germplasm collection at Bundaberg Research Station was screened for the presence of cultivars that may make suitable commercial varieties if they had less seeds. Emphasis was place on finding mandarin cultivars that had good size, colour and eating quality. Nine cultivars were identified as candidates for irradiation. *Fremont* was bred in the USA and was at the time becoming a significant commercial variety, particularly in Queensland (commercial interest has recently declined). It was included because of its excellent colour, eating quality and on-tree storage. The major problem, apart from very high seed numbers, was its small size. *Daisy* was also a USA bred variety and has attracted a lot of attention because of its excellent colour, size and good flavour. It is very seedy and so a good candidate for irradiation. It is very susceptible to Alternaria, which limits its usefulness in subtropical and coastal areas. *IM111* is a Queensland bred variety that resulted from a cross between Imperial and Murcott. It has never been released commercially, partly on account of its high seed numbers. The variety has good size, colour and excellent skin texture, but only moderate flavour. The availability of a lower seeded version of IM111 may make it more attractive for commercialisation. *Kinnow* is a USA variety that is now grown extensively on the Indian sub-continent. It has high Brix and good flavour, and the potential to achieve good fruit size. It is very seedy, and so a lower seeded version may make it more attractive to commercial orchardists. *Ellenor* is a Queensland bred variety that is grown commercially on a small scale. It has excellent flavour and good colour, but is limited by high seed numbers and coarse skin. *Afourer* is originally from Morocco, and is currently of considerable interest to southern growers (Queensland growers have gone off it after great initial enthusiasm). It has good colour and is productive, but the taste is variable. Reduced seed numbers in this variety would make it more commercially appealing. Some *Pummelo* varieties were also irradiated in the hope of producing less-seedy selections. Small numbers of the DPI&F low-seeded Murcott selections *IrM1* and *IrM2* were also re-irradiated.

Budwood from the above cultivars was irradiated in 2000 and budded to Troyer citrange rootstock. The resulting trees were planted-out in late 2001 and allowed to develop without any pruning. As fruit production commenced, all limbs on all trees where checked for the presence of low-seeded fruit. Any such limbs were tagged and then reassessed the following season. Budwood was taken from promising limbs in November 2003, and daughter trees propagated for further evaluation.

5.3.2 Results and discussion

Significant difficulties were experienced in budding success rates of this irradiated material. This was despite the use of radiation levels that we had previously shown to be relatively harmless to the buds viability. Subsequently it was found that other budwood taken from the arboretum and not irradiated also had very low viability. This problem had a negative impact on the number of trees that were generated in the irradiation program, and increased the workload. The problem persisted despite experimentation with a range of budwood treatments, such as fungicides and anti-transpirant dips. Budwood obtained from AusCitrus at the same time produced normal success rates (>90%), indicating that the problem was something related to the arboretum trees from which the budwood for irradiation was collected. This low budding success reoccurred as a problem at Bundaberg in the 2003 season when we propagated selections from a different breeding program. As in 2000, success rates were very low (~20%) and this dramatically increased workload as budwood from multiple selections had to be re-collected and re-budded. It none-the-less provided an important opportunity to understand why this problem had occurred in 2000 (between 2000 and 2003 budding success rates had been normal). An examination of field management records as well as nursery spray records for the two problem seasons provided some possible answers. It is now believed that the problem was probably caused by the application of phosphorus acid and/or spray oil close to when the budwood was cut, as well as the foliar spraying of low-biuret urea on rootstock plants shortly before they were budded. We have not as yet had time to confirm this hypothesis through experimentation but until this is done we will ensure that no sprays of urea, phosphorus acid or spray oil are applied to either budwood source trees or rootstock trees within a month of budding.

Table 5.6 shows the number of irradiation-derived trees that were established for each of the different cultivars. Because these were never pruned, they developed multiple branches close to the original budding position, and each of these branches was checked for the presence of low-seeded fruit. On average about 10 branches needed to be checked on each tree. The first fruit were produced on some trees in 2002 and these were checked for seed number. In 2003, trees fruited profusely and all the branches were checked for low-seeded fruit. Branches on 36 trees were noted as low-seeded, and were reassessed (with a particular interest in examining fruit size) to determine which were most suitable for propagating daughter trees from. Sixteen branches were considered worthy of propagation, and budwood from these branches was budded to Troyer and Benton rootstock in November 2003.

Trees again fruited profusely in 2004, but the extremely high incidence of Nematospora (see section 4.4 this report) within the orchard made the assessment of seed number meaningless. The Nematospora yeast severely disrupts seed formation within the fruit and can cause normally seedy varieties to be completely seedless (as well as distorted and inedible). As an example, this problem was so severe at Bundaberg during 2004 that 400 rootstock seed trees (used to produce seed for the commercial nursery industry) were not harvested because the fruit contained practically no seed.

The trees will be assessed for seed number for the final time in 2005, and will then be removed. Particular attention will be paid to the Pummelo material as this has been slow to commence fruit production (this is normal for *Citrus maxima* and its hybrids) and so very few branches have been accurately assessed to date. Branches used to supply budwood for the daughter trees will also be

carefully monitored to ensure results are consistent with 2003. The daughter trees propagated on the strength of 2003 results will be field planted in early 2005, and assessed for consistency of fruit characteristics.

Table 5.6. Number of field-planted trees derived from irradiated buds of 9 citrus cultivars, the number of branches with low-seeded fruit, and the number of branches finally chosen for propagation of daughter trees. Trees planted 2001, seed number assessed 2003, BRS.

Cultivar	Trees planted	Preliminary selections	Selections propagated
Afourer	41	8	4
Daisy	41	3	3
Ellenor	58	4	2
Fremont	39	12	3
IM111	24	3	2
IrM1	20	6	2
IrM2	2	0	0
Kinnow	80	0	0
Pummelo	38	0	0
Total	343	36	16

6. Project-wide activities

As a coordinated project, a number of activities were undertaken during the course of CT00012 that were common across the four main research components of diploid and triploid hybridisation, mutation and gene technology research. These activities are reported in brief in this chapter. More details concerning project-wide activities are to be found in milestone reports, publications and other documents produced as a result of undertaking a specific task.

6.1 The citrus scion breeding reference committee

An initiative put in place towards the end of project CT96014 was the establishment of the Citrus Scion Breeding Reference Committee (CSBRC) consisting of industry, CSIRO, QDPI&F and HAL representatives. The overall aim of this committee has been to act as an industry/agency steering group operating in a consultative manner to ensure that the breeding program remains focused on short- and long-term strategic industry priorities. The committee assists in setting targets for the breeding program so outcomes are defined clearly and understood by all parties. The committee has assisted in communicating activities, research outputs and industry outcomes to the wider citrus industry.

The CSBRC held its first meeting on March 9, 2000 via telephone conference before CT000012 commenced. At this meeting, the outputs and outcomes proposed for Project CT00012 were discussed along with other issues concerning the breeding program and industry members of the committee endorsed the outputs and outcomes listed in the project application as appropriate.

During the course of the project the CSBRC met either by telephone conference or in person at least twice a year and assisted the breeding team in meeting objectives. The dealings of the committee are recorded in the minutes, which are circulated to the committee members and endorsed at subsequent meetings.

Over the course of the project the CSBRC has been particularly valuable to the breeding team with regard to the development and subsequent endorsement for the Breeding Plan, which is discussed later. In developing and acting on the breeding plan, the CSBRC has provided a valuable forum for developing a commercialisation strategy for the release of new varieties from the breeding program.

6.2 Project teleconferences

Quarterly meetings via telephone conference were held throughout the project between the principal researchers to report on progress, discuss directions and plan other activities. Minutes were taken for all these meetings and circulated to HAL, the CSBRC and appropriate personnel in the research agencies.

6.3 Industry meetings

At the start of CT00012 it was anticipated that an annual report would be presented and tabled at the ACIA/Auscitrus meeting that had normally been held during November. This occurred during 2000 and 2001, although the meeting was re-scheduled on both occasions for August. Since then there have been no invitations from ACIA/Auscitrus for scientists from the breeding program to attend its meeting and present an update on progress.

6.4 The breeding plan

A formal plan for breeding new Australian citrus varieties has been developed and addresses breeding goals, the methods to achieve them, and a commercialisation strategy. The plan was prepared by project scientists and endorsed by the Citrus Scion Breeding Reference Committee. It is an extensive and constantly evolving document. In drawing up the plan it was agreed that detailed product specifications should be documented as part of the breeding objectives for the whole project. Grower members of the CSBRC, however, agreed it must be realised that the chances of breeding something totally novel should not be sacrificed by being too prescriptive in setting goals and specifying products from the program. They believed that the serendipity factor must not be lost from the breeding program.

For the sake of brevity, the plan is not presented in this final report. Priorities for breeding are given below and a copy of the plan can be obtained if needed. In addition, an article printed in the Australian Citrus News provides a summary of the plan [see Sykes, S.R. (2004) Breeding new Australian varieties – where we are headed. Australian Citrus News Vol. 80 (Feb/March 2004), 6-7.].

The key priorities for the National Citrus Scion Breeding Program highlighted in the Breeding Plan.

Priority 1 - Seedless, easy-peel, juicy and sweet fruits

- Very early and early-maturing varieties for export and also regions that are frost prone.
- A mid-season replacement for Ellendale in Queensland.
- A general requirement for new, good coloured, sweet, juicy and seedless mandarins for export and domestic sales.
- Late-maturing varieties for export to specific marketing windows in the August-to-October period.

Priority 2 - Sweet, seedless easy-to peel sweet oranges:

- Dual purpose (fresh fruit and juice) for late winter/spring period.
- Earlier Valencia types for niche markets, eg Japan.
- Sweet oranges with high Brix (12º+) regardless of Brix:Acid ratio.

Priority 3 - Sweet grapefruits for juicing.

- White-flesh types that are sweet and not bitter

6.5 Overseas collaboration

At the first meeting of the CSBRC in March 2000, it was agreed that possible collaboration with Spanish researchers should be explored to enhance the overall project, including the gene transformation research, and capitalise on any advantages that may result from Northern vs. Southern Hemisphere interactions. The breeding team undertook this suggestion and entered into discussions with the group at Moncado led by Dr. Luis Navarro. Various documents and agreements were prepared and discussed, but in the end it became clear to the group that unless a less formal system could be devised, a whole of project-to-whole of project approach would become stifled by bureaucracy. Thus it was decided that specific components of the research could be addressed via a collaborative approach. One such area envisaged was the gene technology research. The suspension of this area of work, however, in mid-2003 has resulted in no further activity as far as Spain is concerned. Future collaboration with Spain may occur when the conventional hybridisation component of the program is ready to start testing selection overseas.

Efforts to establish an overseas collaboration have more recently been focused on the Peoples' Republic of China and CSIRO has been exploring possibilities for mutually beneficial collaborative research with Professor Deng Xiuxin's group at Huazhong Agricultural University, Wuhan.

6.6 Annual breeding and evaluation meeting

At least one member of the research team attended the annual meeting in 2000-2003. Attendees were as follows:

- August 2000, Koltunow, Smith and Sykes (in addition a meeting of the CSBRC was scheduled for the day before the meeting, which allowed committee members to attend the annual meeting as well.
- August 2001, Smith and Sykes
- July 2002, Smith and Sykes
- June 2003, Smith and Sykes

6.7 Extension activities

Various extension activities were undertaken during the course of CT00012. These activities included:

- A range of posters were produced and used at industry field days and ACG conferences. The posters were also provided to HAL for use at industry meetings that the breeding team were unable to attend.
- Local and national field days were attended whenever possible and details concerning the breeding program were made available to attendees. These included the annual Mildura field days where the breeding program was presented as a part of the Riverlink display.
- Aspects of the breeding program were discussed at the National Citrus Varieties Day held in Renmark during June 2003. The principal researchers presented PowerPoint talks at the day and there were also displays of fruits from the breeding program (see fig 3.5). In a similar

manner, fruits from the program have been displayed to growers at field walks and on less formal occasions, often on a one-to-one basis.

- Information concerning the breeding program has been extended to industry via the Australian Citrus News and regional grower newsletters. These have included an annual contribution to the ACN special edition Citrus Insight. Articles in industry print media during the project have included:

Sykes, S.R. (2001) Evaluation of CSIRO-bred hybrids from the National Citrus Scion Breeding Program – CT00012. Proceedings – Citrus Breeding & Evaluation Workshop, NSW Agriculture Dareton.

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- Information concerning the breeding program has been extended to industry as other opportunities have arisen. For example, CSIRO hosted a meeting for growers and other industry representatives at Merbein during November 2002 as part of an exercise to present a vision for citrus improvement to the citrus industry. On other occasions, for example, information concerning the program has been presented on a less formal basis to visiting industry groups both at Merbein and Bundaberg.

6.8 Citrus breeding roadshow

A major extension exercise involving a series of citrus industry/grower briefings was held during the period August to October, 2003 in the major citrus production regions of SA's Riverland, Sunraysia, the MIA and Queensland's central Burnett. The briefings were presented by Alan Whyte, who is Sunraysia's grower representative on the CSBRC, Malcolm Smith and Steve Sykes. The aims of these briefings were to provide levy payers (primarily citrus growers) with an overview of the National Citrus Scion Breeding Program (CT00012), give an update on progress, the outcomes of the review held by Luis Navarro during February 2003 and present a summary of the formal breeding plan developed by the Citrus Scion Breeding Reference Committee including the directions being taken in developing a commercialisation strategy. The briefings followed on from the presentations given at the New Varieties Day held June 19 at Renmark, where an overview of program goals was delivered. It was anticipated that the series of briefings in August-October would capture growers who did not attend the New Varieties Day at Renmark.

An important output sought from the briefings was to receive feedback from industry at grass-roots level on the appropriateness of the breeding program's goals. In addition, it was hoped that industry would comment on other aspects of the breeding program.

The meetings were organised largely with the help of Cittgroup coordinators and were advertised via Cittgroup networks, ie through email, fax and word-of-mouth, and via local media, print and radio.

A detailed report was prepared and submitted to HAL and the CSBRC following the briefings. This report included industry's responses to a questionnaire handed out at the meetings. While further details can be obtained by reference to the report the key points to arise from feedback after the briefings were:

- A majority (60%) of citrus growers attending were aware of the breeding program.
- Over 95% of growers attending understood the objectives of the program.

- Over 95% of growers who provided feedback agreed with the goals and aims of the program and the products targeted by the research.
- Over 95% of growers who provided feedback believed that the program was on the right track.
- Growers in the MIA suggested increased emphasis could be placed on sweet orange varieties.

6.9 Project review February 2003

The National Citrus Scion Breeding Program is a long-term project and has been reviewed every three years. In February 2003, Dr. Luis Navarro undertook a very detailed review of the program.

This exercise required a good deal of preparation by the breeding team and Dr. Navarro who visited Merbein, Adelaide and Bundaberg over a period of 10 days. At the end of his visit, Dr. Navarro presented HAL with his report that addressed the terms of reference for the review. Many aspects of the program were praised in the report and Dr. Navarro recommended that the breeding program be maintained. He also suggested some areas that could be tackled in a different way and the breeding team have taken these on board and tried to accommodate his suggestions where practicable. One significant change as already described was the recommendation to suspend further work on genes for seedlessness until a discovery is made on how to use these genes to modify commercial mandarin varieties. For the sake of brevity, the reader is referred to the review report for further details.

APPENDIX 1

Molecular Breeding for Seedlessness in Citrus

July 1992-June 2003

Achievements, issues and future prospects concerning molecular breeding for seedlessness for Australia's Citrus Industry.

A report prepared by Anna Koltunow upon the suspension of funding from HAL for the biotechnology component of CT00012

Molecular breeding for seedlessness in Citrus

July 1992-June 2003.

Achievements, issues and future prospects concerning molecular breeding for seedlessness for Australia's Citrus Industry.

This report contains a summary of work carried out at CSIRO Plant Industry's Adelaide Laboratory from July 1992-June 2003. This project pioneered molecular breeding of citrus in Australia where none existed previously. It established procedures to regenerate transgenic citrus (West Indian lime) plants and examine the utility of the introduced genes for citrus crop improvement. It successfully identified, produced and tested genes that can prevent seed formation or reduce seed size and number in citrus fruit. Proof of concept has been successfully obtained in West Indian Lime for all but one gene that requires further tailoring. Transformation and regeneration of mandarins and mandarin hybrids has not been successful. This project, part of the citrus scion breeding program will not be funded after June 2003 at this stage. Therefore, the intent of this document is to provide a clear statement of where the work is at, the utility of the genes, their ownership, current bottlenecks in citrus molecular improvement and routes to market for varieties developed in the future utilizing these genes.

Project Coordinator: Prof. Anna Koltunow
Report submitted May 13th 2003.

Contents

Summary

Quality characteristics that make fresh citrus fruits desirable include few or preferably no seeds, ease of peeling, juiciness, size and flavour. Identifying and bringing all of these characteristics together by traditional breeding is difficult because citrus is highly heterozygous and has a long generation time compared with annuals. Introducing genes into existing commercial varieties that confer individual quality traits and thus enhance their value is an attractive option. We have focused on the identification, design and testing of genes that can be used to induce seedlessness.
The initiation of seed and fruit development normally requires pollination and fertilization and the two processes are normally linked in plants, i.e. if seeds don't start to grow neither will the fruit. But some citrus cultivars have a natural capacity to form a fruit even though fertilization and seed development does not occur. This capacity is highly prized in citrus fruit and is a trait called parthenocarpy. Some times parthenocarpy is masked because the flower has fertile pollen so fertilization can occur. In such varieties one way to promote parthenocarpy is to stop pollen formation in the plant and to limit the chance of fertilization by good orchard design. Many citrus varieties are, however, completely dependent on pollination and fertilization to set both seed and fruit and here we have to stop the growth of the seeds soon after fertilization occurs and limit seed growth. An alternative is to identify and/or tailor genes that can confer the trait of parthenocarpy itself and make fruit growth independent of pollination and fertilization when introduced into that variety.

We have successfully developed new genes and tested existing genes for their efficiency in inhibiting pollen and seed development. Initial tests of different tailored versions of these genes were performed in annual plants to guide selection of those most likely to succeed in citrus. We had to establish procedures to introduce genes into citrus from scratch because none were available in Australia and the entire technology was just developing world wide in 1992. We are, and remain, world leaders in controlling seed development in fruits. To this day citrus transformation is not easy and the capacity to do it at all, or with a particular efficiency, is cultivar dependent, i.e. one method does not fit all.

We have now thoroughly examined how these genes work in our test citrus West Indian lime, selected for proof of concept studies because it is very seedy, flowers and fruits quickly and it is not parthenocarpic. Total seed numbers were reduced from an average of 17/fruit in controls to as few as 2/fruit in plants containing the genes. Average seed size was also decreased. From crosspollination with wild type plants we know the strengths and weaknesses of these genes and can predict where they will and won't be useful. Despite significant efforts we have been unsuccessful to date in transforming mandarins, and mandarin hybrids a bottleneck experienced in laboratories around the world until very recently. Some work will continue with students that will study in the Koltunow laboratory.

We have discovered a gene in Arabidopsis that appears to confer the trait of parthenocarpy when it is altered in structure. Inducing parthenocarpy in citrus is not a matter of inserting the Arabidopsis gene. We have had to find the citrus counterpart of the Arabidopsis gene and alter its function in an appropriate manner. Here we are being guided by information concerning the examination of the function of the Arabidopsis gene from the linked Aushort project. We will have some preliminary tailored genes tested in fast growing annual plants by June 30th 2003. The male sterility gene still needs linkage to the final citrus gene and proof of concept in citrus will then need to be obtained. We will attempt to introduce the constructs into lime by mid August 2003 with the utilization of additional CSIRO resources.

The application of these genes individually or in combination for decreasing seed number and inducing seedlessness in commercial seedy varieties is discussed and an update on citrus transformation technology is provided. Their future use in conventional citrus breeding programs should also provide unprecedented flexibility to amalgamate other fruit quality characteristics to develop new citrus cultivars.

Background on pathways of fruit development in citrus - clues for the kinds of genes needed for seedlessness.

The initiation of seed and fruit development in most flowering plants requires pollination and fertilization. The subsequent growth of both seeds and fruit is generally interdependent, i.e. if some seeds don't start to grow neither will the fruit! In some cases fruit size might also be smaller if only a few seeds form. However, seedless cultivars exist, are selected for in breeding programs and are maintained by vegetative propagation.

Our fundamental scientific studies of the developmental physiology of fruit and seed growth in a range of plant species has provided some understanding of the reproductive features that are required to enable the expression of the seedless character. We do not have a genetic blue print mapping out all of the plant genes and their appropriate interconnections that control flowering and all aspects of fruit development as yet. However, using the knowledge available, my strategy has been to develop a set of genes that when introduced in the right combinations in different citrus cultivars should affect the appropriate development of the flower, fruit and seed such that the fruit contains fewer, smaller seeds or preferably no seeds at all. To make this point clearer in the context of generating seedless citrus fruit, I have provided some examples of what we know about fruit and seed formation in different kinds of citrus cultivars and listed the kinds of genes controlling particular reproductive features that are needed to enable seedless fruit development.

Murcott mandarin like many citrus cultivars is absolutely dependent on fertilization and seed initiation for fruit set. It sets more than 20 seeds/fruit. To decrease seed number an introduced gene would have to stop the formation and growth of seeds soon after they initiate. Alternatively a gene would have to be introduced that uncouples the strong linkage between fruit and seed growth so that parthenocarpic fruit development can occur in the absence of seed initiation. Murcott makes pollen so that would also need to be stopped at the same time so that there is no chance for fertilization and seed development.

Clementine mandarin (self-incompatible form) will set seedless or seeded fruit depending on how it is managed on farm in relation to the other citrus cultivars around it. Grown in blocks isolated from other pollen fertile citrus varieties, Clementine will set a seedless fruit. This is because Clementine makes fertile pollen, but its own pollen is not able to fertilize and produce seeds. The mere act of its pollen touching the female part of the flower, however, is sufficient to trigger seedless fruit development. This is called stimulatory parthenocarpy and it gives rise to seedless fruit. However if the Clementine is pollinated by foreign citrus pollen, Valencia for example, it will set seedy fruit. Thus, unlike Murcott, Clementine has in its genetic background two useful features; its own pollen is defective in making seeds, and it has genes that facilitate fruit development in the absence of fertilization. But Clementine is not a bullet-proof seedless cultivar because if pollen from other citrus cultivars is not kept away it will set seedy fruit.

Washington navel and Satsuma mandarin are consistently seedless. They have in-built mechanisms that stop fertilization and they both have that all important genetic capacity for parthenocarpy that enables the fruit to form even though the seed will not grow.

Washington Navel cannot make pollen very well, particularly in cool conditions and the growth of the navel, which is a deformity that starts during flower formation, physically prevents access of the pollen for fertilization of seeds.

Satsuma mandarin does not make pollen and it also has genes that enable parthenocarpic fruit development.

Rationale for four gene types for the production of seedless citrus.

Given what we know about the reproductive features of different citrus cultivars described above, to maximize the chance of success of inducing seedlessness using gene technologies, I reasoned, utilizing accumulating scientific knowledge, that a minimum portfolio of four types of genes was required to induce seedless fruit formation in a range of citrus cultivars (see references 1-5).

1. A gene to inhibit pollen formation and function. This gene is required to stop fertilization and seed initiation. It would also be useful in uncovering the genetic capacity for parthenocarpy in those cultivars that might have it but where it lies hidden because pollen fertility leads to seed set. (How do we tell if a citrus cultivar has parthenocarpic ability? Simple answer: stop the fertilization of the flowers and if fruit is set regardless and it is seedless then the cultivar has natural parthenocarpic ability. There are two ways to do this. Manually remove the male parts of flowers on the tree and bagging these emasculated flowers to isolate them from bees/insects carrying pollen from adjacent citrus trees. Slade Lee's team (QDPI) found Murcott was not parthenocarpic using this method. Caging of pre-flowering trees with an insect-proof mesh cage to prevent mass access of insects that do the work of transferring the heavy and sticky citrus pollen from the male to the female floral organs is another way of testing for parthenocarpy. Sykes and Possingham found that Imperial, Ellendale and Kara mandarin are parthenocarpic using combinations of the caging and manual emasculation methods).

An early version of this gene was recreated from research work I carried out on pollen development in the USA, in collaboration with PGS (now the global biotechnology company AVENTIS), and tested in annual plants. The technology and permission to use the original gene under licence by CSIRO was obtained from Aventis. The gene (TA29/BARNase) inhibits pollen formation because the TA29 part originally from tobacco allows switching on of the linked BARNase in a specific group of pollen forming cells and it stops their growth. The capacity of this gene to inhibit pollen development had not been tested in citrus when we came up with the concept for citrus seedlessness.

2. Seed abortion (75%). A gene to stop seed formation soon after fertilization that targets the growth of internal parts of the seed and stops growth and expansion of 75% of the seed when one copy is introduced. This gene would be useful in reduction of seed number in those cultivars that lack parthenocarpy and need to retain some seeds per fruit for set.

Two versions of the gene were made and performed well in tested in annual plants. Version 1 is called CG1/BARNase. The CG1 part from soybean was obtained from Bob Goldberg (University of California in Los Angeles under an MTA). CG1/BARNase stopped growth in embryo and endosperm cells of annual plants. Surprisingly we found this one gene could induce both male sterility and decreased seed numbers. The combination of the seed abortion and different levels of male fertility was a useful in terms of seed manipulation. It told us to expect a range of reduction in seed numbers from 50-75% or more in citrus. Furthermore, pollen transmission of genes is an issue of GM crops but we found from cross-pollination studies that even in partially fertile plants

transmission of the introduced gene to the environment and surrounding plants was going to be unlikely or reduced (see references 12, 14). Version 2 had an extra piece 400 bases long inserted into the CG1 part from pea. These were obtained from TJ Higgins CSIRO Plant Industry and version 2 was called CG1400BARNase. This gene was made because the CG1/BARNase gene's mode of action in stopping formation in embryo structures restricted the plant regeneration pathway to those where shoots formed. The extra 400 base piece was tested to see if it would extend regeneration to include an embryogenic route because information at the time suggested it might. It did not, and the action of version 2 was the same as version 1 in tested annual plants (refs 12, 14). Other citrus seed switches were examined (reference 9). The effort to develop them outweighed their advantage compared to those already tested. (Simpler progressive summaries of this information are in references 11, 13, 15, 17)

3. Seed abortion (100%). A gene to stop seed formation soon after fertilization in 100% of seeds because it stops growth of the female structures that immediately surround parts of the developing seed. The concept here was to let fruit and seed set begin but if seed formation could be stopped in all seeds soon afterwards a relatively imperceptible soft trace might remain while fruit development progressed.

A range of 5 promoter or switch signals from seeds of different plants were linked to the BARNase portion and tested in annual plants to see if they could exert the desired effect over the course of the project. All but GLUB1 from rice did not. GLUB1 was obtained from Fumio Takaiwa (National Institute of Agrobiological Resources, Tsukuba. Japan). A set of truncated GLUB1 promoter fragments were also tested. One of these - GLUB1.3/BARNase caused 100% seed abortion in tobacco and the formation of soft, gummy seed traces. Other switches from orchid were also examined in a different way by an honours student (ref 8) but proved too general.

4. Identification and tailoring of genes to induce parthenocarpy (refs 16, 17, 19, 22, 23, 24). Genes involved in the events of parthenocarpy were completely unknown. Abed Chaudhury at CSIRO devised a screening method in Arabidopsis to find mutants. One of these was examined by Adam Vivian-Smith during his PhD. HAL contributed to this project. A key gene involved in fruit initiation was identified. It is a complex gene in structure and in the range of functions it performs in plants. The mutation in the Arabidopsis gene does not prevent the protein being made but it is altered in its activity. This we have found out by studying the activity of the Arabidopsis gene in the Aushort project. We initially thought we could use this Arabidopsis gene to confer parthenocarpy in citrus but we will need to use the equivalent citrus gene and alter its function. We have now isolated the citrus counterpart of the Arabidopsis gene. We are being guided by information concerning the examination of the function of the Arabidopsis gene from the linked Aushort project as to how to tailor the citrus gene. We will have some preliminary tailored genes tested in fast growing annual plants by June 30th 2003. The male sterility gene still needs linkage to the final citrus gene and proof of concept in citrus will then need to be obtained. Additional resources have been obtained from CSIRO to do this until mid August 2003, but we certainly do not have the resources in the Aushort project to carry out any further citrus transformation.

Aushort and Citrus: It should be made clear that the Aushort project is a large gene discovery project looking for genes in Arabidopsis that might affect fruit and vegetable colour, shape and form. The parthenocarpy gene is but a small part of this project and is being used as a bait to find other genes that will be useful in altering fruit form. The Arabidopsis genes can be used to find genes in particular crops if industries align with the project and specify what they want done in their crops and provide the linked resources to do so. For more information contact Russell Soderlund at HAL.

The functional utility of 3 of the genes in citrus: proof of concept of their action in West Indian lime and their potential for reducing or eliminating seeds in other cultivars.

Male Sterility Gene TA29BARNase in WI Lime:

1. Causes pollen sterility when introduced to WI lime.
2. We found it has a dual function of limiting seed set if the plant containing it is pollinated by foreign pollen. Instead of the expected 20 seeds/fruit only 4-6 form! It provides added protection against seeds forming from stray pollen!
3. Is a useful gene to partner with the citrus parthenocarpy induction gene being developed to ensure seedless fruit formation.

Applications for commercial citrus:

1. A very useful gene for introducing to seedy cultivars that have parthenocarpic ability. Examples include Kara, Imperial, Ellendale and seedy parthenocarpic mandarin x sweet orange hybrids coming out of the scion breeding program.
2. This gene in combination with the citrus parthenocarpy gene being developed might produce seedless Murcott or seedless versions of cultivars that currently lack parthenocarpic ability.

Below: Pictures of West Indian lime flowers without the male sterility gene (top) and containing the gene (bottom). Note lack of yellow pollen in the bottom flower.

Reducing seed numbers

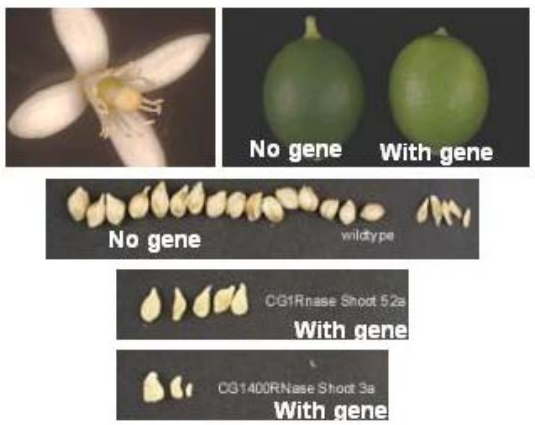
CG1BARNase/CG1400BARNase in Lime:

1. These genes performed in WI Lime just as in the tested annuals. They have a dual function to stop seed growth and also reduce pollen fertility. Some trees were partially fertile, others male sterile and seed number was reduced from 20 to 8, 5 or three per fruit depending on the tree. Importantly many seeds were flattened. Fruit size was fine and brix:acid ratio as per control trees.

Applications for commercial citrus:

1. A very useful gene for reducing seed numbers in varieties that have no parthenocarpic ability and need some seeds per fruit so the tree crops well commercially (eg Murcott).
2. Use of these genes in particular varieties will require selection of regenerated plants to select those forming fruits with very low seed numbers.
3. The reduction in pollen fertility will restrict flow of the gene to the environment and other plants. This is another plus for its use in commercial cultivars.

Below: Male sterile (left) and partially fertile flowers are produced after the introduction of the genes. Normal size fruit forms in plants. West Indian lime is very seedy and has 20 or more fat seeds (No gene panel). Different trees containing the gene produce fruit with reduced numbers of seed and many seeds are flattened and reduced in size. 8, 5 or as few as 3 seeds per fruit have been observed (with gene panels).



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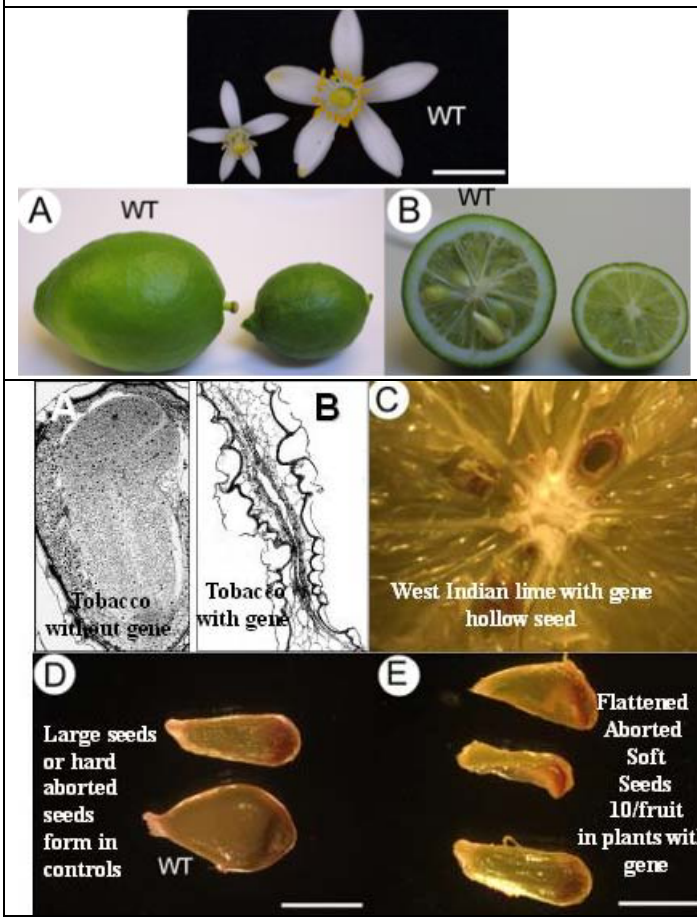

Reducing seed numbers

GLUB1/BARNase in Lime:

1. Plants containing the construction were very difficult to regenerate. The trees were stunted and formed smaller flowers and fruit.
2. The gene caused pollen and seed sterility. Very few large seeds were formed and the traces of medium size (10/fruit) were generally soft and gummy.

Applications for commercial citrus:

1. Not as useful as the two other genes. Significant effort would need to be placed in regenerating plants.
2. May have benefit in decreasing size of larger seedy pummelo or grapefruits and novel hybrids.
3. Plant performance in the field would need to be assessed. Suspect that plants with this gene may be sensitive to stressful growth conditions.

Gene introduction and plant regeneration procedures in citrus: state of the art

Citrus transformation procedures depend on the explant or the tissue that will be subjected to the introduction of the gene and the mode by which the gene is introduced. The source of tissue used to take up the gene and the route by which tissues containing the gene are regenerated will determine whether the tissue comes out juvenile and thus slow to flower and fruit or if it will have a mature character and flower and fruit within 18 months to 2 years. The same source tissue does not work for each cultivar. A rate limiting step is also the capacity to regenerate cells so that they develop into viable tissues containing the introduced gene efficiently - this is often a major bottleneck that is also cultivar dependent.

There are two methods of gene introduction into the cells of citrus:

1. DNA can be introduced by shooting tiny microscopic pellets coated with the gene (called particle gun bombardment). This is not very efficient in citrus. We pioneered this method for citrus in the early to mid 90's when transformation methods were first being worked out for all

citrus cultivars. We switched to the method below as it was the only way we could get genes into West Indian lime. Everyone uses the method below now.

2. Agrobacterium-mediated transformation is the most successful means. It relies on first putting the gene into the bacterium in a special region flanked by particular “border sequences”. Agrobacterium by virtue of its biology does the job of putting the gene between the border sequences into the plant genome.

Source tissues, routes of regeneration and the maturity status of regenerated tissues.

1. **Seed epicotyl explants**. This method is only good for polyembryonic cultivars that produce nucellar seedlings and NOT for monoembryonics. (ie West Indian lime, Satsuma, rootstocks, Kara, etc). You germinate the seeds in the dark which makes this tissue more amenable to taking up the DNA. You cut up the long epicotyls and dip then into a solution of bacterium containing your gene. Regeneration occurs by shoots growing out of the ends. The percentage of shoots containing the gene is very variable amongst cultivars. They are cut off and allowed to form roots, or micrografted in culture. The tissue regenerated is also juvenile and will take a long time to flower unless the plant has a naturally short juvenility period. Grafting onto mature rootstocks might help. We use this method for lime and were instrumental in developing the growing in the dark method with our Japanese colleagues. Most people in the world use this method now including the Florida teams (see below). No one has yet transformed mandarins using this method.

2. **Internode explants from mature tissues**. This method should work for BOTH mono and polyembryonic cultivars. Tissue is grafted and you wait some two or three months for soft new growth to occur. The pieces are cut off, sterilized, recut and dipped into Agrobacterium solution then cultured on growing media for shoots to form. The shoot formation takes many months and not all shoots may contain the gene. A single experiment, once material is ready, may take 6-9 months. The recipes for shoot regeneration are not universal and not known for all cultivars. Some cultivars do not make shoots readily using this method when placed on media. It requires a lot of trial and error to determine which cultivars can best form shoots from freshly grafted material to have any hope of making transgenics, and then follows the battle with their capacity to survive and grow. This is the method developed by Luis Navarro’s lab and other labs are now using it in Brazil and Japan. To date no one has worked out the right culture media for mandarins using this method. Luis’ lab is trying different recipes for Clementine. The advantage of this method apart from its utility for both monoembryonic and polyembryonic cultivars is that the material regenerated is mature and the plants should flower and fruit within 18 months to two years. It requires a large back up of grafted material growing in a glasshouse at the ready and a large space devoted to each cultivar of interest. Each grafted plant can only be cut back twice and used before the procedure fails completely - even in sweet orange where the method works well in labs that have the procedure up and running. Thus grafts need to be kept constantly growing. Recently a method using smaller, thinner explants of sweet orange has been successful.

3. **Embryogenic callus**. This method is mainly applicable to polyembryonic cultivars. It requires the establishment of an undifferentiated white mass of cells that is capable of forming embryos and the polyembryonic cultivars are the source of such material as they arise from nucellus. This callus needs to be sub cultured and the process is labour intensive. The callus often needs to be regenerated from scratch as it loses its embryogenic capacity. We used embryogenic callus in our original studies trying to develop plant transformation methods coupled with particle-gun bombardment. The callus can be washed in a solution containing the bacterium to introduce the gene and then allowed to make embryos by placing it on a medium. These embryos are allowed

to germinate into seedling-like structures capable of forming shoots and roots. The plants regenerated are juvenile. The process is lengthy because of the extended regeneration route. Recently Deng and his co-workers in China transformed Ponkan mandarin using this method. They have regenerated small plantlets containing the male sterility gene (published late in 2002) I have been invited to visit this laboratory in November 2003. These are the only people who have transformed mandarin.

4. **Protoplasts**. Protoplasts are plant cells with their walls removed by a mix of enzymes. They are very fragile and subject to shock and rupture in response to changing media compositions. Genes can be introduced to them by electroporation which is a change in current causing openings to form in the membranes or they can be treated with Agrobacterium. Embryogenic callus is the typical source of protoplasts (see 3 above) as it is difficult to make protoplasts from citrus leaves so this method is often restricted to polyembryonic cultivars. The cell wall needs to be allowed to grow back before they can form callus, then embryos and then shoots. Again, plants regenerated via this procedure are juvenile. Very few laboratories use this method as it is very labour intensive and the efficiency is low. To date no-one has transformed mandarins using this method.

Plusses and minuses of transformation procedures in relation to the genes for seedlessness and seed reduction we have developed.

The gene constructions that contain a BARNase portion would kill cells if they are inadvertently leaky or turned on at a critical time in plant regeneration. On the other hand there is also severe selection pressure to silence the gene or to rearrange it to protect the cell. Deng and co-workers have put the male sterility gene into Ponkan and regenerated plantlets. They have not carried out experiments (southerns) to ensure the gene was inserted intact as we have done. Even in our own experiments we noticed some West Indian lime plants containing this gene only had a portion of it and it was non-functional.

CG1BARNase/CG1400BARNase and GLUB1/BARNase CANNOT be used in transformation and regeneration procedures that utilize an embryo phase as the gene switches on in embryos and the regeneration procedure cannot occur. Our studies have shown this is the case. They need to be introduced by methods 1 (etiolated seedlings) and 2 (mature internode tissue above).

In the review of our project in 1999 the review team recommended that to avoid problems with juvenility and with potential leakiness we try and transform the commercial citrus cultivars by developing or using a mature tissue transformation method. Appropriate methods had not been developed at that time. (For all of the recommendations at that time see the section “Recommendations of the three reviews.” below).

Our attempts and difficulties in regenerating and mandarins and mandarin hybrids containing the genes.

The 1999 reviewers were well aware of our previous methodologies and attempts to transform mandarin embryogenic callus cultures - and that no-one had as yet transformed mandarin. In accordance with the 1999 review we set about attempting mature tissue transformation in addition to the ongoing work. There was also a requirement to balance this work with proving the functional efficiency of the genes in a citrus model for proof of concept.

An avenue to learn the latest developments in mature tissue transformation occurred with the organization of a two month visit of Sandra Protopsaltis to Spain to the laboratory of Luis Navarro at IVIA in late 1999. This was co-funded by CSIRO and HAL. IVIA experiments with sweet orange showed that the tissue needed to be freshly grafted and could only be harvested twice before regrafting began. We prepared hybrid constructs prior to the visit as Dr Navarro recommended that the genes be fused also to green fluorescent protein (GFP) to help identify transformed shoots. We obtained the version of the gene recommended and did this. One transformation experiment normally takes 6-9 months and others are started as possible in the interim material permitting. During her visit Sandra was exposed to the procedure in terms of transforming mature lime and also sweet orange in a staggered fashion that overlapped with experiments they were doing in Spain. She bought back explants from Spain under AQIS quarantine. The shoots began to grow and were GFP positive but died back. The experiment was repeated a number of times using Australian sourced explants and the same happened. As the genes without the GFP had given rise to transgenic West Indian limes we sequenced the version of the GFP that was provided and found that it was being targeted to the inappropriate compartment of the cell, causing toxicity. In respect to consumers and sales the associated presence of the glowing GFP in the plant would not be a desirable feature and the use of the GFP was terminated. The issue of what cultivars the gene should be introduced into was discussed with our advisory committee and the Fremont mandarin came up as it had parthenocarpic tendencies and performed well in warmer climates. Experiments introducing this gene via the seed method failed, Fremont was not shooting efficiently and none of the shoots obtained contained the gene. A questionnaire and survey at one of the ACG meetings failed to identify a suitable consensus concerning a cultivar for transformation, this is still the situation.

Following further discussions with the research team and advisory committee members it was agreed to focus on transforming the mandarin hybrid cultivars arising from the breeding program that were very promising but seedy and had a parthenocarpic tendency. The introduction of the male sterility gene would therefore add value to the outcomes of the breeding program.

The cultivars - 8 in all - were sent to Adelaide in conjunction with various activities associated with trialling on grower properties. Explants taken from these plants, that had been recently grafted, showed the plants had variable shooting efficiencies. We knew we could not transform all of them, but those that produced the most shoots would stand the best chance.

The amount of the material had to be scaled up and there was a delay for two reasons, firstly these are not released commercial cultivars and the plant numbers were increased manually on site to avoid problems with PVR etc. Secondly and importantly, one PC2 glasshouse was demolished to rebuild a new, larger facility. Interim arrangements, with the requirement to structurally alter the culture facility, resulted in mite infestations and delayed many experiments for a number of agencies at the CSIRO site. These were resolved but required reestablishment of material and stocks and the experiments continued.

Sandra Protopsaltis took maternity leave in July 2002, and to maintain continuity she was quickly replaced by Adrienne Gregg to keep the work on track. This required some retraining but three cultivars were identified that showed the most promising shooting efficiencies: 2916cc, 2336cc, and 2514cc.

Experiments to transform and introduce the male sterility gene into these have not yet proven successful in the set of experiments prior to the 2003 review. A larger scale experiment is underway now. Modifications to each set of experiments to increase shooting efficiency and potential for transformant regeneration have occurred throughout all of our procedures. The current experiment

is utilizing a set of modifications reported to increase transgenic shoots from mature sweet orange explants twofold. An indication of the success of this should be obtained by September 2003.

Citrus transformation services elsewhere

Obviously different laboratories are focused on different aspects of citrus improvement at the molecular level. In most cases the work concerning gene development has been run in parallel with appropriate systems to test the efficacy of the candidate genes. Commercial production is a different issue. While there are services in Horticulture that offer micro-propagation, problems with citrus transformation - particularly the capability to transform mandarin cultivars - means that there are methodologies that still need to be established for the process to be commercially viable. Currently citrus transformation is carried out within the infrastructure of the research laboratories focused on different aspects of citrus crop improvement.

The following information was supplied to me by Dr Jude Grosser of the University of Florida via Email (jwg@lal.ufl.edu) concerning the University's Core Citrus Transformation laboratory that recently opened for business. So far it is only providing services to University of Florida scientists (costs/service listed on the website).
http://www.lal.ufl.edu/core/index/htm

The Core Citrus Transformation laboratory is developing plans for outside use and the cost structure for that has yet to be determined. The facility has two full time staff and one part time staff member. It is managed by Dr Orbovic who is not a permanent staff member and they may lose him if this does not occur. The University of Florida has been supporting the lab on soft money and this will need to change in the long run.

The laboratory uses seedling material for transformation. The same procedure that we use for West Indian lime described above (see seed epicotyl explants and Agrobacterium above). This means that polyembryonic varieties are the main targets and that juvenile transgenic material is regenerated. The time to flower and fruit is dependent on the length of the juvenility period for the given variety. So far they can transform any(?) sweet orange or grapefruit cultivar and some rootstocks including Swingle, Carrizo.

Ownership of genes and the potential to trade them

The TA29BARNase gene is licensed from Aventis and its commercial use will have to be negotiated.

The CG1BARNase and GLUB1BARNase genes were constructed from parts obtained from other sources, some being freely available but others obtained under material transfer agreement (MTA) with freedom to operate for research purposes only. The genes were pieced together under CSIRO/HAL funds and research described and proof of concept demonstrated. Thus if the work will proceed to commercial endpoint, an IP audit will need to be conducted and negotiations with all involved parties will have to occur to obtain freedom to use the genes under a commercial licence. Movement of the constructed genes for research purposes can occur if the research agency requesting them agrees to the stipulations of an accompanying MTA.

The Arabidopsis gene with the potential for parthenocarpic induction is in the public domain and for reasons discussed with HAL the gene will not be patented. The exact mode of how it can best be

used to induce parthenocarpy is being deduced for an appropriate construction. This know how is being developed as part of Aushort. The citrus counterpart is being developed to the extent already stated above - MTAs and the need to negotiate agreements for commercial use apply.

Routes to market if transformation technology is developed for designated commercial cultivar

In order to produce a variety by gene technology and get it to market the following elements are required:

1. A gene capable of conferring the desired trait is available and ready for introduction.
2. Target cultivars for improvement have been identified and prioritised by industry and these are backed by market considerations.
3. An appropriate gene introduction and plant regeneration method has been developed for the target cultivars. This might be via a mature tissue transformation procedure (red in the scheme below) or by a procedure that gives rise to juvenile plants that take longer to flower and fruit. (If not, a strategy to develop appropriate methodologies in a given timeframe needs to be negotiated with appropriate parties and agencies)
4. An up to date IP audit has been conducted and appropriate licences and agreements negotiated for the commercial use of the gene and the appropriate method of transformation technology are in place to ensure commercial release of the cultivar is not blocked.

If 1-4 are in place then the table below, modified from the document CT00012 National Citrus Scion Breeding Program Breeding Plan provides a set of likely timeframes for variety release. The management of the variety, royalty return and other aspects are as described in that document for the release of cultivars bred by traditional hybridization. An appropriate marketing plan scheduled to time with variety release is left to the discretion of industry.

Timeline for plant transformation program from gene introduction to variety naming and release.

Operation	Year																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Introduce gene																	
Regenerate plants, check for gene and graft plants																	
Grow plants with internode checking until flowering-and fruiting																	
First Phase of evaluation of fruits																	
Select and propagate								**									
2nd phase evaluation of multiplied selections																	
Variety release																	

The timeline depicted by red shading assumes there is a mature tissue transformation system already in place for the variety that will be improved by inserting that gene. The timeline will be extended if an embryogenic or juvenile tissue transformation system has to be used and there will be an extended time period while trees reach phase change whereby flowering and fruiting occurs (green shading). However, if the gene has been introduced into a variety where management and performance conditions are well known the second phase evaluations may not be so critical, and it might be possible to begin variety release in year 8 (***). Thus, depending on the variety being improved and the tissue type transformed: the naming and release of a new variety may take from 8-to-11-to-17 years.

Recommendations of the three reviews of the citrus scion breeding program with respect to molecular breeding and the actions taken.

Final reports for all HRDC and HAL funded projects are also available through Gerard McEvilly HAL in addition to the final reports of three external reviews of this project commissioned by HRDC/HAL. HAL also has a record of the minutes for the regular researcher teleconferences and the subsequent milestones reports summarized from them.

The November 1995 Review:

Progress: Development of fundamental tools for the initiation of the project. Generated and maintained embryogenic Kara and Murcott callus and capacity to regenerate plantlets from it. This is highly labour intensive. Plants are juvenile, taking 6 or more years to flower and fruit. Insertion of a male sterility gene into genome of Kara callus by biolistics (particle-gun bombardment) but plants could not be regenerated. Tested reconstructed male sterility gene in tobacco, worked. Tested one seed destruction gene, did not reduce seed size. Carried out work to identify some gene promoters active earlier in development. Constructs made and will be tested in tobacco and Arabidopsis. The regeneration method relied on using callus. As these genes are designed to cause cell death in the appropriate tissues, leakage would not enable regeneration. So there were two problems: problems with the transformation and problems with testing the genes in citrus. Need to be sure the genes will work. Preliminary experiments with etiolated stem segments showed the mandarins had a low shooting efficiency. May be able to develop West Indian lime as a test system as they regenerated well. Could focus on Agrobacterium transformation.

1995 Review Recommendations:
Technical review to be carried out in a further 3 years to assess progress in overcoming 2 main obstacles:

1. Development and testing of genes to target seed formation in model plants Arabidopsis and tobacco.
2. Consideration should be given to develop West Indian lime as a citrus test system in addition to Arabidopsis as it would lead to greater confidence in gene function and the capacity to yield a seedless mandarin. WI Lime has a shorter juvenile period.

Concluded that the work ranks with the best in the world. May not achieve the results of seedless mandarins required in the time frame expected by the citrus industry.

The February 1999 Review

Progress against 1995 recommendations:

Obstacle 1 was overcome: Successfully developed genes in three categories that might induce seedlessness and tested them in model Arabidopsis and tobacco. In addition: New gene parts from orchid and also citrus seed storage proteins assessed by Matthew Lynch, an honours student and deemed unsuitable. An Arabidopsis mutant donated by CSIRO's Abed Chhaudhury which exhibits parthenocarpy is being characterized by a PhD student-Adam Vivian-Smith. The gene has been mapped. Researchers future projects discussed were: considering development of two additional gene types to increase the portfolio of genes for seedlessness and cloning the gene mutated in the parthenocarpic Arabidopsis plant.

Obstacle 2 was overcome: Procedures were developed to transform and regenerate transgenic West Indian lime plants to act as a test system for the currently developed genes. Some transgenic plants containing these genes had been regenerated. Gene stability and vegetative plant performance after grafting was being assessed by PCR. (Obviously none of the West Indian lime plants had flowered for assessment.) Researcher future projects discussed were: increasing the primary West Indian lime transformants for each gene to 20 different plants per gene and producing 10 grafted replica plants of these.

1999 Review Recommendations:

1. Dr Koltunow to produce the full set of 20 individual primary West Indian lime transformants for each construct for assessment, under GMAC conditions, of the effectiveness of these genes in producing seedless citrus fruits. If GMAC approved the transgenic lines could be sent to Bundaberg to assist flowering.
2. Continue with the difficult task of transforming mandarin, lemon and sweet orange varieties. Best achieved by transformation of mature tissue to avoid the long juvenile period before flowering.
3. Funds to allow the further development of collaborations with the Spanish group would have benefit to Australia
4. Using the Arabidopsis model system is undoubtedly the most powerful way of identifying genes of possible commercial value in citrus. This is less directly applied, but fundamental to any future progress in citrus breeding by biotechnological approaches.
5. New research is needed to better understand what genes are available for incorporation into citrus breeding programs. This research is expensive and requires funds other than direct industry funds. HRDC and the citrus industry should consider the possibility of funding PhD scholarships in this area-perhaps the ARC/SPIRIT awards.

Concluded: This program is of the highest scientific quality and the group is at the forefront in woody plant transformation and is backed by extensive experience in plant molecular genetics. Genetic manipulation of woody perennials is in its infancy and Dr Koltunow is a world leader who has the best-available chance of putting the Australian citrus industry significantly ahead of the rest. However, this is still a risky area, much more so than the classical hybridization program.

The Feb 2003 Review

Progress against 1999 recommendations:

1. Primary West Indian lime transformants and replicates were produced for the three kinds of introduced genes as specified. Significant troubles regenerating the GLUB1Rnase transgenics because of leakiness. New data specific to citrus was obtained with the genes and it has been summarized in the preceding sections. This was difficult because all the trees are in the greenhouse, crosspollination was required and flowering and fruiting was not synchronous. Nevertheless significant data on hundreds of fruit samples counted with respect to seed number and juice quality (brix:acid). The transgenics are stable during plant growth and also following repeated grafting. Proof of concept of the utility of the genes in citrus now unequivocally demonstrated. Plants not sent to Bundaberg due to complexity of the process and the requirement for insect proof screenhouse not present.
2. Sandra Protopsaltis spent 2 months (Late October-December, 1999) in Valencia Spain being taught the mature tissue transformation method. Not sufficient to undergo the entire procedure

but enough to learn key points on sweet orange. CSIRO funded half and half came from the funded grant. Half of material left in Spain and half material returned did not regenerate plantlets, in either location. The GFP marker construct recommended by the Spanish targeted to wrong region and proved toxic. Subsequent experiments done without it. We began setting up the grafts and material in 2000 for the mature method. Attempts to transform Mandarin sweet orange hybrids, pineapple sweet orange and Fremont via the mature tissue method failed. We have not been able to effectively establish this methodology in the Adelaide lab. This technique is only effectively practiced in the Navarro lab and now in a Brazilian laboratory (only on oranges). Similarly Luis Navarro's laboratory has not yet transformed mandarins using the mature tissue methodology. Focus specifically on transformation would be required to get the genes into an industry specified cultivar but, depending on the cultivar, we could not guarantee success.

3. Avenues for a collaborative agreement with Spain were supported by the Citrus Scion Breeding Industry advisory group. A collaborative agreement between CSIRO and INIA (IVIA's parent body) delayed and considered unworkable. A new agreement between CSIRO and the Laboratory of Luis Navarro is being finalized. Steve Sykes will be the principal CSIRO contact.
4. The FWF gene was cloned by the PhD student Adam Vivian-Smith in partnership with Anna Koltunow and other CSIRO staff. CSIRO sought legal advice on the route to patent at significant cost to CSIRO. While proof of concept that FWF had the potential to optimize fruit and seed set had been demonstrated in Arabidopsis, evidence was not available at the time it could sustain the development of parthenocarpic fruit in other than the model plant. As FWF is a large and complex gene required for a range of plant growth phenomena this is an easy task- our initial hypotheses have not proven correct and this is requiring a set of new gene constructions. Search for the citrus FWF genomic clone and gene continued as part of the citrus project. Remarkable progress has been made on the citrus gene.
5. Expansion of the use of the Arabidopsis model system as a means to isolate key genes modulating fruit and seed set, fruit and vegetable colour, growth, shape and form was developed as a larger cross-disciplinary Aushort project funded by HAL. Anna Koltunow played a lead role in the preparation and establishment of the project, won by competitive tender. Understanding the mode of action of FWF is only a very small part of AusHort's "Key Genes for Horticultural Markets" project. It is however providing the know how concerning how to most effectively manipulate the citrus counterpart of the Arabidopsis FWF gene to induce parthenocarpy. Other genes influencing fruit set and growth are coming out of the AusHort initiative that will benefit a range of Horticultural crops.

Reviewer Recommendations: Luis Navarro has prepared his review to be tabled to HAL - an extract follows:

4.6. Genetic transformation at Adelaide

This project has focused on the development of three genes and on the method for transforming mandarin.

a) The project has been very successful in demonstrating that transformed West Indian lime containing a male sterility gene (MS1) do not produce viable pollen. This has been observed both in the original plants held in Adelaide and in propagated plants grown under containment in an approved screenhouse facility at Merbein. The usefulness of this gene for citrus improvement is very clear. Examples of current male-sterile citrus varieties are navel and Satsuma. It would be ideally applied to parthenocarpic, autocompatible varieties (such as some of the Merbein progeny). The gene is privately owned and used under licence.

b) The project has also been successful in demonstrating that a gene targeted to stop seed growth after fertilization reduces the number and size of seeds produced by the citrus model plant West Indian lime. It is not clear yet if this gene affects the growth of zygotic and/or nucellar embryos. This gene can also be very interesting for citrus breeding if the reduction of seed number production also occurs in the same proportion in low seeded cultivars.

c) Another important objective of the project was the application of an Arabidopsis gene to induce parthenocarpy in citrus. This work has experienced some delay in relation to the anticipated milestones, because of the unexpected complexity of the gene. Nevertheless a genomic clone and a near full length cDNA clone for the citrus homologue in Valencia sweet orange has been obtained and partially sequenced. The development of the parthenocarpy-inducing gene has required the production of a modified Arabidopsis clone. This construction will be introduced in Arabidopsis next June and if it works properly, it will be later introduced into the citrus model plant West Indian lime. (A good model because it is not parthenocarpic). If this gene proves its efficiency in inducing parthenocarpy in Citrus it will be an important achievement, and it will have an additional value because its IP rights will belong to Australia.

d) However, the project failed to establish transformation protocols for mandarins or tangors, despite the efforts and resources addressed to solve this problem. This was a necessary step to prove that transformation technologies could play an important practical role in the National Citrus Breeding Plan. The inability to transform mandarins is not a surprise, since work in other laboratories, including IVIA, also has failed so far to transform this species. The situation in Spain is that mature Clementine cells can be transformed, but they do not regenerate or do so with low efficiency. The problem may be solved quickly or require several years of intensive work, based on experience with genotypes of citrus and other woody species.

The transformation work in Adelaide also was faced with an additional problem of small greenhouse space without the best conditions to grow citrus and an inadequate periodical supply of new plants. Under these conditions the material produced was not probably of the best quality and this may have precluded the group from successfully achieving the transformation protocol for mature sweet oranges that was used as control.

An alternative approach to transform mandarins is by protoplast transformation. This approach is being used at Dr. J. Grosser's laboratory in Florida, Dr. Deng's laboratory in China and in my laboratory in Spain. This approach can only used for polyembryonic genotypes for which nucellar embryogenic callus is available. In addition, the resulting plants have juvenile characters and an expert on protoplast technology is essential to carry out the work. This approach is too complex at the moment to be used as breeding routine tool.

Recommendations February 2003 review

In this situation and taking into account the limitation of the resources it is difficult to make specific recommendations, because there are different alternative possibilities for continuation of the work at Adelaide that should be discussed among parties involved.

- From the point of view of the citrus breeding program the logical approach could be to concentrate all efforts in mandarin transformation, in order to be able to use the technology generated during several years to produce new improved selections. This approach will require a careful selection of the genotypes to be used. However, the establishment of a transformation protocol cannot be guarantee in three years. International collaboration does

not seem feasible at this stage because efficient transformation protocols for mandarin transformation are not yet available at any laboratory and the complexity of IP rights.

- In the case that citrus transformation work will be stopped, provisions should be made for a quick restart if an efficient protocol for mandarin transformation became available from other laboratories.

- Continue working only with the parthenocarpy gene to investigate if it is effective to induce parthenocarpy in the citrus model plant West Indian lime. This work should be done only if the constructions underway prove its efficiency in the already planned work in Arabidopsis. This approach will allow finishing the work that has been done with the gene over the last three years. In addition, it is quite inexpensive, since it will need only one or two transformation experiments, the molecular characterization of no more than ten independent transgenic lines and their analysis for parthenocarpic traits that can be done easily at Merbein. If the gene is proven effective, Australia will hold its patent rights and eventually it could be used to obtain improved genotypes.

- Change the objectives of the Adelaide team towards the genomic characterization of the available breeding progenies at Merbein by DNA microarrays and other genomic technologies. This could be very interesting, particularly if an international consortium on citrus genomics is established in the coming meeting in Valencia. However, this is a very preliminary proposal made by Dr. Koltunow during discussions at Adelaide and it is beyond the commitments of the present review.

Where to from here? CSIRO perspective.

HAL has subsequently informed us by letter that funds to the citrus biotechnology project will not be provided in the 2003/04 financial year.

The CSIRO position stated in ACIL Consulting's document on Plant Breeding Investments with special reference to Citrus, potatoes and strawberries (16 March 1999 page 10) has not changed:

"CSIRO make it abundantly clear, that in the absence of HRDC [HAL] funding its resources would be redirected, particularly the biotechnology work, to other areas outside citrus….. To maintain a citrus focus, citrus industry funds seem to be needed". Funds in the AusHort gene discovery project cover proof of concept in Arabidopsis and rapidly growing model plants. There are insufficient resources in the Key Genes project to cover citrus or any other crop unless the industry aligns with the work for specific purposes as apple has done. The citrus industry had that linkage in this project aimed at improving citrus via gene transformation. The citrus molecular improvement program would only be reactivated following negotiations with industry, that would be dependent upon the outcomes required, the capability to deliver them by CSIRO and the availability of appropriate funds.

Nevertheless given the investment of the industry in this research program and the sudden stoppage of the work a number of activities have been put in place to maximize their completion with the remaining funds and some additional CSIRO resources. Other activities were being put in place and will occur in the following 12 month period because of commitments CSIRO has made to the students or visitors from external agencies. The various activities are summarized below.

- CSIRO Plant Industry is investing funds to cover a postdoc until mid August 2003 to ensure that the citrus parthenocarpy gene progresses to an appropriate stage for tests in annuals and West Indian lime.

- An Iranian Masters student is arriving in October 2003 for a period of 9 months and will investigate further aspects of the mandarin hybrid transformation work after September 2003. He is being trained in transformation and will be involved in introductions of any modified versions of the citrus parthenocarpy gene into West Indian lime for proof of concept. This will be funded by Iran and CSIRO sources. Plantlets generated will be looked after by CSIRO until they flower.

- Prof Koltunow will be visiting Prof. Deng's laboratory in China in late October 2003 (SARS status permitting) to give lectures under the invitation Prof Deng. He has put the male sterility gene into Ponkan. This work began after discussions with Koltunow and Deng at the First International Citrus Biotechnology Congress in Eilat Israel where both Deng and Koltunow were invited speakers. To do this Koltunow informed Deng he would need to obtain permission from Aventis and presumably he has done so.

- A Chinese postdoc from the laboratory of Prof. Deng who has recently regenerated transformed Ponkan mandarin is seeking to come to work in the Prof. Koltunow's laboratory and is particularly interested in gaining fundamental molecular knowledge on seed and fruit development if the if funds can be obtained from Chinese government sources. The period would be a maximum of one year. The project would be co-funded by CSIRO and the nature of it would need to be discussed.

- An Iranian PhD student fully funded by the University of Iran for tuition and by CSIRO for operating expenses has been attracted to work in the Koltunow laboratory in partnership with the University of Adelaide. His project would extend the knowledge base and use of genes involved in fruit initiation in citrus. His acceptance by the university is contingent on obtaining passes in English and he is to undergo the relevant examinations. His final project will also be dependent upon discussions with his University of Adelaide Academic supervisor Prof Margaret Sedgley.

- CSIRO is currently redrafting the collaborative agreement with the laboratory of Dr Luis Navarro of IVIA in Spain to promote exchange and collaboration on citrus crop improvement. Dr Steve Sykes (CSIRO PI Merbein) as the coordinator of the Citrus scion breeding program will be the main contact for the organization of meetings, telephone hook ups etc. Dr Sykes convenes regular meetings with the Citrus Scion Breeding Industry advisory committee. Updates on matters pertaining to the above items can be tabled as agenda items and reported as required in these steering committee meetings.

Given that funds to the project are to cease in the 2003/04 financial year, the current project staff, Adrienne Gregg (substituting for Sandra Protopsaltis while on maternity leave) and Miva Splavinski will be terminated. Transgenic lime replicate trees will be destroyed according to OGTR guidelines once the manuscripts relating to the generation of these plants have been accepted. This will also be the case with replicate plants currently housed at Merbein. Specific knowledge concerning citrus molecular improvement resides with Prof. Koltunow and a technician Sandra Protopsaltis. Sandra will be involved in some of the interim training of the Iranian Masters student but will be shifted onto other research projects as part of her core activities. Any citrus plants regenerated as part of the Iranian Masters and PhD students will be maintained until they flower.

Various genes and DNA stocks, libraries, primers, DNA samples and tools are safely maintained in long-term storage at CSIRO Plant Industry's Adelaide laboratory. Various indicative tools, etc are found in Appendix A.

Appendix A: Stock take of Genes and tools for citrus research.

(not exhaustive)

1. Suite of gene constructions:

In plasmid AND Agrobacterium vectors (appropriate primer sets for identification)
TA29RNASE (Aventis), truncated EVTA29RNASE, truncated EVTA29DTA.
CG1RNASE, CG1400RNASE, (and versions fused to GUS) GLUB1RNASE,GLU3ARNASE, GLUB1248RNASE (and versions fused to GUS)
SERKRNASE, ProlamineRNASE (and versions fused to GUS)

2. Citrus libraries

Ellendale genomic library, Valencia genomic library, Valencia pistil cDNA library, Valencia small seed cDNA library, Valencia large seed cDNA library.

3. Proteins and antibodies

Purified globulin seed storage protein
Purified 22kD and 33kD subunits of citrus seed globulin proteins
Antibodies to the 22kD and 33kD subunits of citrus globulin proteins

4. Genomic clones

11.1 and 12.2 citrus FWF genomic clones

5. cDNA clones

Early citrin seed storage protein gene D3
Two other later citrus seed storage protein cDNA clones
cDNA clone 800bp of FWF

6. Citrus DNA for analysis by PCR

Genomic citrus DNA purified from 15 different citrus cultivars and
Genomic citrus DNA from 50 different progeny segregating for parthenocarpy in the Merbein collection.

7. A+mRNA stocks from Valencia leaf, flower, root seedling, fruit and seed.

Appendix B: Letter regarding FWF to Russell Soderlund.

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Adelaide Laboratory

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PW5760
NSS/mam

2 November 2001

Mr R Soderlund
Commercialization and New Business
Development Manager
Horticulture Australia Limited
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SYDNEY NSW 2000

Dear Russell

AusHort Gene Discovery Initiative

The discussions between CSIRO and HAL last year (?) concerning the establishment of AusHort contemplated the application of an emerging technology on fruit without fertilization as a means to uncouple fruit formation from fertilization to produce seedless fruit and in the long-term to modulate fruit set using a gene called FWF (fruit without fertilization).

Earlier this year we had initiated action through our patent attorney for a new Australian provisional patent application to be developed around FWF. Proof of concept that this had the potential to optimise fruit and seed set had been demonstrated in *Arabidopsis thaliana*. Evidence was not available at the time to sustain the development of parthenocarpic fruit in other than the model plant.

Subsequent to our instructions to our patent attorney to develop a provisional patent specification we had sought further advice on the value of proceeding with the provisional in view of the fact that there may be insufficient available information warranting a patent application in this area, as the claims would be speculative only and not enabling. The advice from Davies Collison Cave in this instance confirmed our view and it was decided not to proceed any further with the provisional application. Accordingly, action has been taken to terminate any development of the patent application with a preference to publication of the results.

The decision to terminate patent protection in this instance is not seen as any way to the detriment of our AusHort projects in the area of modulation of yield, shape, texture and seed content. Nor does it alter our intention to seek appropriate protection in plant species of commercial importance. Our efforts will continue to focus on the ability to control fruit set in horticultural areas of commercial interest.

For your information.

Regards,

N.S. Scott
Deputy Chief

SCO\aushortlet.doc

Appendix C: ACG position on GMOs

The Australian Citrus Growers Inc. Position on Gene Technology

April 2002

No genetically modified organisms are used in the production of Australian citrus fruit.

The Australian citrus industry produces some of the best citrus in the world. It is a vibrant, export-oriented industry with a strong interest in utilising cutting edge technologies to remain internationally competitive. In 2000-01 the value of Australian citrus exports had grown to $191 million.

ACG Inc is committed to exploring new developments in all areas of science and apply these where there are clear benefits to consumers and acceptance by society.

As part of the ongoing, long-term citrus research program, Australian scientists are using plant gene technology to develop the ability to introduce seedlessness into existing citrus varieties. Conventional breeding technologies are also being utilised to develop new varieties. Similar organisations in the USA, Spain, Japan, China, and Israel are also investigating the use of gene technology for potential application in citrus production.

At present this work is at the research stage and further development and rigorous testing is required before any commercial production could be considered.

Citrus growers are monitoring this research with interest, as well as the safety, openness and quality assurance aspects. Australian citrus growers encourage an ongoing dialogue between industry, scientists, government, regulators and consumers. Commercial adoption of this research would only occur after a thorough assessment of the technology, its products and benefits.

Acknowledgements

The project arose from early discussions with Michael Keenan and David Pullar of the then HRDC with John Possingham, Nigel Scott, Anna Koltunow and Steve Sykes of CSIRO. The project captured the attention of the industry panel and Andrew Green was instrumental in becoming familiar with the concepts of the technology and communicating it to the panel before the project was funded. It gained early attention from its audacious goal and its high operating costs relative to other more field oriented projects. Its per annum cost has remained relatively stable over the funding period and Jolyon Burnett and Gerard McEvilly maintained a watchful eye ensuring it ran to a tight budget and in line with industry expectations. We are indebted to the Australian Citrus Industry for their vision in supporting this project and for the camaraderie of the Citrus researchers across Australia. We would particularly like to thank Ian Tolley of Tolley's Nurseries in SA and Chris Nathaniel of Tropiculture NT for their unfailing supply of lime seed and to Ivor Caudle (Caudles citrus nursery) for troyer seeds and assistance with grafting, Bob Shaw (Queensland Citrus Improvement Committee) for troyer seed, and Colin and Maria Gardner at Gardner's nurseries at Merbein for citrange rootstocks. Finally, Dr Abed Chaudhury (CSIRO) for his inspiration in devising mutational screens for parthenocarpy in the model plant Arabidopsis so that insight into the regulation of this process so central to seedless fruit development could finally begin to be dissected.

CSIRO staff, students and visiting scholars associated with the project

CSIRO staff directly associated with the project:

Prof. Anna Koltunow, Dr Steve Sykes, Kathleen Soltys (technician), Dr James Bond (postdoc), Peter Brennan (Experimental Scientist), Sandra Protopsaltis (technician), Georgina Smith (technician), Miva Splawinski (technician), Adrienne Gregg (technician) Matthew Tucker (postdoc). (Current staff in bold).

Students trained in association with the project:

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Dr Adam Vivian-Smith PhD University of Adelaide 2002.

Visiting Scientists/International collaborators with major input:

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Dr Tetsushi Hidaka. Okitsu Japan.
Dr Omura. Okitsu Japan.
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Prof. Robert Goldberg. University of California, Los Angeles. USA.
Luis Navarro IVIA, Valencia Spain.
Behrooz Golyan, Iran (Visiting Oct 2003-June 2004).

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Appendix 2

Tables referred to in Chapter 4 - Triploidy (DPI&F, Bundaberg)

Table 4.1a Number of pollinations, and percentage fruit set, for crosses performed in the 1998 season, BRS.

Female Parent (seed)	Male Parent (pollen)											
	Emperor 4X		Murcott 4X		Joppa 4X		Parramatta 4X		4X Pummelo A		Total	
	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set
Aurora	100	*9*	30	*10*	100	*7*	34	*9*	58	*2*	322	*7*
Aust Clem	21	*71*	20	*25*	42	*36*	35	*63*	3	*0*	121	*39*
Burndale	23	*9*	10	*0*	37	*16*	22	*5*			92	*7*
De Nules	20	*50*	17	*47*	17	*82*	20	*50*			74	*57*
Ellendale	111	*13*	40	*15*	144	*15*	30	*17*	50	*2*	375	*12*
Fina	21	*29*	20	*45*	26	*38*	20	*50*	15	*0*	102	*32*
Fremont	47	*45*	19	*37*	35	*31*	40	*33*	20	*5*	161	*30*
Hickson	60	*2*	25	*8*	78	*8*	41	*2*	4	*0*	208	*4*
IM111	100	*20*	30	*40*	100	*17*	30	*20*	59	*8*	319	*21*
Imperial	100	*47*	30	*47*	99	*21*	62	*29*	20	*20*	311	*33*
Kinnow	100	*36*	30	*23*	100	*36*	40	*40*	60	*7*	330	*28*
Marisol	10	*20*	10	*0*	13	*31*	20	*30*	2	*50*	55	*26*
Nova	42	*29*	11	*18*	35	*26*	31	*29*	4	*0*	123	*20*
Oroval	10	*50*	10	*30*	15	*40*	10	*0*			45	*30*
Umatilla	106	*30*	40	*33*	123	*24*	43	*42*	20	*10*	332	*28*
Wilking	41	*32*	32	*44*	63	*33*	43	*9*	12	*8*	191	*25*
Total	912	*31*	374	*26*	1027	*29*	521	*27*	327	*9*	3161	*25*

Table 4.1b Number of pollinations, and percentage fruit set, for crosses performed in the 1999 season, BRS.

Female Parent (seed)	Male Parent (pollen)																					
	Emperor 4X		Murcott 4X		PummeloA 4X		PummeloB 4X		Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Total	
	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set
Aust.Clem	60	*50*	60	*30*	60	*5*	100	*7*													280	*23*
de Nules	100	*38*	100	*28*	100	*5*	100	*6*													400	*19*
Ellendale			100	*26*																	100	*26*
Hickson	100	*13*	100	*8*	100	*0*	100	*0*													400	*5*
IM111	100	*38*	100	*27*	100	*19*	100	*10*													400	*24*
Imperial	100	*39*	100	*10*	100	*30*	100	*42*													400	*30*
Wilking	100	*40*	60	*38*	100	*16*	100	*11*													360	*26*
PummeloA 4X			4	*0*					2	*0*	1	*100*	1	*0*	2	*0*	4	*0*	1	*0*	15	*14*
PummeloB 4X			2	*0*					2	*0*	2	*50*	2	*0*	2	*0*	50	*24*	2	*50*	62	*18*
Total	560	*36*	626	*19*	560	*13*	600	*13*	4	*0*	3	*75*	3	*0*	4	*0*	54	*12*	3	*25*	2417	*21*

Table 4.1c Number of pollinations, and percentage fruit set, for crosses performed in the 2000 season, BRS.

Female Parent	Male Parent (pollen)																														
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fortune		Total	
(Seed)	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set
Arrufatina	30	*40*	2	*0*	30	*40*	30	*43*	30	*57*	30	*67*																			152	*41*
Aust.Clem	50	*64*	30	*7*	50	*14*	50	*18*																							180	*26*
Burndale	40	*18*	23	*4*	50	*2*	40	*3*																							153	*7*
Corsica 1	5	*80*	5	*80*	5	*80*	7	*86*	5	*80*	5	*100*																			32	*84*
Corsica 2	20	*70*			10	*60*	10	*40*	15	*73*	11	*36*																			66	*56*
Daisy	40	*18*	38	*24*	30	*7*	30	*7*	30	*47*	32	*38*																			200	*23*
deNules	100	*64*	20	*0*	100	*14*	100	*16*																							320	*24*
Ellendale	100	*31*	25	*0*	100	*9*	100	*13*																							325	*13*
Encore	30	*30*	35	*6*	30	*20*	30	*17*	30	*33*	30	*27*																			185	*22*
Fallglo	10	*50*			10	*20*	10	*40*	10	*0*	10	*0*																			50	*22*
Fortune	100	*6*	10	*0*	100	*0*	100	*0*	100	*2*	100	*1*																			510	*2*
Hickson	100	*12*	40	*18*	100	*5*	100	*4*																							340	*10*
IM111	100	*42*	55	*36*	100	*11*	100	*9*																							355	*25*
Imperial	100	*66*	30	*20*	100	*31*	100	*22*																							330	*35*
Katherine 5[1]	20	*N/A*	8	*0*	20	*N/A*	20	*N/A*	20	*N/A*	20	*N/A*																			108	*0*
Katherine 6[1]	15	*N/A*					10	*N/A*			20	*N/A*																			45	
Katherine 9[1]							3	*N/A*	10	*N/A*	12	*N/A*																			25	
Wilking	100	*N/A*	22	*0*	100	*N/A*	100	*N/A*																							322	*0*
4X PumeloA													43	*2*	37	*8*	39	*0*	21	*0*	68	*7*	44	*2*	41	*12*	35	*3*	47	*9*	375	*5*
4X PumeloB													35	*29*	34	*18*	27	*33*	25	*24*	46	*24*	27	*15*	26	*50*	16	*31*	29	*34*	265	*29*
Total	960	*42*	343	*14*	935	*22*	940	*23*	250	*42*	270	*38*	78	*15*	71	*13*	66	*17*	46	*12*	114	*16*	71	*9*	67	*31*	51	*17*	76	*21*	4338	*23*

1 Numbers are branch numbers not flower numbers for these cultivars, multiple pollinations per branch.

Table 4.1d Number of pollinations, and percentage fruit set, for crosses performed in the 2001 season, BRS.

Female Parent (Seed)	Male Parent (Pollen)																					
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		4X Ortanique		4X Burgess		4X Wilking x Mur4X 95		4X Wilking x Joppa4X 2		4X Wilking x Mur4X 96	
	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set
Arrufatina	25	*60*	25	*24*	25	*36*	25	*36*	25	*44*	25	*68*	10	*10*								
Aust Clem													10	*10*								
Corsica 1	20	*60*	20	*25*	20	*5*	20	*20*	20	*55*	30	*42*	10	*0*								
Corsica 2	20	*35*	20	*70*	20	*10*	30	*13*	30	*27*	30	*7*	10	*0*								
Daisy	30	*47*	25	*28*	30	*13*	30	*7*	25	*4*	25	*4*	10	*0*	13	*23*	5	*20*	5	*0*	8	*13*
De Nules													10	*10*			7	*0*				
Ellendale	30	*77*	25	*8*	30	*23*	30	*7*					10	*0*	6	*0*						
Encore	50	*2*	44	*0*	30	*3*	30	*0*	30	*3*	30	*7*	10	*10*			5	*0*				
Fallglo	25	*8*	25	*0*	25	*0*	30	*0*	30	*0*	25	*0*	10	*0*								
Fortune	30	*0*	30	*0*	30	*0*	30	*0*	24	*0*	30	*3*	10	*0*								
Hickson	30	*23*	30	*13*	30	*3*	30	*0*					10	*0*								
IM111	30	*47*	30	*17*	30	*10*	30	*10*					10	*10*			12	*33*				
Imperial	30	*63*	30	*67*	30	*23*	30	*30*					10	*0*								
Wilking	30	*23*	30	*10*	30	*10*	30	*10*					10	*20*								
4X PummeloA																						
4X PummeloB																						
Total	350	*37*	334	*22*	330	*11*	345	*11*	184	*19*	195	*19*	140	*5*	19	*12*	29	*13*	5	*0*	8	*13*

Continued…

Female Parent (Seed)	Male Parent (Pollen)																					
	Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fallglo		Fortune		Ellendale		Total	
	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set	No	% set
Arrufatina																							160	*38*
Aust Clem																							10	*10*
Corsica 1																							140	*30*
Corsica 2																							160	*23*
Daisy																							206	*14*
De Nules																							17	*5*
Ellendale																							131	*19*
Encore																							229	*3*
Fallglo																							170	*1*
Fortune																							184	*0*
Hickson																							130	*8*
IM111																							142	*21*
Imperial																							130	*37*
Wilking																							130	*15*
4X PummeloA	11	*9*	10	*0*	45	*4*	10	*0*	10	*20*	15	*13*	10	*20*	11	*0*	13	*0*	10	*0*	7	*29*	152	*8*
4X PummeloB	50	*18*	36	*0*	50	*4*	50	*2*	20	*0*	50	*8*	25	*4*	20	*5*	20	*10*	50	*8*			371	*6*
Total	61	*14*	46	*0*	95	*4*	60	*1*	30	*10*	65	*11*	35	*12*	31	*3*	33	*5*	60	*4*	7	*29*	2462	*15*

Table 4.1e Number of pollinations, and percentage fruit set, for crosses performed in the 2002 season, BRS.

Female Parent	Male Parent (pollen)																													
	4X WilkingxMurcott 83		4X Fremont		4X Emperor		4X Murcott		4X WilkingxMurcott4X95		4X Minneola		4X Burgess		4X Orlando		4X EllendalexJoppa4X0396		4X EllendalexMurcott4X78		4X EllendalexMurcott4X79		4X WilkingxJoppa4X109		4X WilkingxJoppa4X2		4X WilkingxJoppa4X35		4X WilkingxJoppa4X73		4X WilkingxMurcott4X96	
(seed)	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set
Aust.Clem					42	*48*	42	*5*	15	*13*	42	*38*	42	*45*											42	*0*					55	*22*
Corsica1					50	*54*	6	*50*																								
Corsica2					21	*24*	30	*27*	4	*25*			20	*40*											20	*45*					20	*45*
Daisy	11	*0*	10	*10*	42	*29*	42	*29*	40	*38*	20	*30*	42	*5*			20	*20*	20	*5*	3	*0*	20	*15*	40	*13*	9	*11*	20	*5*	42	*0*
DeNules	9	*22*			25	*44*	30	*30*	30	*40*	30	*37*	40	*48*			10	*0*	10	*20*			10	*40*	30	*33*			10	*10*	50	*32*
Ellendale			?	*N/A*	40	*28*	35	*14*	30	*17*	30	*0*	30	*17*			20	*0*	20	*40*			20	*20*	28	*29*					28	*4*
IM111					30	*47*	35	*20*	30	*17*	30	*30*	30	*37*			10	*30*	10	*20*			10	*30*	30	*27*					30	*37*
Imperial					30	*40*			30	*0*	30	*70*	30	*27*	4	*100*							2	*0*	30	*30*					30	*27*
Temple					14	*7*	10	*0*	21	*0*	20	*0*	15	*0*											20	*5*					20	*0*
Wilking	10	*40*	15	*40*	50	*24*	40	*25*	35	*17*	40	*18*	50	*22*			33	*3*	26	*15*			25	*32*	50	*16*			20	*5*	50	*0*
4X PummeloA																																
4X PummeloB																																
4X Wilking x 4X Murcott No 95																																
Total	30	*21*	25	*25*	344	*34*	270	*22*	235	*18*	242	*28*	299	*27*	4	*100*	93	*11*	86	*20*	3	*0*	87	*23*	290	*22*	9	*11*	50	*7*	325	*18*

Continued….

Female Parent	Male Parent (pollen)																													
	98N246		98N501		98N613		Afourer(Pressler1)		Daisy		Dancy		Ellendale		Fremont		Fortune		Murcott		Page		Sunburst		Temple		Wilking		Total	
(seed)	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set	No	%set
Aust.Clem																													280	*24*
Corsica1																													56	*52*
Corsica2																													115	*34*
Daisy	5	*0*																											386	*13*
DeNules																													284	*30*
Ellendale																													281	*17*
IM111																													245	*29*
Imperial																													186	*37*
Temple																													120	*2*
Wilking			7	*0*	6	*0*																							457	*17*
4X PummeloA							30	*13*	22	*9*			21	*14*			16	*25*	14	*21*	22	*0*					23	*35*	148	*17*
4X PummeloB													35	*17*											11	*9*			46	*13*
4X Wilking x 4X Murcott No 95									20	*20*	21	*19*			40	*18*	20	*10*	25	*0*			20	*0*			20	*15*	166	*12*
Total	5	*0*	7	*0*	6	*0*	30	*13*	42	*15*	21	*19*	56	*16*	40	*18*	36	*18*	39	*11*	22	*0*	20	*0*	11	*9*	43	*25*	2770	*23*

Table 4.1f Number of pollinations, and percentage fruit set, for crosses performed in the 2003 season, BRS.

Female Parent (seed)	Male Parent (pollen)																			
	4X WilkingxMurcott 83		4X Fremont		4X Emperor		4X Murcott		4X WilkingxMurcott4X95		4X Minneola		4X Burgess		4X Orlando		4X PummeloB		Total	
	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No	%set	No	%set
Arrufatina [1]	20	*25*	24	*42*	30	*40*	35	*26*	10	*40*	10	*60*	32	*56*	10	*60*			171	*44*
Aust Clem[1]			39	*21*	70	*33*	40	*13*	50	*18*			50	*14*					249	*20*
Corsica 1[1]			24	*0*	50	*20*	35	*20*	50	*10*	8	*75*	50	*10*	7	*14*			224	*21*
Daisy	50	*22*	50	*2*	50	*34*	50	*8*	50	*8*	50	*0*	50	*28*	50	*18*			400	*15*
DeNules[1]	48	*4*	50	*8*	50	*2*	50	*6*	50	*6*	35	*6*	50	*6*	35	*11*			368	*6*
Ellendale	35	*9*	36	*8*	50	*6*	50	*4*	50	*6*	50	*12*	50	*4*	26	*15*			347	*8*
Fallglo							20	*0*									30	*0*	50	*0*
Fortune							50	*0*									50	*0*	100	*0*
Hickson	50	*2*	50	*4*	50	*4*	50	*6*	50	*4*	50	*2*	50	*2*	50	*4*			400	*4*
IM111	50	*12*	50	*8*	50	*4*	50	*2*	50	*6*	50	*6*	50	*8*	50	*0*			400	*6*
Imperial	10	*10*	30	*17*	50	*16*	50	*20*	50	*16*	30	*23*	50	*22*	30	*20*			300	*18*
Temple	20	*10*	30	*0*	30	*0*	30	*0*	30	*0*	30	*0*	30	*17*	30	*0*			230	*3*
Wilking[1]	10	*0*	15	*13*	50	*12*	35	*9*	50	*14*	40	*23*	50	*14*	10	*0*			260	*11*
Total	293	*10*	398	*11*	530	*16*	545	*9*	490	*12*	353	*21*	512	*16*	298	*14*	80	*0*	3499	*12*

1 Dry fruit and/or seed contamination in these cultivars.

Table 4.1g Number of pollinations for crosses performed in the 2004 season, BRS. % set to be determined in 2005.

Female Parent	Male Parent (pollen)																													
	4X WilkingxMurcott4X 83		4X Fremont		4X Emperor No1		4X Emperor No2		4X Murcott		4X WilkingxMurcott4X 95		4X WilkingxMurcott4X 96		4X Minneola		4X Burgess		4X Orlando No1		4X Orlando No2		4X Excelsior		4X Dancy		3X/4X Hansen		4X Bakers Sweet		Total	
(seed)	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set	No.	%set
Arrufatina	25		14				30		30		30		30		24		30				40		10				30				293	
Aust.Clem	20		30		35				30		30		30		30		30				25						30				290	
Corsica1	30		30		30				25		30		30		30		30										30				265	
Daisy	30		27				30		30		30		30		30		30				30		30		5		30		30		362	
DeNules			30				30				30		45		30		30				25						30				250	
Ellendale	14		22		30				30		30		30		30		30		30				20				30		30		326	
Encore	20		20		30				30		30		30		9						30										199	
Fallglo							19		20						14		30														83	
Fortune	30		30				30		30		16		10		15		30				30						10				231	
Hickson	30		20		40				30		48		50		40		40		35				10				60				403	
IM111	30		30				30		30		30		30		30		30				30						30				300	
Imperial	12		23		30				7		30		40		30		30		12		20						30				264	
Temple	20		19		20		20		23		20		25		20		20		20								20				227	
Wilking	10		30				27		20		30		30		30		35		29								17				258	
Total	271		325		215		216		335		384		410		362		395		126		230		70		5		347		60		3751	

Table 4.2a Numbers of plump seeds sown and total embryos rescued, for crosses performed in the 1998 season, BRS. (All material embryo-rescued except where indicated).

Female Parent (seed)	Male Parent (pollen)											
	Emperor 4X		Murcott 4X		Joppa 4X		Parramatta 4X		4X Pummelo A		Total	
	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued
Aurora	10	19	2	6	6	37	5	11	0	1	23	74
Aust Clem	6	145	0	45	11	200	28	220			45	610
Burndale	8	7			2	35	0	4			10	46
De Nules	5	81	1	144	12	210	3	96			21	531
Ellendale	8	109	2	60	9	156	8	32	0	3	27	360
Fina	5	40	1	79	15	70	10	48			31	237
Fremont	9	112	3	30	11	87	7	111	7	30	37	370
Hickson	0	13	1	12	5	43	0	3			6	71
IM111	49	122	21	68	36	152	22	43	5	18	133	403
Imperial	40	203	11	56	12	67	18	84	1	4	82	414
Kinnow	**100**	178, **410**	0	106	**7**	247,**?**	10	234	29	97	146	437
Marisol	0	12			0	40	0	31	0	0	0	83
Nova	5	88	0	18	17	89	10	42			32	237
Oroval	2	26	0	25	1	28					3	79
Umatilla	17	26	2	7	23	45	16	24	3	0	61	102
Wilking	22	91	27	129	33	109	49	171	5	5	136	505
Total	286	1094	71	785	200	1368	186	1154	50	158	793	4559

Bold, 'Plump' column: some seed sown
Bold, 'Rescue' column: flat seed sown

Table 4.2b Numbers of plump seeds sown and total embryos rescued, for crosses performed in the 1999 season, BRS. (All material embryo-rescued except where indicated).

Female Parent (seed)	Male Parent (pollen)																				Total	
	Emperor 4X		Murcott 4X		PummeloA 4X		PummeloB 4X		Fremont		Page		Kinnow		Ellenor		Daisy		Nova			
	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued	Plump	Rescued
Aust.Clem	250	502	22	269	12	12	7	36													291	819
de Nules	121	948	15	579	2	44	4	70													142	1641
Ellendale			7	386																	7	386
Hickson	0	54	0	41																	0	95
IM111	**155**	78	*50*	153	41	71	**17**	27, **9**													263	302
Imperial	70	211	5	36	17	56	40	123													132	426
Wilking	**145**	242	**55**	143	*51*	97	**42**	42													293	524
PummeloA 4X											10	14									10	14
PummeloB 4X[1]											7	17					*52*	14	*25*	10, **15**	84	31
Total	741	2035	154	1607	123	280	110	271	0	0	17	31	0	0	0	0	52	14	25	0	1222	4238

1 Daisy X Pummelo B 4X: seed number underestimated
Bold, 'Plump' column: all seed sown
Bold, 'Rescued' column: flat seed sown
Italics, 'Plump' column: some seed sown

Table 4.2c Numbers of seed sown and embryos rescued, for crosses performed in the 2000 season, BRS. (Only small embryos rescued, remaining material sown).

Female Parent (Seed)	Male Parent (pollen)																													
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fortune		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina	9	97			28	43	2	60	7	86	17	224																	63	510
Aust.Clem	**117**	286	**5**	20	**7**	23	**20**	13																					149	342
Burndale	8	30	0	1	0	1	1	2																					9	34
Corsica 1	0	70	0	18	0	19	0	25	**1**	87	**2**	90																	3	309
Corsica 2	**2**	236			**4**	30	**3**	26	**5**	208	**2**	57																	16	557
Daisy	8	37	4	85	0	3	3	1	25	92	18	109																	58	327
deNules	**137**	1012			**16**	65	**19**	59																					172	1136
Ellendale	54	135			**5**	25	3	12																					62	172
Encore	1	16	1	1	15	6	4	9	5	37	0	42																	26	111
Fallglo	0	15			0	1	0	6																					0	22
Fortune	6	6							0	2	2	0																	8	8
Hickson	**2**	39	**3**	32	31	11	12	7																					48	89
IM111	141	278	28	204	27	17	18	54																					214	553
Imperial	128	382	3	14	12	61	21	43																					164	500
Katherine 5	239	**607**			9	**98**	0	**19**	356	**638**	77	**819**																	681	2181
Katherine 6	137	**584**					7	**111**			34	**840**																	178	1535
Katherine 9									2	**196**	**16**	77, **177**																	18	196
Wilking	167	**726**			154	**182**	137	**186**																					458	1094
4X PumeloA															29	0					18	**6**	14	**8**	40	**7**			79	**17**	180	38
4X PumeloB													32	0	17	**3**	5	**1**	9	**23**	63	**48**	15	**127**	114	**174**	6	0	70	**51**	331	427
Total	1156	4556	44	375	308	585	250	633	401	1346	168	2181	32	0	46	3	5	1	9	23	81	54	29	135	154	181	6	0	149	68	2838	10141

Bold, 'Sown' column: some plump seed rescued
Bold, 'Rescued' column: flat seed sown

Table 4.2d Numbers of seed sown and embryos rescued, for crosses performed in the 2001 season, BRS. (Only small embryos rescued, remaining material sown).

Female Parent (Seed)	Male Parent (Pollen)																					
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		4X Ortanique		4X Burgess		4X Wilking x Mur4X 95		4X Wilking x Joppa4X 2		4X Wilking x Mur4X 96	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina	3	176	0	48	5	55	1	51	7	115	4	195	3	1								
Aust Clem																						
Corsica 1	5	128	2	51	0	9	0	24	15	93	42	187										
Corsica 2	0	24	5	82	0	0	4	7	23	39	0	16										
Daisy	26	83	10	37	4	6	17	3	0	7	2	6	1	0	7	21	0	6			2	2
De Nules																						
Ellendale	7	188	4	9	1	16	1	9					1	1								
Encore	0	0			2	2			0	0	0	8										
Fallglo	5	3																				
Fortune											1	2										
Hickson	2	60	1	19	1	1							2	0								
IM111	67	164	7	29	7	12	18	10									2	32				
Imperial	23	101	15	74	4	14	4	22					3	0								
Wilking	25	53	1	14	7	5	9	15														
4X PummeloA[1]																						
4X PummeloB[1]																						
Total	163	980	45	363	31	120	54	141	45	254	49	414	10	2	7	21	2	38	0	0	2	2

Continued…

1 All material sown, 'Rescued'=flat seeds.

Female Parent (Seed)	Male Parent (Pollen)																					
	Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fallglo		Fortune		Ellendale		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina																							23	641
Aust Clem																							0	0
Corsica 1																							64	492
Corsica 2																							32	168
Daisy																							69	171
De Nules																							0	0
Ellendale																							14	223
Encore																							2	10
Fallglo																							5	3
Fortune																							1	2
Hickson																							6	80
IM111																							101	247
Imperial																							49	211
Wilking																							42	87
4X PummeloA[1]	0	21			17	5			22	5	37	12	27	34							10	27	113	104
4X PummeloB[1]	114	59			0	0	0	0			31	53	48	9	1	0	21	7	190	128			405	256
Total	114	80	0	0	17	5	0	0	22	5	68	65	75	43	1	0	21	7	190	128	10	27	926	2695

Table 4.2e Numbers of seed sown and embryos rescued, for crosses performed in the 2002 season, BRS. (Only small embryos rescued, remaining material sown).

Female Parent (seed)	Male Parent (pollen)																													
	4X WilkingxMurcott 83		4X Fremont		4X Emperor		4X Murcott		4X WilkingxMurcott4X95		4X Minneola		4X Burgess		4X Orlando		4X EllendalexJoppa4X03'96		4X EllendalexMurcott4X78		4X EllendalexMurcott4X79		4X WilkingxJoppa4X109		4X WilkingxJoppa4X2		4X WilkingxJoppa4X35		4X WilkingxJoppa4X73		4X WilkingxMurcott4X96	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem					25	394	0	**36**	1	31	26	220	33	229																	1	160
Corsica1					7	307	0	40																								
Corsica2					2	39	0	73	0	21			2	92											3	91					2	67
Daisy			1	6	28	104	18	97	19	132	21	35	2	13			34	***5***	2	15			4	33	7	33	0	4	6	0		
DeNules	0	35			18	186	4	196	6	257	20	174	38	293					5	30			11	41	7	133			0	10	7	258
Ellendale			3	117	8	176	2	106	5	47			3	107					3	141			5	76	5	139					1	18
IM111					50	184	12	82	14	36	32	99	29	91			22	6	2	41			6	40	22	87					18	151
Inperial					23	67					23	157	5	36	4	16									12	73					12	68
Temple					0	2																			3	12						
Wilking	7	64	9	81	61	123	24	117	10	64	21	99	58	90			2	1	6	56			35	99	20	75			1	3		
4X PummeloA[1]																																
4X PummeloB[1]																																
4X Wilking x 4X Murcott No 95																																
Total	7	99	13	204	222	1582	60	747	55	588	143	784	170	951	4	16	58	12	18	283	0	0	61	289	79	643	0	4	7	13	41	722

Continued…

Female Parent (seed)	Male Parent (pollen)																													
	98N246		98N501		98N613		Afourer(Pressler1)		Daisy		Dancy		Ellendale		Fremont		Fortune		Murcott		Page		Sunburst		Temple		Wilking		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem																													86	1070
Corsica1																													7	347
Corsica2																													9	383
Daisy																													142	477
DeNules																													116	1613
Ellendale																													35	927
IM111																													207	817
Inperial																													79	417
Temple																													3	14
Wilking																													254	872
4X PummeloA[1]							84	36	35	10			46	57			77	29	12	1							221	102	475	235
4X PummeloB[1]													65	174											4	2			69	176
4X Wilking x 4X Murcott No 95									8	2	4	2			19	8	4	1									10	2	45	15
Total	0	0	0	0	0	0	84	36	43	12	4	2	111	231	19	8	81	30	12	1	0	0	0	0	4	2	231	104	1527	7363

1 All material sown, 'Rescued'=flat seeds. **Bold:** 12 of these 36 flat seeds were sown. ***Bold Italics:*** all 5 flat seeds sown

Table 4.2f Numbers of seed sown and embryos rescued, for crosses performed in the 2003 season, BRS. (Only small embryos rescued, remaining material sown).

Female Parent (seed)	Male Parent (pollen)																			
	4X WilkingxMurcott 83		4X Fremont		4X Emperor		4X Murcott		4X WilkingxMurcott4X95		4X Minneola		4X Burgess		4X Orlando		4X PummeloB		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina[1]	0	39	3	61	7	92	2	55	0	37	2	44	13	162	2	32			29	522
Aust.Clem[1]			1	41	14	142	0	20	0	88			2	52					17	343
Corsica1[1]					0	0	0	0	0	0	0	0	0	2	0	0			0	2
Daisy	6	139	0	0	29	123	1	25	3	30			19	82	11	52			69	451
DeNules[1]	0	14	0	25	0	0	1	15	0	13	1	4	0	23	6	22			8	116
Ellendale	1	20	0	15	6	33	2	32	1	37	3	50	1	17	0	38			14	242
Fallglo																			0	0
Fortune																			0	0
Hickson	0	4	0	4	0	10	1	13	1	9	0	4	0	0	0	3			2	47
IM111	5	48	2	16	0	3	0	0	3	10	7	5	4	25					21	107
Imperial	0	0	2	21	9	29	1	11	8	11	4	25	9	20	1	6			34	123
Temple	1	34											23	36					24	70
Wilking[1]			2	11	16	38	4	15	8	63	20	32	23	28					73	187
Total	13	298	10	194	81	470	12	186	24	298	37	164	94	447	20	153	0	0	291	2210

1 Dry fruit and/or seed contamination in these cultivars.

Table 4.3a Numbers of field-planted hybrids, and whether they were derived from embryo-rescue or seed sowing, for crosses performed in the 1998 season, BRS.

Female Parent (seed)	Male Parent (pollen)											
	Emperor 4X		Murcott 4X		Joppa 4X		Parramatta 4X		4X Pummelo A		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aurora		4		0		4		6		0	0	14
Aust Clem		18		4		39		56			0	117
Burndale		2				3		0			0	5
De Nules		35		18		41		24			0	118
Ellendale		7		9		31		9		0	0	56
Fina		2		0		0		6			0	8
Fremont[1]		0		0		0	0	0		0	0	0
Hickson		7		4		12		2			0	25
IM111		31		5		30		5		4	0	75
Imperial		28		9		18		14		0	0	69
Kinnow[2]	0	0		0	0	4		2		0	0	6
Marisol		0				5		12		0	0	17
Nova[1]		0		0		0		0			0	0
Oroval		3		4		7					0	14
Umatilla		14		3		23		11		0	0	51
Wilking		29		27		68		64		2	0	190
Total	0	180	0	83	0	285	0	211	0	6	0	765

1 polyembryonic seed parent, no zygotic seedlings detected, all material culled
2 polyembryonic seed parent, 6 zygotic seedlings detected, currently in nursery

Table 4.3b Numbers of field-planted hybrids, and whether they were derived from embryo-rescue or seed sowing, for crosses performed in the 1999 season, BRS.

Female Parent (seed)	Male Parent (pollen)																					
	Emperor 4X		Murcott 4X		PummeloA 4X		PummeloB 4X		Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem		22		17		5		7													0	51
de Nules		109		54		7		9													0	179
Ellendale				13																	0	13
Hickson		1		0																	0	1
IM111	69	27	3	22		30	***3***	15													75	94
Imperial		43		4		17	1	48													1	112
Wilking	57	**20**	**34**	3	1	37	7	3													99	63
PummeloA 4X												8									0	8
PummeloB 4X												13					52	6	12	7	64	26
Total	126	222	37	113	1	96	11	82	0	0	0	21	0	0	0	0	52	6	12	7	239	547

Bold: some trees still in nursery
Bold Italics: flat seed sown

Table 4.3c Numbers of field-planted hybrids, and whether they were derived from embryo-rescue or seed sowing, for crosses performed in the 2000 season, BRS.

Female Parent (Seed)	Male Parent (pollen)																												Total	
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fortune			
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina	7	0			2	11	3	9	4	7	9	**17**																	25	44
Aust.Clem	0	**15**	0	3	0	5	0	3																					0	26
Burndale	**5**	**1**		0	0	0	1	0																					6	1
Corsica 1		**1**		0	1	8		0	0	0	0	0																	1	9
Corsica 2	0	8			0	9	0	2	0	**30**	0	**11**																	0	60
Daisy	5	0	3	0		0	0	0	23	**7**	16	**1**																	47	8
deNules	**32**	**100**			5	16	0	18																					37	134
Ellendale	12	**5**			0	2	3	**4**																					15	11
Encore	1	0	0	0	**1**	0	0	0	**2**	0		0																	4	0
Fallglo		0				**1**		0																					0	1
Fortune	5	0							0	0	2	0																	7	0
Hickson	0	2	0	1	0	1	0	6																					0	10
IM111	**110**	**4**	17	**1**	10	4	**13**	4																					150	13
Imperial	**122**	1	**2**	0	8	2	10	1																					142	4
Katherine 5[1]	0				0		0		0	0	0																		0	0
Katherine 6[1]	0						0				0																		0	0
Katherine 9[1]									0	0	0	**3**																	0	3
Wilking	**128**				**40**		**55**	1																					223	1
4X PummeloA															18		5				6		1		18				**25**		73	0
4X PummeloB													18		**14**		1		3		14	0	8		**92**		3		40		193	0
Total	420	137	22	5	65	48	82	39	25	37	18	15	18	0	32	0	6	0	3	0	20	0	9	0	110	0	3	0	65	0	898	281

1 polyembryonic seed parent, only putative zygotic seedlings retained, and included in calculations

Bold: some trees still in nursery

Table 4.3d Numbers of field-planted hybrids, and whether they were derived from embryo-rescue or seed sowing, for crosses performed in the 2001 season, BRS.

Female Parent (seed)	Male Parent (pollen)																					
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		4X Ortanique		4X Burgess		4X Wilking x Mur4X 95		4X Wilking x Joppa4X 2		4X Wilking x Mur4X 96	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina	2	**20**		**5**	2	**53**	6	**8**	**5**	**43**	4	**73**	**3**	0								
Aust Clem																						
Corsica 1	4	0	2	0		0		3	**9**	3	**24**	**4**										
Corsica 2	***1***	**2**	0	**2**			0	0	***1***	0		0										
Daisy	**20**	**6**	**8**	0	**4**	0	2	2		0	***2***	0	***1***		7	0		0			0	0
De Nules																						
Ellendale	7	***5***	**4**	0	1	0	1	***3***					0	***1***								
Encore					1	0						***2***										
Fallglo	2	1			0																	
Fortune	0	0			0						1	0										
Hickson	***2***	0	***1***	***2***	***1***	0							0									
IM111	**39**	**6**	4	1	0	**9**	2	**2**									0	***1***				
Imperial	**23**	0	**11**	0	**4**	0	**4**	0					**2**									
Wilking	***17***	***5***	***2***	0	***5***	0	***5***	***7***														
4X PummeloA																						
4X PummeloB																						
Total	117	45	32	10	18	62	20	25	15	46	31	79	6	1	7	0	0	1	0	0	0	0

Continued….

Bold: some trees in nursery
Bold italics: all trees in nursery

Female Parent (seed)	Male Parent (pollen)																					
	Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fallglo		Fortune		Ellendale		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina																							22	202
Aust Clem																							0	0
Corsica 1																							39	10
Corsica 2																							2	4
Daisy																							44	8
De Nules																							0	0
Ellendale																							13	9
Encore																							1	2
Fallglo																							2	1
Fortune																							1	0
Hickson																							4	2
IM111																							45	19
Imperial																							44	0
Wilking																							29	12
4X PummeloA					***9***				***22***		***35***		***16***								***15***		97	0
4X PummeloB	**103**										***30***		***41***		***1***		***20***		***162***				357	0
Total	103	0	0	0	9	0	0	0	22	0	65	0	57	0	1	0	20	0	162	0	15	0	700	269

Table 4.3e Numbers of hybrid nursery trees, and whether they were derived from embryo-rescue or seed sowing, for crosses performed in the 2002 season, BRS.

Female Parent (seed)	Male Parent (pollen)																													
	4X WilkingxMurcott 83		4X Fremont		4X Emperor		4X Murcott		4X WilkingxMurcott4X95		4X Minneola		4X Burgess		4X Orlando		4X EllendalexJoppa4X03'96		4X EllendalexMurcott4X78		4X EllendalexMurcott4X79		4X WilkingxJoppa4X109		4X WilkingxJoppa4X2		4X WilkingxJoppa4X35		4X WilkingxJoppa4X73		4X WilkingxMurcott4X96	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem					14	15		2	1	2	20	17	22	5																1	21
Corsica1					1	3		0																								
Corsica2					0	0		0		0			0	0											1	3					0	2
Daisy			1	0	28	3	16	1	16	8	10	4	1	0			36	0	2	0			2	2	8	2		0	4	0		
DeNules		14			10	7	0	15	9	22	8	15	29	24					1	5			2	6	1	24			0	0	6	58
Ellendale			2	8	6	2	2	0	0	0			3	7					1	1			5	1	2	8					5	0
IM111					**48**	1	12	0	13	0	20	3	16	1			17	0	1	3			6	2	17	9					17	9
Inperial					31	2					15	3	2	0	4	0									8	6					11	8
Temple						0																			2	0						
Wilking	6	0	7	0	57	0	17	1	9	0	22	1	53	1			2	0	2	0			31	0	12	2			2	0		
4X PummeloA																																
4X PummeloB																																
4X Wilking x 4X Murcott No 95																																
Total	6	14	10	8	195	33	47	19	48	32	95	43	126	38	4	0	55	0	7	9	0	0	46	11	51	54	0	0	6	0	40	98

Continued…

Bold: some trees field-planted
Italics: some trees from sown flat seed

Female Parent (seed)	Male Parent (pollen)																													
	98N246		98N501		98N613		Afourer(Pressler1)		Daisy		Dancy		Ellendale		Fremont		Fortune		Murcott		Page		Sunburst		Temple		Wilking		Total	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem																													58	62
Corsica1																													1	3
Corsica2																													1	5
Daisy																													124	20
DeNules																													66	190
Ellendale																													26	27
IM111																													167	28
Inperial																													71	19
Temple																													2	0
Wilking																													220	5
4X PummeloA							73		20				50				69		9								*141*		362	0
4X PummeloB													37												4				41	0
4X Wilking x 4X Murcott No 95									0	0	0	0			0	0	0	0									0	0	0	0
Total	0	0	0	0	0	0	73	0	20	0	0	0	87	0	0	0	69	0	9	0	0	0	0	0	4	0	141	0	1139	359

Table 4.4a Percentage of pollinations resulting in a field-planted hybrid, for crosses performed in the 1998 season, BRS.

Female Parent (seed)	Male Parent (pollen)					
	Emperor 4X	Murcott 4X	Joppa 4X	Parramatta 4X	4X PummeloA	Average
	% poll	% poll	% poll	% poll	% poll	% poll
Aurora	4	0	4	18	0	5
Aust Clem	86	20	93	160	0	72
Burndale	9	0	8	0		4
De Nules	175	106	241	120		161
Ellendale	6	23	22	30	0	16
Fina	10	0	0	30	0	8
Fremont	0	0	0	0	0	0
Hickson	12	16	15	5	0	10
IM111	31	17	30	17	7	20
Imperial	28	30	18	23	0	20
Kinnow	0	0	4	5	0	2
Marisol	0	0	38	60	0	20
Nova	0	0	0	0	0	0
Oroval	30	40	47	0		29
Umatilla	13	8	19	26	0	13
Wilking	71	84	108	149	17	86
Average	30	21	40	40	2	29

Table 4.4b Percentage of pollinations resulting in a field-planted hybrid, and whether this hybrid was from a small embryo (embryo-rescued) or from a plump seed (sown), for crosses performed in the 1999 season, BRS.

Female Parent (seed)	Male Parent (pollen)																				Average	
	Emperor 4X		Murcott 4X		PummeloA 4X		PummeloB 4X		Fremont		Page		Kinnow		Ellenor		Daisy		Nova			
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem		**37**		**28**		**8**		**7**														20
de Nules		**109**	1	**53**		**7**		**9**														45
Ellendale				**13**																		13
Hickson		**1**		0		0		0														0
IM111	69	27	3	**22**		**30**	***3***	**15**													25	24
Imperial		**43**		**4**		**17**		**49**														28
Wilking	57	20	57	5	1	**37**	7	3													30	16
PummeloA 4X				0						0		**800**		0		0		0		0		114
PummeloB 4X				0						0		**650**		0		0	104	**12**	***600***	**350**	352	145
Average	63	39	20	14	1	17	5	14		0		725		0		0	104	6	600	175	136	45

Bold: includes all plump seed rescued
Bold Italics: includes flat seed sown.

Table 4.4c Percentage of pollinations resulting in a field-planted hybrid, and whether this hybrid was from a small embryo (embryo-rescued) or from a plump seed (sown), for crosses performed in the 2000 season, BRS.

Female Parent (seed)	Male Parent (pollen)																													
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fortune		Average	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina	23	0	0	0	7	37	10	30	13	23	30	57																	14	24
Aust.Clem	0	**30**	0	**10**	0	10	0	**6**																					0	14
Burndale	13	3	0	0	0	0	3	0																					4	1
Corsica 1	0	20	0	0	20	160	0	0	0	0	0	0																	3	30
Corsica 2	0	**40**			0	**90**	0	20	0	**200**	0	**100**																	0	90
Daisy	13	0	8	0	0	0	0	0	77	23	50	3																	25	4
deNules	32	**100**	0	0	5	**16**	0	**18**																					9	34
Ellendale	12	5	0	0	0	**2**	3	4																					4	3
Encore	3	0	0	0	3	0	0	0	7	0	0	0																	2	0
Fallglo	0	0			0	10	0	0	0	0	0	0																	0	2
Fortune	5	0	0	0	0	0	0	0	0	0	2	0																	1	0
Hickson	0	**2**	0	**3**	0	1	0	6																					0	3
IM111	110	4	31	2	10	4	13	4																					41	3
Imperial	122	1	7	0	8	2	10	1																					37	1
Katherine 5[1]	0		0	0	0		0		0		0																		0	0
Katherine 6[1]	0						0				0																		0	
Katherine 9[1]							0	0	0		0	25																	0	13
Wilking	***128***		0	0	**40**		**55**																						56	0
4X PummeloA													0		49		13		0	0	***9***		***2***		***44***		0		**53**		19	0
4X PummeloB													51		***41***		***4***		***12***		**30**		***30***		**354**		19		**138**		75	
Average	27	15	3	1	6	24	5	6	11	35	8	23	26		45		8		6	0	20		16		199		9		96		14	12

1. polyembryonic seed parents, only putative zygotic seedlings included in calculations

Bold: includes some plump seeds rescued.

Bold Italics: includes flat seeds sown.

Table 4.4d Percentage of pollinations resulting in a field-planted hybrid, and whether this hybrid was from a small embryo (embryo-rescued) or from a plump seed (sown), for crosses performed in the 2001 season, BRS.

Female Parent (seed)	Male Parent (pollen)																					
	4X Emperor		4X Murcott		4X PummeloA		4X PummeloB		4X Parra		4X Joppa		4X Ortanique		4X Burgess		4X Wilking x Mur4X 95		4X Wilking x Joppa4X 2		4X Wilking x Mur4X 96	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina	8	80	0	20	8	212	24	32	20	172	16	292	30	0								
Aust Clem													0	0								
Corsica 1	20	0	10	0	0	0	0	15	45	15	80	13	0	0								
Corsica 2	5	10	0	10	0	0	0	0	3	0	0	0	0	0								
Daisy	67	20	32	0	13	0	7	7	0	0	8	0	10	0	54	0	0	0	0	0	0	0
De Nules													0	0			0	0				
Ellendale	23	17	16	0	3	0	3	10					0	10	0	0						
Encore	0	0	0	0	3	0	0	0	0	0	0	7	0	0			0	0				
Fallglo	8	4	0	0	0	0	0	0	0	0	0	0	0	0								
Fortune	0	0	0	0	0	0	0	0	0	0	3	0	0	0								
Hickson	7	0	3	7	3	0	0	0					0	0								
IM111	130	20	13	3	0	30	7	7					0	0			0	8				
Imperial	77	0	37	0	13	0	13	0					20	0								
Wilking	57	17	7	0	17	0	17	23					0	0								
4X PummeloA[1]																						
4X PummeloB[1]																						
Average	33	14	10	3	5	20	6	8	10	27	15	45	4	1	27	0	0	2	0	0	0	0

Continued…

1. sown number includes all flat seed.

Female Parent (seed)	Male Parent (pollen)																					
	Fremont		Page		Kinnow		Ellenor		Daisy		Nova		Murcott		Encore		Fallglo		Fortune		Ellendale		Average	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Arrufatina																							15	115
Aust Clem																							0	0
Corsica 1																							22	6
Corsica 2																							1	3
Daisy																							17	2
De Nules																							0	0
Ellendale																							8	6
Encore																							0	1
Fallglo																							1	1
Fortune																							0	0
Hickson																							3	1
IM111																							25	11
Imperial																							32	0
Wilking																							19	8
4X PummeloA[1]	0		0		20		0		220		233		160		0		0		0		214		77	
4X PummeloB[1]	206		0		0		0		0		60		164		5		100		324				86	
Average	103		0		10		0		110		147		162		3		50		162		214		19	11

Table 4.4e Percentage of pollinations resulting in a hybrid nursery tree, and whether this hybrid was from a small embryo (embryo-rescued) or from a plump seed (sown), for crosses performed in the 2002 season, BRS.

Female Parent (seed)	Male Parent (pollen)																													
	4X WilkingxMurcott 83		4X Fremont		4X Emperor		4X Murcott		4X WilkingxMurcott4X95		4X Minneola		4X Burgess		4X Orlando		4X EllendalexJoppa4X03'96		4X EllendalexMurcott4X78		4X EllendalexMurcott4X79		4X WilkingxJoppa4X109		4X WilkingxJoppa4X2		4X WilkingxJoppa4X35		4X WilkingxJoppa4X73		4X WilkingxMurcott4X96	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem					33	36	0	5	7	13	48	40	52	12											0	0					2	38
Corsica1					2	6	0	0																								
Corsica2					0	0	0	0	0	0			0	0											5	15					0	10
Daisy	0	0	10	0	67	7	38	2	40	20	50	20	2	0			180	0	10	0	0	0	10	10	20	5	0	0	20	0	0	0
DeNules	0	156			40	28	0	50	30	73	27	50	73	60			0	0	10	50			20	60	3	80			0	0	12	116
Ellendale			N/A	N/A	15	5	6	0	0	0	0	0	10	23			0	0	5	5			25	5	7	29					18	0
IM111					160	3	34	0	43	0	67	10	53	3			170	0	10	30			60	20	57	30					57	30
Inperial					103	7			0	0	50	10	7	0	100	0							0	0	27	20					37	27
Temple					0	0	0	0	0	0	0	0	0	0											10	0					0	0
Wilking	60	0	47	0	114	0	43	3	26	0	55	3	106	2			6	0	8	0			124	0	24	4			10	0	0	0
4X PummeloA[1]																																
4X PummeloB[1]																																
4X Wilking x 4X Murcott No 95																																
Average	20	51.9	28.3	0	53.4	9.19	13.4	6.63	16.2	11.9	37	16.6	33.7	11.2	100	0	71.2	0	8.54	17	0	0	39.8	15.8	17	20.3	0	0	10	0	13.9	24.5

Continued…

1. sown number includes all flat seed.

Female Parent (seed)	Male Parent (pollen)																													
	98N246		98N501		98N613		Afourer(Pressler1)		Daisy		Dancy		Ellendale		Fremont		Fortune		Murcott		Page		Sunburst		Temple		Wilking		Average	
	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued	Sown	Rescued
Aust.Clem																													20	21
Corsica1																													1	3
Corsica2																													1	4
Daisy	0	0																											28	4
DeNules																													18	60
Ellendale																													9	7
IM111																													71	13
Inperial																													40	8
Temple																													1	0
Wilking			0	0	0	0																							41	1
4X PummeloA[1]							243	0	91	0			238	0			431	0	64	0	0	0					613	0	240	0
4X PummeloB[1]													106	0											36	0			71	0
4X Wilking x 4X Murcott No 95									0	0	0	0			0	0	0	0	0	0			0	0			0	0	0	0
Average	0	0	0	0	0	0	243	0	45.5	0	0	0	172	0	0	0	216	0	32.1	0	0	0	0	0	36.4	0	307	0	41.7	9.24

Table 4.9a Field-assessed fruit characteristics of 18 tetraploid hybrids of various parentages, Top-worked Block BRS. Assessed August 2003, mostly first year of fruiting.

Tree ID	Size	Shape	Texture	Peel	Rag	Brix	Emb	Ext. Colour	Int. Colour	Comments
Wilking x Murcott 95	medium	flat	slight coarse	easy	some	11	poly	deep orange/red	good	Puffy, thick skin. Taste fair. Fair appearance.
Wilking x Murcott 96	large/medium	flat	coarse	easy	raggy	10.5-11	poly	pale orange	orange	Thick skin, granulation. Taste fair. Poor appearance.
Wilking x Joppa 2	medium/large	pear/tangelo	very coarse	easy	very raggy	11	mono	deep orange	yellow	Very thick skin, soft, ugly, orange taste.
Clem x Murcott 82	medium	flat bottom, irregular	very coarse	chunky	very raggy	12.5	mono	patchy deep orange	poor	Very thick skin, granulated, ugly.
Wilking x Murcott 83	medium	rounded, flat bottom	finely coarse	v. hard, chunky	??too hard to peel	14	poly???	good orange-red	excellent	Very thick skin, firm, small navel, Tasty, good appearance
Ellendale x Joppa 03 '96	v. large	rounded irregular	v. coarse	easy	very raggy	8.3	poly	orange poor	poor	Thick skin, puffy, granulation, ugly appearance, taste orange-poor.
Wilking x Joppa 73	medium	flat, concave, irregular	coarse	easy	raggy	11.5	mono??	orange	poor	Thick skin, puffy, granulation, little flesh once peeled, gluey rind, taste orange-poor, not attractive
Wilking x Joppa 109	large/medium	round	coarse	easy, chunky	raggy	12.5	mono	deep orange	orange, ordinary	Thick skin, juicy, not attractive.
Wilking x Murcott 18	medium	Murcott shape	coarse	easy, chunky	not too bad	10.5	mono	poor	poor	V. thick skin, browning on skin, not attractive, no depth to taste.
Parra x Joppa 319	medium	orange shape	finely coarse	reasonable, chunky	raggy		poly	deep orange	poor	Thick skin, Orange taste-poor, nucellar???
Parra x Joppa 210	medium	orange shape	finely coarse	hard to peel	v. raggy	11	poly	orange	poor	Thick skin, nucellar???
Hamlin x Joppa 118	medium-large	orange shape	finely coarse	easy	raggy	10	poly	orange	poor	Nucellar???
Parra x Joppa 89	medium-large	orange shape	finely coarse	difficult, chunky	raggy	9	poly	deep orange	poor	Thick skin, Taste poor, nucellar???
Parra x Joppa 149	medium-small	orange shape	finely coarse	impossible			poly	deep orange	poor	Thick skin, nucellar???
Parra x Joppa 32	medium	orange shape	coarse	difficult	raggy	10	poly	deep orange	poor	Nucellar???
Hamlin x Joppa 99	medium	orange shape	finely coarse	difficult, chunky	raggy	8	poly	deep orange	yellow	Puffy, Soft, past maturity.
Hamlin x Joppa 61 (1)	medium-small	orange shape	smooth	hard to peel, chunky	raggy	12	poly	deep orange	poor	Thinner skin.
Hamlin x Joppa 44 (a)	medium	round	finely coarse	easy, chunky	raggy	9.5	poly	deep orange	poor	Thick skin.

Table 4.9a Field-assessed fruit characteristics of 16 triploid hybrids of various parentages, Top-worked Block BRS. Assessed August 2003, mostly first year of fruiting.

Tree ID	Size	Shape	Texture	Peel	Rag	Brix	Emb	Ext. Colour	Int. Colour	Comments
Wilking x Joppa 49			v. coarse							Thick skin.
Wilking x Joppa 89	medium	pear shape	coarse					reasonable	pale orange	Thick skin, puffy, strange flavour.
Wilking x Joppa 34	medium	flat	slightly coarse	hard to peel		11.5	mono	good	orange	Lump fruit, Poor flavour.
Wilking x Joppa 188	medium-large	round	coarse	like orange		10	poly	deep orange	poor	Thick skin, puffy, flavour poor.
Wilking x Joppa 159	medium-small	Clem shape					poly	deep orange	yellow	Thick skin, puffy.
Wilking x Joppa 36	large	Clem shape	coarse	chunky			poly	orange	poor	Thick skin, firm, heavy.
Ellendale X Joppa 12		rounded	coarse	easy	raggy		poly??	orange	poor	Thick skin, soft, large navel, mild skunk.
Ellendale x Joppa 82	large	Clem shape	coarse	hard to peel			poly		poor	Thick skin, puffy.
Ellendale x Joppa 73 '96	medium-large	rounded	coarse				mono??			Puffy, not good.
IM3 Site 32	large-medium		slightly coarse				mono			Puffy, shiny, past maturity.
IH2 Site 29	large		coarse			10				Very puffy, very thick skin, very very dry.
Ellendale x Murcott 80	small	Murcott shape	smooth	easy	no rag	11-12.5	poly	good	good	Thin skin, very juicy.
IE2 Site 23	large	irregular shape	lumpy							Ugly, Very dry, puffy.
M12 Site 17	medium		slightly coarse	easy	little rag	10-11.5	mono??	reasonable		Puffy.
M12 Site 16	medium		slightly coarse	easy	little rag	10-11.6	mono??	reasonable		Puffy, acid.
IM1	large		smooth				mono	poor	poor	Imperial smell, shiny, puffy, lumpy, dry, past maturity.
IV1 Site6	medium	irregular shape, orange					poly??	good		Puffy, lumpy skin, well past maturity, VV dry, thick skin.
IV1Site7	medium	irregular shape, orange					poly??	good		Puffy, lumpy skin, well past maturity, VV dry, thick skin.

Table 4.10a Field-assessed fruit characteristics of 21 citrus hybrids of various parentages, Old-trellis Block BRS. Assessed on 14th July 2004 based on very low fruit numbers in the first year of fruiting of 3-year-old seedlings.

Code	Seed Parent	Pollen Parent	Predict. Ploidy	Fruit Size	Fruit Shape	External Colour	Internal Colour	Skin Texture	Seed No.	Taste	Comment
98N425	Aurora	4X Parramatta	3X	small	round/necked	reasonable	poor	coarse	1		unattractive
98N824	Aust. Clem.	4X Parramatta	4X	small	round	deep orange	orange	moderate	1	acid	poor
98N552	Aust. Clem.	4X Parramatta	3X	large	pear/necked	excellent	reasonable	slightly coarse	>10	watery	
98N630	Aust. Clem.	4X Emperor	4X	medium	round	orange	poor	coarse	1	ordinary	easy-peel
98N385	Aust. Clem.	4X Murcott	4X	medium	round	orange	reasonable	coarse	1	sweet	no flavour
98N773	DeNules	4X Murcott	4X	small	pear/necked	poor	reasonable	slightly coarse	3	sweet	still acid
98N130	Ellendale	4X Joppa	3X	small-medium	round	semi-green	poor	coarse	0.5	orange-like	peels like orange
98N172	Ellendale	4X Joppa	4X	small	round	poor	poor	smooth	0	sweet	thick rind
98N108	Ellendale	4X Murcott	3X	medium	flat	deep orange	reasonable	smooth	3	nothing special	some potential
98N487	Hickson	4X Joppa	4X	medium	round	poor	orange	smooth	4.5	orange-like	very orange-like
98N382	Hickson	4X Murcott	4X	small-medium	flat	good	good	slightly coarse	1	acid/sweet	easy-peel, some potential
98N048	IM111	4X Joppa	4X	medium	roundish	orange	poor	coarse	1		thick rind
98N367	Imperial	4X Joppa	4X	medium-large	round	poor	poor	coarse	2	ordinary	very juicy
98N406	Marisol	4X Parramatta	4X	small-medium	round	deep orange	poor	smooth	3		thick skin
98N620	Umatilla	4X Joppa	3X	large	flat	good	poor	coarse	>10	too dry	easy-peel
98N616	Umatilla	4X Joppa	3X	small	flat/slight neck	good	reasonable	coarse	>10	no depth	hard to peel
98N643	Umatilla	4X Parramatta	4X	large	round	poor	poor	coarse	0	too dry	orange-like appearance
98N114	Umatilla	4X Emperor	3X	medium-large	round/flat-top & bottom	orange	poor	coarse	0	horrible	easy-peel
98N225	Wilking	4X Joppa	4X	medium	roundish	poor	poor	coarse	5.5		thick skin
98N216	Wilking	4X Emperor									
99N183	Wilking	4X Murcott	4X	medium	flat/slight neck	good	reasonable	coarse	2	very ordinary	easy-peel