Self-incompatibility of Olive

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B.Sc., M.Sc.

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Discipline of Wine and Horticulture

School of Agriculture, Food, and Wine

Faculty of Science

University of Adelaide

May 2008

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Abstract

The olive (*Olea europaea* L.) is one of the most ancient fruit trees and has been cultivated for its oil in the Mediterranean area for thousands of years. Today, the consumption of olive oil and table olives is increasing both in traditional producing countries and the entire world. Most olive cultivars are self-incompatible and do not produce a commercial yield after self-pollination. In this thesis, inflorescence architecture and sexual compatibility relationships of some olive cultivars, and gene expression in olive pistils during flowering were studied.

To study the inflorescence architecture of olive, 45 inflorescences in each of the cultivars Manzanillo, Mission, and Frantoio were checked every morning from flower opening to petal fall. The flower position on the inflorescence had a highly significant effect on the opening day in all cultivars. Terminal flowers and the flowers located on the primary branches opened earlier than flowers located on the secondary branches. Flower position also had a highly significant effect on gender in Manzanillo and Mission. In Manzanillo, the secondary branches had fewer perfect flowers than the primary branches. In Mission, the secondary branches had no perfect flowers at all. In Manzanillo, perfect flowers had significantly longer petal persistence than staminate flowers. To study flower competition within the inflorescence, the distal halves, on which the flowers tend to be perfect, of 120 inflorescences in three trees of Manzanillo were removed about one month before full bloom. This resulted in a highly significant increase in the percentage of perfect flowers on the proximal halves. The effects of shoot orientation and inflorescence location on inflorescence characteristics in the cultivars Frantoio, Kalamata, and Koroneiki were also studied. For each cultivar, inflorescence characteristics in three sections of shoots (top, middle, and base) and four sides of the three selected trees (north, south, east, and west) were recorded. The statistical analysis showed that basal inflorescences were shorter and with fewer flowers but with the same percentage of perfect flowers. Shoot orientation did not have any influence on these characteristics in any of the cultivars.

Sexual compatibility was assessed using two methods. In the first method, controlled crossings were performed in the cultivars Frantoio, Koroneiki, and Kalamata. The pistils were harvested one week after hand pollination and stained with 0.1% aniline blue. The styles and ovules were separated, mounted in 80% glycerol, and

observed under a fluorescence microscope. In Frantoio and Koroneiki, the number of ovules penetrated by a pollen tube was used to estimate the level of sexual compatibility. In Kalamata, the numbers of ovules penetrated by pollen tubes were not significantly different between treatments; therefore, the number of pollen tubes in the lower style was used. All the cultivars studied were self-incompatible. Frantoio (as a host) was incompatible with Koroneiki and Barnea but partially compatible with Mission. Koroneiki (as a host) was incompatible with Barnea but partially compatible with Frantoio and Mission. Kalamata (as a host) was compatible with Barnea, incompatible with Mission and Koroneiki in 2004, but partially compatible with them in 2005. In the second method, eight microsatellite markers were used for genotyping three Kalamata mother trees, 40 embryos per mother tree, and all the potential pollen donors. Genotyping data were analysed using FaMoz software, and the number of embryos assigned to each putative pollen donor was determined. Paternity analysis showed that Kalamata (as a host) was self-incompatible, compatible with Barnea, Benito, and Katsourela, but incompatible with Arbequina, Azapa, and Picual.

To study the gene expression in olive pistils during flowering, a genomic approach was initiated using cDNA subtractive array analysis. Total RNA was isolated from olive pistils at two developmental stages, where self-incompatibility (SI) genes are expected to be differentially expressed: 1) small green flower buds (expression of SI genes not expected) and 2) large white flower buds containing receptive pistils just prior to opening (expression of SI genes expected). From each stage, cDNA libraries were prepared and put through forward and reverse subtractive hybridisations to enrich for differentially expressed cDNAs in stage 2. Macroarrays were prepared by printing 2304 differentially expressed cDNAs onto nylon membranes and hybridised with forward- and reverse-subtracted probes. The analysis identified 90 up-regulated cDNA clones highly expressed in receptive pistils. Further subtracted and unsubtracted hybridisations confirmed up-regulation of the majority of these cDNAs. Gene expression profiles across different tissues showed that most of the genes were pistil-specific. The expression pattern of the genes showed high similarity in Kalamata, Frantoio, Barnea, and Pendolino. All the screened genes were sequenced and their similarities were searched in the NCBI database. The most redundant and interesting up-regulated clones were those similar to a receptor protein kinase-like protein. Some versions of this protein play a role in the sporophytic SI system of *Brassica* and the gametophytic SI system of *Papaver* and rye.

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Acknowledgments

I would like to express my sincere gratitude to my supervisors Dr Brent Kaiser, Dr Jenny Guerin, and Professor Margaret Sedgley for their supervision, advice, criticism, and support during my candidature.

I would also like to sincerely thank Dr Chris Ford, Dr Michelle Wirthensohn, Dr Sunita Ramesh, Dr Tricia Franks, and Mr Bob Barrett for their continual support and technical advice. Special thanks also to all staff and students of the School of Agriculture, Food, and Wine and particularly the Discipline of Wine and Horticulture especially Iraj Tavassolian, Jana Kolesik, Nenah Mackenzie, Patrick Loughlin, Scott Carter, Nasser Abbasspour, Wendy Sullivan, and Rebecca Vandeleur for their support and friendship.

My gratitude extends to the staff of Australian Genome Research Facility (AGRF), especially Nicole Burtt, for their help in DNA genotyping and sequencing, to the staff of Australian Centre for Plant Functional Genomics (ACPFG), especially Natalia Tikhomirov, for their help in colony printing, to Janine Jones and Kathy Haskard from Biometrics SA for statistical advice, to Dr Sophie Gerber for advice on using FaMoz software, and to Milan Pavlic for generously allowing us to sample the trees on his property (Milano Olives Collection).

I acknowledge my appreciation to the Gorgan University of Agricultural Sciences and Natural Resources and the Ministry of Science, Research and Technology of Iran for awarding me a scholarship to undertake this study.

Finally, this thesis would not have been completed without the uncompromising support of my wife, Maryam, and my daughter, Nazaninzahra, who shared all my happiness and frustration throughout my study. Also special thanks to my parents for their love, support, and understanding of my study abroad.

List of Abbreviations

%	percent
°	degree
°C	degrees Celsius
µg	microgram
µl	microlitre
3'	3 prime end of a nucleic acid
5'	5 prime end of a nucleic acid
A_E	effective number of alleles
AFLP	amplified fragment length polymorphism
AGRF	Australian Genome Research Facility
al	Allele
am	before noon
ANOVA	analysis of variance
A_O	observed number of alleles
AOA	Australian Olive Association
ARK	*Arabidopsis* receptor kinase
B1	branch 1
B2	branch 2
B3	branch 3
B4L	branch 4 lateral
B4T	branch 4 terminal
B5L1	branch 5 lateral 1
B5L2	branch 5 lateral 2
B5T	branch 5 terminal
B6L1	branch 6 lateral 1
B6L2	branch 6 lateral 2
B6T	branch 6 terminal
BBCH	Biologische Bundesanstalt, Bundessortenamt, Chemische Industrie
BC	before Christ
BLAST	Basic Local Alignment Search Tool, a computer program
BLASTX	a computer program to search protein databases using a translated nucleotide query
bp	base pair

c	column
Ca^{2+}	ionic solution of calcium
cDNA	complementary DNA
CDP-Star	disodium 2-chloro-5-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo[3.3.1.13,7]decan}-4-yl) phenyl phosphate
cm	centimetre
CTAB	cetyltrimethylammonium bromide
DEF	differentially expressed fragment
df	degrees of freedom
DIG	digoxigenin
DNA	deoxyribonucleic acid.
DNase	deoxyribonuclease
dNTP	a generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP
dUTP	2'-deoxyuridine 5'-triphosphate
E	east
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EMO2	EMO2AJ416320, a microsatellite primer
EP	exclusion probability
EPD	effective pollination distance
EST	expressed sequence tags
et al.	et alia (Latin)
E-value	expect-value
FAM	a fluorescent dye-labelled oligo
FAO	Food and Agriculture Organisation
Fig.	Figure
FS	forward-subtracted
g	gram
Gel Doc	gel documentation system
GML	generalised linear modelling
GSI	gametophytic self-incompatibility
h	hour
ha	hectare
H_E	expected heterozygosity
HEX	a fluorescent dye-labelled oligo

H_O	observed heterozygosity
IP	identity probability
IPI	index of pollen-incompatibility
ISI	index of self-incompatibility
kg	kilogram
km	kilometre
LB	Luria Bertani medium
LOD	log of the odds ratio
LP	all lateral positions
LPF	all lateral perfect flowers
LS	lower style
LSD	least significant difference
LSF	all lateral staminate flowers
LSI	late-acting self-incompatibility
M	molar
m	metre
MAPK	mitogen-activated protein kinase
Max	maximum
mg	milligram
min	minute
Min	minimum
ml	millilitre
mm	millimetre
mM	millimolar
MQ	milli-Q, water that has been purified using an ion exchange cartridge
N	north
n (No)	number
NA	not available/applicable
NCBI	National Centre for Biotechnology Information
ND	no difference
NED	a fluorescent dye-labelled oligo
ng	nanogram
nm	nanometre
NOVA	National Olive Variety Assessment
NP	null allele probability
NR	non-redundant

NS	not significant
®	trade mark
OSI	ovarian self-incompatibility
P	probability
PCR	polymerase chain reaction
PD	power of discrimination
PF	all perfect flowers
pg	picogram
pH	potential of Hydrogen (-log [H^+])
pm	after noon
PR	pathogenesis-related protein
PVP	polyvinylpyrrolidone
r	row
RAPD	random amplified polymorphic DNA
REML	restricted maximum likelihood
RFLP	restriction fragment length polymorphism
RFU	relative fluorescence unit
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RS	reverse-subtracted
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
S	south
SA	South Australia
SCR	S-locus cysteine-rich protein
SDS	sodium dodecyl sulphate
SE	standard error of the mean
SF	all staminate flowers
SI	self-incompatibility
SLF/SFB	S-locus F-box protein
SLG	S-locus glycoprotein
SNP	single nucleotide polymorphism
SP11	S-locus pollen protein 11
sPPase	soluble inorganic pyrophosphatase
SRK	S-locus receptor kinase

SSC	standard saline citrate
SSI	sporophytic self-incompatibility
SSR	simple sequences repeat
SSR14	ssrOeUA-DCA14 AJ279863, a microsatellite primer
SSR3	ssrOeUA-DCA3 AJ279854, a microsatellite primer
SSR4	ssrOeUA-DCA4 AJ279855, a microsatellite primer
SSR9	ssrOeUA-DCA9 AJ279859, a microsatellite primer
subsp.	subspecies
t	tonnes
TBLASTX	a computer program to search translated nucleotide database using a translated nucleotide query
TE	a buffer made of 10 mM Tris HCl, 0.1 mM EDTA, pH 8.0
TF	terminal flower
TIP	tonoplast intrinsic protein
™	trade mark
TP	all terminal positions
TPF	all terminal perfect flowers
TRIS	trishydroxymethylaminomethane
TSF	all terminal staminate flowers
U1	unsubtracted tester control 1
U2	unsubtracted tester control 2
UDO24	UDO99-024, a microsatellite primer
UDO6	UDO99-006, a microsatellite primer
UDO8	UDO99-008, a microsatellite primer
US	upper style
USA	United States of America
UV	ultraviolet
v/v	volume to volume
v/v/v	volume to volume to volume
w/v	weight to volume
WA	Western Australia
X2	chi-square test

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CHAPTER ONE

General Introduction and Literature Review

1.1 Introduction

The olive is one of the oldest cultivated fruit trees and has been the main source of edible oil in the Mediterranean basin for thousands of years (Bertrand, 2002). At present, the demand for olive oil is increasing, even outside the Mediterranean basin, mainly because it has a high level of oleic acid, which is one of the healthiest fatty acids (Orlandi et al., 2003). The world production of olive fruits in 2005 was about 15 million tonnes (FAOSTAT-website, accessed 14 Nov 2007).

The olive industry in Australia is growing rapidly (Kailis and Sweeney, 2002), and the consumption of olive oil and table olives in Australia is the highest outside the Mediterranean area (Kailis and Sweeney, 2002; Kailis and Davies, 2004). During the last 15 years, imports of olive oil and table olives into Australia have doubled (AOA-website, accessed 16 Nov 2007). Australia has suitable climatic conditions, sufficient physical resources, and the horticultural knowledge to support a modern olive industry (Sweeney and Davies, 2004).

One of the most important problems in olive cultivation is pollen-incompatibility. Several studies on the self- and cross-incompatibility of olive cultivars have resulted in variable and conflicting results. Frantoio, for example, was concluded to be self-compatible in some reports (Sharma et al., 1976; Fontanazza and Baldoni, 1990; Fabbri et al., 2004) but self-incompatible in others (Wu et al., 2002; Mookerjee et al., 2005). The variations may be because pollen-incompatibility in olive can be influenced by climatic conditions especially air temperature; therefore, the degree of incompatibility varies from area to area and from year to year (Griggs et al., 1975; Lavee, 1986;

Androulakis and Loupassaki, 1990; Lavee et al., 2002). In spite of great progress in understanding the genetic and molecular mechanisms of self-incompatibility in some plants, there is little information about olive.

This chapter reviews the importance of the olive in the world and in Australia, olive flowering and fruit set, self-incompatibility in general and of olive in particular, and the methods to study self- and cross-incompatibility in olive cultivars, including paternity analysis.

1.2 General description of olive

1.2.1 Botany and taxonomy

The olive (*Olea europaea* L.) is an evergreen subtropical fruit tree characteristic of the Mediterranean area. The olive is a diploid (2n = 2x = 46) (Angiolillo et al., 1999; Reale et al., 2006b) and long-lived species. It can live for more than 500 years, but trees older than 2000 years have also been recorded (Doveri and Baldoni, 2007). The olive is a small to medium-sized tree, with thick, grey-green, and oppositely arranged leaves (Martin and Sibbett, 2005).

The olive belongs to the family Oleaceae and sub-family *Oleideae* (Doveri and Baldoni, 2007). The family Oleaceae also includes some ornamental species such as jasmine, lilac, forsythia, ash, privet, and phyllirea. According to a new revision (Green, 2002), the genus *Olea* has three subgenera: *Paniculatae*, *Tetrapilus*, and *Olea*. *Olea europaea* L., which is the only species of the family with edible fruits (Lavee, 1985; Carriero et al., 2002), is divided into six subspecies based on morphology and geographical distribution:

1) subsp. *europaea*, distributed in the Mediterranean basin;
2) subsp. *cuspidata*, distributed in south west Asia and south east Africa;
3) subsp. *laperrinei*, from the Sahara region;
4) subsp. *maroccana*, from Morocco;
5) subsp. *cerasiformis*, from the Madeira Islands;
6) subsp. *guanchica*, from the Canary Islands.

Subsp. *europaea* includes two botanical varieties: *europaea* (cultivated olive) and *sylvestris* (Mediterranean wild olive). Wild olive, also called oleaster olive (Besnard et al., 2001b), is characterised by its shrubby growth, short leaves, small fruits, thin mesocarp, and low oil content (Zohary, 1994; Vargas and Kadereit, 2001). It is considered to be the genetic stock from which the cultivated olive was developed (Zohary, 1994). A study on wild olive plants in 10 areas of the Mediterranean, using microsatellite markers, showed that they are present in both the eastern and western Mediterranean and are not derived from cultivated olives (Breton et al., 2006).

1.2.2 Origin and domestication

The origin of olive is still unclear, but the main hypothesis suggests that it originated from the eastern shores of the Mediterranean (Lavee, 1985; Rugini and Lavee, 1992). The olive was domesticated in this area about 4000-3000 BC and then distributed into the western areas of the Mediterranean basin (Zohary, 1994; Conner and Fereres, 2005). Cultivated and wild oleaster olives (both from subsp. *europaea*) are closely related to some other subspecies (Zohary, 1994). The first is subsp. *cuspidata* from south west Asia and south east Africa, supporting the hypothesis of the origin of olive in the Middle East. The other subspecies are *laperrinei* from the Sahara region and *cerasiformis* from the Madeira Islands, supporting an alternative hypothesis on the origin of olive in north and north west Africa.

1.2.3 Importance of olive

The olive tree has been cultivated in the Mediterranean basin for thousands of years (Bertrand, 2002). Although 90% of world olive production is used for oil extraction (Colmagro et al., 2001), the consumption of table olives is also growing worldwide. Today, the olive tree is grown commercially within latitudes 30° and 45° in both the northern and southern hemispheres, where climatic conditions are similar to the Mediterranean basin, with mild winters and warm, dry summers (Sanz-Cortes et al., 2002; Connell, 2005). Australia, Argentina, Chile, China, Peru, South Africa, and USA are among the countries that have recently started an olive industry. In 2005, world olive production was about 15 million tonnes; more than half of it (51.71%) in Spain and Italy, with only 0.15% in Australia (FAOSTAT-website, accessed 14 Nov 2007).

Table 1.1 shows the production and area harvested of olive in the world, the 10 leading countries and Australia in 2005.

Table 1.1. Production and area harvested of olive in the world, the 10 leading countries, and Australia in 2005 (FAOSTAT-website, accessed 14 Nov 2007).

NOTE: This table is included on page 4 of the print copy of the thesis held in the University of Adelaide Library.

1.2.4 Olive industry in Australia

The olive was first introduced to Australia at the beginning of the 1800s, when early settlers brought olive cuttings and seedlings to their new home (Booth and Davies, 1996; Spennemann and Allen, 2000a), and today it is cultivated in most states (Spennemann and Allen, 2000b). Due to the health advantages of olive oil in comparison to other vegetable oils, its consumption is increasing worldwide and also in Australia (Fig. 1.1). Australians consume about 1.5 litres of olive oil and 0.7 kg table olives per person annually, the highest outside the Mediterranean basin (Kailis and Sweeney, 2002; Kailis and Davies, 2004). Fig. 1.1 shows that the imports of olive oil and table olives into Australia have doubled during the last 15 years (120.28% and 127.72% growth, respectively) (AOA-website, accessed 16 Nov 2007). Fig. 1.2 shows the Australian areas with climatic conditions similar to traditional olive growing regions in the Mediterranean.

NOTE: This figure is included on page 5 in the print copy of the thesis held in the University of Adelaide Library.

Fig. 1.1. Imports of olive oil and table olives into Australia during the last 15 years (AOA-website, accessed 16 Nov 2007).

NOTE: This figure is included on page 5 in the print copy of the thesis held in the University of Adelaide Library.

Fig. 1.2. Areas (shaded) with climatic conditions similar to traditional olive growing regions in the Mediterranean (Sweeney and Davies, 1998).

1.2.5 Cultivated olive

The olive's ancient origin, easy propagation, and popularity have resulted in the presence of numerous cultivars across the world. Several cultivars may have the same name (homonyms), or the same cultivar may be called different names (synonyms) in different areas (Lanza et al., 1996; Barranco et al., 2000a; Besnard et al., 2001c). Frantoio, for example, is an Italian cultivar with 20 synonyms including: Frantoiano, Infrantoio, Correggiolo, Crognolo, Raggio, Raggiolo, Rajo, Razza, Razzo, Pendaglio, and Pignatello (Barranco et al., 2000b). A study of 300 bibliographical references showed that there are more than 1200 olive cultivars with more than 3000 names in 24 countries (Bartolini et al., 1994). In another reference, the number of olive cultivars has been recorded as up to 2600 (Rugini and Lavee, 1992).

The confusion in naming olive cultivars may create problems for growers and researchers. Morphological and phenological characters are widely used for cultivar identification. Although these markers are useful tools for identification, they are influenced by environmental conditions, and in addition, they require long observation of mature plants (Trujillo et al., 1997; Belaj et al., 1999). Biochemical markers such as isozymes have been used in olive (Ouazzani et al., 1996; Potes et al., 1999; Kamoun et al., 2002). However, since isozymes are products of gene expression, the results may be affected by environmental conditions, type of tissue sample, and other factors (Fabbri et al., 1995).

In recent years, DNA markers have been added to the tools for genotype identification. They are not affected by environmental conditions or different phenological stages and allow a fast and successful approach to distinguish between olive cultivars (Vergari et al., 1998). Random amplified polymorphic DNAs (RAPDs) (Fabbri et al., 1995; Vergari et al., 1998; Weisman et al., 1998; Belaj et al., 1999; Mekuria et al., 1999; Besnard et al., 2001a; Sanz-Cortes et al., 2001; Mekuria et al., 2002; Belaj et al., 2004), amplified fragment length polymorphisms (AFLPs) (Angiolillo et al., 1999), restriction fragment length polymorphisms (RFLPs) (Besnard et al., 2001a; Khadari et al., 2003), and simple sequences repeats (SSRs) or microsatellites (Barranco et al., 2000a; Rallo et al., 2000; Bandelj et al., 2002; Khadari et al., 2003; Diaz et al., 2006a; Omrani-Sabbaghi et al., 2007) have been widely applied

in the genetic discrimination of olive. More recently, SNP-based markers were used to distinguish between olive cultivars (Reale et al., 2006b). Belaj et al. (2003) compared three of these DNA markers among 32 olive cultivars and argued that microsatellites presented a higher level of polymorphism and greater information content than RAPDs and AFLPs.

1.3 Flowering and fruit set

1.3.1 Flowering

An adult olive tree produces about 500,000 flowers a year (Martin et al., 2005). Olive flowers are grouped in inflorescences, called panicles. The inflorescence length depends on the cultivar and varies from 3 to 8 cm (Lavee, 1985). The number of flowers per inflorescence also depends on the cultivar and normally is between 15 and 30 (Martin and Sibbett, 2005) but may vary from year to year, from tree to tree, from shoot to shoot, and from inflorescence to inflorescence (Brooks, 1948; Lavee and Datt, 1978; Lavee, 1985; Cuevas et al., 1994a; Lavee, 1996; Lavee et al., 2002; Reale et al., 2006a). The inflorescences are usually borne at the axil of leaves on shoots developed in the previous season. In some conditions, especially in warmer climates and after a relatively cold winter, inflorescences are also seen at the shoot apex (Lavee, 1996). They may rarely develop on 2- or 3-year-old shoots (Lavee, 1996).

Fabbri and Benelli (2000) reviewed the studies conducted on olive flowering and reported that the results confirm the two-step theory of flower bud induction and differentiation in olive, previously suggested by Lavee (1996). In the first step during spring-summer, likely flower buds are simulated to prevent their differentiation into leaf buds. In the second step during autumn-winter and under further favourable conditions, the candidate buds are simulated again to form the flower structures. The final number of flower buds depends on environmental conditions, nutrition, hormonal balance, and other factors in both steps. It is accepted that olive flower buds need chilling in winter to open properly, but the chilling influence on flower bud induction and differentiation is still in question (Lavee, 1996; Fabbri and Benelli, 2000). Most olive cultivars follow alternate bearing and normally produce small numbers of flowers and fruits after a heavy yield (Lavee, 1985).

1.3.2 Floral morphology

Olive flowers are small and consist of four fused green sepals, four white-yellowish petals, two stamens, and two carpels (Fabbri et al., 2004). The petals are fused at their base and drop as a unit, often with the stamens attached (Martin et al., 2005). The style is short and thick with a relatively large stigma. There are two types of flowers: perfect flowers, which have functional pistils, and staminate flowers, whose pistils have degenerated. The pistil degeneration can occur at any stage of development (Lavee, 1985; Fabbri et al., 2004), and water and nutrient stresses during flower development can lead to abortion (Martin and Sibbett, 2005). The number of staminate flowers is controlled genetically and environmentally; therefore, it may vary from year to year, from tree to tree, from shoot to shoot, and from inflorescence to inflorescence (Badr and Hartmann, 1971; Lavee et al., 2002; Fabbri et al., 2004; Martin and Sibbett, 2005).

The flower position on the inflorescence also affects its gender (Bouranis et al., 1999; Dimassi et al., 1999; Ateyyeh et al., 2000; Cuevas and Polito, 2004; Martin et al., 2005). More perfect than staminate flowers are located at the tip (Brooks, 1948; Griggs et al., 1975; Bouranis et al., 1999; Ateyyeh et al., 2000; Martin et al., 2005) and middle (Dimassi et al., 1999). The percentage of staminate flowers has no significant effect on yield (Lavee et al., 1996), except for some clones of Ascolana in some years with less than 5% of perfect flowers (Lavee et al., 1996; Lavee et al., 2002). Abnormal flowers are also common in some cultivars. Flowers with three or four stamens and five, six, or eight petals have been reported (Lavee, 1985).

1.3.3 Floral phenology

Inflorescence development is slow and usually takes 4 to 6 weeks from inflorescence bud opening to anthesis (Lavee, 1985). When the inflorescences reach a length of about 2 cm, they start to elongate rapidly, and when they are about 2/3 of their final length, the flower buds begin to expand (Lavee, 1996). Anthesis normally takes 2-3 days in an individual inflorescence, 5-6 days in an individual tree, but 10-15 days in cooler seasons and environments (Fabbri et al., 2004). When the temperature is unstable, anthesis may occur in more than one flush, but the first flush usually sets the fruits that reach maturity (Lavee, 1985). The flower position on the inflorescence also

affects its opening. In Mission, for example, the first flowers to open were always located on the primary branches, and the last to open on the secondary branches in 79% of cases (Cuevas and Polito, 2004).

1.3.4 Pollination and fertilisation

Olive flowers produce a large number of pollen grains (Griggs et al., 1975; Lavee, 1986), which are triangular in shape with a netted surface (Lavee, 1985). Although olive flowers are wind-pollinated, they are visited by insects including honeybees, which may assist pollination (Lavee, 1985; Free, 1993). Olive flowers do not produce nectar, and insects collect only pollen grains (Lavee, 1985). Critical conditions such as strong and dry winds, rain, and high temperature affect pollination and may reduce fruit set (Lavee, 1986; Conner and Fereres, 2005). Although olive pollen can be carried by wind as far as 12 km (Fabbri et al., 2004), the effective pollination distance (EPD) has been reported to be 30 m in normal conditions (Ayerza and Coates, 2004; Fabbri et al., 2004; Sibbett and Osgood, 2005). Ten percent of pollenisers within the EPD of the main cultivar is enough to ensure a good yield (Lavee, 1996). Due to the excessive amount of pollen and its high distribution, there is no correlation between the viability of pollen and fruit set (Lavee, 1986). The viability of olive pollen is usually high (Lavee et al., 2002).

Pollen grains land on the stigma and germinate to produce pollen tubes, which grow through style to fertilise the eggs to form embryos. Pollen tubes need appropriate air temperature to grow properly. In cooler temperatures, pollen tubes grow slowly and may fail to reach the ovules or reach them after their degeneration. High temperatures also inhibit pollen germination and slow down or stop the pollen tube (Bartoloni and Guerriero, 1995). On the other hand, hot and dry conditions may shorten the receptivity period of the stigma (Martin et al., 2005). Most pollen tubes are inhibited in the stigma before entering the transmitting tissue in the style, and normally one or two pollen tubes grow towards the ovary, one reaches the carpels, and one penetrates one of the four ovules (Ateyyeh et al., 2000).

1.3.5 Fruit set and development

In a year with normal flowering, 1 to 2% of fruit set is enough for a commercial yield (Lavee, 1986; Lavee, 1996; Fabbri et al., 2004; Martin et al., 2005). In ‘off’ years,

when the flowering is weak, fruit set may increase to 10% (Martin et al., 2005). Normally one fruit per inflorescence develops, except for some cultivars with small fruits such as Koroneiki and Arbequina (Lavee, 1986; Conner and Fereres, 2005), or when a high number of the inflorescences are removed (Lavee et al., 1996). After pollination, the first flowers to drop are staminate flowers. Then, unfertilised perfect flowers and fertilised young fruits are dropped as a result of competition. In Manzanillo, the two drops overlapped, but their peaks were 8 and 13-15 days after full bloom, respectively. Twenty-five days after full bloom, the number of fruits stabilises, and only a few may subsequently drop as a result of competition (Rapoport and Rallo, 1991).

The olive fruit is a drupe with a mesocarp containing oil (22%), water (50%), proteins (1.6%), carbohydrates (19.1%), cellulose (5.8%), and minerals (1.5%) (Doveri and Baldoni, 2007). Fruit growth follows a double sigmoid curve like other drupes. It usually consists of three separate stages: growth as a result of cell division, hardening of the endocarp when mesocarp growth stops, and the second growth phase due to cell enlargement (Lavee, 1986; Fabbri et al., 2004). In normal conditions, only fertilised ovaries remain on the inflorescences, but under some conditions, especially on inflorescences with no normal fruits, some parthenocarpic fruits (shot berries) may develop (Lavee, 1996). Cross-pollination is reported to decrease the number of shot berries (Griggs et al., 1975; Fernandez-Escobar and Gomez-Valledor, 1985). They are small and round (regardless of the normal fruit shape of the cultivar) and reach maturity earlier than other fruits (Lavee, 1986; Lavee, 1996).

1.4 Self-incompatibility (SI)

Successful sexual reproduction in angiosperms depends on a series of events, in which a pollen grain attaches to a receptive stigma, adheres and hydrates, germinates, and produces a tube that grows through the style and towards the ovary to fertilise the eggs and form an embryo. Outbreeding (cross-fertilisation) increases genetic variability and consequently imparts strong evolutionary potential. Different mechanisms have evolved in flowering plants to promote outbreeding such as SI, monoecy, dioecy, dichogamy, and male sterility. SI is the most widespread mechanism (Franklin-Tong and Franklin, 2003; Hiscock and McInnis, 2003) and is estimated to be present in more

than half of the species of angiosperms (de Nettancourt, 1977; McClure and Franklin-Tong, 2006).

SI is defined as "the inability of a fertile hermaphrodite seed-plant to produce zygotes after self-pollination" (de Nettancourt, 1977; de Nettancourt, 2001). SI prevents inbreeding (self-fertilisation) by recognition and rejection of self or self-related pollen (Hiscock and McInnis, 2003). It seems that SI arose later than the separation of families because some closely-related families have different systems of SI. Solanaceae and Convolvulaceae, for example, are two closely-related families with gametophytic and sporophytic systems, respectively (Kowyama et al., 2000). Steinbachs and Holsinger (2002) argued that SI has evolved at least 21 times during the evolution of flowering plants.

SI systems are classified on the basis of floral morphology, genetic control of the pollen phenotype, and the inhibition site. There are two kinds of SI based on floral morphology: heteromorphic SI, in which the flowers of the interbreeding plants have different structures, and homomorphic SI, in which the flowers of the interbreeding plants have the same visible morphology. On the basis of genetic control of the pollen phenotype, homomorphic SI is divided into two groups: sporophytic and gametophytic systems. There is also another kind of SI called late-acting or ovarian, in which inhibition does not happen in the stigma or in the style but later in the ovary (Sedgley, 1994).

1.4.1 Heteromorphic SI

Heteromorphic SI occurs in some 24 plant families, such as Connaraceae, Oxalidaceae, Plumbaginaceae, Primulaceae, Rubiaceae, and Saxifragaceae, and over 164 genera of angiosperms (Ganders, 1979). Heteromorphism (heterostyly) has two types: distyly and tristyly. Distylous plants have two morphs, and the classic example is *Primula*. The long-style morph (called pin) has long styles and short stamens, and the short-style morph (called thrum) has short styles and long stamens. There are also some differences in pollen grain size and colour, stigmatic papillae and corolla size. Compatible pollination is possible only between the morphs and not within them. Thus, long stamen flowers pollinate and fertilise the long style flowers and short stamen

flowers the short style flowers (Ganders, 1979; Gibbs, 1986). Tristyly is less common and present in only three families: Lythraceae, Oxalidaceae, and Pontederiaceae. In tristylous plants, three morphs are present: long-, mid-, and short-style, each morph with stamens at the two other levels. For example, the long-style morph has mid-level and short stamens and is fertilised only by long stamen flowers (Ganders, 1979; Gibbs, 1986).

SI does not result solely from the differences in style and stamen lengths. Heteromorphic SI also involves a sporophytic system, in which the reaction of the pollen is determined by the genotype of the parent sporophyte. There is an accepted view that the SI mechanism evolved first, and then the morphological features accumulated (Gibbs, 1986).

1.4.2 Sporophytic SI (SSI)

SSI has been found in Brassicaceae (cabbage, radish, turnip, broccoli, and cauliflower), Asteraceae (*Senecio* and *Cosmos*), Convolvulaceae (sweet potato), and Betulaceae (hazelnut) (Sedgley, 1994; McClure and Franklin-Tong, 2006). In this system, the SI phenotype of germinating pollen is determined by the diploid genotype of the pollen donor (sporophyte), and self or self-related pollen is rejected before or just after germination (Newbigin et al., 1993; McCubbin and Kao, 2000). It is assumed that the nutritive inner wall of the pollen sac (tapetum, a sporophytic tissue) supplies the proteins responsible for SI to the pollen coating (Hiscock, 2002). SSI is associated with dry stigmas, tri-nucleate pollens, and dominance effects of alleles (de Nettancourt, 1997; Hiscock and McInnis, 2003). A pollen grain is incompatible when the dominant allele of the pollen donor plant matches the dominant allele of the pistil (Hiscock and McInnis, 2003). In *Brassica*, most of the S-haplotypes examined in stigmas are codominant, but codominance in pollens is less frequent (Hatakeyama et al., 2001).

SSI in *Brassica* has been studied in detail at the molecular level, and reviewed recently by Brugiere et al. (2000), Hiscock (2002), and Hiscock and McInnis (2003). In brief, three principal genes have been identified: S-locus receptor kinase (SRK), which is the female determinant of SSI and spans the plasma membrane of stigmatic papilla cells, cysteine-rich protein (SCR) also called S-locus pollen protein 11 (SP11), which is

the male determinant of SSI and the cognate ligand of SRK, and S-locus glycoprotein (SLG), which has a high sequence similarity to the receptor region of SRK. SLG is not essential for SSI but acts in forming the receptor complex. In an incompatible reaction, SCR is recognised by the receptor domain of SRK and binds to it (activation). The activated SRK then initiates a signalling cascade that results in rejection by preventing pollen adhesion and germination or by stopping pollen tube growth. The exact mechanism by which this occurs is not yet clear (Brugiere et al., 2000; Hiscock, 2002; Hiscock and McInnis, 2003).

1.4.3 Gametophytic SI (GSI)

GSI is the most widespread SI systems in woody horticultural species (Sedgley, 1994) and across all plants (Franklin-Tong and Franklin, 2003). It has been found in Rosaceae (apple, pear, cherry, and almond), Fabaceae, Scrophulariaceae (*Antirrhinum*), Solanaceae (*Nicotiana*, *Petunia*, *Solanum*, and *Lycopersicon*), Campanulaceae (*Campanula*), Cornaceae, Papaveraceae (*Papaver*), Liliaceae, and Poaceae (*Phalaris*, and *Hordeum*) (Franklin-Tong and Franklin, 2003). In this system, the SI phenotype of germinating pollen (gametophyte) is determined by its own haploid genotype (Newbigin et al., 1993; McCubbin and Kao, 2000; de Graaf et al., 2006). In most plants with GSI, incompatible pollen germinates successfully on the stigma and grows into the style, but pollen tube growth is inhibited in the style (Newbigin et al., 1993). In an incompatible reaction, the pattern of pollen tube growth is initially similar to a compatible one, but at some stage, the growth becomes irregular, the tube walls become thicker, and a large amount of callose deposits at its tip, and finally the tube tip may swell and burst (Newbigin et al., 1993). GSI is associated with wet stigmas and bi-nucleate pollen (de Nettancourt, 1997). There is no dominance effect between the alleles; therefore, a compatible cross occurs when the allele carried by the haploid pollen is different from either of the alleles carried by the diploid style (Newbigin et al., 1993; de Graaf et al., 2006).

Two different GSI mechanisms have been investigated in detail at the molecular level and reviewed recently by Franklin-Tong and Franklin (2003), de Graaf et al. (2006), and McClure and Franklin-Tong (2006):

1) S-RNase-based mechanism: This is the most widespread GSI mechanism and has been found in Solanaceae, Rosaceae, Scrophulariaceae, and Campanulaceae (Franklin-Tong and Franklin, 2003). It has also been reviewed by Roalson and McCubbin (2003) and Kao and Tsukamoto (2004). In this mechanism, the main pistil component is S-RNase, a glycoprotein with ribonuclease activity. The pollen component is an S-Locus F-box protein called SLF and sometimes SFB (McClure and Franklin-Tong, 2006). Both S-RNase and SLF/SFB have a specific binding site and a non-specific binding site. In an incompatible reaction, specific sites of S-RNase and SLF/SFB bind to each other and degrade the pollen RNA leading to pollen tube rejection. In a compatible reaction, the non-specific site of SLF/SFB binds the non-specific site of S-RNase, inhibits its cytotoxic activity, and as a result the pollen tube can grow (de Graaf et al., 2006).

2) *Papaver* mechanism: This mechanism is found in the Papaveraceae (Franklin-Tong and Franklin, 2003). It has been reviewed by Thomas et al. (2003) and Franklin-Tong (2007). In field poppy (*Papaver*), the inhibition morphology is similar to SSI and pollen tube arrest occurs close to the stigma surface (Newbigin et al., 1993). In this mechanism, the pistil gene products, called S-proteins, are specifically expressed in the stigma. In pollen tubes, p26 is a cytosolic protein and includes Pr-p26a and Pr-p26b, which are soluble inorganic pyrophosphatase (sPPase) homologues. In an incompatible reaction, an allele-specific interaction (binding of the S-protein to a pollen receptor) triggers a large-scale Ca^{2+} influx into the pollen tube and, as a result, Pr-p26 shows an increase in phosphorylation, its sPPase activity is reduced, and F-actin is reorganised and depolymerised, leading to the inhibition of pollen tube growth (de Graaf et al., 2006). A mitogen-activated protein kinase, called p56-MAPK, has been suggested to be involved in a programmed cell death signalling cascade several minutes after initial rapid arrest of incompatible pollen tubes (de Graaf et al., 2006; McClure and Franklin-Tong, 2006; Li et al., 2007).

1.4.4 Ovarian SI (OSI)

OSI, also called late-acting SI (LSI) or late-acting OSI, refers to the situation where self pollen tubes grow successfully in the style and reach the ovule, but no fruit or seed is set (Aguilar and Bernardello, 2001; LaDoux and Friar, 2006). The importance of this system of SI has been underestimated and was believed to be rare (de

Nettancourt, 2001). Seavey and Bawa (1986) reviewed 25 species with OSI. Since then, many more species have been reported to have OSI including: *Ipomopsis tenuifolia* (LaDoux and Friar, 2006) and *I. aggregata* (Sage et al., 2006) (Polemoniaceae), *Jacaranda racemosa* (Bignoniaceae) (Bittencourt and Semir, 2006), three species of *Tabebuia* (Bignoniaceae) (Bittencourt and Semir, 2005), *Sophora fernandeziana* (Fabaceae) (Bernardello et al., 2004), *Pseudowintera axillaris* (Winteraceae) (Sage and Sampson, 2003), *Lycium cestrides* (Solanaceae) (Aguilar and Bernardello, 2001), and several species of *Crocus* (Iridaceae) (Chichiricco, 1996).

OSI can be pre- or post-zygotic. In pre-zygotic cases, self pollen tubes may enter the ovules without penetrating the embryo sacs, or they may penetrate the embryo sacs without completing double fertilisation. In post-zygotic cases, double fertilisation occurs, but zygotes never divide (Sage et al., 2006). Some authors believe that post-zygotic OSI and early-acting inbreeding depression are the same phenomenon, but more studies are suggested to discriminate between them (Bittencourt and Semir, 2006).

1.5 SI in olives

Most olive cultivars are self-incompatible or partially self-compatible and need to be fertilised by compatible pollenisers to ensure a commercial yield (Lavee, 1986; Lavee, 1990; Besnard et al., 1999; Dimassi et al., 1999; Moutier, 2002; Fabbri et al., 2004; Conner and Fereres, 2005). They may set some fruits in a monocultivar culture but benefit greatly from cross-pollination. Many studies have been conducted on the SI of olive cultivars and according to their results olive cultivars have been classified into three groups: self-incompatible, partially self-compatible, and self-compatible. From 547 olive cultivars classified by FAO (FAO-website, accessed 20 Nov 2007), according to the degree of SI, 348 cultivars (63.62%) have been recorded as self-incompatible, 94 cultivars (17.18%) as partially self-compatible, and 105 cultivars (19.20%) as self-compatible.

Previous studies reported Koroneiki as a self-compatible cultivar (Lavee, 1986; Lavee et al., 2002) and Picholine, Leccino, Kalamata, Manzanillo, Picual, and Arbequina as self-incompatible or partially self-compatible cultivars (Griggs et al.,

1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Dimassi et al., 1999; Ghrisi et al., 1999; Wu et al., 2002; Mookerjee et al., 2005; Diaz et al., 2006b). Furthermore, some cultivars are cross-incompatible and cannot fertilise each other (Griggs et al., 1975; Cuevas and Polito, 1997; Martin et al., 2005; Mookerjee et al., 2005). Ascolana and Mission were shown to be cross-incompatible with Manzanillo (as a host) and Barouni with Sevillano (as a host) (Martin et al., 2005).

Cross-incompatibility has been reported to be reciprocal or bidirectional in some pairs of cultivars such as Manzanillo and Mission (Griggs et al., 1975; Cuevas and Polito, 1997; Martin et al., 2005), while some other authors showed that it is not always reciprocal (Moutier et al., 2001; Lavee et al., 2002). Moutier et al. (2002) reported that Picholine is incompatible with Manzanillo (as a host) but not in the opposite direction. Lavee et al. (2002) could not find reciprocal consistency after their long-term (12 years) and large-scale (36 cultivars) experiment.

Several studies on the SI of olive cultivars have resulted in variable and conflicting results. Frantoio, for example, was concluded to be self-compatible in some reports (Sharma et al., 1976; Fontanazza and Baldoni, 1990; Fabbri et al., 2004) but self-incompatible in others (Wu et al., 2002; Mookerjee et al., 2005). Koroneiki also has been reported to be self-compatible by Lavee (1986) and Lavee et al. (2002) but self-incompatible by Mookerjee et al. (2005). The variation of results might be due to confusion in cultivar identity and the use of homonyms and synonyms (Bartolini et al., 1994; Lanza et al., 1996; Mekuria et al., 1999), contamination that may happen during pollen collection, flowering shoot isolation, and hand pollination in controlled crossing, annual changes in the total number of flowers in trees (Lavee et al., 2002), and climatic conditions (Lanza et al., 1996; Mekuria et al., 1999).

Pollen-incompatibility in olives is widely influenced by climatic conditions; thus, it may vary from area to area and from year to year (Griggs et al., 1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Lavee et al., 2002). It has been shown that pollen tubes grow more vigorously following cross-pollination (Ghrisi et al., 1999; Cuevas et al., 2001). High temperatures inhibit self pollen tube growth in the style (Griggs et al., 1975; Fernandez-Escobar et al., 1983), while cross-compatible pollen tubes are less

affected (Lavee et al., 2002). Therefore, in high temperatures cross-fertilisation is more likely to happen than self-fertilisation.

The presence of SI in olive cultivars obliges growers to plant more than one cultivar in their orchard. Fabbri et al. (2004) recommended three or four cultivars in a commercial orchard to guarantee good fruit set even if the main cultivar is considered self-compatible. Because olive cultivars might be cross-incompatible, it is important to know which cultivars are best to grow together. The selected cultivars, also, must have overlapping anthesis. Some cultivars have been reported to be efficient pollenisers for a wide range of cultivars, such as Uovo di Piccione for Manzanillo, Mission, Ascolana, and many others (Griggs et al., 1975; Lavee and Datt, 1978) and Frantoio for Manzanillo, Kalamata, Pendolino, and Picual (Wu et al., 2002). Some other advantages of planting more cultivars in the orchard are diversification of oil quality, spreading harvest requirements, minimising risk from environmental variability and changing market preferences, and ensuring annual commercial yields despite the alternate bearing habit of olive trees (Archer, 1996; Conner and Fereres, 2005).

1.5.1 Methods of study

Four different methods have been used to study the self- and cross-incompatibility of olive cultivars:

1) Measurement of fruit set after controlled self- and cross-pollination in comparison to open pollination (Fernandez-Escobar and Gomez-Valledor, 1985; Rallo et al., 1990; Bartoloni and Guerriero, 1995; Cuevas and Polito, 1997; Cuevas et al., 2001; Moutier, 2002; Quero et al., 2002).

2) Pollen tube observation using a fluorescence microscope after controlled crossing (Bartoloni and Guerriero, 1995; Cuevas et al., 2001; Wu et al., 2002).

3) Measurement of *in vitro* pollen germination and pollen tube growth in a culture sometimes containing the pistil extracts of other cultivars (Lavee and Datt, 1978; Fernandez-Escobar et al., 1983; Ghrisi et al., 1999).

4) Paternity analysis using molecular markers (de la Rosa et al., 2004; Mookerjee et al., 2005; Diaz et al., 2006b).

1.5.2 Paternity analysis

Paternity analysis is a test to find the biological father of an individual using molecular markers. In animals and plants, it is used to study the gene and pollen flow into the next generation or other populations. In plants, it can also be used to study sexual compatibility between individuals. Isozymes have been used for paternity analysis in sweet cherry (Brant et al., 1999), wild radish (Ellstrand et al., 1989), and almond (Jackson and Clarke, 1991). Brant et al. (1999) used isozyme markers to trace the pollenisers for sweet cherry cultivars and showed that the cultivar Summit was predominantly pollinated by Stella, and Stella by Van. In recent years, DNA markers such as RAPDs (Delaporte et al., 2001; Goto et al., 2002; Joung and Roh, 2004), AFLPs (Krauss and Peakall, 1998; Krauss, 1999; Gerber et al., 2000; Krauss, 2000; Joung and Roh, 2004), and microsatellites (Dow and Ashley, 1998; Streiff et al., 1999; Chaix et al., 2003; Oddou-Muratorio et al., 2003; Schueler et al., 2003; Isagi et al., 2004; Robledo-Arnuncio and Gil, 2005) have been used.

Microsatellites are ideal markers for paternity analysis because they are highly polymorphic and codominant (Queller et al., 1993). Gerber et al. (2000) compared AFLPs as dominant and microsatellites as codominant markers and argued that AFLPs are less efficient than microsatellites for paternity analysis but can still be used with good confidence. In olives, microsatellites have been used in paternity analysis to find the probable contamination in breeding programs (de la Rosa et al., 2004) and to study sexual compatibility between cultivars (Mookerjee et al., 2005; Diaz et al., 2006b).

Three methods have been used to analyse population patterns of paternity (Devlin et al., 1988):

1) Simple exclusion: In this method, the genotypes of the progeny and mother are compared, the maternal contribution is subtracted, and the father is distinguished according to the remaining paternal gametic contribution.

2) Most likely method: In this method, the likelihood of paternity is calculated for all possible fathers, and the one with the highest likelihood value is assigned as the true father. Gerber et al. (2003) have developed a computer program, called FaMoz, to calculate the likelihood value. In this program, a simulation is performed using an excessive number of progeny to estimate a threshold value (Gerber et al., 2000), and a genotyping error rate is used for both the simulation and the likelihood calculation.

3) Fractional method: In this method, the likelihood of paternity is calculated similar to the previous method, but a proportion of the paternal likelihood of each male to the paternal likelihood of all other males is used to assign the father.

1.6 Research aims

Pollen-incompatibility is one of the most important problems in olive cultivation. The Australian olive industry started about 200 years ago (Booth and Davies, 1996; Spennemann and Allen, 2000a), and today about 200 cultivars are grown, with commercial production of a smaller number in most states (Burr, 1999). Little information is available on self- and cross-incompatibility relationships under the various Australian climatic conditions. Moreover, since these conditions, particularly high temperatures, influence SI greatly (Griggs et al., 1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Lavee et al., 2002), more studies are necessary.

An adult olive tree produces about 500,000 flowers in ‘on’ years (Martin et al., 2005). Large variations have been observed in the number of inflorescences per shoot and tree, the number of flowers per inflorescence, inflorescence structure, percent staminate flowers in the inflorescence, shoot, and tree, and their positions on the inflorescences. On the other hand, these variations may change from cultivar to cultivar, from year to year, and from area to area. Understanding the floral biology and knowing the timing of floral anthesis in different cultivars are important for optimising cross-pollination and improving fruit set and production.

The mechanism regulating SI in olive is unknown, and no studies have been conducted at the molecular level. Evaluating gene expression in tissues where SI occurs

is important to elucidate the genetic control of this process. In most biological systems, genes are either repressed or de-repressed in response to internal and external cues. Identification, cloning, and studying the activity of these genes will ultimately lead to a better understanding of the molecular mechanisms occurring in these tissues. Techniques to enrich for differentially expressed genes in olive pistils during flowering such as subtractive cDNA hybridisation will be important to make headway in this research field. cDNA macroarray synthesis with the enriched genes and their hybridisation with different probes will hopefully narrow the field of potential genes and focus on the mechanisms regulating SI.

The objectives of this study, therefore, are:

1) To study the floral biology and inflorescence architecture in some olive cultivars.
2) To study the self- and cross-incompatibility of some olive cultivars using pollen tube observation.
3) To study the SI of Kalamata and to identify compatible pollenisers for this cultivar using microsatellite markers and paternity analysis.
4) To analyse gene expression in olive pistils during flowering as a tool to identify olive SI genes.

CHAPTER TWO

Orchard Layout, Plant Materials, and Climatic Recordings

2.1 Introduction

Several factors influence the sexual reproduction of plants, and one of the most important is climate. Olive trees usually grow in areas with a Mediterranean climate (with mild winters and warm, dry summers), which are found between 30° and 45° north and south latitudes (Sanz-Cortes et al., 2002; Connell, 2005). Climatic conditions in general and temperature in particular have been reported to affect different stages of olive flowering and fruit set including floral induction, ovary abortion, anthesis, pollination, and SI (Griggs et al., 1975; Ghrisi et al., 1999; Lavee et al., 2002; Sanz-Cortes et al., 2002; Ayerza and Coates, 2004; Martin et al., 2005; Reale et al., 2006a). This chapter describes the experimental sites used in this study including pertinent information on climatic conditions and plant material.

2.2 National Olive Variety Assessment (NOVA) collection

The NOVA collection (Fig. 2.1), located at the Roseworthy Campus, University of Adelaide, SA, Australia at an elevation of 68 m above sea level, latitude 34.52 S, and longitude 138.68 E, was established in 1998. The collection consists of three replicates of two tree plots of 100 accessions (600 trees). Tree spacing is 6 m within rows by 7 m between rows. Irrigation is applied by in-line drippers with a 3.6 litres per hour flow rate. The irrigation schedule is based on soil moisture monitoring using EnviroSCAN® probes and is applied before crop water stress occurs. Annual leaf tissue tests in January monitor tree nutrient levels, and appropriate fertilisers are applied. Weeds are controlled along the tree rows using contact and residual herbicides. Rye corn is sown between the

tree rows each winter as a cover crop and slashed in November to control weeds and increase soil organic matter.

All 600 trees were DNA fingerprinted using RAPDs to ensure their true varietal naming (Guerin et al., 2002). The fingerprints were compared to standard cultivars obtained from international and Australian collections. Of the 100 NOVA accessions tested, which were planted as 87 different cultivars, only 55 different genotypes were detected (Table 2.1). Forty-two of these genotypes matched with international standards, seven genotypes did not match with international standards but did with some of the other genotypes forming groups 1 to 7, and six genotypes did not match with any standards or groups including Blanquette–Early, Katsourela, Mission (WA), Pigale, Queen of Spain, and Verdale (Blackwood).

Fig. 2.2 shows the bloom times of the cultivars. The start of bloom (when 10% of flowers bloomed), full bloom (when 80% of flowers bloomed), and the end of bloom (when 80% of flowers dropped their petals) were measured over two years (2002 and 2003) and used to calculate the mean bloom time (Sweeney, S., personal communication, 2004).

2.2.1 Climatic conditions

Roseworthy has a Mediterranean-type climate with an average annual rainfall of 440.3 mm with 328.8 mm (about 75%) occurring between April and October. Fig. 2.3 shows the climatic data in 2004, 2005, and the long-term averages, and Fig. 2.4 shows wind direction at the NOVA collection in November (olive anthesis time). Table 2.2 shows the temperature, humidity, and rainfall at the NOVA collection during the flowering and sampling periods of 2004 and 2005. Data were collected from Roseworthy Agricultural College Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology).

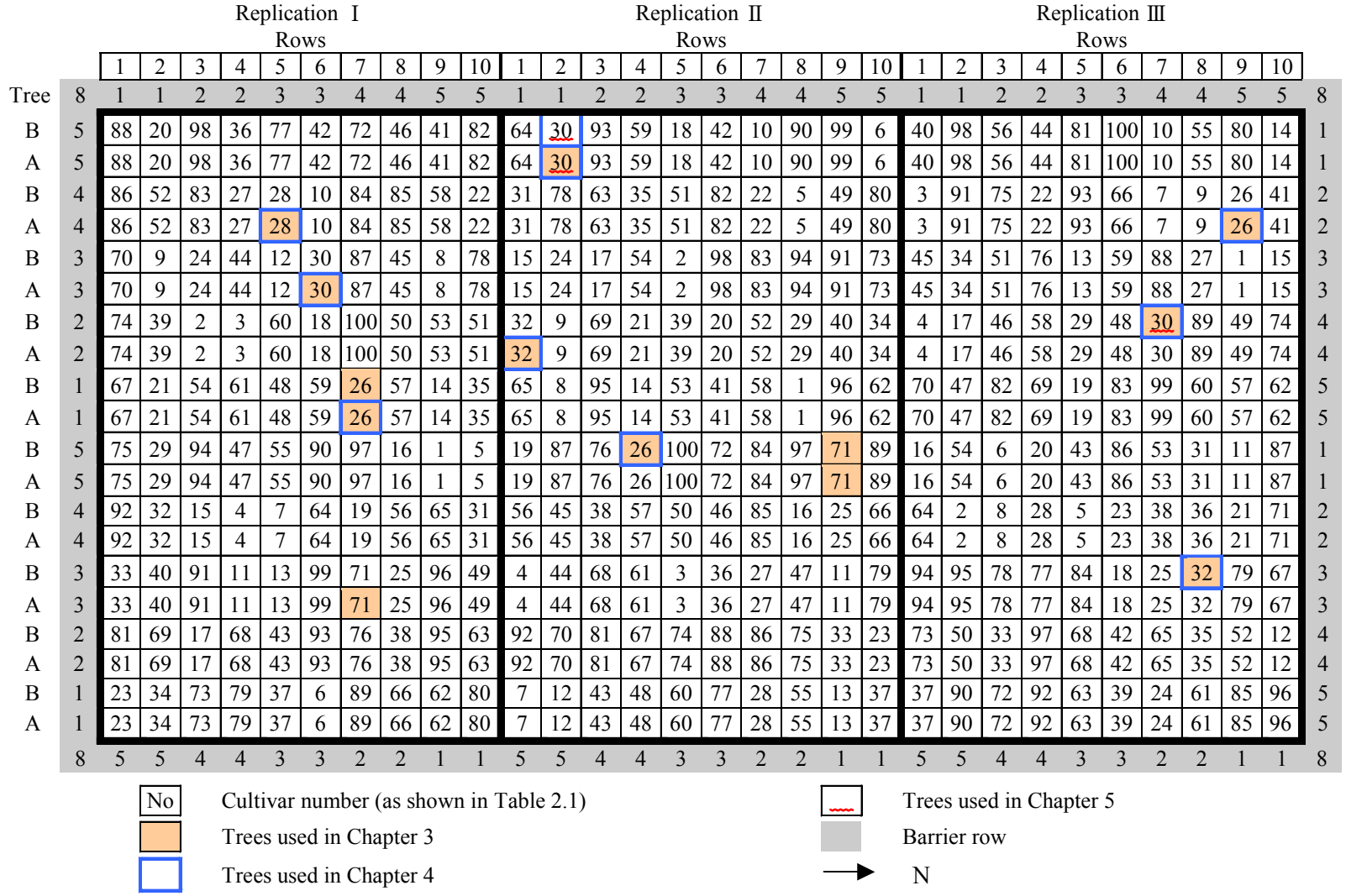


Fig. 2.1. Field map of the NOVA collection showing the trees used in the study (highlighted).

Table 2.1. Cultivars in the NOVA collection.

Tree number	Cultivar	Tree number	Cultivar
1	Frantoio	51	Columella
2	Picual	52	Frantoio
3	Barnea	53	Sevillano
4	Manzanillo	54	Group 7
5	Arbequina	55	Dr Fiasci
6	Leccino	56	Group 6
7	Pendolino	57	Frantoio
8	Hoji Blanca	58	FS17
9	Coratina	59	Group 5
10	Mission (WA)	60	Gros Reddeneau
11	177	61	Verdale Aglandau[z]
12	Picual	62	Institute
13	Frantoio	63	Group 3
14	Manaiki	64	Group 3
15	Barouni	65	Large Pickling
16	Manzanillo	66	Frantoio
17	Verdale	67	Group 5
18	Sevillano	68	Frantoio
19	Sevillano	69	Verdale Aglandau[z]
20	UC13A6	70	Group 1
21	Verdale	71	Mission
22	Azapa	72	Frantoio
23	Benito	73	Group 5
24	Verdale	74	Nevadillo Blanco
25	Jumbo Kalamata	75	Group 7
26	Frantoio	76	Oblitza
27	Queen of Spain	77	Hoji Blanca
28	Koroneiki	78	Group 6
29	Frantoio	79	Group 3
30	Kalamata	80	Group 1
31	Katsourela	81	Frantoio
32	Koroneiki	82	Group 5
33	Souri	83	Pigale
34	Amelon	84	Group 1
35	Areccuzo	85	Praecox
36	Ascolana	86	Frantoio
37	Atro Rubens	87	Regalise de Languedoc
38	Atroviolacea Brun Ribier	88	Rouget
39	Mission	89	Group 4
40	Frantoio	90	Verdale Aglandau[z]
41	Arbequina	91	Verdale Aglandau[z]
42	Black Italian	92	Group 2
43	Group 4	93	Verdale (Blackwood)
44	Blanquette - Early	94	Volos
45	Group 2	95	Frantoio
46	Frantoio	96	Picual
47	Group 5	97	Barnea
48	Group 2	98	Manzanillo
49	Frantoio	99	Arbequina
50	Buchine	100	Hoji Blanca

Verdale Aglandau[z] is also called Hardy's Mammoth in Australia.

	October			November																									
Cultivar	29	30	31	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	No of trees[z]
Amelon																															6
Arbequina																															36
Areccuzo																															12
Ascolana																															12
Atro Rubens																															10
Atroviolacea Brun Ribier																															8
Azapa																															12
Barnea																															24
Barouni																															12
Benito																															12
Black Italian																															12
Blanquette - Early																															4
Buchine																									03						4
Columella																															12
Coratina																															6
Dr Fiasci																															8
Frantoio																															161
FS17																															12
Sevillano																															35
Gros Reddeneau																															12
Group 1																															35
Group 2																															30
Group 3																															34
Group 4																															24
Group 5																															57
Group 6																															12
Group 7																															22
Hojiblanca																															34
I77																															11

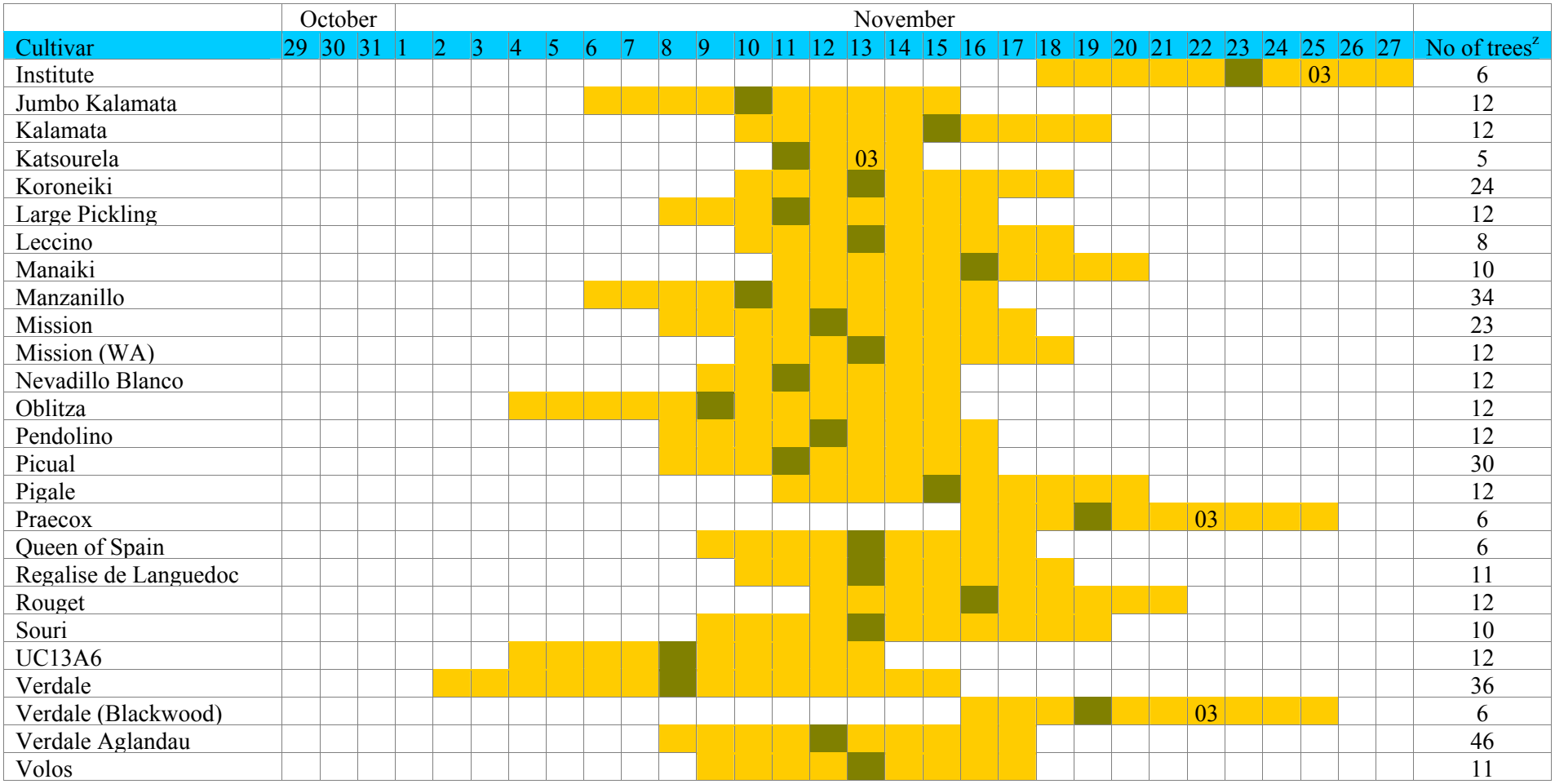
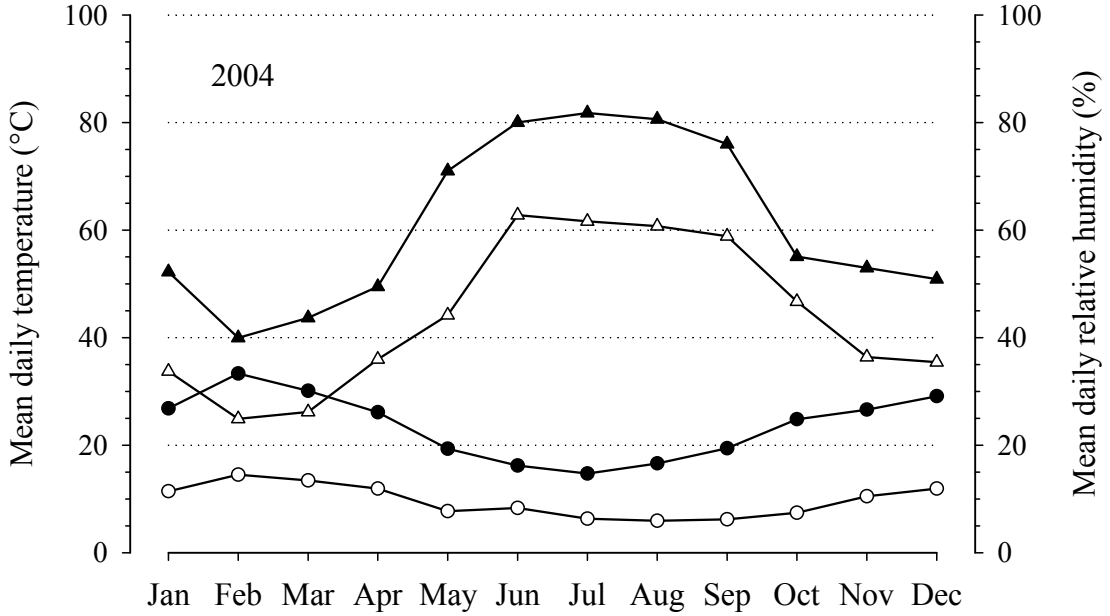
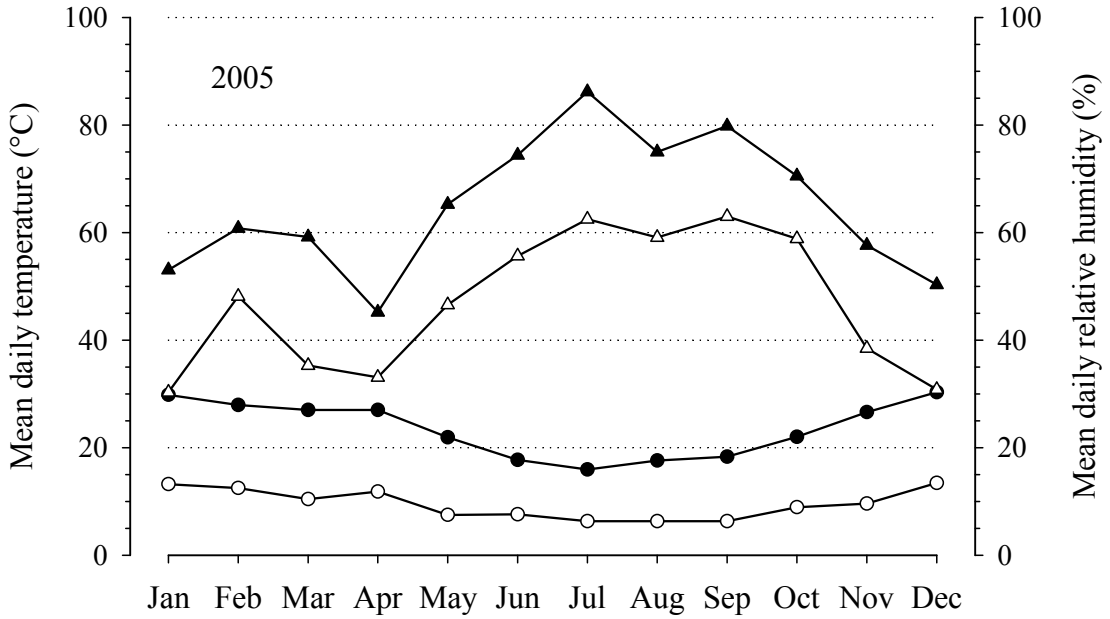


Fig. 2.2. Bloom times of the cultivars in the NOVA collection (mean of 2002 and 2003). [z]: Shows the total number of trees used in two years. In cultivars Buchine, Institute, Katsourela, Praecox, and Verdale (Blackwood) (indicated by 03), only the data from 2003 were used.

Full bloom


2004
Mean daily temperature (°C)
Mean daily relative humidity (%)
0
20
40
60
80
100
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec


2005
Mean daily temperature (°C)
Mean daily relative humidity (%)
0
20
40
60
80
100
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

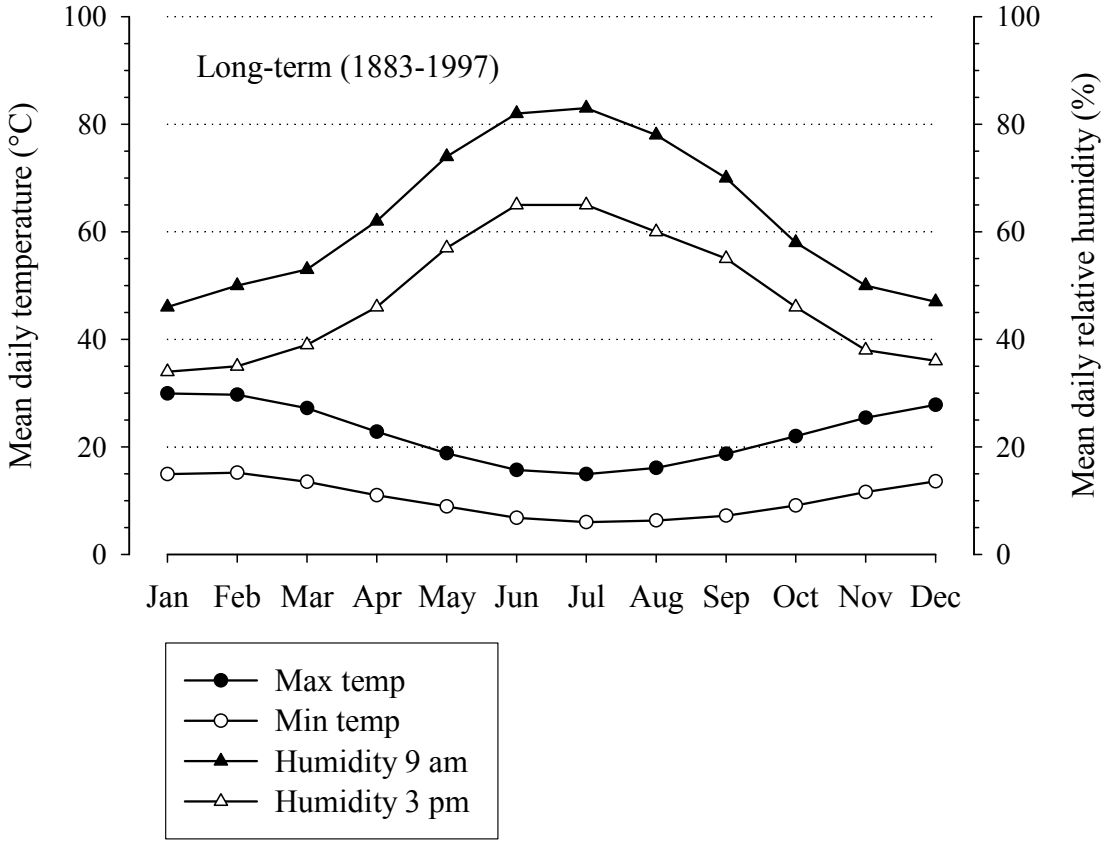


Fig. 2.3. Monthly means of daily maximum and minimum temperatures and monthly mean of relative humidity at 9 am and 3 pm at the NOVA collection for 2004, 2005, and the long-term averages. Data were collected from the Roseworthy Agricultural College Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology).

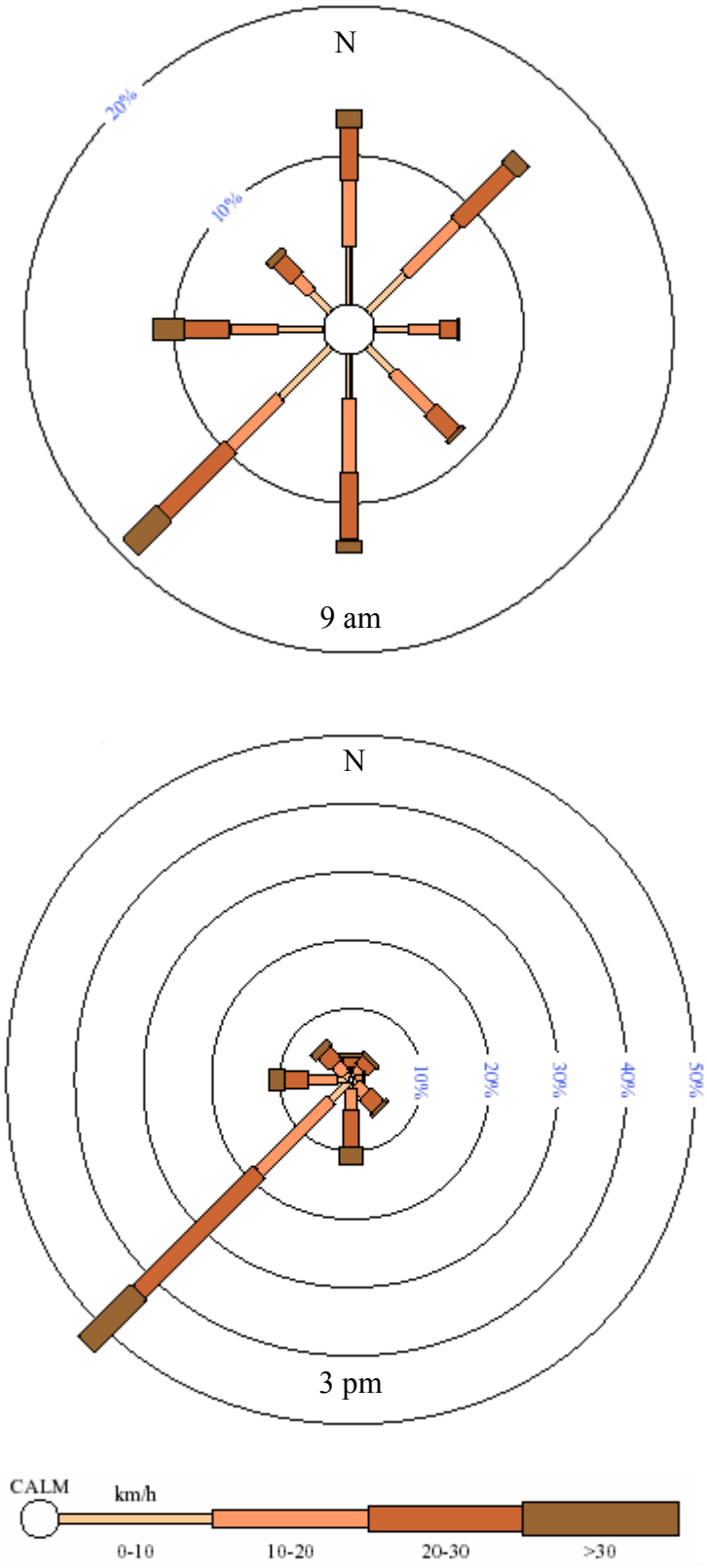


Fig. 2.4. Wind direction at the NOVA collection in November (olive anthesis time) at 9 am and 3 pm [long-term (1955-1997)]. Data were collected from the Roseworthy Agricultural College Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology). Percent of calm days at 9 am was 8% and at 3 pm was 2%.

Table 2.2. Temperature, humidity, and rainfall at the NOVA collection during the flowering and sampling periods of 2004 and 2005.

Year	Date	Temperature (°C)		Relative humidity (%)		Rainfall (mm)
		Max	Min	9 am	3 pm	
2004	25 Oct 2004	NA	4.5	28	12	0.0
	26 Oct 2004	30.3	11.4	33	74	0.0
	27 Oct 2004	17.8	7.1	48	42	5.2
	28 Oct 2004	21.3	6.6	55	56	0.0
	29 Oct 2004	24.1	2.7	47	18	0.0
	30 Oct 2004	NA	3.9	21	9	0.0
	31 Oct 2004	34.9	11.8	13	9	0.0
	01 Nov 2004	25.9	10.8	66	33	0.0
	02 Nov 2004	17.3	9.5	88	39	4.2
	03 Nov 2004	23.2	1.7	53	18	1.0
	04 Nov 2004	20.7	10.0	90	61	10.6
	05 Nov 2004	16.1	10.5	89	84	9.2
	06 Nov 2004	17.2	6.7	88	81	10.4
	07 Nov 2004	19.3	3.2	68	54	3.0
	08 Nov 2004	22.6	3.7	51	33	0.0
	09 Nov 2004	NA	4.2	41	17	0.0
	10 Nov 2004	28.6	13.6	68	19	0.2
2005	29 Oct 2005	19.0	15.0	94	86	0.0
	30 Oct 2005	NA	9.0	77	NA	NA
	31 Oct 2005	33.6	NA	NA	43	NA
	01 Nov 2005	34.3	10.6	70	41	0.0
	02 Nov 2005	32.0	16.2	53	38	0.0
	03 Nov 2005	19.6	12.2	83	46	6.4
	04 Nov 2005	25.6	6.3	70	40	0.0
	05 Nov 2005	32.6	10.7	42	17	0.0
	06 Nov 2005	26.3	17.8	39	72	0.0
	07 Nov 2005	21.1	13.4	88	94	18.2
	08 Nov 2005	26.0	14.5	94	60	25.4
	09 Nov 2005	21.5	15.0	88	67	0.2
	10 Nov 2005	17.6	8.7	81	57	1.6
	11 Nov 2005	20.4	5.1	72	48	1.8
	12 Nov 2005	20.9	4.5	67	48	0.0
	13 Nov 2005	26.4	6.4	68	38	0.0

Data were collected from the Roseworthy Agricultural College Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology). NA: Not available.

2.3 Waite collection

This collection (Fig. 2.5) is located in the Birksgate orchard at the Adelaide foothills in the Waite Campus, University of Adelaide, SA, Australia with an elevation of 115 m above sea level, latitude 34.96 S, and longitude 138.63 E. Ten cultivars were planted in this collection in 1999 at a spacing of 7 by 7 m (except for some trees in row 5). The trees received standard fertilizer, water, and cultivation. The cultivar identity was verified using RAPDs, and the cultivars matched with international standards (Guerin, J., personal communication, 2004).

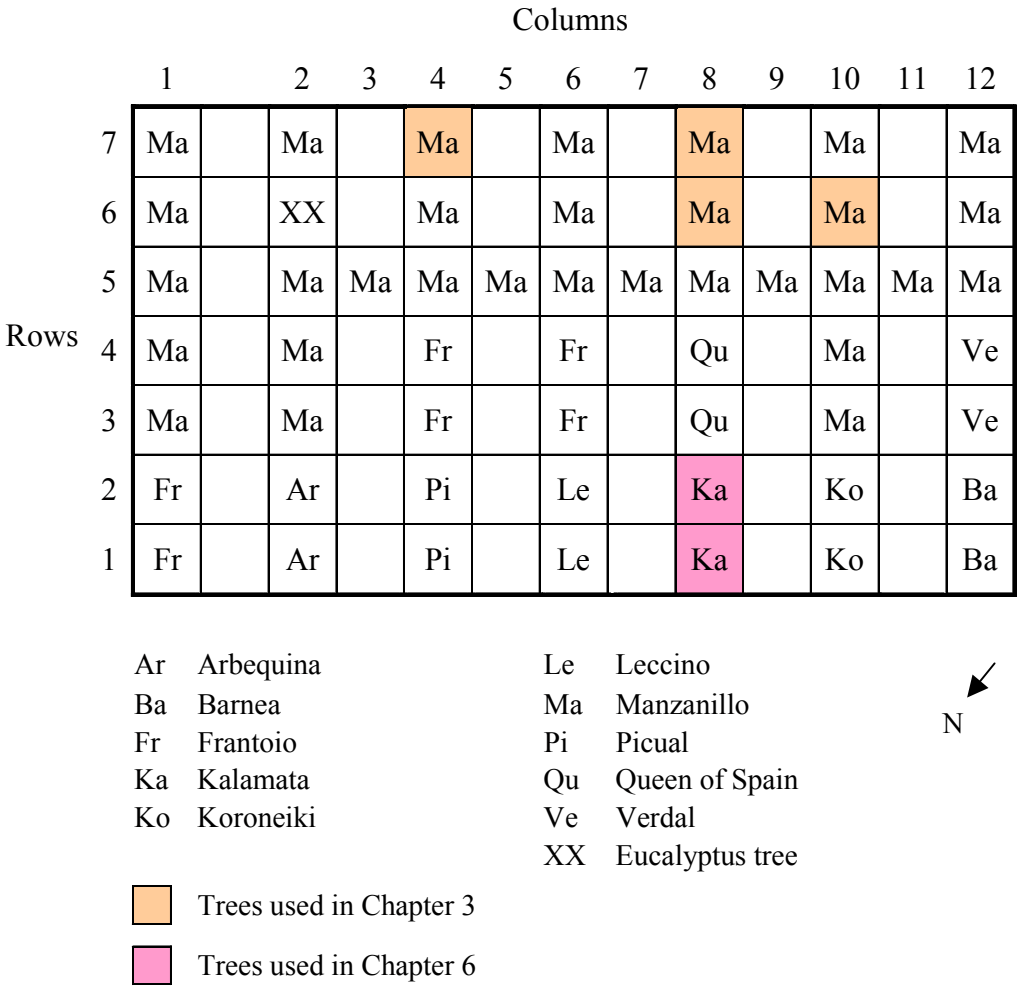
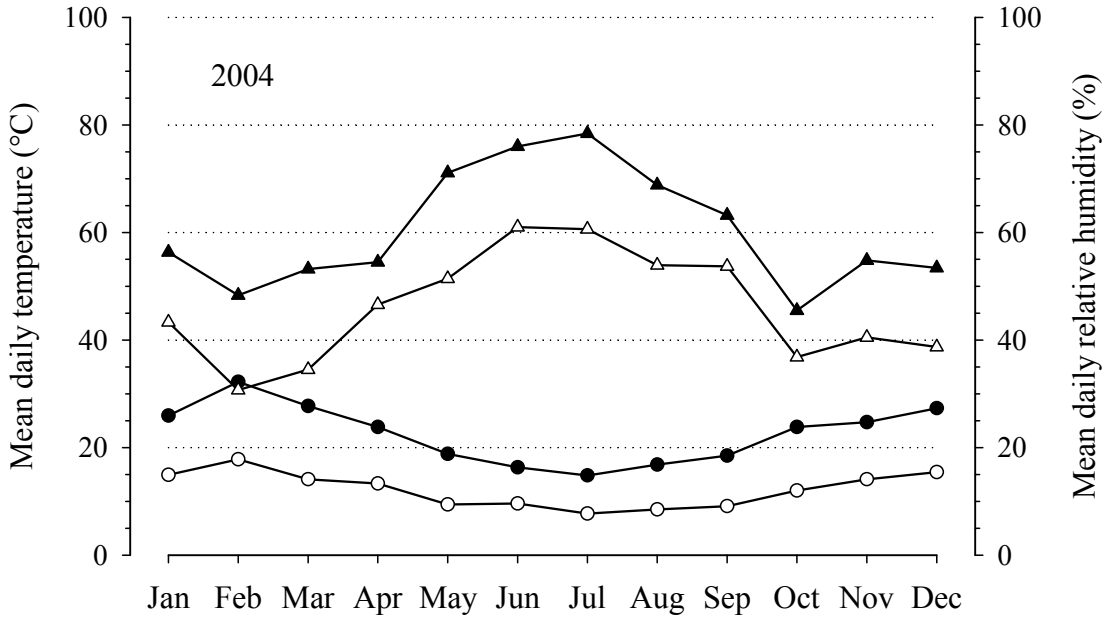


Fig. 2.5. Field map of the Waite collection showing the trees used in this study. Two or three digits were used for introducing the trees (eg. 78 and 610). The first digits represent the row numbers and the second and third represent the column numbers.

2.3.1 Climatic conditions

Waite has an average annual rainfall of 621.8 mm with 484.2 mm (about 78%) between April and October. Fig. 2.6 shows climatic data at the Waite collection in 2004, 2005, and the long-term averages. Table 2.3 shows the temperature, humidity, and rainfall at this collection during the flowering and sampling periods of 2005. Data were collected from the Waite Institute Weather Station (34.97 S, 138.63 E) for the long-term averages and from the Kent Town Weather Station (34.51 S, 138.68 E) for 2004 and 2005 (Australian Bureau of Meteorology). The Kent Town Weather Station, which is 5 km north of the Waite collection at an elevation of 48 m above sea level, was used instead of the Waite Institute Weather Station that ceased observations in 2000.



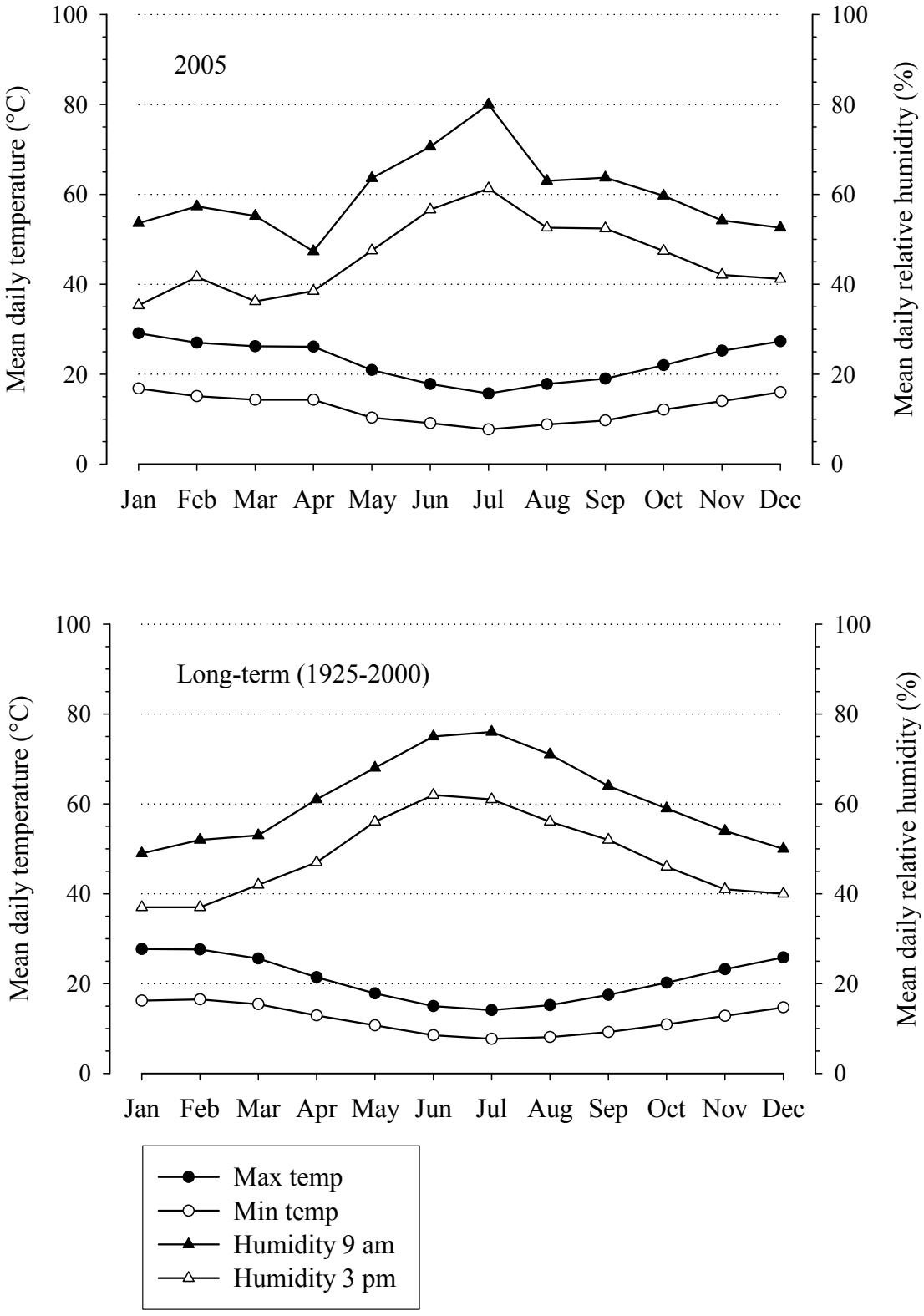


Fig. 2.6. Monthly means of daily maximum and minimum temperatures and monthly mean of relative humidity at 9 am and 3 pm at the Waite collection for 2004, 2005, and the long-term averages. Data were collected from the Waite Institute Weather Station (34.97 S, 138.63 E) for the long-term averages and from the Kent Town Weather Station (34.51 S, 138.68 E) for 2004 and 2005 (Australian Bureau of Meteorology).

Table 2.3. Temperature, humidity, and rainfall at the Waite collection during the flowering and sampling periods of 2005.

Date	Temperature (°C)		Relative humidity (%)		Rainfall (mm)
	Max	Min	9 am	3 pm	
25 Sep 2005	15.8	12.3	77	85	1.2
26 Sep 2005	17.7	11.5	66	54	9.2
27 Sep 2005	21.9	7.2	57	39	0.0
28 Sep 2005	19.3	14.5	73	52	2.0
29 Sep 2005	18.2	8.5	64	50	2.0
30 Sep 2005	23.4	9.7	38	24	0.0
01 Oct 2005	25.8	12.3	70	18	0.0
02 Oct 2005	26.6	11.6	79	27	0.0
03 Oct 2005	18.6	14.4	58	49	0.0
04 Oct 2005	27.9	9.7	43	32	0.0
05 Oct 2005	21.4	16.2	74	52	0.0
06 Oct 2005	20.4	11.1	53	50	0.6
07 Oct 2005	16.9	9.4	89	57	18
08 Oct 2005	14.8	9.1	75	72	9.8
09 Oct 2005	17.6	9.6	58	45	14.6
10 Oct 2005	22.9	7.0	40	42	0.0
11 Oct 2005	16.9	10.2	55	38	2.0
12 Oct 2005	20.8	6.8	51	33	0.0
13 Oct 2005	18.4	7.7	45	47	0.0
14 Oct 2005	19.2	4.7	44	41	0.0
15 Oct 2005	26.0	8.0	35	36	0.0
16 Oct 2005	27.2	13.9	29	31	0.4
17 Oct 2005	27.3	19.9	22	20	0.0
18 Oct 2005	25.2	20.0	34	41	0.0
19 Oct 2005	20.4	15.5	87	88	8.6
20 Oct 2005	21.9	14.2	73	52	12.2
21 Oct 2005	19.3	14.5	72	63	1.6
22 Oct 2005	24.0	13.6	66	50	0.6
23 Oct 2005	18.3	13.9	60	76	0.2
24 Oct 2005	18.2	13.2	87	70	11.6
25 Oct 2005	24.8	13.1	73	42	1.0
26 Oct 2005	19.6	13.5	63	49	0.6
27 Oct 2005	22.7	12.3	59	47	0.2
28 Oct 2005	29.9	12.0	37	27	0.0
29 Oct 2005	18.5	16.3	95	91	3.2
30 Oct 2005	23.2	12.5	65	40	3.2
31 Oct 2005	26.2	10.1	61	42	0.0
01 Nov 2005	30.9	15.2	52	38	0.0
02 Nov 2005	31.2	21.2	42	46	0.0
03 Nov 2005	19.2	13.6	65	54	10.2
04 Nov 2005	25.3	9.9	56	29	0.0
05 Nov 2005	30.7	15.9	29	18	0.0
06 Nov 2005	27.7	23.5	31	47	0.0
07 Nov 2005	20.9	14.3	93	65	15.0
08 Nov 2005	24.6	14.7	96	76	44.8

Table 2.3. Continued.

Date	Temperature (°C)		Relative humidity (%)		Rainfall (mm)
	Max	Min	9 am	3 pm	
09 Nov 2005	20.5	15.2	84	62	1.0
10 Nov 2005	17.0	11.1	59	57	4.0
11 Nov 2005	19.5	8.7	71	42	4.4
12 Nov 2005	20.5	8.9	72	43	0.0
13 Nov 2005	24.9	9.3	55	42	0.0
14 Nov 2005	32.7	13.5	28	13	0.0
15 Nov 2005	22.0	13.8	52	31	0.0
16 Nov 2005	27.6	11.6	45	31	0.0
17 Nov 2005	33.3	16.2	18	11	0.0
18 Nov 2005	27.2	22.8	40	66	0.0
19 Nov 2005	20.4	15.8	60	47	0.2
20 Nov 2005	21.4	13.7	61	53	0.0
21 Nov 2005	24.7	11.8	62	51	0.0
22 Nov 2005	23.8	11.3	56	43	0.0
23 Nov 2005	26.8	15.1	44	32	0.0

Data were collected from the Kent Town Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology).

2.4 Milano collection

The Milano collection (Fig. 2.7) in Gumeracha is located in the Adelaide Hills, SA, Australia at an elevation of 355 m above sea level, latitude 34.49 S, and longitude 138.52 E. The trees used in this study were planted in 1998 except for Barnea and Frantoio, which were planted in 2000. The orchard was irrigated using tree sprinklers every two weeks for 10 to 12 hours at 35 litres per hour in the growing season. Other cultivation methods were standard. All trees were fingerprinted using five microsatellite markers, and the identity of most trees was confirmed by comparison with standard samples (Mookerjee et al., 2005).

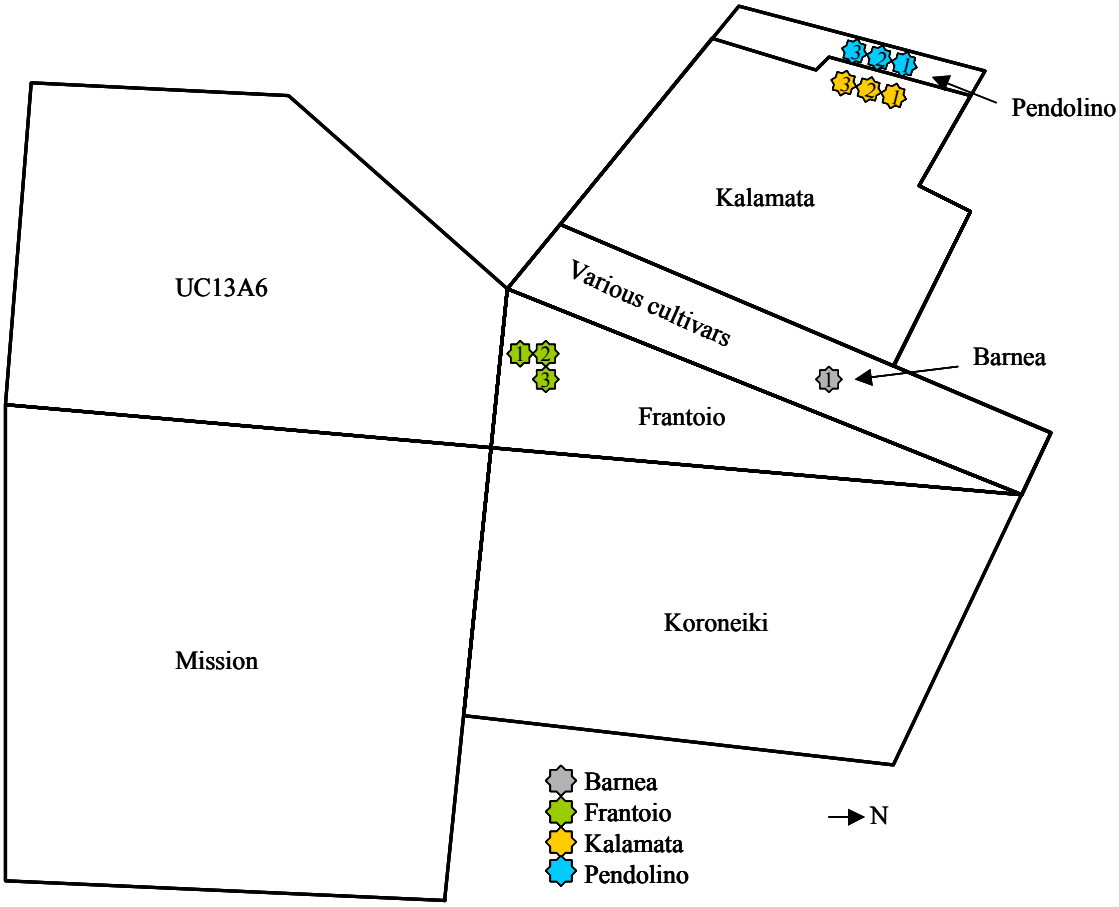


Fig. 2.7. Field map of the Milano collection, showing the trees used in Chapter 6.

2.4.1 Climatic conditions

The average annual rainfall at this collection is 775.7 mm with 607.2 mm (about 78%) between April and October. Fig. 2.8 shows climatic data at the Milano collection in 2006 and the long-term averages. Table 2.4 shows the temperature, humidity, and rainfall at this collection during the sampling period in 2006. Data were collected from the Mount Crawford Weather Station (34.71 S, 138.95 E), which is 11.5 km north east of Gumeracha at an elevation of 395 m above sea level (Australian Bureau of Meteorology).

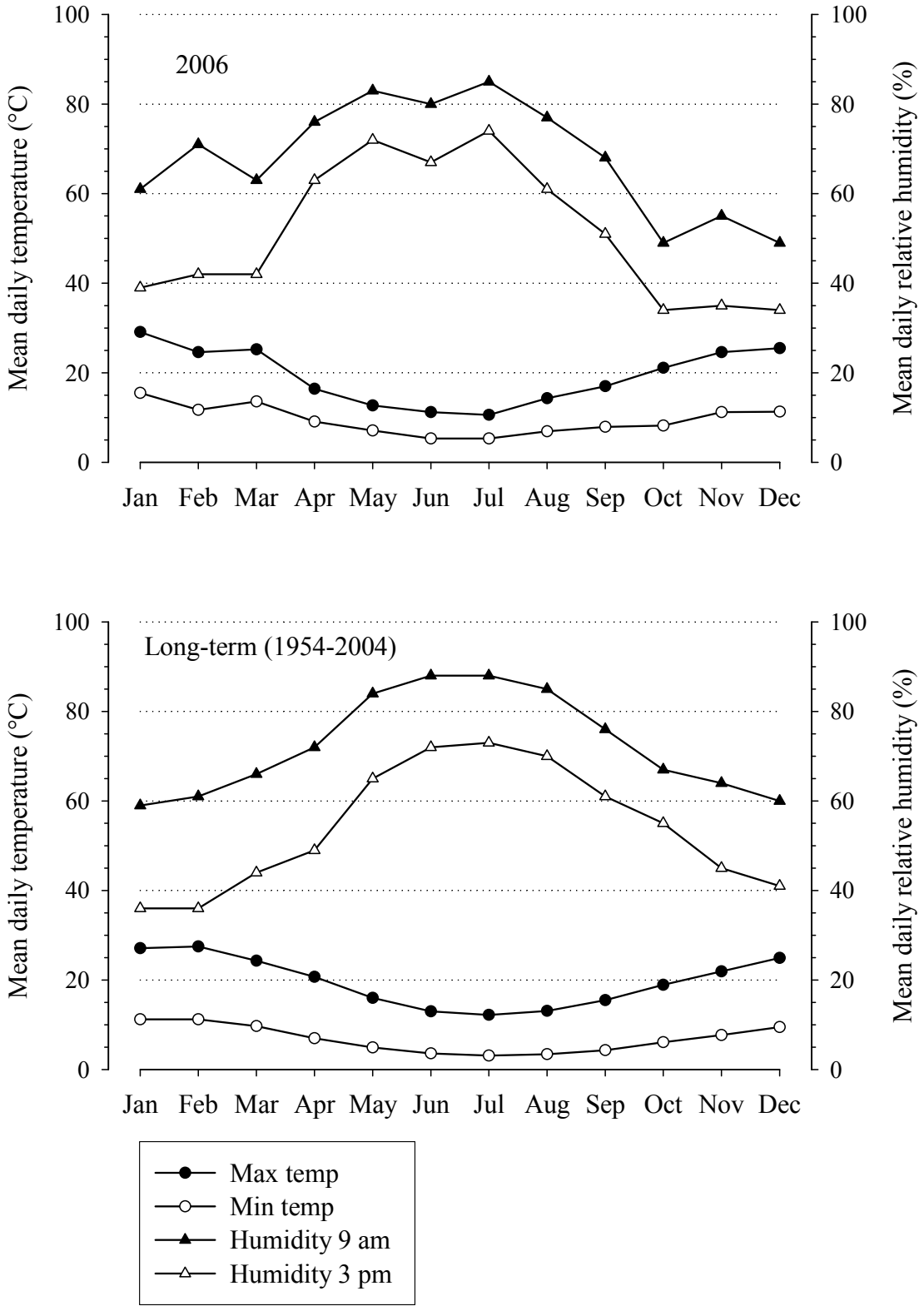


Fig. 2.8. Monthly means of daily maximum and minimum temperatures and monthly mean of relative humidity at 9 am and 3 pm at the Milano collection for 2006 and the long-term averages. Data were collected from the Mount Crawford Weather Station (34.71 S, 138.95 E) (Australian Bureau of Meteorology).

Table 2.4. Temperature, humidity, and rainfall at the Milano collection during the sampling period of 2006.

Date	Temperature (°C)		Relative humidity (%)		Rainfall (mm)
	Max	Min	9 am	3 pm	
27 Oct 2005	16.0	4.6	65	65	0.0
28 Oct 2005	12.5	5.2	63	66	0.0
29 Oct 2005	18.4	3.3	60	33	0.0
30 Oct 2005	23.5	4.4	54	22	0.0
31 Oct 2005	25.3	8.9	34	34	0.0
01 Nov 2005	26.8	10.8	39	21	0.0
02 Nov 2005	16.3	12.5	84	59	1.4
03 Nov 2005	18.6	7.6	79	54	0.0
04 Nov 2005	21.7	8.4	86	53	0.0
05 Nov 2005	19.8	8.8	82	42	0.0
06 Nov 2005	18.4	8.1	71	46	0.0
07 Nov 2005	18.0	7.6	77	48	0.0
08 Nov 2005	18.5	4.9	69	28	0.0
09 Nov 2005	24.2	5.1	64	26	0.0
10 Nov 2005	27.1	11.0	25	15	0.0
11 Nov 2005	28.4	13.6	19	41	0.0
12 Nov 2005	21.7	13.1	99	65	28.2
13 Nov 2005	15.7	10.2	93	52	0.2
14 Nov 2005	15.7	4.3	77	54	0.2
15 Nov 2005	13.3	6.1	72	47	1.8
16 Nov 2005	16.4	4.2	74	43	0.0
17 Nov 2005	23.7	5.7	61	24	0.0
18 Nov 2005	29.2	11.1	20	11	0.0
19 Nov 2005	32.0	20.3	12	9	0.0
20 Nov 2005	34.0	24.1	11	9	0.0

Data were collected from the Mount Crawford Weather Station (34.71 S, 138.95 E) (Australian Bureau of Meteorology).

CHAPTER THREE

Inflorescence Architecture

3.1 Introduction

A mature olive tree produces about 500,000 flowers but only 1-2% of them set fruits that reach maturity (Griggs et al., 1975; Martin, 1990; Lavee et al., 1996). Olive flowers are borne on inflorescences termed panicles. The inflorescences are developed mostly at leaf axils and have a central axis, terminated by a flower. The primary branches grow on the central axis and may also have secondary branches. In some cultivars, tertiary branches are found (Lavee, 1985; Weis et al., 1988; Weis et al., 1991). The number of flowers and their distribution on the inflorescence are specific for each cultivar but can change from year to year (Lavee, 1996).

The olive flower is small and white. It consists of four fused green sepals, four white petals, two stamens each with a large yellow anther, and two carpels each with two ovules (Lavee, 1996; Fabbri et al., 2004). The flowers are either perfect (hermaphrodite) or staminate (male) (Fig. 3.1). The perfect flowers have a plump green pistil with a short, thick style and a large stigma. The staminate flowers have no pistil or only a small yellow aborted one (Griggs et al., 1975; Free, 1993; Fabbri et al., 2004). The proportion of perfect and staminate flowers is genetically determined but also depends on climatic conditions and the level of fruit production in the previous year (Lavee, 1996; Lavee et al., 2002); therefore, it may vary from year to year, from tree to tree, from shoot to shoot, and from inflorescence to inflorescence (Badr and Hartmann, 1971; Fabbri et al., 2004; Martin and Sibbett, 2005).

In an individual inflorescence, the flower position affects both time of opening and gender (Cuevas and Polito, 2004). Cuevas and Polito (2004) reported that in

Mission the first flowers to open were always located on the primary branches and the last to open on the secondary branches in 79% of cases. They also showed that the probability of developing as perfect was 0.85 for terminal flowers, 0.65 for flowers at the first four primary branches from the tip, and 0.31 for flowers at the fifth primary branch and its corresponding secondary branches.

Olive growers need to optimise cross-pollination in order to control the quantity and quality of fruits. To do this, studying floral biology and phenology are important. The objective of this work was to study the biology of flowers arising at different positions on the inflorescence and on different inflorescences of the tree. Manzanillo, Mission, Frantoio, Kalamata, and Koroneiki, which are all cultivars in widespread use, were used for this study.

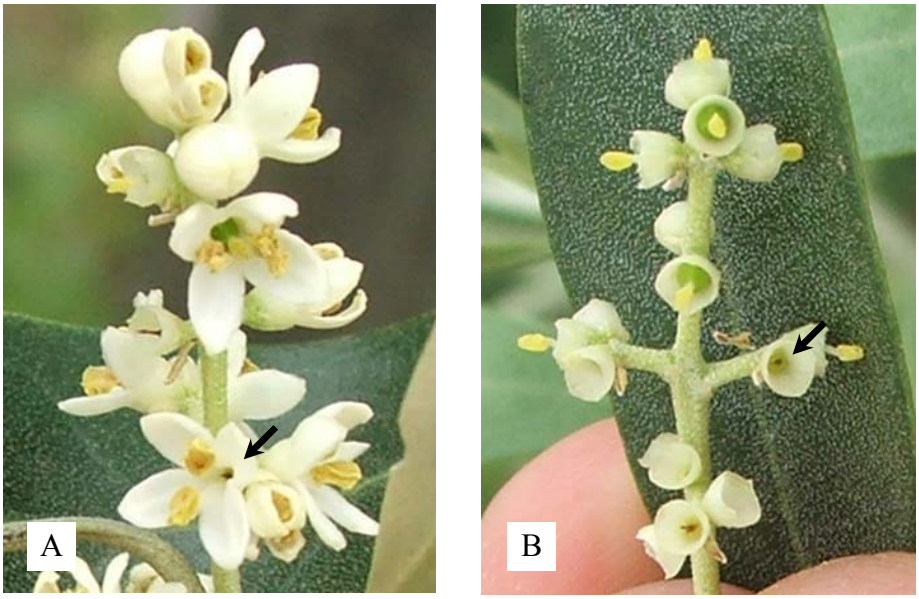


Fig. 3.1. Olive perfect and staminate (arrows) flowers before (A) and after petal abscission (B).

3.2 Materials and methods

3.2.1 Inflorescence architecture

The study was carried out in 2005 on three cultivars: Manzanillo, Mission, and Frantoio. Manzanillo trees 74, 78, and 610 at the Waite collection (Fig. 2.5), Mission trees 71 I A, 71 II A, and 71 II B, and Frantoio trees 26 I A, 26 I B, and 26 II B at the

NOVA collection (Fig. 2.1) were used. All trees were in good physiological and cultural condition, and the cultivar identities had been confirmed using RAPDs (Guerin et al., 2002; Guerin, J., personal communication, 2004). On the north side of each tree, five inflorescences from the middle of three shoots, which had just started to bloom, were selected (in total 2184 flowers) and checked every morning from flower opening to petal fall. The structure of all inflorescences, and the gender, opening day, and petal fall of all flowers were recorded. The detachment of the corolla from the flower base was considered as petal fall. Table 3.1 shows inflorescence structure, with all possible branches and flowers, and the full and abbreviated names used for them in this study. Mixed modelling using Restricted Maximum Likelihood (REML), which can deal with unbalanced data, was performed for all statistical analyses (GenStat version 8). Data were suitably transformed to satisfy the assumptions of normality and constant variance prior to analysis. Some fixed effects were removed due to zero variation.

3.2.2 Effects of shoot orientation and inflorescence location on inflorescence

The study was conducted in 2004 on cultivars Frantoio, Kalamata, and Koroneiki. Frantoio trees 26 Ⅰ A, 26 Ⅱ B, and 26 Ⅲ A, Kalamata trees 30 Ⅰ A, 30 Ⅱ A, and 30 Ⅲ B, and Koroneiki trees 28 Ⅰ A, 32 Ⅱ A, and 32 Ⅲ B were selected from the NOVA collection (Fig. 2.1). All trees were in good physiological condition and their genetic identities had been confirmed using RAPDs (Guerin et al., 2002). At full bloom, four flowering shoots from the four cardinal directions of each tree (north, south, east, and west) and in each shoot three inflorescence locations including tip, middle, and base were selected. In each part, the length (cm) and the numbers of total and perfect flowers in three inflorescences were recorded. The data were analysed statistically in a split-plot design (GenStat version 8). These characteristics were also recorded for 10 apical inflorescences in each tree if present.

3.2.3 Morphology of the axillary and apical inflorescence

The characteristics obtained from the two last experiments were used to compare the axillary inflorescences of five cultivars. Due to the unequal number of samples (108 inflorescences per cultivar from 2004 and 45 inflorescences per cultivar from 2005), data were analysed in an unbalanced design using GenStat regression facilities called Unbalanced Treatment Structure (GenStat version 8). In Koroneiki, 20 apical

inflorescences from 20 shoots in two trees (32ⅡA and 32ⅢB at the NOVA collection) were compared in 2004 with the equivalent number of axillary inflorescences from four shoots in the same trees using ANOVA and unpaired t-test (GenStat version 8). Frantoio, Kalamata, and the third tree of Koroneiki did not have any apical inflorescences.

3.2.4 Effect of inflorescence pruning on the percentage of perfect flowers

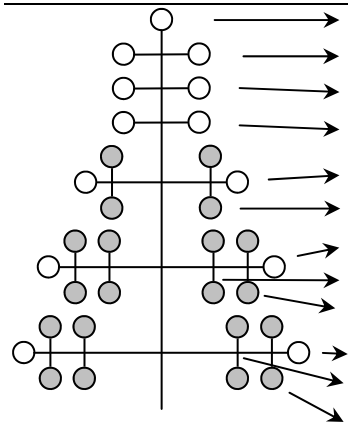
To study the effect of removing the distal half of the inflorescence, on which the flowers tend to be perfect (Bouranis et al., 1999; Ateyyeh et al., 2000; Martin et al., 2005), on flower gender in the proximal half, three Manzanillo trees at the Waite collection (Fig. 2.5) were selected (trees 74, 78, and 68) in 2005. The identities of all trees had been confirmed using RAPDs (Guerin, J., personal communication, 2004). In each tree, 10 inflorescences from four flowering shoots were pruned approximately 30 days before full bloom. The pruning location was above the first secondary branch (Fig. 3.5). The numbers of total and perfect flowers were recorded at full bloom in the pruned inflorescences and also in the proximal half of the equivalent number of untreated controls. Data were analysed statistically using ANOVA, t-test, and the non-parametric Mann Whitney test (GenStat version 8).

3.3 Results

3.3.1 Inflorescence architecture

In each cultivar, 45 inflorescences were observed but not all of them had the same structure. In other words, the number of positions and flowers differed from one inflorescence to another (Table 3.1). All inflorescences in all three cultivars had terminal flower (TF), branch 1 (B1), branch 2 (B2), branch 3 (B3), and branch 4 terminal (B4T) (100%). The least common positions were branch 5 lateral 2 (B5L2) in Manzanillo (4%) and branch 6 terminal (B6T), branch 6 lateral 1 (B6L1), and branch 6 lateral 2 (B6L2) in Mission (2%) and Frantoio (2%). Every position could potentially accommodate one, two, or four flowers, but not all of them existed. For example, TF had all possible flowers (100%) in all 45 inflorescences of all cultivars, while B5L2 had only 2 out of 8 possible flowers (25%) in Manzanillo, 27 out of 36 possible flowers (75%) in Mission, and 12 out of 20 possible flowers (60%) in Frantoio.

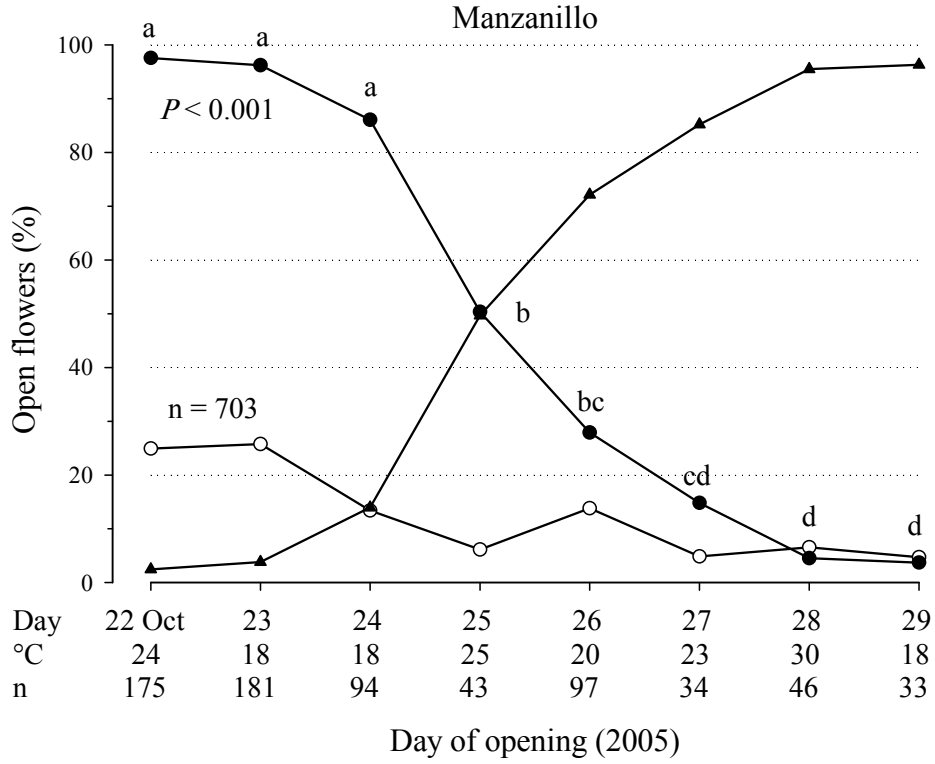
Table 3.1. Number (%) of positions and flowers in 45 inflorescences.

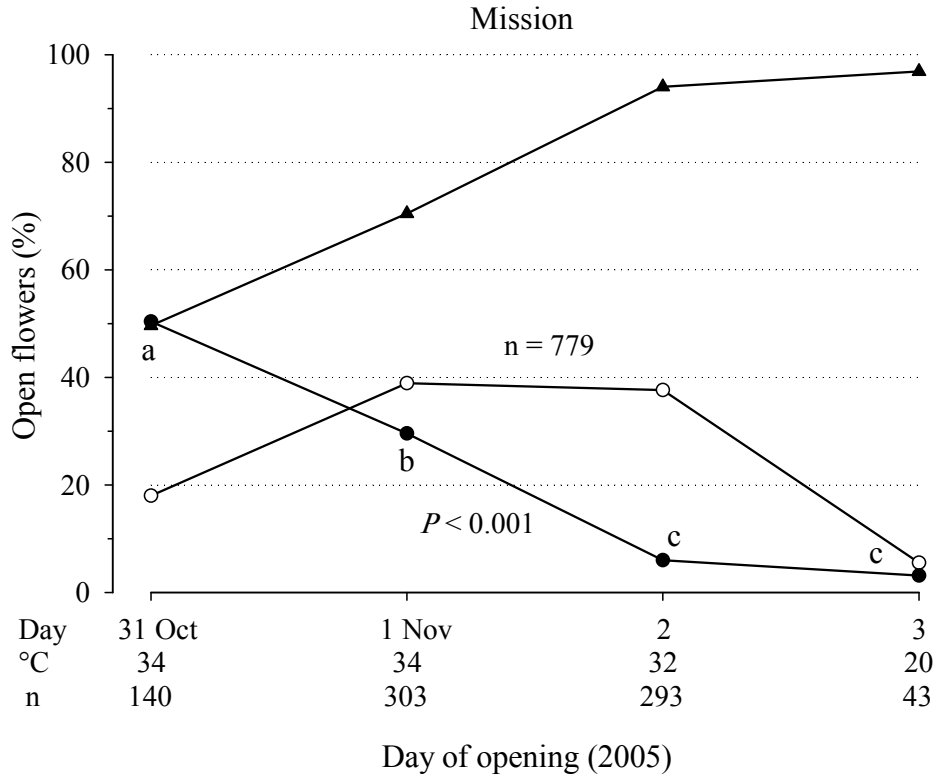
Position	Abbreviated name	Full name	Possible Number of flowers	Manzanillo		Mission		Frantoio	
				Position[z] No (%)	Flower[y] No (%)	Position[z] No (%)	Flower[y] No (%)	Position[z] No (%)	Flower[y] No (%)
	TF	Terminal flower	1	45 (100)	45 (100)	45 (100)	45 (100)	45 (100)	45 (100)
	B1	Branch 1	1-2	45 (100)	83 (92)	45 (100)	89 (99)	45 (100)	86 (96)
	B2	Branch 2	1-2	45 (100)	89 (99)	45 (100)	90 (100)	45 (100)	89 (99)
	B3	Branch 3	1-2	45 (100)	89 (99)	45 (100)	89 (99)	45 (100)	89 (99)
	B4T	Branch 4 terminal	1-2	45 (100)	89 (99)	45 (100)	90 (100)	45 (100)	88 (98)
	B4L	Branch 4 lateral	1-4	17 (38)	48 (71)	38 (84)	124 (82)	29 (64)	93 (80)
	B5T	Branch 5 terminal	1-2	35 (78)	70 (100)	37 (82)	73 (99)	32 (71)	61 (95)
	B5L1	Branch 5 lateral 1	1-4	35 (78)	120 (86)	37 (82)	143 (97)	32 (71)	123 (96)
	B5L2	Branch 5 lateral 2	1-4	2 (4)	2 (25)	9 (20)	27 (75)	5 (11)	12 (60)
	B6T	Branch 6 terminal	1-2	9 (20)	17 (94)	1 (0)	2 (100)	2 (4)	4 (100)
	B6L1	Branch 6 lateral 1	1-4	9 (20)	32 (89)	1 (0)	4 (100)	2 (4)	6 (75)
	B6L2	Branch 6 lateral 2	1-4	7 (16)	19 (68)	1 (0)	4 (100)	2 (4)	6 (75)
	TP	All terminal positions			482 (69)		478 (61)		462 (66)
	LP	All lateral positions			221 (31)		301 (39)		240 (34)
	PF	All perfect flowers			495 (70)		190 (24)		689 (98)
	SF	All staminate flowers			208 (30)		589 (76)		13 (2)
	TPF	All terminal perfect flowers			436 (62)		190 (24)		455 (65)
	TSF	All terminal staminate flowers			46 (7)		288 (37)		7 (1)
	LPF	All lateral perfect flowers			59 (8)		0 (0)		234 (33)
	LSF	All lateral staminate flowers			162 (23)		301 (39)		6 (1)
		Total flowers			703		779		702

Unshaded circles show the terminal positions including TF, B1, B2, B3, B4T, B5T, and B6T, and shaded circles show the lateral positions including B4L, B5L1, B5L2, B6L1, and B6L2. [z] and [y]: Percentage of existing positions and flowers are based on the highest possible number of positions (45) and flowers (number of existing position × 1-4), respectively.

3.3.1.1 Opening pattern of flowers

The opening period (duration between the opening of the first flower to the last) took eight days for Manzanillo at the Waite collection and four days for Mission and Frantoio at the NOVA collection.





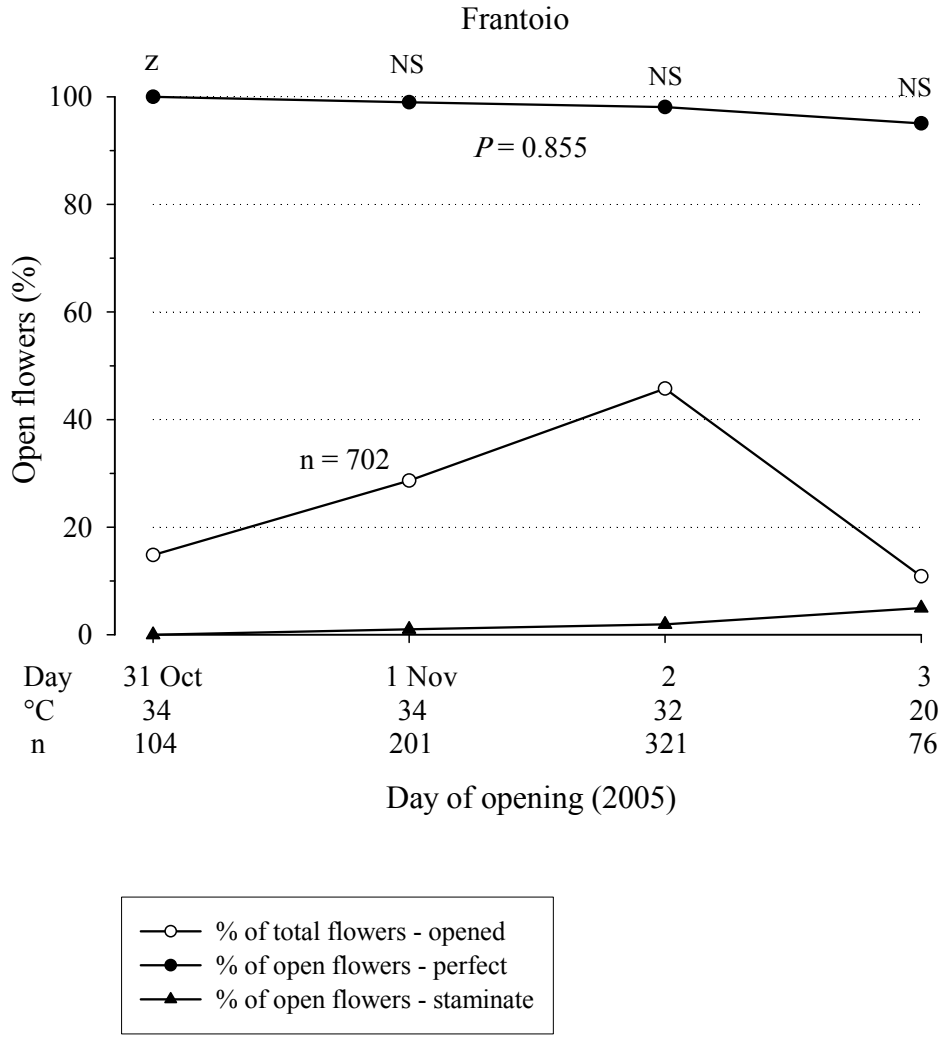


Fig. 3.2. Opening pattern of total, perfect, and staminate flowers. The percentage of total open flowers is based on the total number of flowers. The percentages of open perfect and staminate flowers are based on the total number of open flowers on each day (the numbers have been represented under the *X*-axis). Different letters represent significant differences at $P = 0.01$, LSD test. NS means non-significant difference. In Manzanillo, and Mission, data were transformed to arcsine prior to analysis. In Frantoio, data did not satisfy the assumption of constant variance even after arcsine transformation; therefore, the counts of five inflorescences were aggregated within the shoot and then transformed to arcsine. z: Data were not analysed statistically due to zero variation.

The mean daily maximum temperatures for the opening period were 22 °C at the Waite collection and 30 °C at the NOVA collection. Most flowers opened in the middle of the opening period except for Manzanillo, in which the flowers mainly opened over the first two days (Fig. 3.2). The statistical analyses were done only on the percentage of perfect flowers, calculated in each inflorescence individually. The percentage of

perfect flowers reduced significantly towards the end of period in Manzanillo ($P < 0.001$) and Mission ($P < 0.001$) but not in Frantoio ($P = 0.855$). Not all the inflorescences completed flower opening at the same time. In Mission, for example, one inflorescence (out of 45) completed opening in two days, 28 inflorescences in three days, and 16 inflorescences in four days (Table 3.2).

Table 3.2. Number of inflorescences completing flower opening (n = 45).

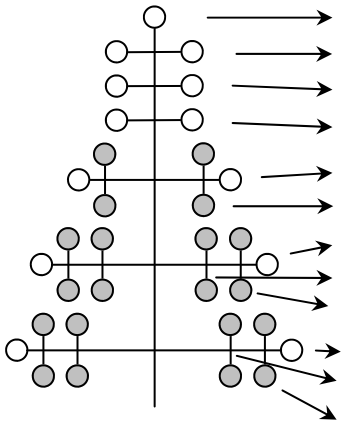
Cultivar	Opening period (Day)	Number of inflorescences that completed opening in			
		1/4 of duration	2/4 of duration	3/4 of duration	4/4 of duration
Manzanillo	8	0	0	12	33
Mission	4	0	1	28	16
Frantoio	4	0	0	18	27

In all cultivars, different positions had significantly different opening days ($P < 0.001$). In Manzanillo, B4T was the first position to open (Table 3.3). The mean opening day of flowers in this position was 1.2. It shows that most flowers at this position (74 out of 89 flowers) were open on day 1 and the others on day 2. B4T was also the first position to open in Mission with the mean opening day 1.4. In Frantoio, B4T and two other terminal positions (B5T and B6T) were the first positions to open. The last position to open in all cultivars was B5L2 with the mean opening day 6.5 in Manzanillo, 3.2 in Mission, and 3.7 in Frantoio. The difference between PF and SF was significant in Manzanillo ($P < 0.001$) and Mission ($P = 0.010$) but not in Frantoio ($P = 0.056$). In other words, the perfect flowers opened earlier than staminate flowers in Manzanillo and Mission. TP also opened earlier than LP in all cultivars ($P < 0.001$). Multiple comparison of positions (LSD, 0.01) showed that in all cultivars terminal positions opened earlier than laterals except for B1 that opened later than some of the laterals. Statistical analysis also showed that TPF opened earlier than the other flowers in all cultivars (in Mission simultaneously with TSF). In Manzanillo, TSF and LPF opened at the same time but before LSF.

Table 3.3 shows an inflorescence that has all possible positions, although such an inflorescence did not exist (according to Table 3.9, which shows seven inflorescence structures and their frequencies). Table 3.4 shows the opening days of terminal

positions observed in structures 2, 3, 4, and 5 (68.1% of the all inflorescence) and in structure 1 (23.0% of the all inflorescence). In structures 2, 3, 4, and 5, the first position to open in all cultivars was B4T (in Mission statistically simultaneous with B5T) and the last was B1. In structure 1, which did not have B5T and B6T, the first position to open in all cultivars was B3 (statistically simultaneous with TF and B4T in Manzanillo and with B4T in Mission).

Table 3.3. Opening days at different positions.

Position		Opening day					
		Manzanillo		Mission		Frantoio	
		$P < 0.001$		$P < 0.001$		$P < 0.001$	
	TF	1.6 ± 0.1	b	2.0 ± 0.1	c	2.3 ± 0.1	d
	B1	4.5 ± 0.2	e	2.8 ± 0.1	d	3.2 ± 0.1	ef
	B2	2.2 ± 0.1	c	2.2 ± 0.1	c	2.5 ± 0.1	d
	B3	1.9 ± 0.1	b	1.7 ± 0.1	b	1.9 ± 0.1	c
	B4T	1.2 ± 0.0	a	1.4 ± 0.1	a	1.4 ± 0.1	a
	B4L	5.4 ± 0.3	f	2.9 ± 0.0	d	3.3 ± 0.1	ef
	B5T	1.6 ± 0.1	b	1.6 ± 0.1	ab	1.7 ± 0.1	b
	B5L1	5.8 ± 0.2	f	2.8 ± 0.0	d	3.1 ± 0.0	e
	B5L2	6.5 ± 0.5	f	3.2 ± 0.1	e	3.7 ± 0.2	f
	B6T	1.9 ± 0.2	b	1.5 ± 0.5	ab	2.5 ± 0.3	d
	B6L1	3.8 ± 0.2	d	2.5 ± 0.3	d	3.0	z
	B6L2	5.7 ± 0.4	f	3.0	z	3.7 ± 0.2	f
		$P < 0.001$		$P < 0.001$		$P < 0.001$	
	TP	2.2 ± 0.1		1.9 ± 0.0		2.2 ± 0.0	
	LP	5.4 ± 0.1		2.9 ± 0.0		3.2 ± 0.0	
		$P < 0.001$		$P = 0.010$		$P = 0.056$	
	PF	2.2 ± 0.1		1.7 ± 0.1		2.5 ± 0.0	
	SF	5.6 ± 0.1		2.5 ± 0.0		3.1 ± 0.2	
		$P < 0.001$		$P < 0.001$		$P < 0.001$	
	TPF	2.0 ± 0.1	a	1.7 ± 0.0	a	2.2 ± 0.0	a
	TSF	4.5 ± 0.3	b	2.1 ± 0.0	a	3.0 ± 0.3	b
	LPF	4.2 ± 0.2	b	NA		3.2 ± 0.0	b
	LSF	5.9 ± 0.1	c	2.9 ± 0.0	b	3.2 ± 0.2	b

Unshaded and shaded circles show the terminal and lateral positions, respectively. Mean values ± SE (number of flowers shown in Table 3.1). SE could not be calculated when the variation was zero. z: Data were not analysed statistically due to zero variation. Different letters within each column represent statistical differences at $P < 0.01$, LSD test. All data were transformed to square roots prior to analysis. NA: Not applicable.

Table 3.4. Opening days of terminal positions in some inflorescence structures.

Terminal positions	Opening day											
	In structures 2, 3, 4, and 5						In structure 1					
	Manzanillo		Mission		Frantoio		Manzanillo		Mission		Frantoio	
	$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$		$P < 0.001$	
TF	1.7	b	2.1	c	2.5	d	1.4	ab	1.8	b	1.9	b
B1	5.0	d	3.0	d	3.4	e	3.0	c	2.1	b	2.8	c
B2	2.3	c	2.3	c	2.7	d	1.7	b	2.0	b	2.0	b
B3	2.0	b	1.8	b	2.2	c	1.1	a	1.1	a	1.3	a
B4T	1.1	a	1.4	a	1.6	a	1.2	ab	1.2	a	1.8	b
B5T	1.7	b	1.6	ab	1.9	b	NA		NA		NA	

Different letters within each column represent statistical differences at $P < 0.01$, LSD test. All data were transformed to square roots prior to analysis. NA: Not applicable.

3.3.1.2 Position of perfect and staminate flowers on the inflorescence

Statistical analysis showed that there was a highly significant difference in the percent of perfect flowers among different positions in Manzanillo ($P < 0.001$) and Mission ($P < 0.001$) but not in Frantoio ($P = 0.590$). In Manzanillo, TF, B2, B4T, and B5T had the highest percentage of perfect flowers (Fig. 3.3). B3 and B6T had 100% perfect flowers; therefore, they were not compared to others due to zero variation. Among the terminal positions only B1 had a few perfect flowers (58.9%) and was more similar to B6L1 as a lateral position (66.7%). B4L, B5L1, and B6L2 had the lowest percentages of perfect flowers and B5L2 did not have any. In Mission, most terminal positions including TF, B2, B3, B4T, and B5T had the highest percentage of perfect flowers. B6T was not analysed statistically due to zero variation but had approximately the same percentage of perfect flowers as B4T and B5T. In this cultivar like Manzanillo, B1 had the lowest percentage of perfect flowers (24.4%) at terminal positions. The lateral positions including B4L, B5L1, B5L2, B6L1, and B6L2 produced only staminate flowers. In Frantoio, there was no significant difference between terminal and lateral positions perhaps due to the high percentage of perfect flowers (98.2%).

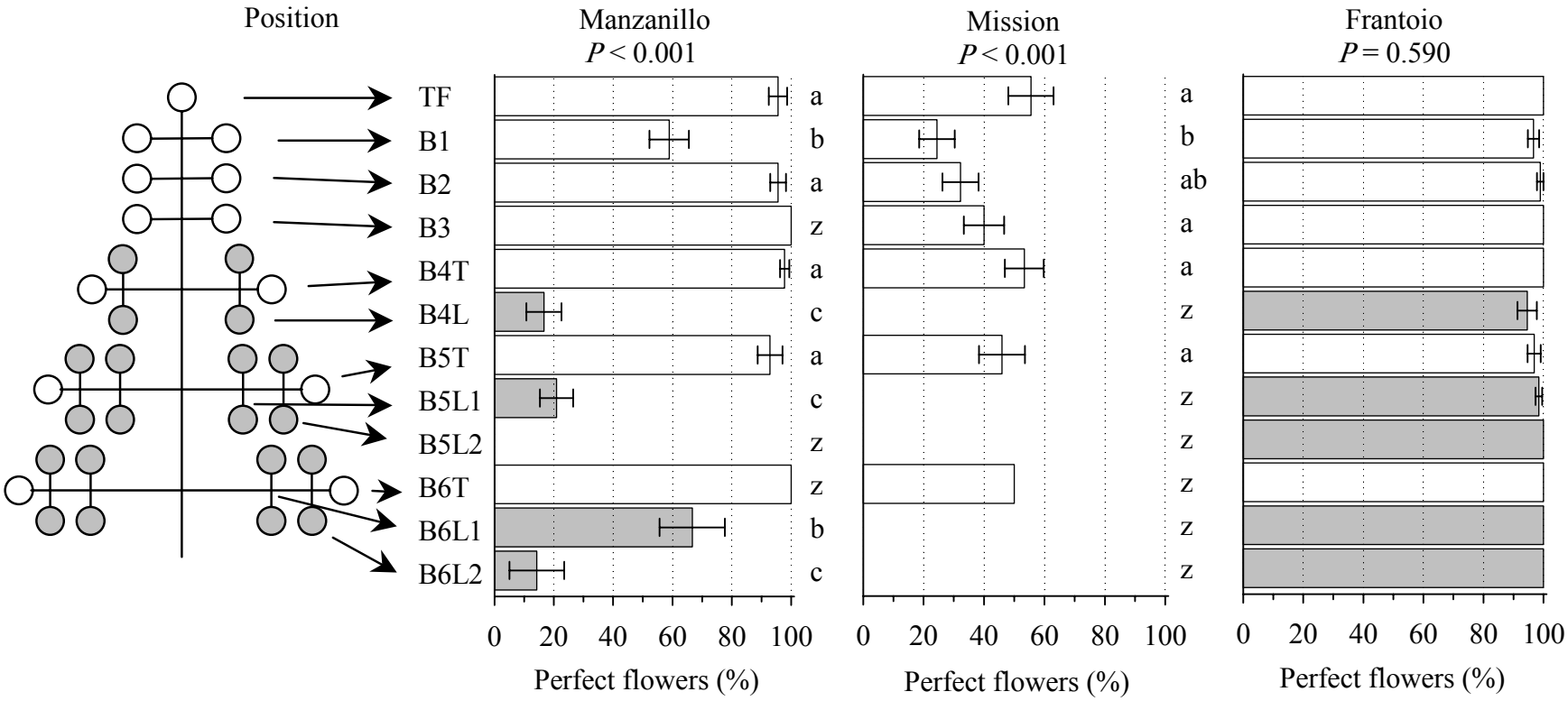


Fig. 3.3. Percentage of perfect flowers at different positions. Unshaded and shaded circles show the terminal and lateral positions, respectively. Error bars indicate ±SE and could not be calculated when the variation was zero (numbers of positions shown in Table 3.1). Different letters within each cultivar represent significant differences at $P = 0.01$, LSD test. In Manzanillo and Mission, data were transformed to arcsine prior to analysis. In Frantoio, initial arcsine transformation did not satisfy the assumption of constant variance; therefore, the counts of five inflorescences were aggregated within the shoot and then transformed to arcsine. z: Data were not analysed statistically due to zero variation.

3.3.1.3 Petal persistence at different positions of the inflorescence

The mean petal persistence in Manzanillo (2.4 days) was higher than in Mission (1.7 days) and Frantoio (1.4 days). The higher temperature at the NOVA collection, as documented in 3.3.1.1, was probably the reason for the difference. The day of opening had a highly significant effect on petal persistence in all cultivars ($P < 0.001$). Fig. 3.4 shows that petal persistence reduced gradually towards the end of the opening period, unless there was a sharp fall in temperature. In Manzanillo, for example, petal persistence was 2.5 days on 22 October 2005. It increased to 2.8 when the temperature changed from 24 °C to 18 °C. After 23 October, petal persistence reduced again until 26 October when the temperature fell, and after 27 October petal persistence reduced until the end of the opening period. On the last day of flower opening (29 October), there was no increase in petal persistence despite a very sharp reduction of temperature. On that day, 15 of the last 33 flowers, which were closed on the previous day, had no petals or only faded and separated corollas; therefore, petal persistence was considered zero. In Mission and Frantoio, petal persistence showed a gradual decrease from 31 October to 2 November and then an increase on 3 November when the temperature fell sharply.

Table 3.5 shows that different positions had significantly different petal persistence in Manzanillo ($P < 0.001$), Mission ($P = 0.021$), and Frantoio ($P < 0.001$). Positions with the lowest petal persistence were B4L, B5L1, and B5L2 in Manzanillo, TF and B6L1 in Mission, and TF in Frantoio. In Manzanillo, PF and TP had significantly higher petal persistence than SF and LP, respectively ($P < 0.001$ in both). TPF and LPF had the same petal persistence but higher than TSF and LSF, respectively. In Mission and Frantoio, there were no significant differences in petal persistence between PF and SF, between TP and LP, and between TPF, TSF, LPF, and LSF.

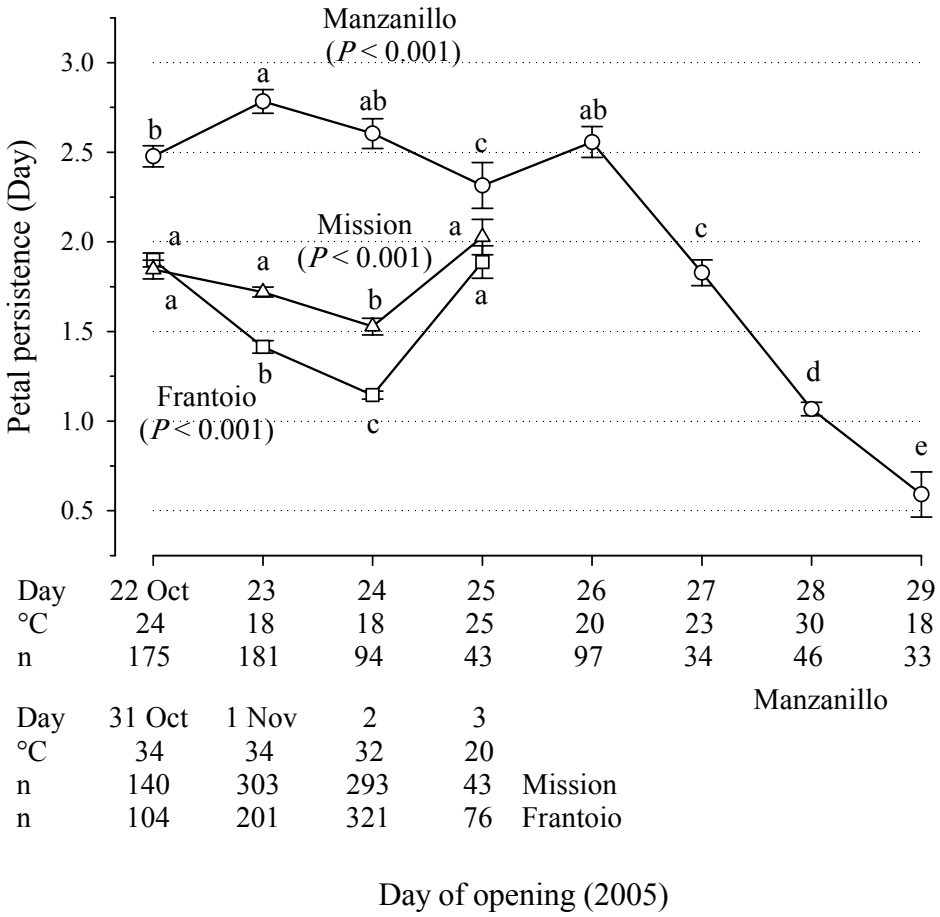
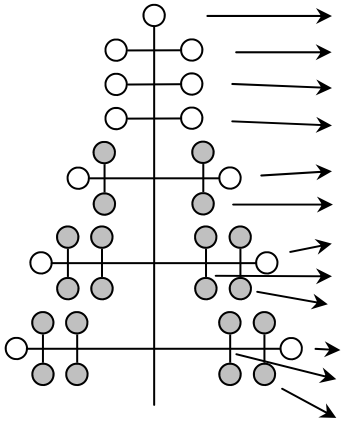


Fig. 3.4. Petal persistence during opening period. Error bars indicate ±SE. Different letters represent significant differences at P = 0.01, LSD test. In Manzanillo and Mission, data were transformed to square roots prior to analysis. In Frantoio, data did not satisfy the assumption of constant variance even after square root transformation; therefore, the counts of five inflorescences were averaged within the shoot and then transformed to square roots.

Table 3.5. Petal persistence at different positions.

Position		Petal persistence					
		Manzanillo		Mission		Frantoio	
		$P < 0.001$		$P < 0.001$		$P < 0.001$	
	TF	2.5 ± 0.1	ab	1.4 ± 0.1	b	1.2 ± 0.1	b
	B1	2.3 ± 0.1	b	1.8 ± 0.1	a	1.4 ± 0.1	ab
	B2	2.7 ± 0.1	a	1.6 ± 0.1	ab	1.4 ± 0.1	ab
	B3	2.6 ± 0.1	ab	1.7 ± 0.1	a	1.4 ± 0.1	ab
	B4T	2.5 ± 0.1	ab	1.6 ± 0.1	ab	1.6 ± 0.1	a
	B4L	1.8 ± 0.1	c	1.7 ± 0.1	ab	1.5 ± 0.1	ab
	B5T	2.5 ± 0.1	ab	1.6 ± 0.1	ab	1.4 ± 0.1	ab
	B5L1	1.8 ± 0.1	c	1.8 ± 0.1	a	1.3 ± 0.0	ab
	B5L2	1.5 ± 0.5	c	1.7 ± 0.2	ab	1.7 ± 0.2	a
	B6T	2.4 ± 0.2	ab	2.0	z	1.0	z
	B6L1	2.1 ± 0.1	b	1.2 ± 0.2	b	1.0	z
	B6L2	2.5 ± 0.1	ab	1.0	z	1.3 ± 0.2	ab
		$P < 0.001$		$P = 0.132$		$P = 0.119$	
	TP	2.5 ± 0.0		1.7 ± 0.0		1.5 ± 0.0	
	LP	1.9 ± 0.1		1.7 ± 0.0		1.4 ± 0.0	
		$P < 0.001$		$P = 0.801$		$P = 0.274$	
	PF	2.6 ± 0.0		1.6 ± 0.0		1.4 ± 0.0	
	SF	1.8 ± 0.1		1.7 ± 0.0		1.2 ± 0.2	
		$P < 0.001$		$P = 0.269$		$P = 0.320$	
	TPF	2.6 ± 0.0	a	1.6 ± 0.0		1.5 ± 0.0	
	TSF	2.1 ± 0.2	bc	1.7 ± 0.0		1.6 ± 0.2	
	LPF	2.3 ± 0.1	ab	NA		1.4 ± 0.0	
	LSF	1.8 ± 0.1	c	1.7 ± 0.0		1.0	z

Unshaded and shaded circles show the terminal and lateral positions, respectively. Mean values ± SE (number of flowers shown in Table 3.1). SE could not be calculated when the variation was zero. Different letters within each column represent statistical differences at $P < 0.01$, LSD test. z: Data were not analysed statistically due to zero variation. All data were transformed to square roots prior to analysis. NA: Not applicable.

3.3.2 Effects of shoot orientation and inflorescence location on the inflorescence

Table 3.6 shows the inflorescence characteristics in four different cardinal directions on the trees and three different locations on shoots, statistically analysed for three cultivars together. Shoot orientation had a significant effect on the percentage of perfect flowers ($P = 0.048$) but not on the length of the inflorescence ($P = 0.293$) and number of flowers per inflorescence ($P = 0.576$). The inflorescences located on the east side of trees had higher percentages of perfect flowers than those on the south. Three

different inflorescence locations including tip, middle, and base parts of shoots had a significant effect on the length of the inflorescence ($P < 0.001$) and the number of flowers per inflorescence ($P < 0.001$) but not on the percentage of perfect flowers ($P = 0.719$). The inflorescences located at the tip and middle were longer and had more flowers than those at the base. The results also showed that Frantoio, Kalamata, and Koroneiki were statistically different in all characteristics. Frantoio had the longest inflorescence (4.1 cm), and Kalamata had the highest number of flowers per inflorescence (22.3). By contrast, Koroneiki had the shortest inflorescence (3.0 cm) and lowest number of flowers (17.1).

Table 3.6. Inflorescence characteristics in different directions, locations, and cultivars.

	n	Length (cm)		No of flowers[z]		% perfect flowers[y]	
Direction		$P = 0.293$		$P = 0.576$		$P = 0.048$	
North	81	3.4 ± 0.1		20.3 ± 0.7		50.0 ± 4.0	ab
South	81	3.4 ± 0.1		19.2 ± 0.6		44.8 ± 4.3	b
East	81	3.6 ± 0.1		20.4 ± 0.8		59.8 ± 3.7	a
West	81	3.2 ± 0.1		17.7 ± 0.6		47.4 ± 4.5	ab
Location		$P < 0.001$		$P < 0.001$		$P = 0.719$	
Tip	108	3.7 ± 0.1	a	20.7 ± 0.5	a	49.6 ± 3.6	
Middle	108	3.6 ± 0.1	a	20.8 ± 0.5	a	51.3 ± 3.5	
Base	108	2.9 ± 0.1	b	16.8 ± 0.7	b	50.7 ± 3.8	
Cultivar		$P < 0.001$		$P < 0.001$		$P < 0.001$	
Frantoio	108	4.1 ± 0.1	a	18.9 ± 0.6	b	92.1 ± 1.6	a
Kalamata	108	3.2 ± 0.1	b	22.3 ± 0.6	a	23.2 ± 1.9	c
Koroneiki	108	3.0 ± 0.1	c	17.1 ± 0.5	c	36.3 ± 2.9	b

Mean values ±SE. z and y: Data were transformed to square roots and arcsine, respectively. Different letters within each column represent significant differences at $P = 0.05$, Tukey's test.

The data were also analysed separately for each cultivar (Table 3.7) and showed that shoot orientation had no significant effect on any characteristic in any cultivar, while location had significant effects on the length of the inflorescence and number of flowers in all cultivars but not on the percentage of perfect flowers. It seems that the base inflorescences were shorter and had fewer flowers in all cultivars. In Koroneiki, the differences were not significant between the base and middle inflorescences in length and between the base and tip inflorescences in the number of flowers.

Table 3.7. Effects of shoot orientation and inflorescence location on inflorescence characteristics in Frantoio, Kalamata, and Koroneiki.

Cultivar	n	Frantoio			Kalamata			Koroneiki		
		Length (cm)	No of flowers [z]	% perfect flowers [y]	Length (cm)	No of flowers [z]	% perfect flowers [y]	Length (cm)	No of flowers [z]	% perfect flowers [y]
Direction		$P = 0.874$	$P = 0.660$	$P = 0.607$	$P = 0.321$	$P = 0.392$	$P = 0.188$	$P = 0.389$	$P = 0.284$	$P = 0.617$
North	27	3.9	17.3	93.9	3.2	24.6	11.7	3.2	18.6	44.6
South	27	4.1	20.0	84.2	3.2	21.6	20.5	2.7	16.1	29.6
East	27	4.4	21.3	96.6	3.6	23.6	39.0	3.0	16.3	44.0
West	27	4.0	17.2	93.56	3.0	19.3	21.5	2.7	16.7	27.2
Location		$P < 0.001$	$P = 0.003$	$P = 0.449$	$P < 0.001$	$P = 0.004$	$P = 0.085$	$P = 0.034$	$P = 0.030$	$P = 0.451$
Tip	36	4.4 a	20.8 a	91.6	3.4 a	23.4 a	21.2	3.2 a	17.8 ab	35.9
Middle	36	4.3 a	19.5 a	95.2	3.5 a	24.3 a	27.4	3.0 ab	18.5 a	31.3
Base	36	3.5 b	16.5 b	89.4	2.7 b	19.1 b	20.9	2.5 b	14.4 b	41.8

[z] and [y]: Data were transformed to square roots and arcsine, respectively. Different letters within each column represent significant differences at $P = 0.05$, Tukey's test.

3.3.3 Morphology of axillary and apical inflorescences

The statistical analysis showed highly significant differences among cultivars in the number of flowers per inflorescence ($P < 0.001$) and percentage of perfect flowers ($P < 0.001$) (Table 3.8). The mean number of flowers per inflorescence varied from 15.6 in Manzanillo (2005) to 22.3 in Kalamata (2004). On the other hand, the number of flowers per individual inflorescence ranged from four in Frantoio (2004) to 49 in Kalamata (2004). In Frantoio, the number of flowers per inflorescence and the percentage of perfect flowers did not change over two years. Frantoio (2004) and Frantoio (2005) had the highest percentage of perfect flowers among the cultivars. In Frantoio (2004), Frantoio (2005), and Manzanillo (2005), all the inflorescences were female fertile. In other words, they had at least one perfect flower per inflorescence and therefore the ability to set fruit. On the other hand, there were no perfect inflorescences (inflorescences with 100% perfect flowers) in Kalamata (2004), Manzanillo (2005), and Mission (2005). The statistical analysis also showed that the number of flowers was significantly different among the inflorescences of each shoot ($P = 0.002$), among the shoots of each tree ($P < 0.001$), and among the trees of each cultivar ($P < 0.001$). By contrast, the percentage of perfect flowers was not significantly different among the inflorescences of each shoot and among the trees of each cultivar but it was among the shoots of each tree.

Inflorescences may also be different in structure. Table 3.9 shows all the structures observed and their frequencies in Manzanillo, Mission, and Frantoio in 2005. The most common structures were 2 in Manzanillo and 3 in Mission and Frantoio. Structure 4 was not found in Manzanillo and structure 6 was not in Mission and Frantoio. The least common structures were 5 and 6 in Manzanillo, 7 in Mission, and 5 and 7 in Frantoio.

In olive some flowering shoots produce apical inflorescences in addition to the axillary inflorescences. Two trees of Koroneiki had some apical inflorescences in 2004, while Kalamata, Frantoio, and the third tree of Koroneiki did not have any. Table 3.10 shows the characteristics of apical inflorescences in comparison with the equivalent number of axillary inflorescences in Koroneiki. Apical inflorescences had significantly more flowers ($P < 0.001$) but the same length and percentage of perfect flowers.

Table 3.8. Inflorescence characteristics of some olive cultivars.

Cultivar	n	Flower per inflorescence			Perfect flowers			No (%) of inflorescences	
		No[z]		Range	%[y]		Range	Female fertile[x]	Perfect[w]
Kalamata (2004)	108	22.3 ± 0.6	a	12-49	23.2 ± 1.9	c	0.0-75.0	85 (78.7)	0 (0.0)
Koroneiki (2004)	108	17.1 ± 0.5	b	6-33	36.3 ± 2.9	c	0.0-100	87 (80.6)	3 (2.8)
Frantoio (2004)	108	18.9 ± 0.6	ab	4-43	92.1 ± 1.6	a	6.9-100	108 (100)	74 (68.5)
Frantoio (2005)	45	15.8 ± 0.4	b	10-24	98.2 ± 0.7	a	73.7-100	45 (100)	37 (34.3)
Manzanillo (2005)	45	15.6 ± 0.6	b	11-25	69.6 ± 2.1	b	16.7-93.3	45 (100)	0 (0.0)
Mission (2005)	45	17.3 ± 0.5	ab	11-25	24.3 ± 3.2	c	0.0-69.2	31 (68.9)	0 (0.0)
Cultivar		$P < 0.001$			$P < 0.001$				
Cultivar: Tree		$P < 0.001$			$P = 0.498$				
Cultivar: Tree: Shoot		$P < 0.001$			$P = 0.010$				
Cultivar: Tree: Shoot: Inflorescence		$P = 0.002$			$P = 0.998$				

Mean values ±SE. [z] and [y]: Data were transformed to square roots and arcsine, respectively. [x] and [w]: Inflorescences with at least one perfect flower and 100% perfect flowers, respectively. Different letters within each column represent significant differences at $P = 0.05$, LSD test.

Table 3.9. Number (%) of inflorescence structures.

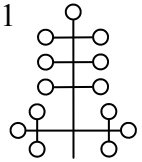
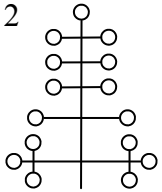
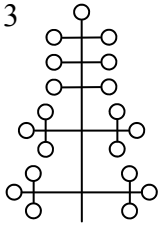
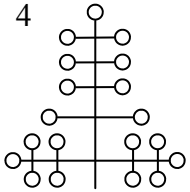
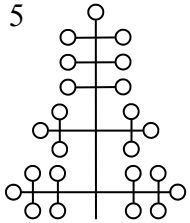
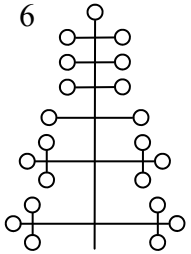
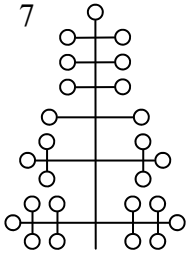
Structures	n	1 	2 	3 	4 	5 	6 	7 
Manzanillo	45	10 (22.2)	19 (42.2)	5 (11.1)	0 (0.0)	2 (4.4)	2 (4.4)	7 (15.6)
Mission	45	8 (17.8)	5 (11.1)	22 (48.9)	0 (0.0)	9 (20.0)	0 (0.0)	1 (2.2)
Frantoio	45	13 (28.9)	11 (24.4)	14 (31.1)	3 (6.7)	2 (4.4)	0 (0.0)	2 (4.4)
Mean percent		23.0	25.9	30.4	2.2	9.6	1.5	7.4

Table 3.10. Characteristics of some apical and axillary inflorescences in Koroneiki.

Inflorescence	n	Length (cm)	No of flowers[z]	% perfect flowers[y]
Apical	20	3.4 ± 0.2	23.4 ± 1.0	34.2 ± 6.1
Axillary	20	3.1 ± 0.2	16.9 ± 1.3	41.8 ± 7.3
ANOVA		$P = 0.186$	$P < 0.001$	$P = 0.390$
T-test		$P = 0.263$	$P < 0.001$	$P = 0.537$

Mean values ±SE. [z] and [y]: Data were transformed to square roots and arcsine, respectively.

3.3.4 Effect of inflorescence pruning on the percentage of perfect flowers

Removing the distal half of inflorescences in Manzanillo increased the percentage of perfect flowers in the proximal half (Fig. 3.5). The percentage of perfect flowers in the pruned inflorescences was 48.4% in comparison with 34.7% in the similar part of the untreated controls. ANOVA, t-test, and the non-parametric Mann Whitney test indicated that the difference is highly significant ($P < 0.001$ in all tests). Data were transformed to arcsine before the first two tests.

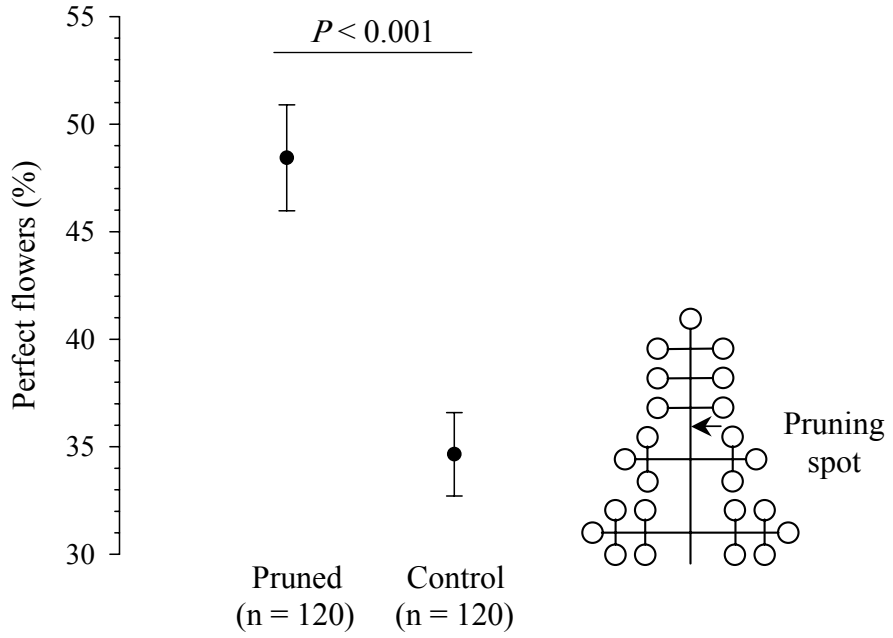


Fig. 3.5. Effect of inflorescence pruning on the percentage of perfect flowers in Manzanillo. Error bars indicate ±SE (n = 120).

3.4 Discussion

The length of the anthesis period depends on the cultivar and climatic conditions (Lavee et al., 2002). It is usually 5-6 days (Dimassi et al., 1999; Fabbri et al., 2004) but can take 10-15 days in cooler areas (Fabbri et al., 2004; Conner and Fereres, 2005; Reale et al., 2006a). Under the climatic conditions of this study, the opening period was eight days for Manzanillo in the Waite collection and four days for Mission and Frantoio in the NOVA collection, which is located in the warmer area of Roseworthy. Individual inflorescences can bloom in 2-3 days (Fabbri et al., 2004), as in this study 28 (out of 45) Mission inflorescences bloomed in three days and one inflorescence in only two days.

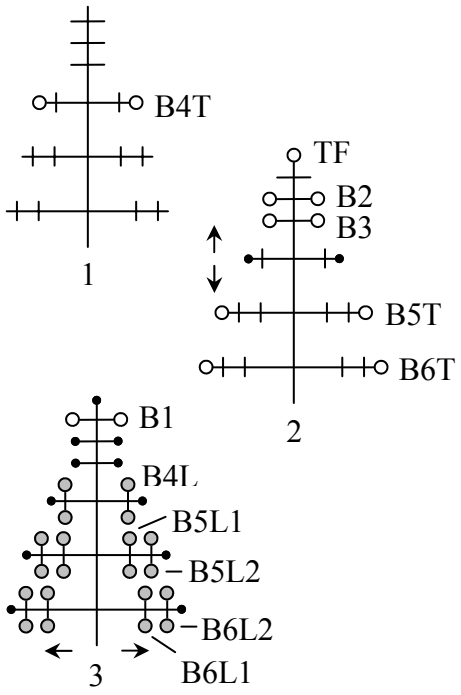
The present study also showed that in all cultivars the percentage of perfect flowers was higher in the beginning of the opening period and reduced towards the end of the period though the reduction was not statistically significant in Frantoio. In Manzanillo and Mission, PF opened significantly earlier than SF. In Frantoio, there were only 13 staminate flowers that opened shortly after the PF, but the effect was not significant. These results confirm the findings of other workers that the perfect flowers tend to bloom before the staminate flowers (Brooks, 1948; Cuevas and Polito, 2004).

It was previously reported that the flower position on the inflorescence affected the opening day (Cuevas and Polito, 2004). Cuevas and Polito (2004) showed that the first flowers to open were on primary branches and the last to open were on secondary branches in 79% of cases and primary branches in 20% of cases (most commonly the flowers arising immediately basal to the terminal flowers; B1 in this study). These results correspond well with the observations of this study, which showed that in all cultivars studied TP (primary branches plus TF) opened earlier than LP (secondary branches), TPF were the first flowers to open, LSF were the last, and TSF and LPF opened at the same time. B4T was the first position to open in all cultivars (in Frantoio simultaneously with B5T and B6T), and B5L2 was the last.

In Table 3.11 flower opening was categorised in three stages according to the results of this study. The first position to open was B4T (stage 1); then the other terminal positions opened gradually and in a trend towards two directions (the vertical

arrows in stage 2). At nearly the same time as B1 (stage 3), the first laterals (B4L, B5L1, and B6L1) opened and after them B5L2 and B6L2 (in a trend that the horizontal arrows show). An exception for this opening pattern was B4T in Mission (stage 1). It was not statistically different from B5T and B6T (stage 2) in the opening day. TF was another exception that did not follow the vertical trend of opening (stage 2) and opened earlier than B1 and B2. These findings are in contrast with those of Lavee (1985) who suggested that there is no determined order of opening within the inflorescence, although there were some inflorescences that did not match this pattern entirely.

Table 3.11. Opening stages of flowers in olive.

Opening stage		Position	Opening day					
			Manzanillo $P < 0.001$		Mission $P < 0.001$		Frantoio $P < 0.001$	
	1	B4T	1.2	a	1.4	a[y]	1.4	a
	2	TF	1.6	b	2.0	c	2.3	d
		B2	2.2	c	2.2	c	2.5	d
		B3	1.9	b	1.7	b	1.9	c
		B5T	1.6	b	1.6	ab[y]	1.7	b
		B6T	1.9	b	1.5	ab[y]	2.5	d
	3	B1	4.5	e	2.8	d	3.2	ef
		B4L	5.4	f	2.9	d	3.3	ef
		B5L1	5.8	f	2.8	d	3.1	e
		B5L2	6.5	f	3.2	e	3.7	f
		B6L1	3.8	d	2.5	d	3.0	z
		B6L2	5.7	f	3.0	z	3.7	f

Unshaded and shaded circles show the terminal and lateral positions open at the current stage, respectively, small black circles show the positions open at the previous stages, and the lines with no circle show unopened positions. z: Data were not analysed statistically due to zero variation. Different letters within each column represent statistical differences at $P < 0.01$, LSD test (from Table 3.3). [y]: Positions that were not different statistically but placed in different opening stages.

Previous studies showed that flower position on the inflorescence also affected the gender of that flower (Bouranis et al., 1999; Dimassi et al., 1999; Ateyyeh et al., 2000; Cuevas and Polito, 2004; Martin et al., 2005). Perfect flowers were observed more at the tip of the inflorescence (Brooks, 1948; Bouranis et al., 1999; Ateyyeh et al., 2000; Martin et al., 2005) and in the middle (Dimassi et al., 1999). On the other hand, within

the typical triple-flower groups on the inflorescence the middle flower was generally perfect (Brooks, 1948; Martin et al., 2005). Cuevas and Polito (2004) also found that the probability of developing as perfect was 85% for TF, 65% for B1, B2, B3, and B4T, and 31% for B5T and B5L1. These results agree with the findings of the present study in Manzanillo and Mission. In these two cultivars, there were highly significant differences in the percentage of perfect flowers between positions but not in Frantoio. In Manzanillo, the terminal positions had a higher percentage of perfect flowers than laterals except for B1, which had the same percentage of perfect flowers as B6L1 but more than the other laterals. In Mission, the terminal positions had 40.1% of perfect flowers, while the laterals were all staminate. In this cultivar, B1 had the lowest percentage of perfect flowers (26.6%) between the terminal positions.

Cuevas and Polito (2004) have hypothesised that perfect flowers tend to develop on primary branches because of their better nurtured positions. Furthermore, early blooming is also associated with nutrient availability and is not inherent to the perfect flowers. The results of Frantoio support this hypothesis. In this cultivar, 98.2% of flowers were perfect, and there was no significant difference between the positions, while different positions had the same opening pattern as Manzanillo and Mission. In other words, in Frantoio the lateral positions bloomed later, while they had the same probability to be perfect as terminals.

According to the results of this study, petal persistence depends on the daily temperature during flower opening. It was longer at the Waite collection (2.4 days in Manzanillo) than the NOVA, which experienced higher temperatures (1.7 days in Mission and 1.4 days in Frantoio). A comparison made on Mission (Cuevas and Polito, 2004) showed that the perfect flowers were heavier than the staminate flowers. They had heavier pistils and petals but the stamens were the same weight. They were similar in some characteristics such as the number of pollen grains per flower, pollen grain diameter, percentage pollen viability and germination, and pollen tube growth rate. The findings of this study showed that in Mission and Frantoio PF and TP had the same petal persistence as SF and LP, respectively, while in Manzanillo PF and TP had significantly longer petal persistence than SF and LP, respectively. This may be due to the cooler climate or be a cultivar effect. The opening day and petal persistence of different flowers of Manzanillo showed that the flowers that opened earlier had longer

petal persistence. For example, TPF and LSF were the first and last flowers to open with the longest and shortest petal persistence, respectively. In Mission and Frantoio, flowers did not follow this trend maybe because of their different environmental conditions and shorter opening periods.

Dimassi et al. (1999) found that shoot orientation had a significant effect on the percentage of perfect flowers. In most cultivars observed by them, shoots located on the south and north of trees had the highest and lowest percentage of perfect flowers, respectively. The results of this study showed that shoot orientation did not have any effect on the length of the inflorescence, the number of flowers per inflorescence, and the percentage of perfect flowers in Frantoio, Kalamata, and Koroneiki. When all cultivars were analysed together, the effect of shoot orientation on the percentage of perfect flowers was significant. The shoots located on the east side had more perfect flowers than the shoots on the south in the NOVA collection.

The effect of inflorescence location on the shoot on inflorescence characteristics has been reported previously (Lavee, 1996; Dimassi et al., 1999; Ateyyeh et al., 2000; Cuevas and Polito, 2004). According to the results presented here, inflorescence location on shoots did not have any effect on the percentage of perfect flowers. This does not agree with the findings of Ateyyeh et al. (2000) and Dimassi et al. (1999), although they are in contrast with each other as Ateyyeh et al. (2000) reported a lower percentage of perfect flowers at the base of shoots and Dimassi et al. (1999) at the tip. The present study also showed that the inflorescences located at the base of shoots were smaller and with a lower number of flowers, which agrees with the observation of Ateyyeh et al. (2000) and Lavee (1996).

Inflorescence characteristics are specific for each cultivar. Under the conditions of this study, Manzanillo had approximately the same number of flowers per inflorescence and percentage of perfect flowers as previously recorded for this cultivar (Brooks, 1948; Lavee and Datt, 1978; Cuevas et al., 1994a; Lavee et al., 1996; Lavee et al., 2002; Wu et al., 2002), but Mission had a lower percentage of perfect flowers than recorded previously (Brooks, 1948; Cuevas and Polito, 2004). Kalamata and Frantoio had approximately the same percentage of perfect flowers as previously observed at the same location but slightly fewer flowers per inflorescence (Wu et al., 2002). The

cultivar with a higher percentage of perfect flowers, Frantoio (2004 and 2005) also had a higher percentage of female fertile and perfect inflorescences. Manzanillo had more female fertile inflorescences than previously recorded (Cuevas et al., 1994a), but Mission had fewer female fertile and perfect inflorescences than recorded previously (Cuevas and Polito, 2004). The number of flowers per inflorescence and percentage of perfect flowers are genetically determined and vary from cultivar to cultivar (Lavee, 1996). They may also vary from year to year (Lavee, 1985; Cuevas et al., 1994a; Lavee, 1996; Lavee et al., 2002; Reale et al., 2006a), from tree to tree (Lavee et al., 2002), from shoot to shoot (Brooks, 1948), and from inflorescence to inflorescence (Lavee and Datt, 1978; Lavee et al., 2002). In this study, the number of flowers per inflorescence and percentage of perfect flowers did not change significantly from 2004 to 2005 in Frantoio. It was also observed that the number of flowers per inflorescence varied from tree to tree, from shoot to shoot, and from inflorescence to inflorescence, while the percentage of perfect flowers varied only from shoot to shoot.

Previous workers reported that inflorescences play a sink role in the olive, and therefore nutritional conditions have an important effect on pistil abortion (Uriu, 1959; Cuevas et al., 1994a; Perica et al., 2001; Levin and Lavee, 2005). The findings of the present study showed that removing the distal half of the inflorescence, in which the flowers tend to be perfect, 30 days before full bloom decreased pistil abortion in the remaining flowers. On the one hand, this result showed competition within the inflorescence. On the other hand, it confirmed the main time of pistil abortion previously suggested by Cuevas et al. (1999) and Uriu (1959) as about one month before bloom.

The results lead to the conclusion that in olive trees with a large number of staminate flowers, the perfect flowers open in the first days of the opening period mainly in terminal positions, and the staminate flowers open later mainly on the laterals. Olive flowers compete within the inflorescence and may develop to be staminate especially in poorer nurtured positions such as lateral positions including B4L, B5L1, B5L2, B6L1, and B6L2 in competition with their corresponding terminals (B4T, B5T, and B6T, respectively) and B1 (a terminal position) in competition with TF. More studies are needed to understand why some flowers tend to dry and fall before opening, and which flowers are more likely to set fruit and remain until harvest.

CHAPTER FOUR

Sexual Compatibility of Olive: Assessed by Pollen Tube Observation

4.1 Introduction

Most olive cultivars are self-incompatible or partially self-compatible and need to be fertilised by compatible pollenisers to ensure acceptable production (Lavee, 1986; Lavee, 1990; Besnard et al., 1999; Dimassi et al., 1999; Moutier, 2002; Fabbri et al., 2004; Conner and Fereres, 2005). Furthermore, some cultivars are cross-incompatible and cannot fertilise each other (Griggs et al., 1975; Cuevas and Polito, 1997; Martin et al., 2005; Mookerjee et al., 2005). Manzanillo (as a host), for example, is cross-incompatible with Mission and Ascolana (Cuevas and Polito, 1997). The degree of SI in olive is widely influenced by climatic conditions and therefore varies from environment to environment and from year to year (Griggs et al., 1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Lavee et al., 2002). SI is a genetically controlled mechanism that prevents self-fertilisation in about half of angiosperm species (McClure and Franklin-Tong, 2006). The gametophytic system is the most widespread SI system (Franklin-Tong and Franklin, 2003) and is proposed to control the SI of olive (Ateyyeh et al., 2000).

Pollen tube observation using the fluorescence microscope is an important method to study SI (Sedgley, 1994). During pollen tube growth, callose is deposited within the tube plugs as well as on the pollen tube wall (Dumas and Knox, 1983; Majewska-Sawka et al., 2002). The deposition occurs at the tube tip only when growth is inhibited by the incompatibility response (Dumas and Knox, 1983). In olive, the pollen tubes normally produce less than four callose plugs; therefore, staining and observing the pollen tubes

depends on the callose deposited on their walls (Cuevas et al., 1994b; Majewska-Sawka et al., 2002).

The present work aimed to study the incompatibility relationships between Frantoio, Kalamata, and Koroneiki, as the host cultivars, and Barnea, Mission, Koroneiki, and Frantoio, as the pollen donors, under the climatic conditions of Roseworthy, SA, Australia. Frantoio, Kalamata, Koroneiki, Barnea, and Mission are all cultivars in widespread use in Australia (Kailis and Davies, 2004; Sweeney and Davies, 2004).

4.2 Materials and methods

4.2.1 Plant materials

The study was performed in 2004 on three trees for each of the cultivars Frantoio, Koroneiki, and Kalamata in the NOVA Collection. The trees selected were Frantoio 26ⅠA, 26ⅡB, and 26ⅢA, Koroneiki 28ⅠA, 32ⅡA, and 32ⅢB, and Kalamata 30ⅠA, 30ⅡA, and 30ⅢB (Fig. 2.1). In 2005, the study was repeated on the same trees of Kalamata. All trees were seven years old (in 2004), in good physiological condition, and grown with supplementary irrigation and fertilisation. The pollen was prepared from two olive collections: the NOVA and the Waite collections. The identities of all selected trees in both collections had been confirmed by DNA fingerprinting using RAPDs (Guerin et al., 2002; Guerin, J., personal communication, 2004).

4.2.2 Controlled crossing

Pollen collection was carried out by bagging flowering shoots before anthesis and shaking them after full bloom. The collected pollen was stored in small glass bottles at 4 °C until use. Five flowering shoots per host tree were chosen for five different crosses. Twenty-five to 30 flowers per shoot were selected just before opening [large white flower buds in the phenological stage 60 based on BBCH scale (Sanz-Cortes et al., 2002)], opened with forceps, and crossed using a fine paintbrush. To avoid unwanted pollen, hands and all equipment were washed with 70% ethanol after every cross. The

pollinated shoots were isolated using small paper bags. The selected shoots for open-pollination were left untreated to receive airborne pollen.

4.2.3 Pollen tube observation

The pistils were harvested seven days after crossing (Wu et al., 2002; Martin et al., 2005), fixed using Carnoy's fluid (ethanol:chloroform:glacial acetic acid, 6:3:1) for 24 h, and stored in 70% ethanol for 30 min and then in 90% ethanol at 4 °C until use. Twenty fixed pistils per cross were hydrated via an ethanol series through 70%, 50%, and 30% ethanol to MQ water (30 min for each ethanol dilution and 2 × 30 min for MQ water), softened with 0.8 M NaOH for 6 hours at room temperature, washed in running water overnight, and stained with 0.1% aniline blue (Martin, 1959) in alkaline phosphate buffer (pH 11.5) overnight at 4 °C.

The styles and ovules were separated under the dissecting microscope, mounted in 80% glycerol, and observed under reflected UV light using a Zeiss photomicroscope equipped with exciter filters 395-440 nm, interference beam splitter FT 460, and barrier filter LP 470. In total, 2400 slides were prepared and the number of pollen grains and pollen tubes on the stigma, pollen tubes in the upper and lower style (the points where the style joined the stigma and ovary, respectively), and pollen tubes penetrating the ovules were recorded. Fig. 4.1 displays the inhibition site of incompatible pollen tubes in the upper style (A) and the penetration of a successful compatible pollen tube into the lower style (A) and ovule (B).

4.2.4 Statistical analysis of data

Pollen tube penetration into the ovule can be considered a sign of fertilisation as a result of sexual compatibility. Since this is a rare event, it was decided to consider the assessment of each pistil as presence or absence of at least one pollen tube penetrating an ovule. The presence of one or more pollen tubes per pistil was allocated one, and the absence of pollen tubes was allocated zero. The assessment was binary (presence and absence); therefore, these data could be modelled using a form of Generalised Linear Modelling (GML) assuming a binomial distribution with a logit link function. This is otherwise known as logistic regression. The model fitted would be:

$$\text{logit}(p_{ij}) = \log\left(\frac{p_{ij}}{1-p_{ij}}\right) = \mu + (host)_i + (donor)_j + (host.donor)_{ij}$$

Where:

i = 1, . . . , 3 corresponding to each cultivar as a host.

j = 1, . . . , 6 corresponding to each cultivar as a pollen donor.

pij is the probability that a flower has a pollen tube in the ovule ($0 < pij < 1$).

1 - *pij* is the probability that a flower does not have a pollen tube in the ovule.

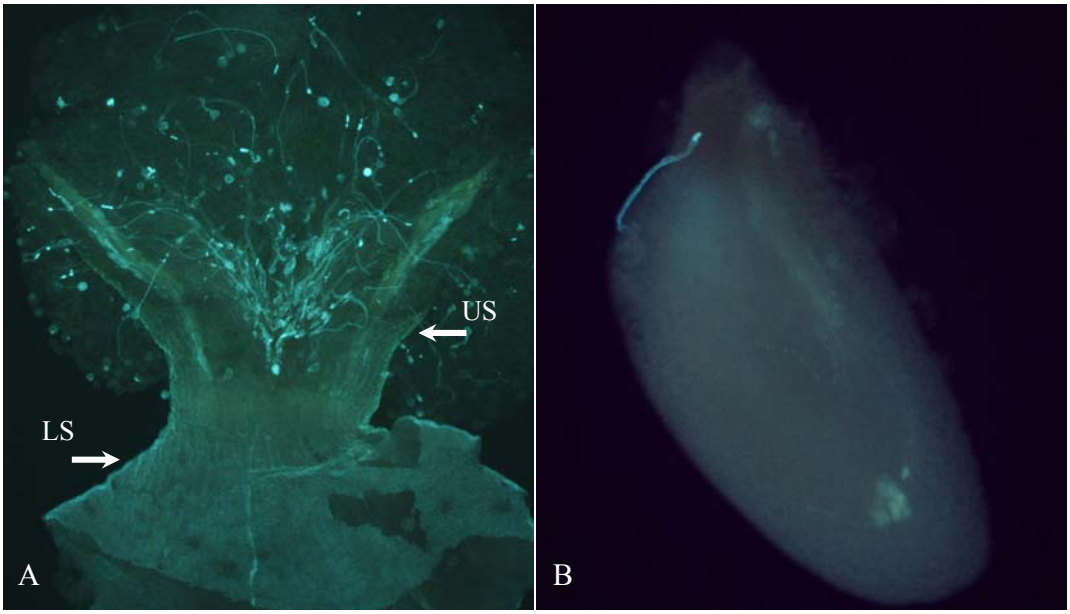


Fig. 4.1. The inhibition site of incompatible pollen tubes in the upper style (A) and the penetration of a successful compatible pollen tube into the lower style (A) and ovule (B). Arrows (US and LS) show the upper and lower style regions, respectively. Pistils squashed, stained with aniline blue, and observed under UV light.

The importance of cross (or combination of host and pollen donor) was determined using a likelihood ratio test, which was approximately chi-square distributed. The only problem applying this model was that a number of crosses across replicates had no pollen tubes penetrating the ovule at all. This caused a problem when performing logistic regression. The statistical tests became inaccurate due to many of the expected values becoming close to zero, and the standard errors were incorrect. The problem is analogous to the problem when analysing contingency tables with the chi-

square test. If a cell in a contingency table has low counts, this often causes the expected values to be less than five, which causes the chi-square test to be incorrect.

To test between crosses, contingency tables were set up and Fisher exact test, two-tailed, which is an alternative to the chi-square test, was performed. The chi-square test was not used on the contingency tables due to the expected values in some cells being less than five. To analyse the difference among all pollen donors in each host cultivar, the r × c Fisher exact test (also called Fisher-Freeman-Halton test) using StatsDirect statistical software (version 2.5.7) was used. To test between every two crosses, the 2 × 2 Fisher exact test using GenStat (version 7) were performed.

4.3 Results

The olive pistil normally has two carpels, each containing two ovules, but not all of the ovules are fertilised. The study of 1200 flowers showed that 78.8% and 93.8% of the pistils did not have any pollen tube in the lower style and carpels, respectively (Table 4.1). In Koroneiki (2004), Kalamata (2004), and Kalamata (2005), no pistils were found to have more than one pollen tube in the carpels. In Kalamata (2004), the lower style had the same result while in Kalamata (2005) and Koroneiki (2004) 1.0% and 3.0% of the pistils, respectively, had two pollen tubes in the lower style but never more. Only in Frantoio (2004), 0.3% of the pistils (1/300) had three pollen tubes in the lower style and carpels. Conversely, none of the ovules was found to be penetrated by more than one pollen tube.

Table 4.1. Percentage of pistils with 0, 1, 2, or 3 pollen tubes in the lower style and ovule.

Cultivar	% pistils (n = 300) with 0, 1, 2, or 3 pollen tubes in							
	Lower style				Carpels			
	0	1	2	3	0	1	2	3
Frantoio (2004)	75.0	22.3	2.3	0.3	85.0	14.0	0.7	0.3
Koroneiki (2004)	76.0	23.0	1.0	0.0	92.3	7.7	0.0	0.0
Kalamata (2004)	90.3	9.7	0.0	0.0	98.7	1.3	0.0	0.0
Kalamata (2005)	74.0	23.0	3.0	0.0	99.0	1.0	0.0	0.0
Mean	78.8	19.5	1.6	0.1	93.8	6.0	0.2	0.1

Tables 4.2 to 4.5 show the number of pollen tubes in the upper style, lower style, and ovule in all cultivars. The *P*-value[y] shows the results of the r × c Fisher exact test and statistical differences among all applied pollen donors. Different letters within each column show significant differences according to the 2 × 2 Fisher exact test used to determine the statistical differences between the pollen donors in each cultivar.

The index of SI (ISI) (Zapata and Arroyo, 1978) was applied to evaluate the degree of self- and cross-incompatibility. ISI is the ratio of fruit or seed set after self- or cross-pollination to fruit or seed set after open-pollination, as a potential compatible cross. A ratio equal to or lower than 0.2 indicates an incompatible cross, between 0.2 and 1 a partially compatible cross, and equal to or higher than 1 a compatible cross. Since this ratio was applied to assess both self- and cross-incompatibility, it was designated the index of pollen-incompatibility (IPI) instead of ISI. The ratio has been used to assess incompatibilities in olive in several previous studies (Androulakis and Loupassaki, 1990; Cuevas and Polito, 1997; Cuevas et al., 2001; Moutier et al., 2001; Lavee et al., 2002; Moutier, 2002; Quero et al., 2002). In the present study, for Frantoio (2004) and Koroneiki (2004) the number of pollen tubes in the ovule and for Kalamata (2004) and Kalamata (2005) the number of pollen tubes in the lower style were used to calculate IPI instead of fruit or seed set.

Table 4.2. Number of pollen tubes (n = 60) in different parts of the Frantoio (2004) pistil.

Pollen donors	Number of pollen tubes in						*P*-value[z]	IPI
	Upper style		Lower style		Ovule			
P-value[y]	< 0.001		< 0.001		< 0.001			
Self-pollination	3	b	3	c	0	c	0.212	0
Barnea	4	b	5	c	1	c	0.350	0.05
Mission	41	a	22	b	9	b	< 0.001	0.48
Koroneiki	2	b	2	c	0	c	0.548	0
Open-pollination	44	a	43	a	22	a	< 0.001	NA

P-value[z]: Statistical differences among the upper style, lower style, and ovule. *P*-value[y]: Statistical differences among the pollen donors. Different letters within each column represent the significant differences among the pollen donors according to the 2 × 2 Fisher exact test. NA: Not applicable.

Table 4.3. Number of pollen tubes (n = 60) in different parts of the Koroneiki (2004) pistil.

Pollen donors	Number of pollen tubes in						*P*-value[z]	IPI
	Upper style		Lower style		Ovule			
P-value[y]	< 0.001		< 0.001		< 0.001			
Self-pollination	3	bc	3	bc	0	c	0.249	0
Barnea	0	c	0	c	0	c	ND	0
Mission	33	a	31	a	6	ab	< 0.001	0.43
Frantoio	6	b	6	b	3	bc	0.563	0.21
Open-pollination	34	a	32	a	14	a	< 0.001	NA

ND: No difference (all data were zero). Other abbreviations as in Table 4.2.

Table 4.4. Number of pollen tubes (n = 60) in different parts of the Kalamata (2004) pistil.

Pollen donors	Number of pollen tubes in						*P*-value[z]	IPI
	Upper style		Lower style		Ovule			
P-value[y]	< 0.001		< 0.001		0.132			
Self-pollination	0	d	0	b	0	NS	ND	0
Barnea	36	a	14	a	3	NS	< 0.001	1.08
Mission	0	d	0	b	0	NS	ND	0
Koroneiki	5	c	2	b	0	NS	0.439	0.15
Open-pollination	15	b	13	a	1	NS	0.829	NA

P-value[z]: Statistical differences among the upper and lower style. *P*-value[y]: Statistical differences among the pollen donors. Different letters within each column represent the significant differences among the pollen donors according to the 2 × 2 Fisher exact test. NS: No significant difference. ND: No difference (all data were zero). NA: Not applicable.

Table 4.5. Number of pollen tubes (n = 60) in different parts of the Kalamata (2005) pistil.

Pollen donors	Number of pollen tubes in						*P*-value[z]	IPI
	Upper style		Lower style		Ovule			
P-value[y]	< 0.001		< 0.001		0.515			
Self-pollination	7	c	4	d	0	NS	0.529	0.18
Barnea	59	a	30	a	1	NS	< 0.001	1.36
Mission	9	c	8	cd	0	NS	> 0.999	0.36
Koroneiki	58	a	17	bc	2	NS	< 0.001	0.64
Open-pollination	29	b	22	ab	0	NS	0.268	NA

Abbreviations as in Table 4.4.

The *P*-value[z] shows statistical differences among the number of pollen tubes present in the upper style, lower style, and ovule in Frantoio (2004) and Koroneiki (2004) but only between the upper and lower style in Kalamata (2004) and Kalamata (2005). The statistical analysis shows that in Frantoio (2004) and Koroneiki (2004) there was a highly significant difference ($P < 0.001$) among the pollen donors in all three parts of pistil. The number of pollen tubes in the ovule, which is the most reliable sign of sexual compatibility available, shows that Frantoio (2004) and Koroneiki (2004) were self-incompatible since no ovules were fertilised after self-pollination (IPI = 0.00). Both cultivars were partially compatible with Mission (IPI = 0.48 and 0.43, respectively) but incompatible with Barnea (IPI = 0.05 and 0.00, respectively). Koroneiki, as a pollen donor, was not compatible with Frantoio (IPI = 0.00) while Frantoio, as a pollen donor, was partially compatible with Koroneiki (IPI = 0.21). In Kalamata (2004), there was no significant difference ($P = 0.132$) among the pollen donors when the number of pollen tubes in the ovule was analysed. The repeat of the experiment with this cultivar in 2005 led to the same result ($P = 0.515$); therefore, the number of pollen tubes in the lower style was used for analysis. It indicates that Kalamata was self-incompatible (IPI = 0.00 and 0.18 in 2004 and 2005, respectively). Kalamata was compatible with Barnea (IPI higher than one in both years) but not completely compatible with Mission and Koroneiki. It was incompatible with Mission and Koroneiki in 2004 (IPI = 0.00 and 0.15, respectively) but partially compatible in 2005 (IPI = 0.36 and 0.64, respectively). Tables 4.6 to 4.9 show the *P*-value of all 2×2 Fisher exact tests.

In Frantoio (2004) and Koroneiki (2004) there was a highly significant difference among the number of pollen tubes present in the upper style, lower style, and ovule after crossing with open-pollination ($P < 0.001$) and Mission ($P < 0.001$), as compatible pollen donors, while there was no significant difference when they were crossed with the incompatible pollen donors including Barnea, Frantoio, and Koroneiki. In Kalamata (2004), the upper and lower style were significantly different only after crossing with Barnea ($P < 0.001$), as the most compatible pollen donor, but not after crossing with the other pollen donors. Kalamata (2005) showed the same results apart from the highly significant difference found after crossing with Koroneiki. The results show that the

number of pollen tubes in the upper and lower style may be a reliable indication of fertilisation and therefore pollen-incompatibility in olive cultivars.

Table 4.6. *P*-value of all 2 × 2 Fisher exact tests in Frantoio (2004).

	Pollen donors	Self-pollination	Open-pollination	Barnea	Mission
Upper style	Open-pollination	< 0.001			
	Barnea	0.719	< 0.001		
	Mission	< 0.001	0.555	< 0.001	
	Koroneiki	0.682	< 0.001	0.443	< 0.001
Lower style	Open-pollination	< 0.001			
	Barnea	0.495	< 0.001		
	Mission	< 0.001	< 0.001	< 0.001	
	Koroneiki	0.682	< 0.001	0.276	< 0.001
Ovule	Open-pollination	< 0.001			
	Barnea	0.999	< 0.001		
	Mission	0.003	0.012	0.017	
	Koroneiki	ND	< 0.001	0.999	0.003

ND: No difference (all data were zero).

Table 4.7. *P*-value of all 2 × 2 Fisher exact tests in Koroneiki (2004).

	Pollen donors	Self-pollination	Open-pollination	Barnea	Mission
Upper style	Open-pollination	< 0.001			
	Barnea	0.122	< 0.001		
	Mission	< 0.001	0.857	< 0.001	
	Frantoio	0.327	< 0.001	0.014	< 0.001
Lower style	Open-pollination	< 0.001			
	Barnea	0.122	< 0.001		
	Mission	< 0.001	0.857	< 0.001	
	Frantoio	0.327	< 0.001	0.014	< 0.001
Ovule	Open-pollination	< 0.001			
	Barnea	ND	< 0.001		
	Mission	0.027	0.085	0.027	
	Frantoio	0.244	0.007	0.244	0.491

ND: No difference (all data were zero).

Table 4.8. *P*-value of all 2 × 2 Fisher exact tests in Kalamata (2004).

	Pollen donors	Self-pollination	Open-pollination	Barnea	Mission
Upper style	Open-pollination	< 0.001			
	Barnea	< 0.001	< 0.001		
	Mission	ND	< 0.001	< 0.001	
	Koroneiki	0.029	0.016	< 0.001	0.029
Lower style	Open-pollination	< 0.001			
	Barnea	< 0.001	> 0.999		
	Mission	ND	< 0.001	< 0.001	
	Koroneiki	0.496	0.004	0.002	0.496

ND: No difference (all data were zero).

Table 4.9. *P*-value of all 2 × 2 Fisher exact tests in Kalamata (2005).

	Pollen donors	Self-pollination	Open-pollination	Barnea	Mission
Upper style	Open-pollination	< 0.001			
	Barnea	< 0.001	< 0.001		
	Mission	0.605	< 0.001	< 0.001	
	Koroneiki	< 0.001	< 0.001	0.622	< 0.001
Lower style	Open-pollination	< 0.001			
	Barnea	< 0.001	0.197		
	Mission	0.362	0.006	< 0.001	
	Koroneiki	0.003	0.117	0.024	0.167

Table 4.10 shows the results of the Fisher exact test for determining how the pollen donors behaved when crossed with the different host cultivars. Open-pollination and Frantoio were not significant (P = 0.121 and 0.244), but Barnea and Mission differed significantly ($P < 0.001$ and $P = 0.006$). To determine which host cultivars differed when pollinated with Barnea and Mission, 2 × 2 Fisher exact tests were done. Frantoio (2004) and Koroneiki (2004) had a significantly lower number of pollen tubes when pollinated with Barnea, but Kalamata (2004) had a significantly lower number of pollen tubes when pollinated with Mission.

Table 4.10. Number of pollen tubes (n = 60) after crossing with common pollen donors across all host cultivars.

Host cultivar	Pollen donors				
	Open-pollination	Barnea	Mission		Frantoio
P-value	0.121	< 0.001	0.006		0.244
Frantoio (2004)	22	1 c	9	a	0
Koroneiki (2004)	14	0 c	6	a	3
Kalamata (2004)	13	14 b	0	b	NA
Kalamata (2005)	22	30 a	8	a	NA

Different letters within each column indicate significant differences among the pollen donors according to the 2 × 2 Fisher exact test. NA: Not applicable.

4.4 Discussion

In this study, Frantoio, Koroneiki, and Kalamata were found to be self-incompatible, which confirms the presence of SI in olive as previously reported (Lavee, 1986; Lavee, 1990; Besnard et al., 1999; Dimassi et al., 1999; Lavee et al., 2002; Moutier, 2002; Wu et al., 2002; Fabbri et al., 2004; Conner and Fereres, 2005; Mookerjee et al., 2005).

Under the environmental conditions of this study, Frantoio was self-incompatible (IPI = 0.00), which agrees with the findings of Wu et al. (2002) and Mookerjee et al. (2005), while it was previously recorded as self-compatible in Australia (Sharma et al., 1976) and in some other countries (Fontanazza and Baldoni, 1990; Fabbri et al., 2004). Frantoio (as a host) was also cross-incompatible with Barnea and Koroneiki but partially compatible with Mission. Cross-incompatibility between olive cultivars has previously been suggested by many authors (Griggs et al., 1975; Cuevas and Polito, 1997; Martin et al., 2005; Mookerjee et al., 2005), although Lavee et al. (2002) did not find any true cross-incompatibility after their long-term (12 years) and large-scale (36 cultivars at three sites) experiment. Cross-incompatibility between Frantoio (as a host) and Barnea has also been observed using paternity analysis (Mookerjee et al., 2005), another technique to evaluate pollen-incompatibility.

Koroneiki, a cultivar previously reported as self-compatible (Lavee, 1986; Lavee et al., 2002), showed a high level of SI (IPI = 0.00). The same result was also obtained by Mookerjee et al. (2005), in South Australia. Results from the present study showed

that Koroneiki (as a host) was incompatible with Barnea and partially compatible with Frantoio and Mission while Mookerjee et al. (2005) found incompatibility between Koroneiki (as a host) and Frantoio. They also observed that Mission was the best pollen donor for Koroneiki.

Reciprocal incompatibility, as a character of GSI (Sedgley, 1994), has been observed in some pairs of olive cultivars such as Mission and Manzanillo (Griggs et al., 1975; Cuevas and Polito, 1997; Martin et al., 2005) and Frantoio and Koroneiki (Mookerjee et al., 2005) while some authors showed that reciprocity does not always exist (Moutier et al., 2001; Lavee et al., 2002). In the present study, Koroneiki could not penetrate any ovule of Frantoio, but there were a few penetrations in the opposite direction. Further observation is needed to investigate the true reciprocity between these two cultivars especially because the levels of cross-incompatibility (IPI = 0.00 and 0.21, respectively) were close to each other.

There was no significant difference among the number of pollen tubes in ovules between the two years of study in Kalamata. It may be that seven days was not long enough for pollen tubes to reach the ovules in this cultivar, under the conditions of this study. This is at variance with Martin et al. (2005) and Wu et al. (2002). Kalamata has shown a high level of SI in South Australia (Wu et al., 2002; Mookerjee et al., 2005). This is in general agreement with the result of this study in both years when the number of pollen tubes in the lower style was used to assess incompatibility.

Kalamata (as a host) was compatible with Barnea in both years, while in a previous study by Mookerjee et al. (2005), using paternity analysis, Barnea did not fertilise Kalamata (0/160). Their result may be due to the small number of Barnea trees on their study site, the relatively long distance of Barnea from the host trees, or even the direction of the wind. Kalamata (as a host) was cross-incompatible with Mission and Koroneiki in 2004 but partially compatible with them in the next year. Mission has been previously found not to be a suitable polleniser for Kalamata, but Koroneiki was the best among the pollen donors available (Mookerjee et al., 2005). Further experiments are required to identify pollen donors for Kalamata as it is an important cultivar in Australia.

An olive pistil has two carpels each containing two ovules (Martin and Sibbett, 2005). Ateyyeh et al. (2000) observed that in olive most of the pollen tubes were inhibited in the stigma before entering the transmitting tissue in the style. They showed that only one or two pollen tubes (and rarely three) grew towards the ovary and only one (and rarely two) reached the carpels to penetrate an ovule (rarely two). These results agree with this study, in which only 2.3%, 1.0%, 0.0%, and 3.0% of pistils (n = 300) had two pollen tubes in the lower style in Frantoio, Koroneiki, Kalamata (2004), and Kalamata (2005), respectively. Furthermore, only 0.7% of Frantoio pistils had two pollen tubes in the carpels. An interesting observation was that 0.3% of pistils (1/300) in Frantoio had three pollen tubes in the lower style and carpels, which led to three fertilised ovules. Wu et al. (2002) even found four pollen tubes in the lower style of Picual, but in general they recorded a maximum of two and most commonly zero or one pollen tube in the lower style.

Another interesting observation was that in incompatible crosses such as Frantoio and Koroneiki pollinated by Barnea, Frantoio, and Koroneiki there was no significant difference between the number of pollen tubes present in the upper style, lower style, and ovule. In other words, the incompatible pollen tubes were inhibited in the stigma before entering the style. The same result was found in previous studies (Cuevas and Polito, 1997; Ateyyeh et al., 2000). Ateyyeh et al. (2000) concluded that olive SI is gametophytic. It appears that both recognition and blocking sites of incompatible pollen tubes are located in the stigma, but more studies are necessary to determine whether the incompatibility system present in olive is under gametophytic control.

In conclusion, the results show the presence of self- and cross-incompatibility in Frantoio, Koroneiki, and Kalamata under the environmental conditions of Roseworthy, SA, Australia. These results are in line with those obtained from the studies carried out in other parts of South Australia (Wu et al., 2002; Mookerjee et al., 2005) but not with the results in some other countries (Fontanazza and Baldoni, 1990; Lavee et al., 2002; Fabbri et al., 2004). In view of the level of self- and cross-incompatibility in olive, three to four cultivars should be included in orchards to guarantee good fruit set.

CHAPTER FIVE

Sexual Compatibility of Olive: Assessed by Paternity Analysis

5.1 Introduction

SI is a mechanism to prevent self-fertilisation in plants. Most olive cultivars are self-incompatible or show some level of SI and need to be fertilised by other cultivars for successful fruit set (Lavee, 1986; Lavee, 1990; Besnard et al., 1999; Dimassi et al., 1999; Moutier, 2002; Fabbri et al., 2004; Conner and Fereres, 2005). As a result, SI obliges olive growers to plant more than one cultivar in their orchards to ensure sufficient cross-pollination (Griggs et al., 1975; Cuevas and Polito, 1997; Martin et al., 2005; Mookerjee et al., 2005). Climatic conditions, especially air temperature, have a significant effect on the degree of SI; thus, it changes from environment to environment and from year to year (Griggs et al., 1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Lavee et al., 2002).

Different methods have been used to study the SI of olive: measurement of fruit set (Fernandez-Escobar and Gomez-Valledor, 1985; Rallo et al., 1990; Bartoloni and Guerriero, 1995; Cuevas and Polito, 1997; Cuevas et al., 2001; Moutier, 2002; Quero et al., 2002) and pollen tube observation (Bartoloni and Guerriero, 1995; Cuevas et al., 2001; Wu et al., 2002) after controlled crossing, *in vitro* pollen germination and pollen tube growth in a culture medium sometimes containing pistil extracts of other cultivars (Lavee and Datt, 1978; Fernandez-Escobar et al., 1983; Ghrisi et al., 1999), and paternity analysis (de la Rosa et al., 2004; Mookerjee et al., 2005; Diaz et al., 2006b). In paternity analysis, the genotype of the mother plant is compared to the genotype of offspring to distinguish the father. Microsatellite markers are codominant and highly

polymorphic, two characteristics that make them especially useful for paternity analysis (Queller et al., 1993).

The aim of this study was to assess the SI of Kalamata and the cross-incompatibility between Kalamata (as a host) and other cultivars using eight microsatellite markers in order to select good pollenisers for the climatic conditions of Roseworthy, SA, Australia. Kalamata is one of the most popular table olives grown in Australia (Kailis and Davies, 2004) and can also be used for oil extraction (Barranco et al., 2000b).

5.2 Materials and methods

5.2.1 Plant materials

The study was conducted in 2004 on three trees of Kalamata (trees 30ⅡA, 30ⅡB, and 30ⅢB, as mother trees 1, 2, and 3, respectively) at the NOVA collection (Fig. 2.1). All trees were in good physiological condition, and their genetic identities had been confirmed by DNA fingerprinting using RAPDs (Guerin et al., 2002). Leaf samples were collected from the 95 cultivars present at the NOVA collection for genotyping the mother trees and potential pollen donors. The samples were transferred on ice to the laboratory and kept at 4 °C until use. The other five named cultivars at the collection were excluded, because they were genetic repeats of other cultivars (Guerin et al., 2002). As a result, the 95 DNA samples plus a negative control could be examined in a 96-well plate. Fifteen mature fruits from each cardinal side of the mother trees (north, south, east, and west) were collected for genotyping the embryos. The samples were transferred on ice to the laboratory and kept at 4 °C until use. Forty embryos per mother tree were separated for DNA extraction (10 embryos from each side). To do this, the fruit flesh was removed, the stones were cracked open using a vice, and the embryos were separated from the endosperm using a pair of forceps (Fig. 5.1).

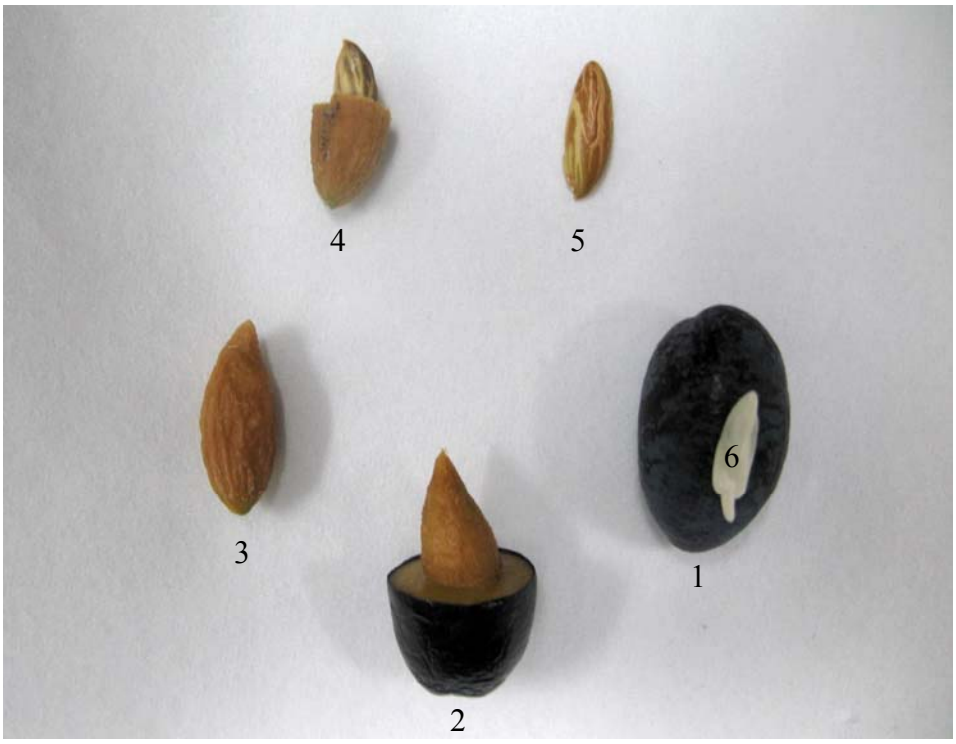


Fig. 5.1. Separation of the embryos from mature olive fruits: 1) mature Kalamata fruit, 2) removing the fruit flesh, 3) stone, 4) cracked open stone, 5) seed, and 6) embryo.

5.2.2 DNA extraction

DNA was extracted from the leaf samples using a modified method of Doyle and Doyle (1990) (Mekuria et al., 1999). In this method, 100 mg of leaf tissue was added to a 2 ml microcentrifuge tube and ground with liquid nitrogen using a small pestle. The fine powder was incubated for 30 min at 60 °C in 500 µl of extraction buffer [1 M Tris-HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 3% (w/v) CTAB, 0.2% (w/v) PVP-40T (added just before use), 0.2% (v/v) 2-mercaptoethanol (added just before use)] and mixed every 10 min by gentle inversion. Contaminants were washed by 500 µl of chloroform/isoamyl alcohol (24:1 v/v) and gentle mixing at room temperature for 10 min. The mixture was centrifuged (8000 rpm/room temperature/20 min), and the upper aqueous layer transferred to a fresh microcentrifuge tube. It was mixed gently with 2/3 volume of cold isopropanol, incubated 20 min on ice, and centrifuged at 8000 rpm for 20 min. The supernatant was decanted, and the DNA was dissolved in 100 µl of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). RNA was removed by adding 1 µl of

10 mg/µl DNase-free RNase A and incubating at 37 °C for 30 min. RNase and other proteins were removed by precipitation with 800 µl of cold ethanol, incubation on ice for 20 min, and centrifugation at 8000 rpm for 20 min. The supernatant was decanted, and the pellet was dried and dissolved in 100 µl of TE buffer. The absorbance of DNA samples was determined, and the quality was calculated by the ratio of absorbance at 260 and 280 nm. DNA samples with absorbance ratios more than 1.8 were used for further analysis and stored at –20 °C.

DNA was extracted from each embryo separately. The embryos were ground in a 2 ml microcentrifuge tube with 500 ml of grinding buffer [100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 4 mg/ml diethyl dithio carbamic acid (added just before use), 100 µg/ml DNase-free RNase A (added just before use)] and incubated at 65 °C for 10 min. After adding 500 ml of lysis buffer [100 mM Tris, pH 8.0, 20 mM EDTA, pH 8.0, 1 M NaCl, 2% (w/v) SDS, 1% (w/v) sodium metabisulphite (added just before use)], the samples were incubated for an extra 30 min at 65 °C. One millilitre of phenol-chloroform-isoamylalcohol (25:24:1 v/v/v) was added, mixed, and centrifuged at 13,200 rpm for 10 min. The supernatant was removed to a fresh tube, and DNA was precipitated by adding 500 µl of isopropanol, mixing, incubating on ice for 15 min, and centrifugation at 13,200 rpm for 5 min. The supernatant was decanted, and DNA was washed with 1 ml of wash buffer [76% (v/v) ethanol, 10 mM ammonium acetate], spun on a daisy wheel for 10 min, and centrifuged for at 13,200 rpm for 5 min. The supernatant was decanted, and the pellet was dried, dissolved in 50 µl TE buffer, and stored at –20 °C.

5.2.3 Genotyping

Eight microsatellite primers were used for genotyping the mother trees, embryos, and potential pollen donors (Table 5.1). FAM- and HEX-labelled primers were obtained from GeneWorks Pty Ltd, Adelaide, SA, Australia, and NED-labelled primers were obtained from Applied Biosystems, USA. The primers were used for amplification in three groups: 1) UDO8, EMO2, and SSR9, 2) UDO24, SSR4, and SSR14, and 3) UDO6 and SSR3. Polymerase chain reaction (PCR) was performed in a volume of 6 µl containing 60 ng DNA of parents or 0.1 µl DNA of embryos (measurement not performed due to the small quantity of the DNA extracted for each embryo), 0.5 mM of each dNTP, 0.15 U of Immolase^{TM} DNA Polymerase (Bioline), 1× ImmoBuffer [16 mM

$(NH_4)_2$ SO4, 670 mM Tris-HCl pH 8.3, 0.1% Tween-20], 2 mM $MgCl_2$, and 0.5 μM of each forward and reverse primer using a MJ Research Tetrad thermal cycler (MJ Research). The PCR program included an initial denaturation at 95 °C for 7 min, 35 cycles of 45 s at 95 °C, 45 s at 55 °C, 45 s at 72 °C, and a final extension at 72 °C for 20 min. The PCR products were diluted 1:100 and 3 μl was separated on an ABI Prism 3730 DNA Analyser (Applied Biosystems) using LIZ 500 standard. The alleles were scored using GeneMapper version 3.7 (Applied Biosystems). Fig. 5.2 shows the electropherograms of an embryo scored by GeneMapper after amplification with HEX-labelled SSR3 (green peaks) and FAM-labelled UDO6 (blue peaks). The genotyping was repeated for embryos with no allele identification.

Table 5.1. Microsatellite loci used for genotyping and paternity analysis.

Locus	Full name (origin)	Fluorescently labelled primer sequences (5′-3′)	Annealing temperature (°C)	Alleles scored (bp)
UDO6	UDO99-006 (Cipriani et al., 2002)	F: **FAM**-TCAGTTTGTTGCCTTTAGTGGA R: TTGTAATATGCCATGTAACTCGAT	57	148, 160, 168, 170, 174, 178, 182
UDO8	UDO99-008 (Cipriani et al., 2002)	F: **HEX**-AAAAACACAACCCGTGCAAT R: AAATTCCTCCAAGCCGATCT	57	156, 162, 164, 166, 172, 178
UDO24	UDO99-024 (Cipriani et al., 2002)	F: **HEX**-GATTTATTAAAAGCAAAACATACAAA R: CAATAACAAATGAGCATGATAAGACA	57	166, 172, 179, 181, 186, 188, 192, 202
EMO2	EMO2AJ416320 (de La Rosa et al., 2002)	F:**NED**-CTCGCACTTTAAATTCATATGGGTAGGT R: GCGTGCTTGGGTGCTTGTTTG	57	202, 208, 212, 216
SSR3	ssrOeUA-DCA3 AJ279854 (Sefc et al., 2000)	F: **HEX**-CCCAAGCGGAGGTGTATATTGTTAC R: TGCTTTTGTCGTGTTTGAGATGTTG	50	230, 235, 237, 241, 243, 247, 251
SSR4	ssrOeUA-DCA4 AJ279855 (Sefc et al., 2000)	F: **NED**-CTTAACTTTGTGCTTCTCCATATCC R: AGTGACAAAAGCAAAAGACTAAAGC	55	130, 136, 140, 156, 162
SSR9	ssrOeUA-DCA9 AJ279859 (Sefc et al., 2000)	F: **FAM**-AATCAAAGTCTTCCTTCTCATTTCG R: GATCCTTCCAAAAGTATAACCTCTC	55	162, 166, 172, 183, 192, 197, 207
SSR14	ssrOeUA-DCA14 AJ279863 (Sefc et al., 2000)	F: **FAM**-AATTTTTTAATGCACTATAATTTAC R: TTGAGGTCTCTATATCTCCCAGGGG	50	172, 176, 178, 180, 184, 188

NOTE: This figure is included on page 83 of the print copy of the thesis held in the University of Adelaide Library.

Fig. 5.2. Electropherograms of a Kalamata embryo by GeneMapper. The green peaks show alleles 230 and 251 produced by HEX-labelled SSR3, and the blue peaks show alleles 160 and 170 produced by FAM-labelled UDO6. The arrow shows a stutter peak in allele 251 of SSR3. Stutter peaks are common in microsatellites and are believed to arise from slippage of DNA polymerase during PCR (Luty et al., 1990).

5.2.4 Data analysis

For each locus allele, frequency and the following genetic parameters in the parent population were calculated:

A_O: The observed number of alleles

A_E: The effective number of alleles, which is a measure of diversity, was calculated according to the formula:

$A_E = 1/\sum p_i^2$ (Morgante et al., 1994),

where *pi* is the frequency of the *i*th allele.

H_O: The observed heterozygosity was calculated as the proportion of heterozygotes over genotypes for each locus.

H_E: The expected heterozygosity or gene diversity reflects the level of polymorphism and was estimated using the formula:

$H_E = 1 - \sum p_i^2$ (Nei, 1973),

where *pi* is the frequency of the *i*th allele.

PD: The power of discrimination was calculated using PowerStats (version 12) software (Promega Corporation) as:

$PD = 1 - \sum p_i^2$ (Kloosterman et al., 1993),

where *pi* is the frequency of the *i*th genotype.

IP: Identity probability represents the probability that two individuals drawn from a population will have the same genotype (Jamieson and Taylor, 1997; Waits et al., 2001). It was computed by FaMoz software (Gerber et al., 2003) to show the probability of wrongly assigning a genotype as the pollen donor.

EP: Exclusion probability was computed by FaMoz software (Gerber et al., 2003) for paternity and shows the capability of the marker system to exclude any given relationship (Jamieson and Taylor, 1997) and in this experiment any unlikely pollen donor.

NP: Null allele probability was estimated according to the formula: $NP = (H_E - H_O)/(1 + H_E)$ (Brookfield, 1996).

Genotyping data were used for paternity analysis using FaMoz (http://www.pierroton.inra.fr/genetics/labo/Software/Famoz/index.html) a software generated by Gerber et al. (2003). FaMoz uses the genotypes of offspring, mother and potential pollen donors to calculate the log of the odds ratio (LOD) scores for any

potential parentage relationship. The genotype with the highest LOD score is considered as the most likely pollen donor (Gerber et al., 2003). To determine the threshold value of the LOD score to choose a genotype as a true pollen donor, simulation was done using 1000 generated offspring from the genotyped parents. Possible genotyping error rate for both simulation and LOD score calculation was considered 0.01 (Gerber et al., 2000). Genotyping errors include scoring errors, false homozygotes owing to null alleles or weak amplifications, and mishandled samples (Blouin, 2003), and it is normally in the range of 0.25% to 2% for microsatellites (Ewen et al., 2000). The r × c Fisher exact test, two-tailed (also called Fisher-Freeman-Halton test) was conducted using StatsDirect statistical software (version 2.5.7) to analyse the difference among the pollen donors with widespread use in Australia.

5.3 Results

Genetic parameters of the eight microsatellite loci used are reported in Table 5.2. The number of alleles per locus (A_O) ranged from four (EMO2) to eight (UDO24), with a mean of 6.3. Expected heterozygosity (H_E), also called gene diversity, (Nei, 1973) varied from 0.643 (UDO24) to 0.838 (SSR3). The cumulative identity probability (IP = 0.0000) showed that the probability of assigning a wrong genotype as the pollen donor was very low. The high cumulative exclusion probability (EP = 0.9968) showed that the marker system was able to exclude almost all (99.68%) unlikely pollen donors for any given offspring. Null allele probability (NP) (Brookfield, 1996) showed that expected heterozygosity (H_E) in SSR3, SSR9, and SSR14 was not different from observed heterozygosity (H_O) statistically; thus, homozygosity was used instead of null heterozygosity. The segregation of the microsatellite markers used in this research had been previously tested by Mookerjee et al. (2005). The only new marker was SSR9, which was tested on the progeny of a cross between Frantoio and Kalamata, and the segregation of the amplification products fitted the expected 1:1:1:1 (X^2 = 7.000, df 3).

NTSYS-pc (version 2.02 k) was used to generate a dendrogram including all 95 genotypes of the NOVA collection and showed the presence of 54 different cultivars (Fig. 5.3), one less than the 55 cultivars, which was previously reported by Guerin et al.

(2002) using RAPD markers. Gros Reddeneau was found to have the same identity as cultivar Verdale Aglandau (also called Hardy's Mammoth in Australia).

Table 5.2. Genetic parameters of eight microsatellite loci in parental population.

Locus	A_O	A_E	H_O	H_E	PD	IP	EP	NP[y]
UDO6	7	4.7	0.481	0.785	0.892	0.0714	0.5768	0.170
UDO8	6	2.9	0.481	0.653	0.795	0.1424	0.4306	0.104
UDO24	8	2.8	0.426	0.643	0.808	0.1257	0.4601	0.132
EMO2	4	3.5	0.222	0.714	0.755	0.2271	0.3191	0.287
SSR3	7	6.2	0.852	0.838	0.933	0.0396	0.6730	**-0.008**
SSR4	5	4.0	0.222	0.748	0.806	0.1199	0.4708	0.301
SSR9	7	5.3	0.833	0.811	0.909	0.0597	0.6079	**-0.012**
SSR14	6	3.2	0.685	0.691	0.858	0.1171	0.4733	**0.003**
All[z]	6.3	4.1	0.525	0.735	0.844	0.0000	0.9968	NA

All[z]: The values are cumulative for IP and EP and mean for the other parameters. NP[y]: The values in bold mean that the test was significant. NA: Not applicable.

The LOD scores for the most likely pollen donors ranged from 2.08 to 4.77. There were some embryos that had LOD scores lower than the threshold calculated in simulation (2.00) (unassigned embryos). There were also some assigned embryos, which had more than one possible pollen donor with the same LOD score. The pollen donors with the same LOD score were those with close genetic distance like Frantoio and Mission (WA), Picual and Azapa, and Verdale and Benito, according to the results of this study and that of Guerin et al. (2002). Such embryos were not used to select the compatible pollenisers. Table 5.3 shows the number of unassigned and assigned embryos. In Kalamata 1, for example, 10 out of 40 embryos did not assign to any pollen donor, 6 assigned embryos had more than one possible pollen donor (with the same LOD score), and 24 assigned embryos were used to select the compatible pollenisers.

Table 5.3. Number of embryos unassigned, assigned, and used to select pollenisers.

Kalamata mother trees	Number of embryos			
	Total	Unassigned	Assigned with more than one possible pollen donor (same LOD score)	Assigned/ used to select pollenisers
1	40	10	6	24
2	40	15	1	24
3	40	15	1	24

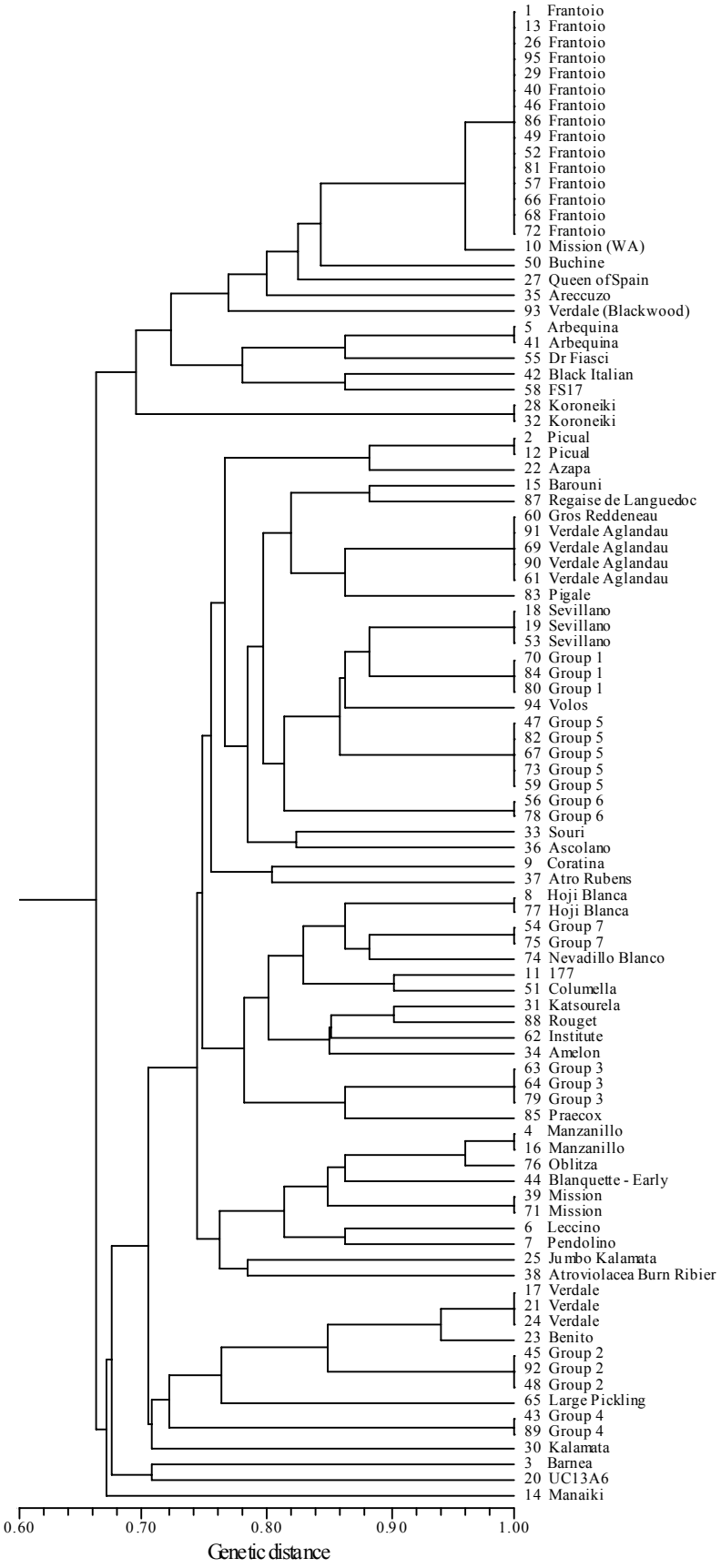


Fig. 5.3. Genetic distance of the 95 genotypes present in the study site.

There were 54 different cultivars in the study site (the NOVA collection). Since only some of them are grown commercially in Australia, they were classified into two groups. One group includes 22 cultivars with widespread use in Australia (Kailis and Sweeney, 2002; Kailis and Davies, 2004; Sweeney and Davies, 2004; Oliveaustralia-website, accessed 02 May 2007), and the other group includes the other 32 cultivars, with limited use in Australia (Table 5.4). The Fisher exact test was performed for the pollen donors with widespread use in Australia and showed a highly significant difference among them ($P < 0.001$). Although the olive pollen grain can be carried by wind as far as 12 km (Fabbri et al., 2004), the EPD has been reported to be 30 m in normal conditions (Ayerza and Coates, 2004; Fabbri et al., 2004; Sibbett and Osgood, 2005). The mother trees, which were within the EPD of the pollen donor, are shown in bold (Table 5.4).

The ISI (Zapata and Arroyo, 1978), which is the ratio of fruit or seed set after self-pollination to fruit or seed set after open-pollination, as a potential compatible cross, was calculated to assess the level of SI. A ratio equal to or lower than 0.2 indicates an incompatible cross, between 0.2 and 1 a partially compatible cross, and equal to or higher than 1 a compatible cross. The number of Kalamata embryos assigned to Kalamata itself was used as the fruit set after self-pollination, and the number of embryos assigned to all other pollen donors used as the fruit set after open-pollination. The ISI of 0.04 showed that Kalamata is a self-incompatible cultivar.

Table 5.5 shows the selected good and poor pollenisers. Barnea was a good polleniser for Kalamata, and is widely planted in Australia. Mission (WA), Benito, and Katsourela were three good pollenisers but have limited use in Australia. The number of embryos assigned to Mission (WA) and Benito were 14 and 6, even though one and none of the mother trees were in the Kalamata EPD, respectively. A pollen donor was considered as a poor polleniser only when all of the mother trees were in the EPD. Frantoio, the most widespread cultivar at the study site, was a poor polleniser for Kalamata, even though Frantoio trees were abundantly located around all of the mother trees, some very close to them (less than 10 m).

Table 5.4. Number of embryos assigned to putative pollen donors in Kalamata.

Pollen donors with widespread use in Australia	Mother trees				Pollen donors with limited use in Australia	Mother trees			
	1	2	3	Total		1	2	3	Total
n	24	24	24	72	n	24	24	24	72
Arbequina	**0**	**0**	**0**	0	Amelon	0	0	0	0
Ascolana	0	0	0	0	Areccuzo	**0**	**0**	0	0
Azapa	**0**	**0**	**0**	0	Atro Rubens	0	0	0	0
Barnea	**2**	**2**	**2**	6	Atroviolacea B. R.[y]	0	0	0	0
Barouni	**0**	**0**	**0**	0	Benito	0	3	3	6
Coratina	**1**	1	**1**	3	Black Italian	**0**	**0**	0	0
Frantoio	**0**	**0**	**0**	0	Blanquette-Early	0	1	1	2
Sevillano	**1**	**0**	**0**	1	Buchine	0	0	0	0
Hoji Blanca	**0**	**0**	**0**	0	Columella	**1**	**0**	**0**	1
Jumbo Kalamata	2	0	0	2	Dr Fiasci	0	1	**1**	2
Kalamata	**1**	**1**	**1**	3	FS17	1	1	**1**	3
Koroneiki	**2**	0	0	2	Group 1	**0**	1	**1**	2
Leccino	0	0	0	0	Group 2	**0**	**0**	**0**	0
Manzanillo	**0**	**0**	**0**	0	Group 3	**0**	**0**	0	0
Mission	0	0	0	0	Group 4	1	1	**1**	3
Nevadillo Blanco	1	0	**0**	1	Group 5	**1**	**1**	**1**	3
Pendolino	0	0	**0**	0	Group 6	**0**	**2**	2	4
Picual	**0**	**0**	**0**	0	Group 7	**0**	**0**	**0**	0
Souri	0	0	0	0	Institute	0	0	**0**	0
UC13A6	0	0	**0**	0	Katsourela	**2**	**2**	**2**	6
Verdale	**0**	**0**	0	0	Large Pickling	1	0	0	1
Verdale A.[z]	**0**	0	**0**	0	Manaiki	2	0	0	2
					Mission (WA)	2	6	**6**	14
					Oblitza	0	0	**0**	0
					Pigale	0	0	**0**	0
					Praecox	**1**	**0**	0	1
					Queen of Spain	1	0	**0**	1
					Regalise de L.[x]	1	1	**1**	3
					Rouget	0	0	**0**	0
					Verdale (Blackwood)	**0**	**0**	**0**	0
					Volos	0	0	0	0
					177	0	0	**0**	0

Boldface shows that the mother trees were within the EPD of the pollen donor (30 m). Verdale A.[z]: The full name is Verdale Aglandau; also called Hardy's Mammoth in Australia. Atroviolacea B. R.[y]: The full name is Atroviolacea Brun Ribier. Regalise de L.[x]: The full name is Regalise de Languedoc.

Table 5.5. Good and poor pollenisers selected for Kalamata.

	Pollenisers (number of embryos assigned)	
	with widespread use in Australia	with limited use in Australia
Good	Barnea (6)	Mission (WA) (14) Benito (6) Katsourela (6)
Poor	Arbequina (0) Azapa (0) Barouni (0) Frantoio (0) Hoji Blanca (0) Manzanillo (0) Picual (0)	Group 7 (0) Verdale (Blackwood) (0)

5.4 Discussion

In this study, the sexual compatibility of some olive cultivars with Kalamata (as a host) was assessed using eight microsatellite markers. Previous studies used four (de la Rosa et al., 2004; Diaz et al., 2006b) and eight microsatellite markers (Mookerjee et al., 2005) for paternity analysis in olive and four (Robledo-Arnuncio and Gil, 2005), five (Isagi et al., 2004; Hansen and Kjaer, 2006), and six microsatellite markers (Chaix et al., 2003; Oddou-Muratorio et al., 2003) for paternity analysis in other trees. The high EP (0.9968) showed that the applied markers were able to exclude almost all (99.68%) unlikely pollen donors for any given offspring.

Null alleles are alleles with no detectable PCR product after electrophoresis. The identification of null alleles is important in paternity analysis. If non-null homozygotes are scored incorrectly as null heterozygotes, false pollen donors can be assigned as likely fathers. For example, if an embryo has the genotype A/A, it will be scored as A/null and may be assigned to false fathers such as B/null and C/null. The NP test (Brookfield, 1996) did not show a significant difference between H_E and H_O in SSR3, SSR9, and SSR14; thus, homozygosity was used instead of null heterozygosity.

Little information is available about the level of SI in Kalamata. It has been reported to have a high level of SI in Crete, Greece, between 1979 and 1983 (ISI = 0.14, 0.14, 0.16, 0.13, and 0.12, respectively) (Androulakis and Loupassaki, 1990) and at

Roseworthy, SA, Australia (Wu et al., 2002). Kalamata was also completely self-incompatible at Gumeracha, SA, Australia in a study using paternity analysis (Mookerjee et al., 2005). The results presented here confirmed the presence of a high level of SI in Kalamata at Roseworthy, SA, Australia (ISI = 0.04). Only three Kalamata embryos were assigned to Kalamata itself (as a pollen donor) from 74 embryos assigned and used for the analysis. According to the conditions of this study the only good polleniser with widespread use in Australia was Barnea (six assigned embryos). Good pollenisers with limited use in Australia were Mission (WA), Benito, and Katsourela (14, 6, and 6 assigned embryos, respectively).

Olive pollen grains may be carried as far as 12 km (Fabbri et al., 2004), but a polleniser cannot be effective from that distance. The EPD in olive has been reported to be 30 m (Ayerza and Coates, 2004; Fabbri et al., 2004; Sibbett and Osgood, 2005). All good and poor pollenisers in this study were within the EPD of all three Kalamata mother trees, except for Mission (WA) and Benito that show high cross-compatibility with Kalamata (as a host). On the other hand, it shows that their pollen grains were transported further than the normal EPD. This may be due to their optimal situation in terms of the predominant wind direction. It is also possible that they have small pollen grains that readily become airborne, or are attractive to insects, but these options have not been investigated. Barnea and Katsourela were two other cross-compatible cultivars with Kalamata (as a host). In a previous study, Barnea and Katsourela did not fertilise any Kalamata embryos (0/160 embryos in both crosses) (Mookerjee et al., 2005). The very small number of Barnea and Katsourela pollen donors (2/1482 and 5/1482 trees in the study site, respectively), and their long distance from the mother trees (> 60 m) were the probable reasons.

Wind is the primary agent of olive pollination, though insects often visit olive flowers to collect pollen (Free, 1993; Martin et al., 2005). Long-term averages of climatic data from the Roseworthy Agricultural College Weather Station (34.51 S, 138.68 E) (Australian Bureau of Meteorology) shows that at the NOVA collection there are some air currents at 9 am during November (olive flowering time) in all directions and at 3 pm especially towards south west (Fig. 2.4).

Among the poor pollenisers, Arbequina, Azapa, and Picual may be considered cross-incompatible with Kalamata, because they had flowers in 2003 (Sweeny, S., personal communication, 2005), had overlapping anthesis (Fig. 2.2), were close to the mother trees (less than 15 m in at least two cases), and none of them was male sterile in 2003 as they were able to fertilise other cultivars (Guerin, J., personal communication, 2006). The other poor pollenisers did not share some of these features and might have had no chance to reach the host flowers. Verdale (Blackwood), for example, did not have overlapping anthesis with Kalamata.

Frantoio was cross-compatible with Kalamata in previous studies in South Australia (Wu et al., 2002; Mookerjee et al., 2005). This is in contrast with the findings of the present study, in which no embryo was assigned to Frantoio. This may be due to differences in air temperature between the study sites at the time of flowering. Pollen-incompatibility is influenced by temperature and varies from environment to environment and from year to year (Griggs et al., 1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Lavee et al., 2002). High temperature during anthesis decreases self-fertilisation by inhibiting pollen tube growth in the style (Griggs et al., 1975; Fernandez-Escobar et al., 1983; Cuevas et al., 1994b), while cross-fertilisation is considerably less affected (Lavee et al., 2002). Another probable reason is the close genetic distance of Frantoio and Mission (WA) (Guerin et al., 2002). FaMoz software showed more than one likely pollen donor (with same LOD score) for eight Kalamata embryos [Frantoio and Mission (WA) in five of them]. Fourteen embryos were assigned to Mission (WA), and Frantoio was the second likely pollen donor in all of them. The mean difference between the LOD scores of Mission (WA) and Frantoio in these 14 embryos was 0.09, much lower than the mean difference between the LOD scores of the first and second likely pollen donor in other assigned embryos (0.43). It seems that it was difficult for the marker system and FaMoz to distinguish between Frantoio and Mission (WA) as pollen donors due to their close genetic relationship, and this may be the reason for the high number of embryos assigned to Mission (WA) (14 embryos), while only one of the Mission (WA) trees was within the EPD of the Kalamata mother trees. More microsatellite markers are suggested to prevent such problems.

In this study, Picual was cross-incompatible with Kalamata, which confirms the results of a previous study in the NOVA collection conducted by pollen tube

observation after controlled crossing (Wu et al., 2002). Mission, Manzanillo, Pendolino, Leccino. Sevillano, and UC13A6 fertilised either no or only a small number of Kalamata embryos in the present study, as was also found by Mookerjee et al. (2005) (less than 1 and 3 embryos, respectively). At both study sites, the pollen donors were located beyond the EPD of the Kalamata mother trees. Mission (Mookerjee et al., 2005), Manzanillo, and Pendolino (Wu et al., 2002) have been reported to be cross-incompatible with Kalamata in South Australia. Verdale, Verdale Aglandau, Group 2, and Group 3 did not fertilize any embryos, and only two cases of each were within the EPD of the Kalamata mother trees. Verdale fertilized only 1/160 Kalamata embryos in the previous study (Mookerjee et al., 2005).

The results presented here suggest that Kalamata was self-incompatible under the climatic conditions of Roseworthy, SA, Australia. It was cross-compatible with Barnea, Benito, and Katsourela but cross-incompatible with Arbequina, Azapa, and Picual. The olive growers of the region should use some of these compatible pollenisers with Kalamata. More studies are needed to investigate the sexual compatibility relationships between Kalamata and Frantoio. The results obtained suggest that a larger microsatellite marker system is required for assessing the paternity analysis of olive when multiple cultivars are present, as in the NOVA collection.

CHAPTER SIX

Analysis of Gene Expression in Olive Pistils during Flowering: Towards Finding Olive Self-incompatibility Genes

6.1 Introduction

Flowering plants have different mechanisms to prevent inbreeding such as SI, which is estimated to be present in more than half of the species of angiosperms (de Nettancourt, 1977; McClure and Franklin-Tong, 2006). SI prevents self-fertilisation by recognition and rejection of self or self-related pollen (Hiscock and McInnis, 2003). Different SI systems have been found in agricultural crops. SSI is present in Brassicaceae (cabbage, broccoli, and cauliflower), Convolvulaceae (sweet potato), and Betulaceae (hazelnut) (Sedgley, 1994; McClure and Franklin-Tong, 2006) and GSI, the most widespread SI system in woody horticultural species (Sedgley, 1994) and all plants (Franklin-Tong and Franklin, 2003), is present in Rosaceae (apple, pear, cherry, and almond), Solanaceae (*Nicotiana*, *Petunia*, *Solanum*, and *Lycopersicon*), and Papaveraceae (*Papaver*) (Franklin-Tong and Franklin, 2003). SSI in *Brassica* and GSI in Solanaceae and Rosaceae (S-RNase-based mechanism) and Papaveraceae (*Papaver* mechanism) have been studied in detail at the molecular level. Some studies have suggested that the SI system of olive is gametophytic (Ateyyeh et al., 2000; Orlandi et al., 2005), as pollen tubes are inhibited in the transmitting tissue, a characteristic of GSI, although the genetic (dominance) behaviour of the pollen, which determines the system of SI to be gametophytic or sporophytic is not clear. No studies have been performed on the olive SI mechanism at the molecular level.

Different strategies have been used to find the SI genes. Nasrallah et al. (1985) used differential screening to find a cDNA clone encoding an S-locus-specific

glycoprotein from *Brassica* mature stigmas. Li et al. (1994) used the same strategy to clone a putative SI gene from the pollen of the grass *Phalaris coerulescens*. SI is a pollen-pistil interaction, taking place in pistils. In all three SI mechanisms, the SI genes were first isolated from pistils. The pollen components of SI have been identified in both the S-RNase-based and *Brassica* mechanisms later but have not been identified in the *Papaver* mechanism (McClure and Franklin-Tong, 2006, Hiscock, 2002; Hiscock and McInnis, 2003).

The aim of this study was to identify genes involved in SI in olive pistils. Kalamata is a popular olive cultivar that shows consistent SI. It has been reported to have a high level of SI in Crete (Androulakis and Loupassaki, 1990) and locally in South Australia (Wu et al., 2002; Mookerjee et al., 2005). To identify SI genes, a subtractive cDNA library was synthesised at two developmental stages, where SI genes are expected to be differentially expressed and interrogated for novel up-regulated SI candidates using nylon macroarray screens.

6.2 Materials and methods

6.2.1 Plant material

6.2.1.1 Subtracted cDNA library synthesis

The study was performed using tissues sampled from two Kalamata trees (tree numbers 18 and 28) from the Waite collection (Fig. 2.5). Both trees were in good physiological condition, and their identities had been confirmed by DNA fingerprinting using RAPDs (Guerin et al., 2002; Guerin, J., personal communication, 2004). To identify the genes expressed in olive pistils, flower buds were sampled in 2004 at two developmental stages (Fig. 6.1), where SI genes are expected to be differentially expressed: 1) small green flower buds (expression of SI genes not expected) - equivalent to stage 56 of the BBCH phenological scale (Sanz-Cortes et al., 2002), and 2) large white flower buds containing receptive pistils just prior to opening and when the first flowers of the inflorescence had started to open (expression of SI genes expected) - equivalent to stage 60 of the BBCH phenological scale (Sanz-Cortes et al., 2002). The samples were collected at 9 am and transferred on ice to the laboratory. The

pistils and other tissues were dissected from the buds, frozen in liquid nitrogen, and stored in microcentrifuge tubes at –80 °C until use.

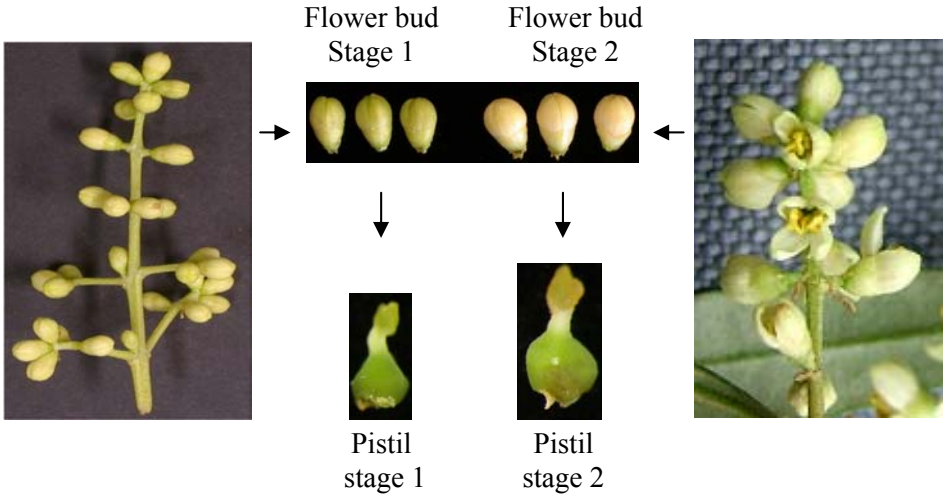


Fig. 6.1. Two developmental stages of olive flower buds used to study differentially expressed genes.

6.2.1.2 Tissue specific expression

To study gene expression across different olive tissues, flower buds and leaves were sampled in 2006 at stage 2 from three Kalamata trees in the Milano collection (tree numbers 1, 2, and 3 in Fig. 2.7). The samples were transferred on ice to the laboratory, where leaves were frozen in liquid nitrogen and stored at –80 °C until use and petals and anthers were dissected from the buds using a pair of forceps, frozen in liquid nitrogen, and stored in microcentrifuge tubes at –80 °C until use.

6.2.1.3 Cultivar specific expression

To study gene expression across different olive cultivars, whole flower buds were sampled in 2006 at stage 2 from three olive cultivars in the Milano collection (Fig. 2.7): Frantoio (tree numbers 1, 2, and 3), Barnea (tree number 1), and Pendolino (tree numbers 1, 2, and 3). The samples were transferred on ice to the laboratory, and then the flower buds (stage 2) were separated from the flower buds at other stages and stored at –80 °C until use. All trees in the Milano collection were in good physiological condition, and their identities had been confirmed by DNA fingerprinting using microsatellites (Mookerjee et al., 2005).

6.2.2 RNA extraction

Pooled samples (100 mg) of individual olive tissues including pistil stages 1 and 2, whole flower buds stage 2, petals + anthers, and leaves were used for RNA extraction. Tissues were ground in a mortar containing liquid nitrogen to a fine powder, transferred to a liquid nitrogen-cooled RNase-free microcentrifuge tube, and total RNA was extracted using the Purelink Micro-to-Midi Total RNA Purification System (Invitrogen) and stored at -80°C.

6.2.3 cDNA library construction

6.2.3.1 cDNA subtraction

RNA samples (3 μg) from stages 1 and 2 were transcribed to produce cDNAs 1 and 2 using a BD SMART^{TM} PCR cDNA Synthesis Kit (BD Biosciences). In order to enrich for pistil-specific cDNA at stages 1 and 2, two subtractions were made using the PCR-Select cDNA Subtraction Kit (Clontech) (Fig. 6.2). In brief, cDNAs 1 and 2 were digested using *Rsa* I to generate short blunt-ended double stranded cDNA fragments, which were optimal for subtraction and necessary for adaptor ligation. In the reverse subtraction (Fig. 6.2, A), cDNA 1 was used to create two populations: testers 1-1 and 1-2, which were ligated to Adaptors 1 (5′-CTAATACGACTCACTATAGGGCTCGAGC GGCCGCCCGGGCAGGT-3′) and 2 (5′-CTAATACGACTCACTATAGGGCAGCGT GGTCGCGGCCGAGGT-3′), respectively. For the first hybridisation, an excess of cDNA 2 (as driver with no adaptor) was added to each tester cDNA, heat denatured (98 °C/1.5 min), and then allowed to anneal (68 °C/8 h). For the second hybridisation, two tester populations were mixed together and another excess of fresh denatured driver cDNA 2 was added for further enrichment. Differentially expressed fragments (DEFs) were selectively amplified with two PCR reactions. In the primary PCR, only double stranded cDNA with different adaptor sequences on each end were exponentially amplified using PCR primer 1 (5′-CTAATACGACTCACTATAGGGC-3′). A secondary nested PCR reaction was performed to further enrich for DEFs using nested PCR primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and 2 (5′-AGCGTGGTCG CGGCCGAGGT-3′). The product was used as reverse-subtracted (RS) cDNA for probe preparation. A portion of both tester cDNA samples were mixed together, amplified with primary and secondary PCRs, and used as unsubtracted tester control 1 (U1). In the forward subtraction (Fig. 6.2, B), the same process was performed with cDNA 2 as

tester and cDNA 1 as driver to enrich for DEFs in pistil stage 2. The product was used as forward-subtracted (FS) template cDNA for macroarray and probe preparation. Unsubtracted tester control 2 (U2) was also made from a mixture of two tester cDNA populations. The efficiency of both subtractions was evaluated using the controls provided in the kit.

6.2.3.2 FS cDNA ligation and transformation

FS cDNA was ligated into pGEM-T Easy (Promega) and transformed into competent *E. coli* cells. Transformed colonies were selected on LB-agar plates containing ampicillin (100 mg/ml) and IPTG/X-Gal (80 µg/ml each). 2304 colonies containing inserts were picked using a colony picking robot (CPAS, BioRad), spotted into six 384-well plates containing LB-ampicillin, and incubated at 37 °C overnight. Plates were replicated twice before storing at –80 °C in liquid LB-ampicillin containing 5% (v/v) glycerol.

6.2.4 Macroarray preparation

The colonies containing FS cDNA fragments were spotted from six 384-well plates onto Hybond N+ nylon membranes (Amersham) resting on LB-agar-ampicillin and incubated at 37 °C overnight. To lyse the colonies, the membranes were placed (colony side facing up) on a series of Whatman 3 mm papers saturated with colony-denaturation solution (1.5 M NaCl, 0.5 M NaOH) at room temperature for 15 min, then transferred to neutralisation solution (1.5 M NaCl, 0.5 M Tris, pH 7.4) at room temperature for 15 min, and finally set in 2× SSC solution (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) at room temperature for 10 min. The denatured DNAs were fixed onto the membrane using a UV crosslinker (Amersham), and the membranes kept at room temperature until use.

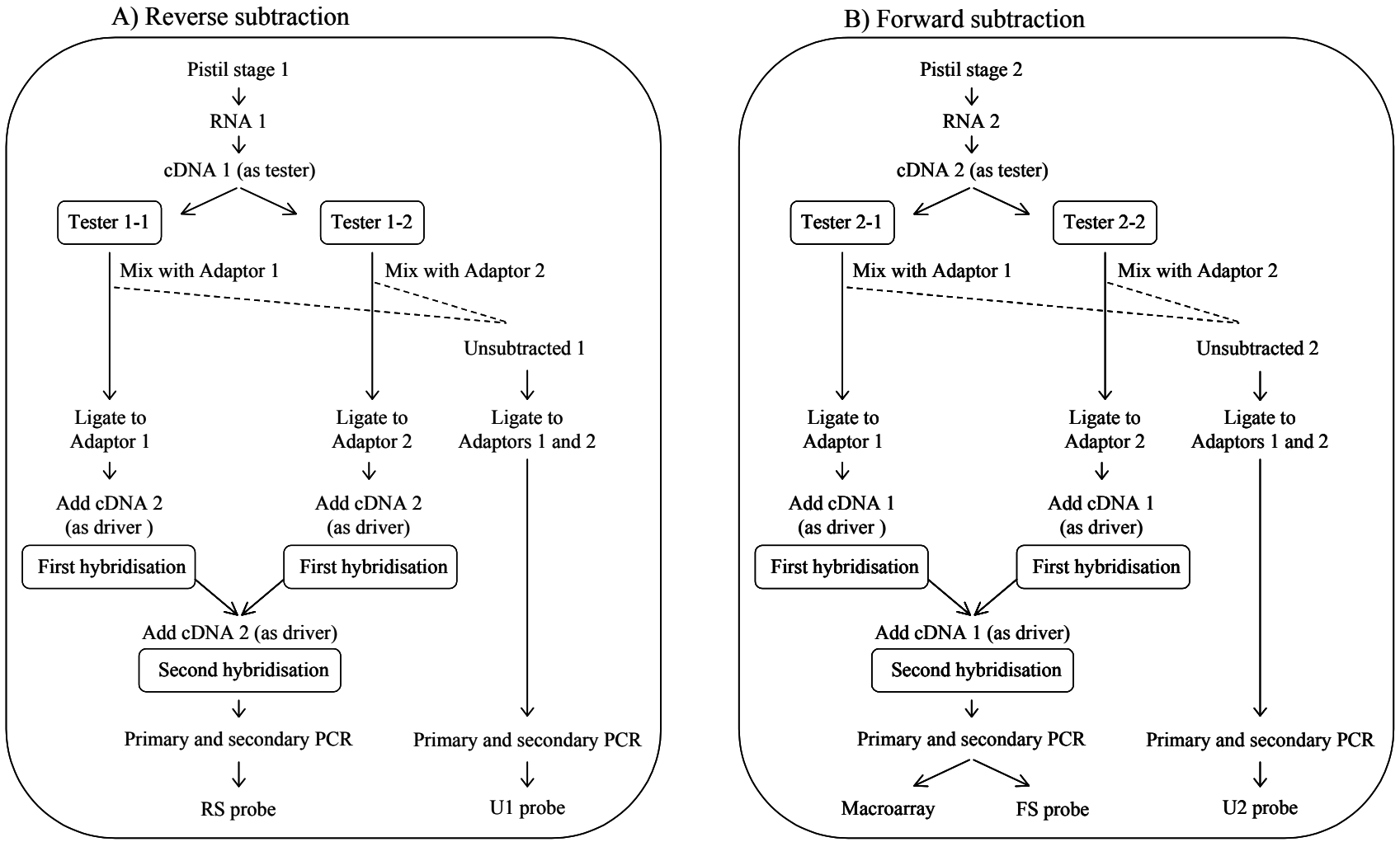


Fig. 6.2. Reverse (A) and forward (B) cDNA subtractions (adopted from PCR-select differential screening kit user manual, Clontech, Catalogue K1808-1, 2001).

6.2.5 DIG-labelled probe preparation

Four sets of DIG labelled cDNA probes were used in this study:

1) RS (down-regulated clones) and FS (up-regulated clones) probes produced from template cDNA produced in the initial cDNA substraction assay.

2) U1 (down-regulated control-cDNA 1) and U2 probes (up-regulated control-cDNA 2).

3) Probes from Kalamata pistil, leaf, and petal + anther (tissue controls).

4) Probes from whole flower bud stage 2 of four different cultivars (cultivar controls).

FS, RS, U1, and U2 probes were synthesized using the PCR DIG Labelling Mix (Roche) on the individual cDNA populations. DIG-labelled cDNA probes were purified using the QIAquick PCR Purification Kit (Qiagen), and their yields were estimated using a nylon membrane spot test against known quantities of DIG-labelled Control DNA (Roche) (Fig. 6.3). A third and fourth set of probes were derived from total RNA extracted from either Kalamata pistil, leaf, petal + anthers, or whole flower buds stage 2 of Kalamata, Frantoio, Barnea, and Pendolino. DIG-labelled single stranded cDNA probes were synthesised directly from total RNA using the microarray cDNA Labelling Kit (Roche) and purified using the Microarray Target Purification Kit (Roche) to remove residual nucleotides, primers, proteins, nucleases, and other reaction components. For each probe set, the incorporation of the DIG steroid was confirmed using gel electrophoresis, where incorporation of the DIG label increases the molecular weight of the labelled DNA relative to an un-labelled DNA control, and test hybridisations against template cDNA spotted on nylon membranes were performed to standardise probe hybridisation reactions. All probes were denatured before hybridisation by boiling at 95 °C for 5 min and rapidly chilling on ice.

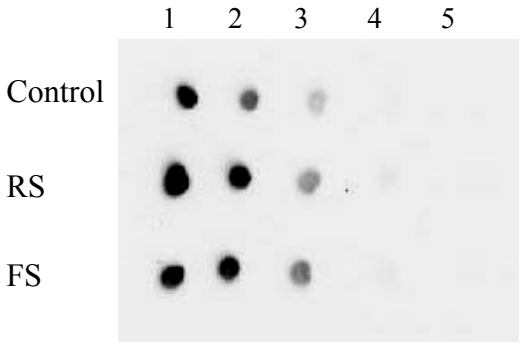


Fig. 6.3. Yield of the DIG-labelled RS and FS probes used in a typical spot test. From left to right each control spot contains: 100, 10, 1, 0.1, and 0.01 pg/µl.

6.2.6 Macroarray hybridisation and scanning

Prior to hybridisation, macroarrays were saturated in 2× SSC solution and then treated using a proteinase K solution (2.5 mg/ml in 2× SSC) at 37 °C for 1 hour to break down the proteins of colony cells. Any remaining cellular debris on the membranes was removed by a gentle pressing with Whatman 3 mm paper (pre-wetted in sterile MQ water). Membranes were transferred into roller bottles, prehybridised with 30 ml of DIG Easy Hyb Solution (Roche), and incubated in a hybridisation oven at 42 °C for 2 h. Prehybridisation buffer was removed and replaced with 20 ml of DIG Easy Hyb solution containing denatured probes and incubated at 42 °C overnight.

After hybridisation, membranes were first washed with a low-stringency wash buffer (2× SSC, 0.1% SDS) at room temperature for 2 × 10 min periods on a rotary shaker followed by 2 × 15 min high-stringency washes (0.2× SSC, 0.1% SDS) at 65 °C in a shaking water bath. The DIG signal was detected by chemiluminescence (Roche). Membranes were submersed in DIG wash buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5, 0.3% (v/v) Tween 20] for 2 min and then incubated with blocking buffer [1% (w/v) blocking reagent (Roche), 0.1 M maleic acid, 0.15 M NaCl, pH 7.5] for 30 min with gentle shaking to prevent non-specific interaction of the antibody with the membranes. Blocking buffer was replaced with anti-DIG-alkaline phosphatase (Roche) (diluted 1:10,000 in blocking solution) and incubated for 30 min. Unbound probe was removed using wash buffer (2 × 15 min). The membranes were transferred to DIG detection buffer (0.1 M NaCl, 0.1 M Tris, pH 9.5) at room temperature for 2 min and then incubated with CDP-Star (Roche) (diluted 1:100 in detection buffer) in a transparent plastic bag at room temperature for 5 min. The chemiluminescent signal was detected in a Gel Doc XRS workstation (BioRad).

6.2.7 Macroarray analysis and DNA sequencing

The macroarray images were analysed using ImaGene 6 software (BioDiscovery). A recognition grid was applied, and the spots inspected to correct the location of the recognition circles. The background (Mean) was corrected locally, following log transformation (Base 2). The normalised signal mean was used to select clones with at least 4-fold up-regulation. From 2304 cDNA clones present on the arrays, 90 up-regulated clones were selected and sequenced. For sequencing, a pure culture of each

colony was obtained by streaking it on an LB-agar-ampicillin plate and incubating at 37 °C overnight. Isolated colonies were transferred into a centrifuge tube containing LB-ampicillin solution and incubated at 37 °C overnight in an orbital mixer incubator (200 rpm). Plasmids were isolated from the colony cultures using GenElute Plasmid Miniprep Kit (SIGMA). Plasmids were partially sequenced from the 5′-end (AGRF sequencing facility, Brisbane, Queensland, Australia). Gene sequences were used in BLASTX searches against the non-redundant (NR) protein database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

After macroarray hybridisation with RS and FS probes, 90 clones were selected, transferred from the 384-well plates to a single 96-well master plate containing LB-ampicillin-5% glycerol solution, and stored at –80 °C until use. With each master plate, six control clones consisting of two empty plasmid controls (C5 and G2), two negative controls (C6 and G3) from clones not expressed in either of the FS or RS hybridisations, and two positive controls (C7 and G4) from the fragments highly expressed in both hybridisations were also added (Fig. 6.5, A). The collection of 96 clones was spotted onto a membrane with four complete replicates. The clones were hybridised again with FS, RS, U1, and U2 cDNA probes as well as probes prepared from different tissues and cultivars. An average signal of the four replicates of each clone was used to identify 4-fold up- and down-regulated clones.

6.3 Results

6.3.1 RS and FS hybridisations

To identify the genes involved in olive SI, a cDNA subtraction library was synthesized from pistils and then evaluated using nylon macroarrays. In order to capture genes up-regulated when pistils become receptive to pollen, two independent cDNA libraries were synthesized from RNA extracted from pistils at stage 1 and stage 2, respectively. The two libraries were combined in reciprocal subtractive hybridisations to enrich for forward (FS – up-regulated) and reverse (RS – down-regulated) cDNA populations. A random population of FS cDNAs were printed onto high-density macroarrays and then probed with equal quantities of DIG-labelled cDNA probes synthesized from either the RS or FS cDNA templates. Digital images of each blot were

captured and analysed using ImaGene software. When RS signals were subtracted from FS signals, 90 clones were identified with at least 4-fold higher pixel intensity than that of the respective RS hybridisation (Fig. 6.4 and Table 6.1). For each up-regulated clone, the respective *E. coli* colony from the original starter plate was used for further study.

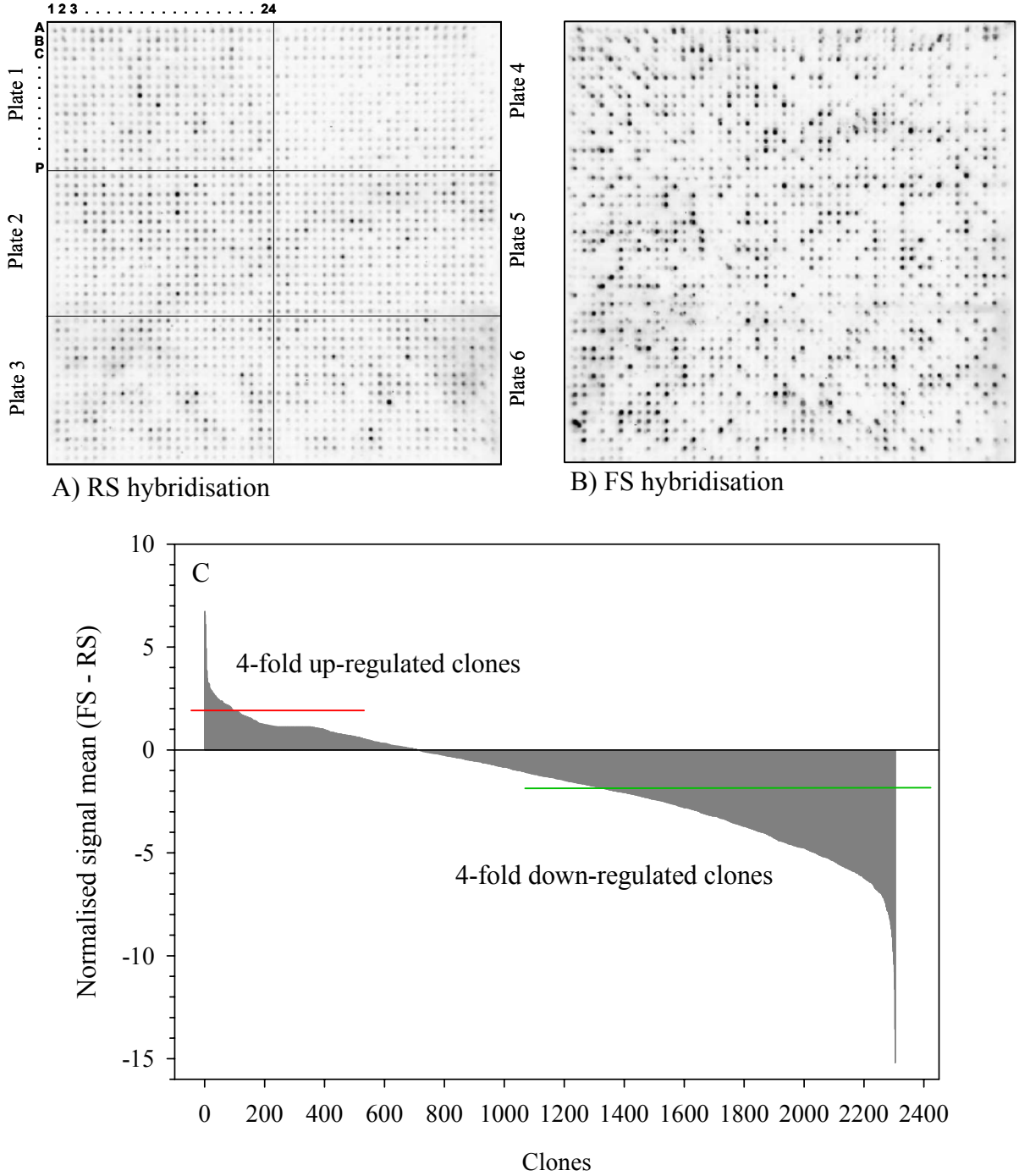


Fig. 6.4. Down- and up-regulated putative FS clones hybridised against RS (A) and FS (B) probes, respectively. C) Normalised signal mean of FS – RS hybridisation. Genes showing 4-fold up- and down-regulation are indicated with the red and green horizontal lines, respectively.

Table 6.1. Selected clones showing 4-fold up-regulation between FS and RS hybridisations.

Clone ID			Normalised signal mean (FS - RS)	Clone ID			Normalised signal mean (FS - RS)
Original (plate/well)		Subcloned (new plate)		Original (plate/well)		Subcloned (new plate)	
1	C23	A1	2.531979	3	M4	E1	2.657486
1	C24	A2	2.451729	4	A23	E2	2.966074
1	D13	A3	2.183719	4	B17	E3	2.174441
1	F8	A4	2.372763	4	B22	E4	2.530508
1	F14	A5	2.756450	4	B23	E5	6.550442
1	H18	A6	2.101447	4	C1	E6	2.168329
1	I9	A7	3.010747	4	D2	E7	2.118554
1	I18	A8	2.180384	4	D23	E8	2.615540
1	I24	A9	3.856681	4	F4	E9	2.453899
1	K6	A10	2.381572	4	G10	E10	2.375866
1	K18	A11	2.859446	4	G12	E11	3.232156
1	L3	A12	2.257437	4	I1	E12	6.073245
1	L23	B1	2.929606	4	K4	F1	2.561847
1	M22	B2	2.478770	4	K17	F2	2.669507
1	M24	B3	2.880826	4	L24	F3	2.758610
1	N1	B4	2.627611	4	M4	F4	2.553829
1	N5	B5	2.681638	4	M5	F5	2.473867
2	B12	B6	2.409829	4	M17	F6	2.873131
2	B24	B7	3.481350	4	N5	F7	2.559196
2	C21	B8	3.887520	4	N12	F8	2.791423
2	G1	B9	3.268068	4	N22	F9	2.473940
2	H4	B10	2.907023	4	O6	F10	2.257699
2	K6	B11	2.373231	4	O14	F11	2.244101
2	L6	B12	3.281515	4	P12	F12	2.425147
2	M4	C1	2.779873	4	P19	G1	2.111104
2	M22	C2	2.722778	NA	NA	G2 (Control[z])	NA
2	N1	C3	3.263175	2	L11	G3 (Control[y])	NA
2	P3	C4	2.159942	6	J13	G4 (Control[x])	NA
NA	NA	C5 (Control[z])	NA	5	B11	G5	2.459628
1	C22	C6 (Control[y])	NA	5	B17	G6	2.372055
2	E14	C7 (Control[x])	NA	5	B19	G7	2.334450
3	B4	C8	6.737300	5	E15	G8	2.267503
3	F7	C9	2.216486	5	F20	G9	2.221747
3	G13	C10	2.790384	5	L5	G10	2.198273
3	H7	C11	4.942508	5	P19	G11	2.988097
3	H13	C12	2.050846	5	P20	G12	3.843140
3	I1	D1	3.240424	5	P24	H1	2.340573
3	I2	D2	2.938248	6	E21	H2	3.229765
3	I11	D3	2.181220	6	H9	H3	6.104152
3	I17	D4	2.163208	6	I18	H4	2.100271
3	I19	D5	2.197121	6	J2	H5	2.256915
3	J1	D6	3.187671	6	J14	H6	2.125202
3	J7	D7	2.398134	6	L10	H7	2.216816
3	K2	D8	2.364501	6	L19	H8	2.135246
3	K6	D9	2.283886	6	M9	H9	2.017791
3	K7	D10	2.625720	6	N10	H10	2.212378
3	L9	D11	2.383733	6	P10	H11	2.145079
3	L11	D12	2.393837	6	P23	H12	2.047694

[z], [y], and [x]: Empty plasmids, negative (clones poorly expressed in both FS and RS hybridisations), and positive controls (clones highly expressed in both FS and RS hybridisations), respectively.

6.3.2 Confirmation of primary macroarrays screens

To assess the reproducibility of the initial macroarray analysis, the 90 up-regulated clones from the first hybridisation experiment were printed onto smaller membranes, in replicates of four. On each membrane, controls were also added consisting of colonies containing empty plasmids (plate regions C5 and G2), clones poorly expressed in both FS and RS hybridisations (negative) (plate regions C6 and G3), and clones highly expressed in both FS and RS hybridisations (positive) (plate regions C7 and G4). Individual membranes were hybridised with RS and FS probes as previously done in the initial screen (Fig. 6.5) and then using replicated blots probed with U1 and U2 probes (Fig. 6.6). Macroarray images were analysed using ImaGene software to calculate pixel intensity across each image. An average signal of the four replicates of each clone was used to estimate the difference between RS and FS signals and U1 and U2 signals to identify 4-fold up-regulated clones. The hybridisation patterns using unsubtracted cDNA probes (U1 and U2) (Fig. 6.6, A and B) were relatively similar to that obtained with the subtracted RS and FS probes (Fig. 6.5, A and B). In the confirming FS-RS macroarrays, 82 (85%) of the clones were found to be up-regulated (4-fold difference), while 14 (15%) clones including A1-2, D8, E10, H9-12, empty plasmids, negative, and positive controls remained unchanged (Fig. 6.5, C). In the U2-U1 macroarrays, fewer clones [71 (74%)] were found to be up-regulated (4-fold) at pistil stage 2 (U2) relative to that of stage 1 (U1), and 25 (26%) clones including A1, D2, D4-6, E1-2, E4, E6, E12, F1-3, G10-11, H9-12, empty plasmids, negative, and positive controls remained unchanged (H12 was down-regulated but not 4-fold) (Fig. 6.6, C). In both FS-RS and U2-U1 hybridisations, empty plasmids (C5 and G2) and H9 showed no signal.

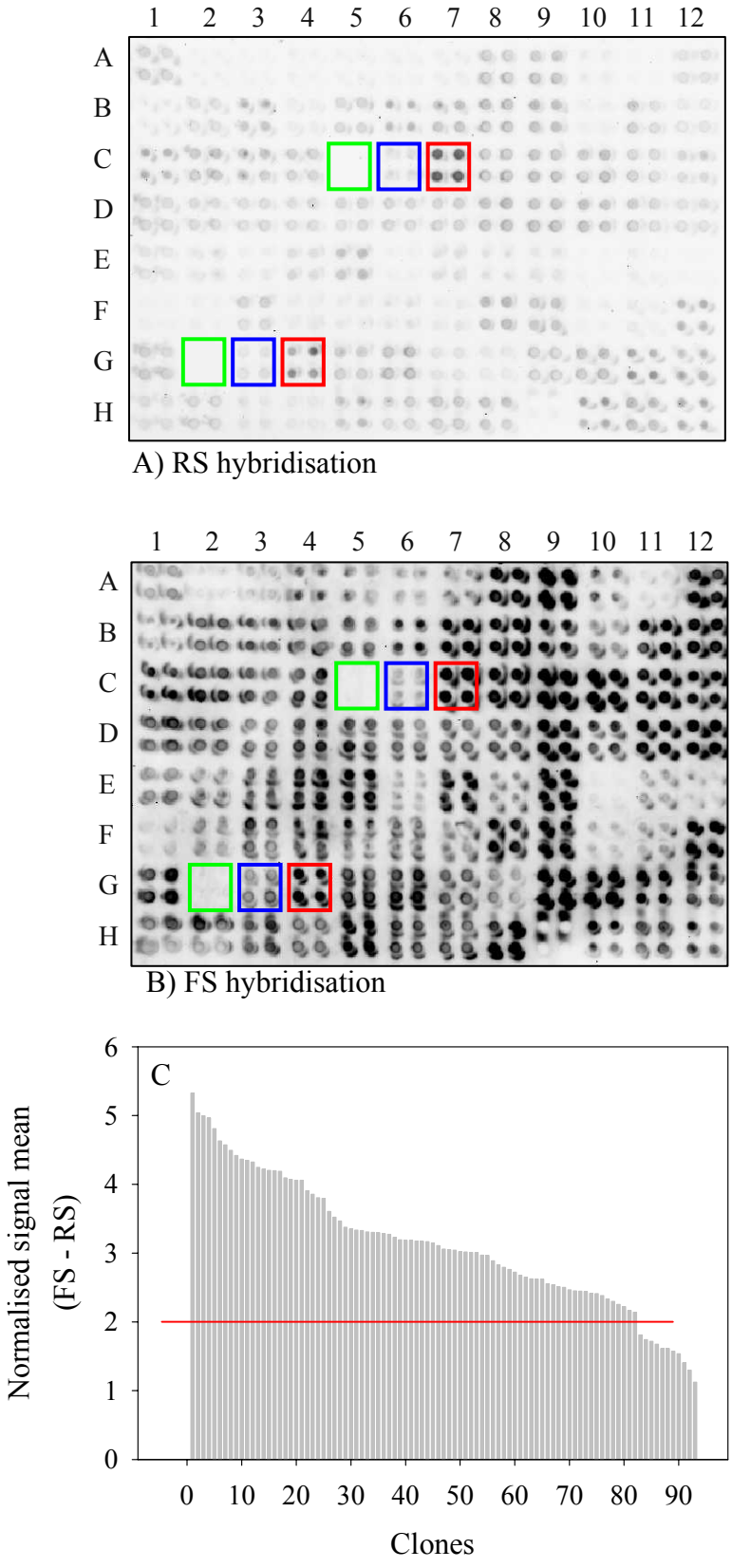


Fig. 6.5. Second round hybridisation of putative up-regulated genes with RS (A) and FS (B) DIG-labelled cDNA probes. C) Normalised signal mean of FS – RS hybridisations. Genes showing 4-fold up-regulation are indicated with a horizontal line. Green, blue, and red squares show empty plasmids, negative, and positive controls, respectively.

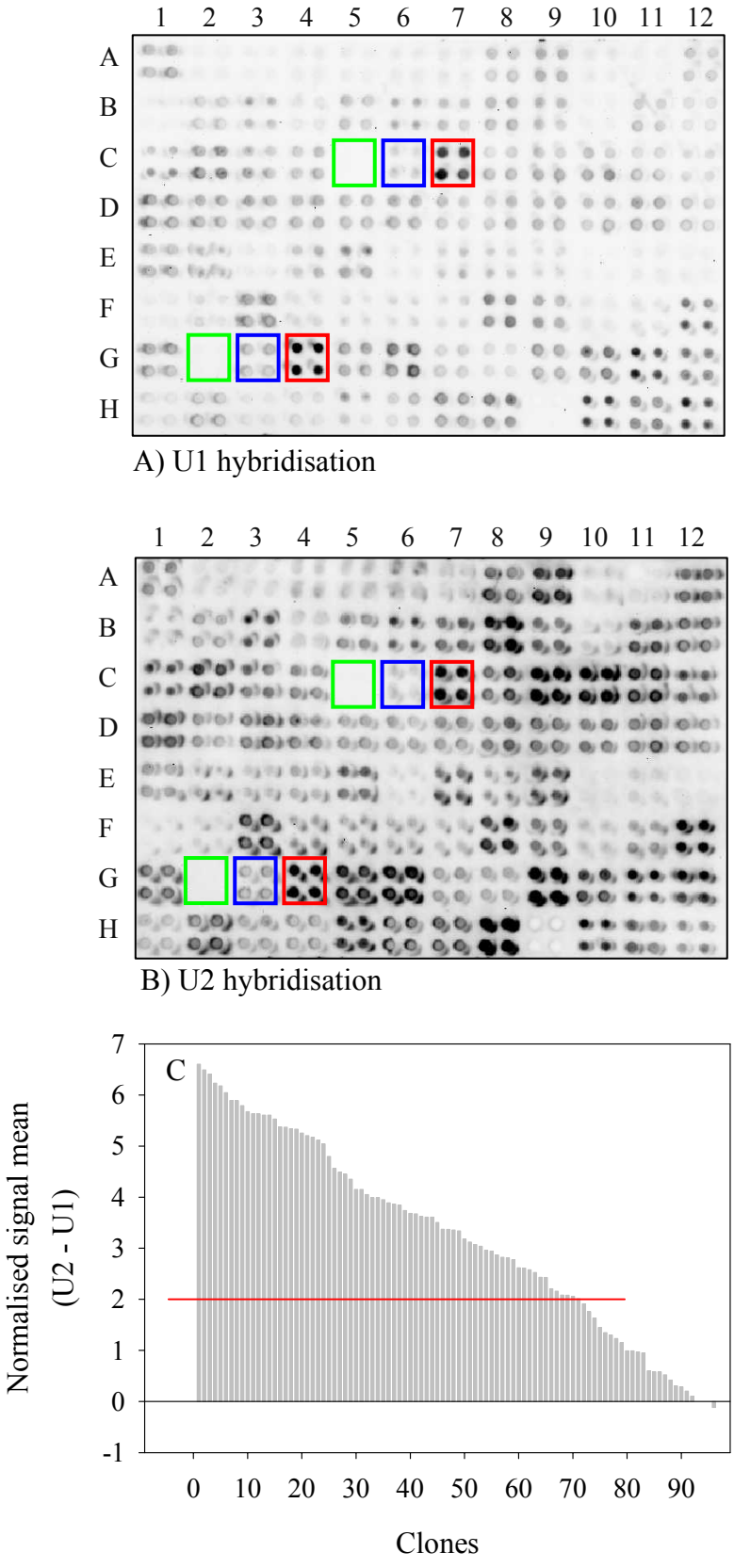
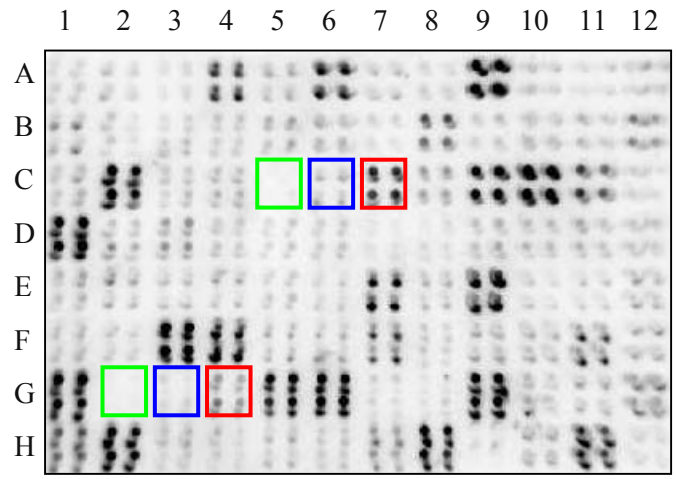


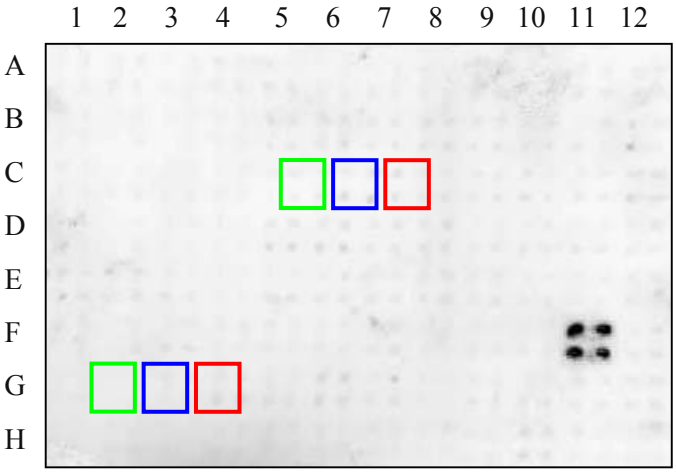
Fig. 6.6. Second round hybridisation of putative up-regulated genes with U1 (A) and U2 (B) DIG-labelled cDNA probes. C) Normalised signal mean of U2 – U1 hybridisations. Genes showing 4-fold up-regulation are indicated with a horizontal line. Green, blue, and red squares show empty plasmids, negative, and positive controls, respectively.

6.3.3 Gene expression profiles across different tissues

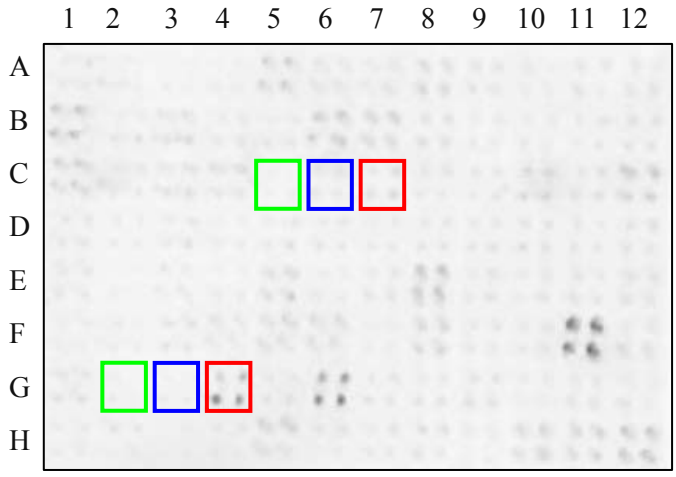
To assess the tissue specificity of each up-regulated clone, three macroarray hybridisations were performed using tissue specific probes prepared from pistil, leaf, and petal + anther (Fig. 6.7). The analysis of pistil versus leaf (Fig. 6.8) showed at least 4-fold up-regulation in pistils for 92 (96%) of the clones including all negative and positive controls. Two empty plasmids (C5 and G2) and H9 remained unchanged (zero signal), and only clone F11 (photosystem II protein) showed 4-fold down-regulation. The analysis of pistil versus petal + anther (Fig. 6.9) showed 4-fold up-regulation in pistils for 72 (75%) clones including C6 and G3 (two negative controls) and C7 (a positive control). Twenty-three (24%) clones including C5 and G2 (two empty plasmids), and G4 (a positive control) were expressed equally in both tissues, and only clone E10 (an unknown gene) was down-regulated 4-fold. The results confirmed that a high percent of selected FS clones were over-expressed in pistil tissue.



A) Pistil



B) Leaf



C) Petal + anther

Fig. 6.7. Gene expression in different tissues of Kalamata. Green, blue, and red squares show empty plasmids, negative, and positive controls, respectively.

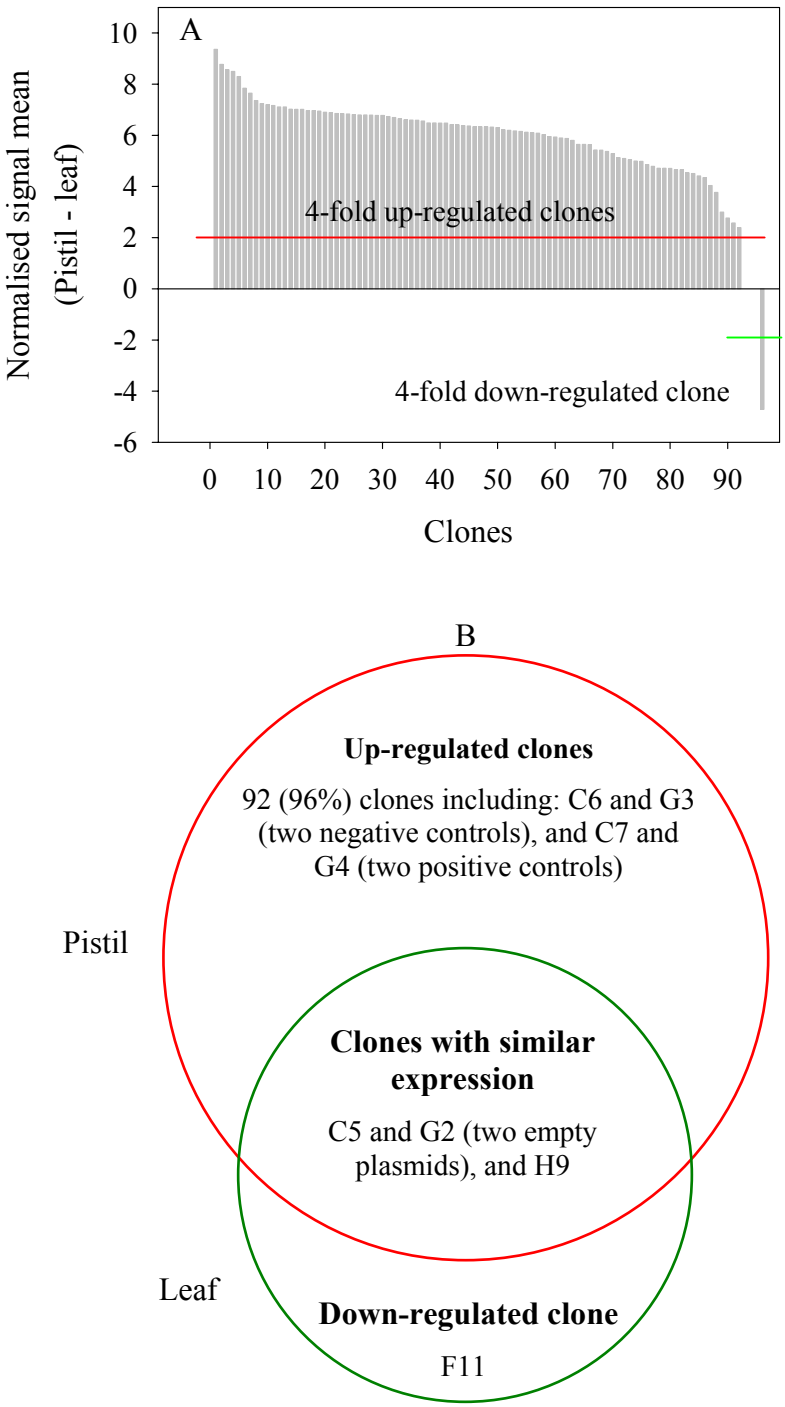
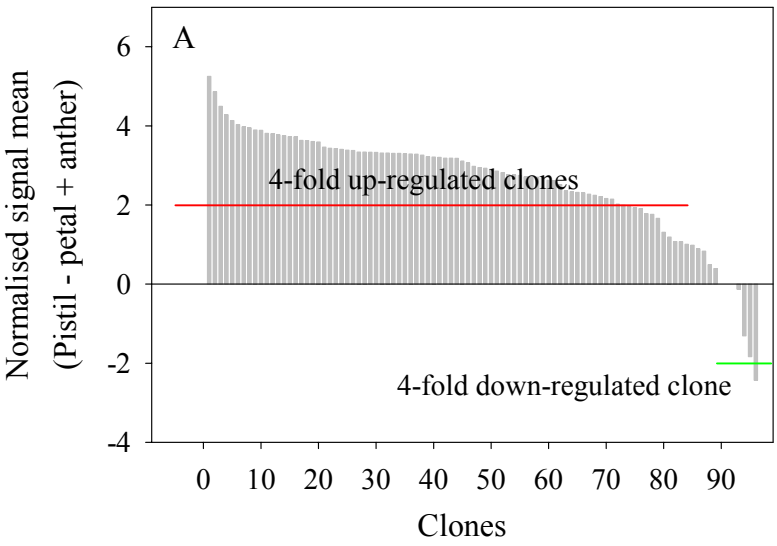


Fig. 6.8. A) Normalised signal mean of pistil – leaf hybridisations. Genes showing 4-fold up- and down-regulation are indicated with the red and green horizontal lines, respectively. B) Specific clones with 4-fold up- and down-regulation or similar expression in Kalamata pistil and leaf.



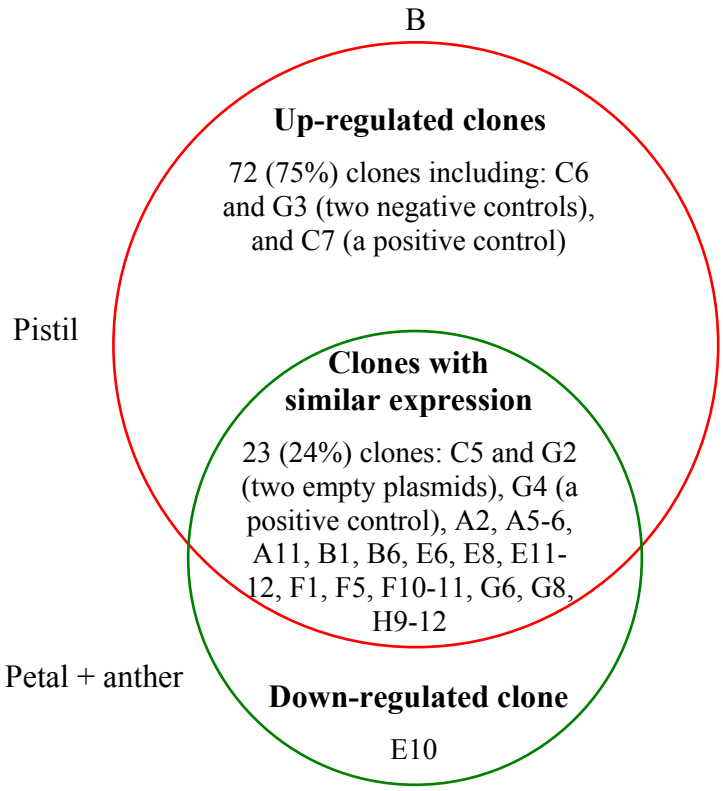


Fig. 6.9. A) Normalised signal mean of pistil – petal + anther hybridisations. Genes showing 4-fold up- and down-regulation are indicated with the red and green horizontal lines, respectively. B) Specific clones with 4-fold up- and down-regulation or similar expression in Kalamata pistil and petal + anther.

6.3.4 Gene expression profiles across different olive cultivars

Each of the FS cDNAs was tested for changes in expression in whole flower buds across different olive cultivars. Total RNA was extracted and converted to DIG-labelled cDNA from whole flower buds (stage 2) of Kalamata, Frantoio (Wu et al., 2002; Mookerjee et al., 2005) and Barnea (results of Chapter 4 and 5) two compatible cultivars with Kalamata, and Pendolino an incompatible cultivar to Kalamata (Wu et al., 2002; Mookerjee et al., 2005). Hybridisation against the initial selection of 90 putative up-regulated FS clones resulted mostly in similar expression patterns across each membrane (Fig. 6.10). However, each cultivar displayed some specific up- and down-regulated clones (Fig. 6.11 to 6.14). For example, clone H12 (a hydrolase) in Frantoio, clones A9 and C12 (two unknown genes) in Barnea, and clones B10 (a beta-1,3-glucanase-like protein) and D10 (a putative reverse transcriptase) in Pendolino had 4-fold higher expression than those found in Kalamata. It was interesting that different repeats of clones within each group were not expressed equally in all the cultivars studied. Group 5, for example, included six repeats of beta-1,3-glucanase-like protein, which showed variable expression. A12, B11, D11, and D12 were expressed equally across all the cultivars, D5 was expressed equally in Kalamata, Barnea, and Pendolino but less in Frantoio, and D6 was expressed equally in Kalamata and Pendolino but less in Frantoio and Barnea.

Fig. 6.14 shows all the up-regulated clones in Kalamata across the other cultivars, and indicates that not all of them were expressed equally across the cultivars. Clones A7, B12, D6, E3, E12, F7, and G12 were expressed equally in Kalamata and Pendolino but less in Frantoio and Barnea; On the other hand, clones A1, B1, D1, D2, F1, and G1 were expressed equally in Kalamata, Frantoio, and Barnea but less in Pendolino. Among them, A7 and D6 matched with beta-1,3-glucanase-like proteins, B1 and E3 matched with receptor protein kinase-like proteins, G12 matched with a putative lipid transfer protein, A1 matched with a postsynaptic protein-related, D2 matched with a thaumatin-like protein, but B12, D1, E12, F1, F7, and G1 had zero or non-significant matches (Table 6.2 and 6.3).

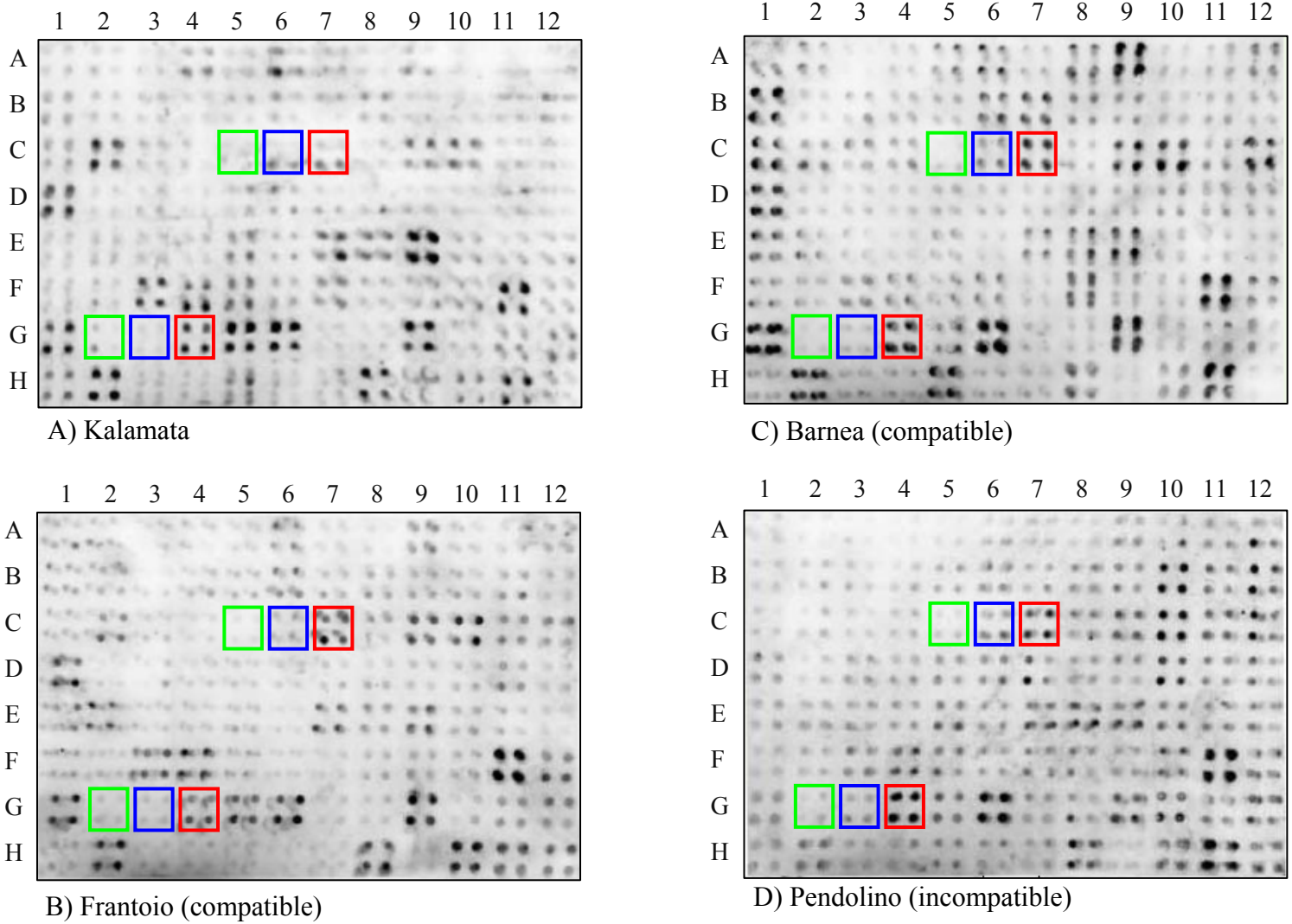
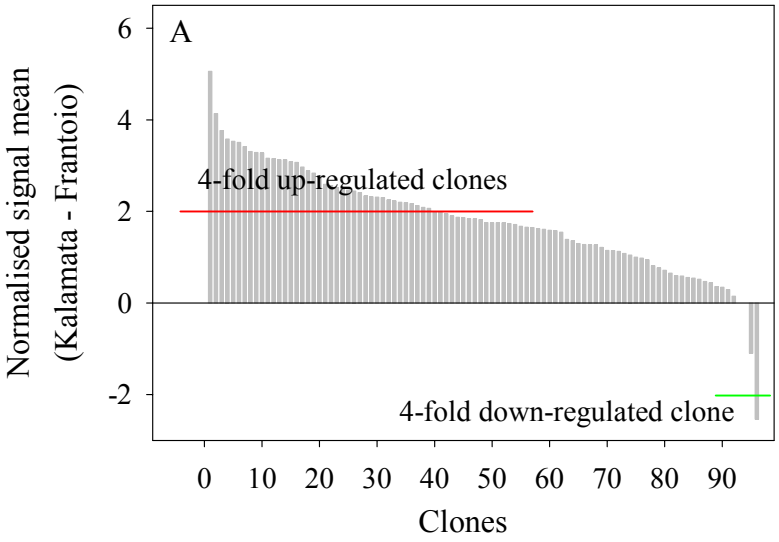


Fig. 6.10. Gene expression in whole flower buds of different olive cultivars. Green, blue, and red squares show empty plasmids, negative, and positive controls, respectively.



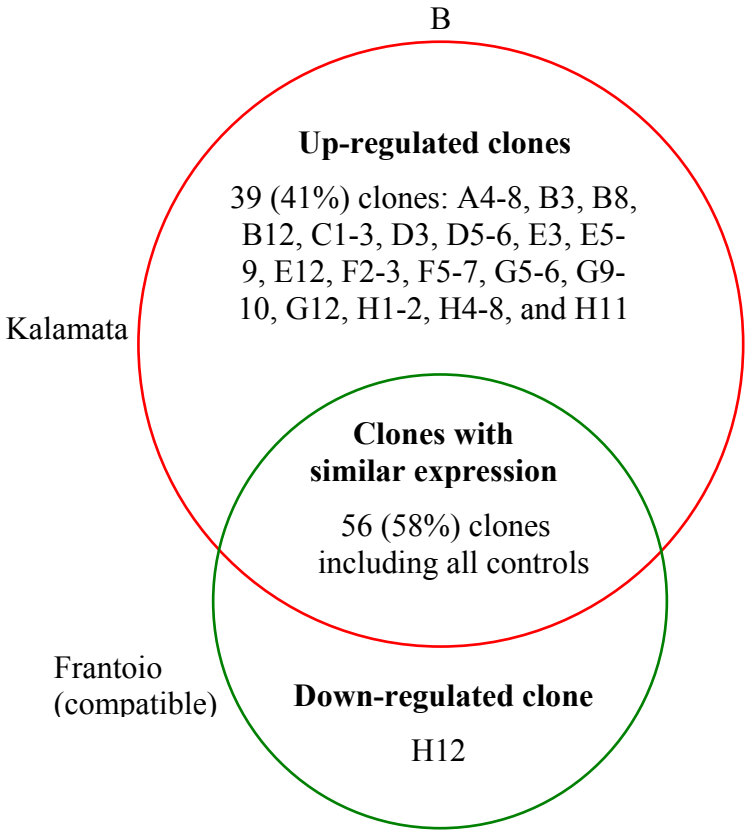
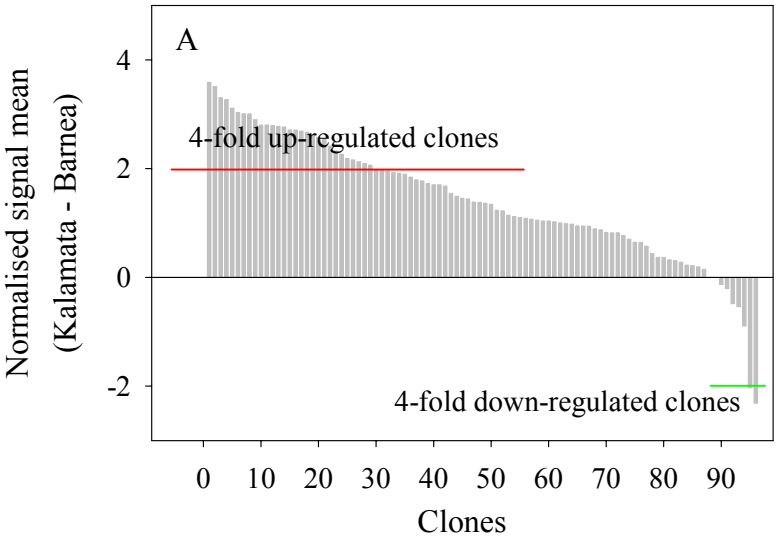


Fig. 6.11. A) Normalised signal mean of whole flower buds of Kalamata – Frantoio (a compatible cultivar) hybridisations. Genes showing 4-fold up- and down-regulation are indicated with red and green horizontal lines, respectively. B) Specific clones with 4-fold up- and down- regulation, or similar expression.



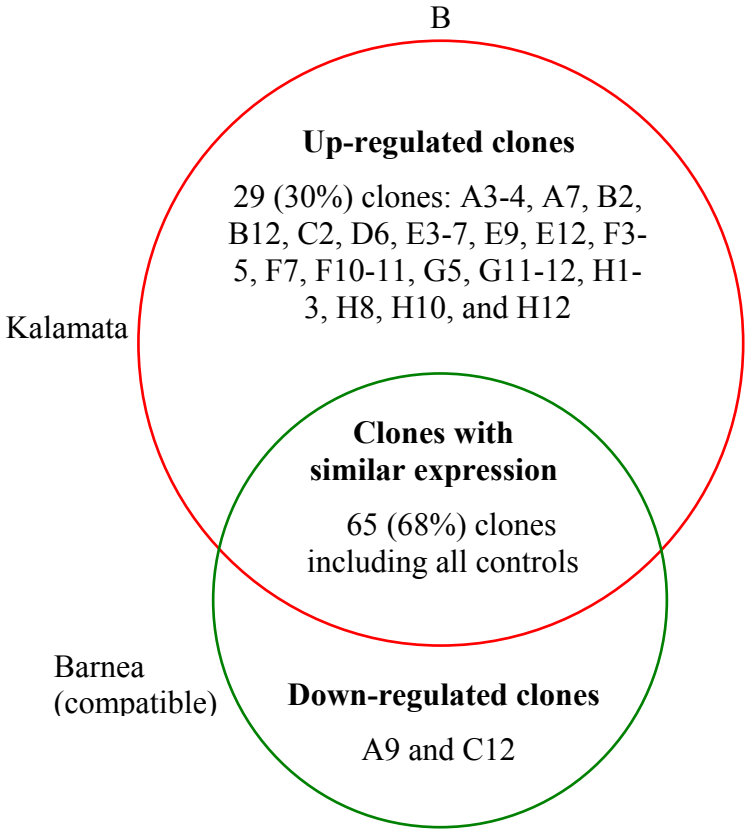
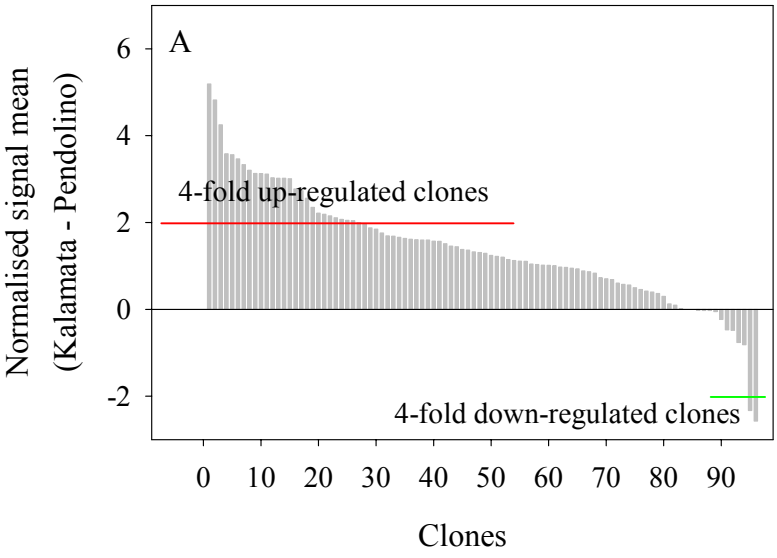


Fig. 6.12. A) Normalised signal mean of whole flower buds of Kalamata – Barnea (a compatible cultivar) hybridisations. Genes showing 4-fold up- and down-regulation are indicated with red and green horizontal lines, respectively. B) Specific clones with 4-fold up- and down- regulation, or similar expression.



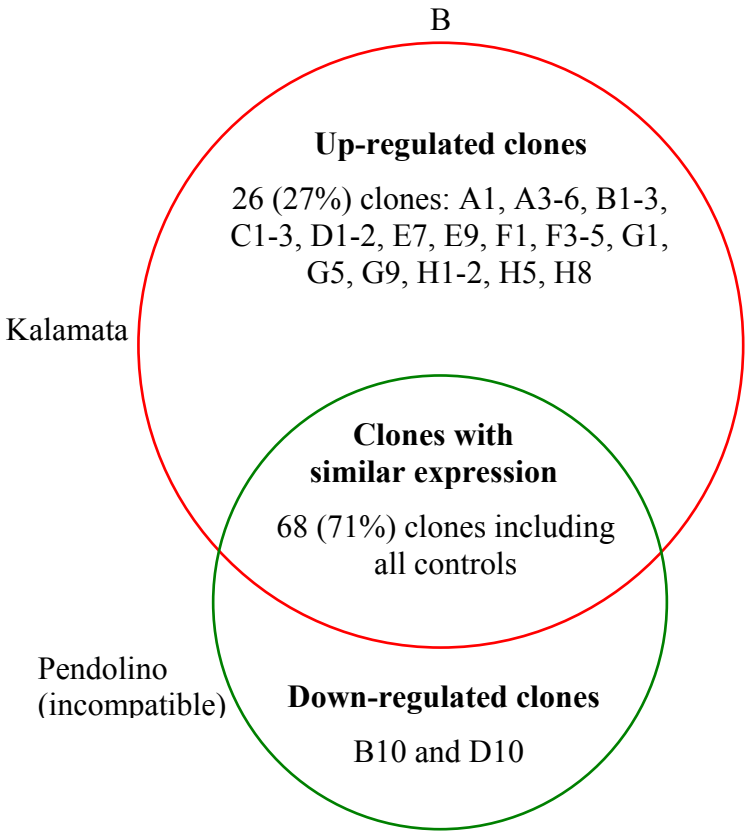


Fig. 6.13. A) Normalised signal mean of whole flower buds of Kalamata – Pendolino (an incompatible cultivar) hybridisations. Genes showing 4-fold up- and down-regulation are indicated with red and green horizontal lines, respectively. B) Specific clones with 4-fold up- and down- regulation, or similar expression.

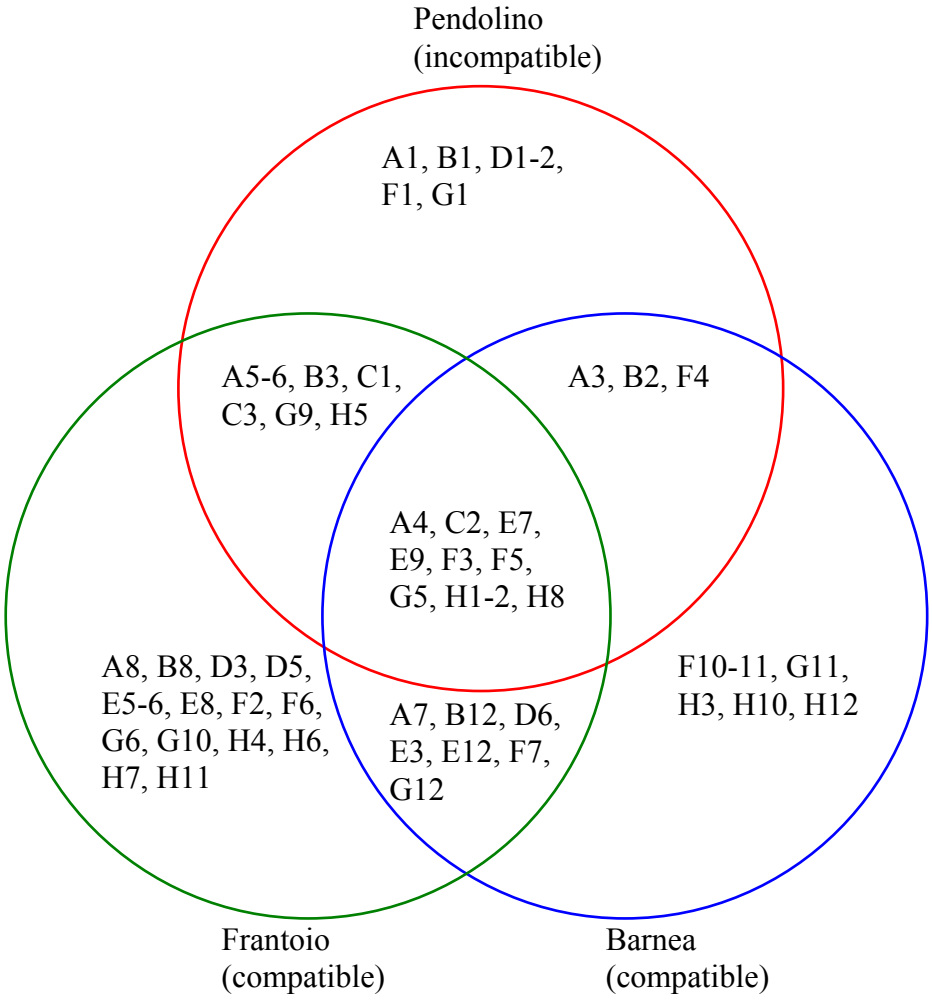


Fig. 6.14. Up-regulated clones in Kalamata across Frantoio (compatible), Barnea (compatible), and Pendolino (incompatible).

6.3.5 Sequence and homology analysis

Eighty-nine of the 90 putative up-regulated cDNAs were sequenced from the 5′-end (F10 could not been sequenced due to the low quantity of plasmid DNA). Six clones, including B5, B9, C2, E2, F7, and H8, had consistently poor sequence results and were not used further. The remaining sequences were compared using clustalW analysis (version 1.83) and presented using a phylogenetic tree constructed with the neighbour joining method and absolute identity algorithms (MacVector 9.5.4). The comparison identified 14 general sequence groups (Table 6.2) with more than 90% identity within each group (in total 42 clones). The consensus sequence of each group was used for homology analysis using the BLASTX program (NCBI) against the NR protein database. With a similarity cut-off (BLASTX E-value < 10^{-10}) (van Doorn et al.,

2003; Lan et al., 2004), half of the groups had significant similarity to known (5 groups) or unknown proteins (2 groups), and the other seven groups did not show any significant similarity (E-value > 10^{-10}). The homology analysis of the remaining 41 clones showed either significant (19 clones with known and three clones with unknown proteins) or non-significant matches to sequences in the NCBI database (Table 6.3). An interesting point is that almost a quarter of the hits were to woody plants. In most cases, the highest similarity in the database was considered the putative function of the sequence, except when these were unknown proteins, for example, clones matched with receptor protein kinase-like protein. Appendix A lists all of the individual sequences except for those with poor quality. Sequences with zero or non-significant matches (37 clones in total) were also analysed against NCBI expressed sequence tags (EST) using TBLASTX; however, none of them showed significant homology (E-value < 10^{-10}) to deposited sequences. It would appear some of these sequences are novel, which have not previously been defined in other organisms (indicated with an asterisk in Appendix A).

Several clones were identified multiple times (groups 1-14) even though the macroarray was constructed from clones randomly chosen from the cDNA libraries. This suggests that the corresponding genes had high expression levels in pistils at stage 2. Consensus sequences of groups 1, 2, and 3 matched significantly with a receptor protein kinase-like protein (Table 6.2). Clones A8, B1, and B3 (Table 6.3) also matched with this protein. The phylogenetic tree (Fig. 6.15, A) shows the similarity of the sequences and some related proteins in *Arabidopsis* and *Brassica*. Among the sequences identified, B3 was more similar to the SRK in *Brassica* species and *Arabidopsis* receptor kinase 2 and 3 (ARK 2 and 3). SRK proteins have previously been reported to be involved in SSI (Nasrallah et al., 1994; Takasaki et al., 2000), and ARK 2 and 3 are similar to SRK at the sequence level (Pastuglia et al., 2002). Groups 4 and 5 (consensus sequences) and clone C8 matched with beta-1,3-glucanase-like protein, a pathogenesis-related protein (PR). The phylogenetic tree (Fig. 6.15, B) reveals they were more similar to the glucanases previously reported in *Nicotiana*, *Ziziphus*, and *Arabidopsis* than those reported in olive. Some other defence/stress related proteins (Lan et al., 2004) found in this study were aluminium induced protein (A2), drought-induced protein (A11), thaumatin-like protein (D2), lipid transfer protein (F5), and abscisic stress ripening protein (H10).

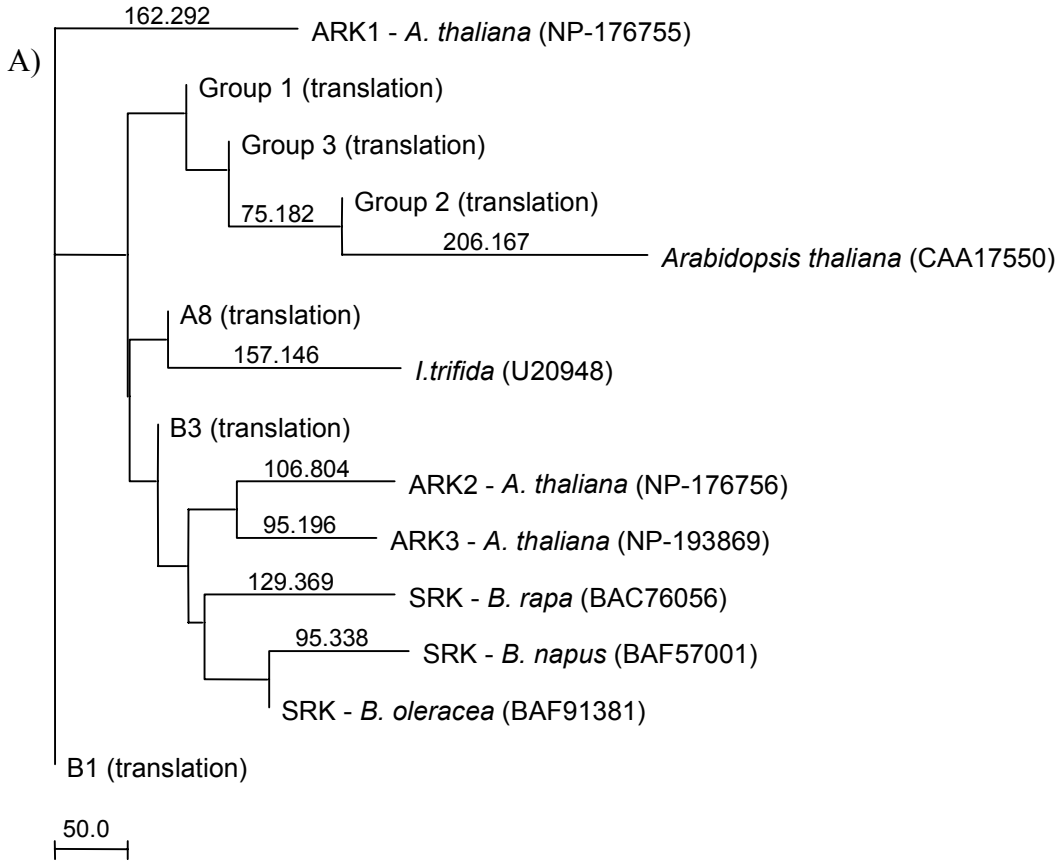
When the sequences were analysed using BLASTX against *Olea* in the NR protein database, only four sequences matched significantly (E-value < 10^{-10}) with known olive proteins (Table 6.4). Clone B10, as the longest sequence among groups 4, 5, and clone C8, matched significantly with three different kinds of glucanase. F11, a clone expressed equally in Kalamata pistil and petal + anther but more in leaf (Fig. 6.7 to 6.9), matched with photosystem II protein. F12 matched significantly with three different aquaporins previously reported in olive. And, H11 matched with beta-glucosidase. Fig. 6.15, B-E shows the absolute difference of these cloned cDNA (translated) and related proteins in olive and some other plants.

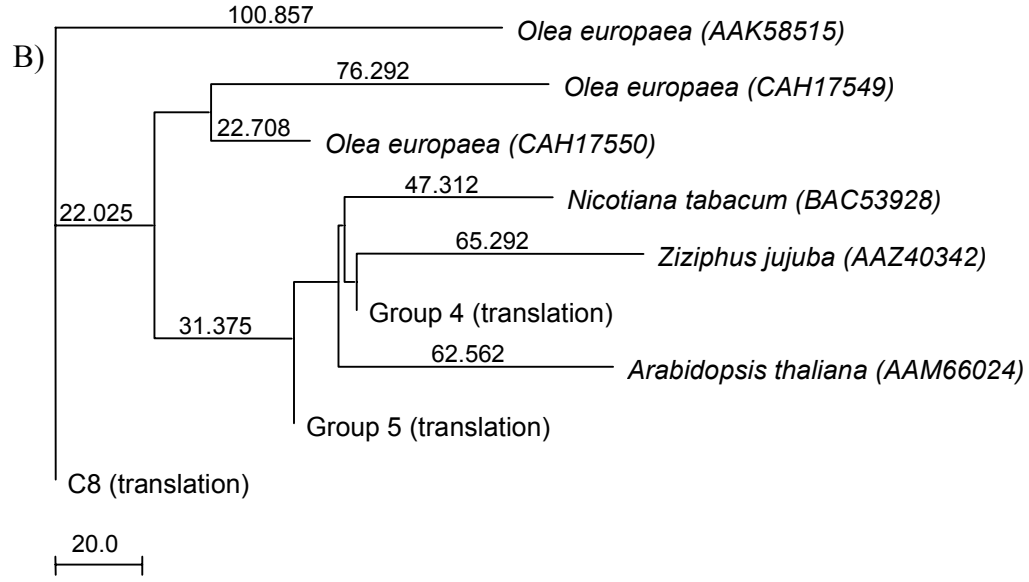
Table 6.2. Gene identification and putative function of the up-regulated (FS) clones with multiple times of expression in olive pistils stage 2.

Group	Clones included	Accession (Hit to NR)	Putative protein function (organism)	E-value	Score	Size (bp)
1	A5, F6	CAA17550	Receptor protein kinase-like protein (*Arabidopsis thaliana*)	3e-35	151	590
2	B7, C4, F8, H5	CAA17550	Receptor protein kinase-like protein (*Arabidopsis thaliana*)	3e-35	137	416
3	B2, C1, C3, E3, H9	CAA17550	Receptor protein kinase-like protein (*Arabidopsis thaliana*)	9e-39	162	583
4	A7, A10, B10	BAC53928	Beta-1,3-glucanase-like protein (*Nicotiana tabacum*)	4e-62	239	463
5	A12, B11, D5, D6, D11, D12	BAC53928	Beta-1,3-glucanase-like protein (*Nicotiana tabacum*)	2e-61	237	452
6	E4, G10	CAO65877	Unknown protein (*Vitis Vinifera*)	3e-40	166	415
7	E1, E5	ABK94059	Unknown protein (*Populus trichocarpa*)	2e-13	78.2	435
8	E6, F2	NA	Non significant matches	$>10^{-10}$	NA	280
9	H3, H4	NA	Non significant matches	$>10^{-10}$	NA	307
10	C11, H1	NA	Non significant matches	$>10^{-10}$	NA	504
11	D4, F1	NA	Non significant matches	$>10^{-10}$	NA	498
12	A9, C9, C10, D1, E7, E9	NA	Non significant matches	$>10^{-10}$	NA	388
13	F3, G1	NA	Non significant matches	$>10^{-10}$	NA	661
14	D9, H7	NA	Non significant matches	$>10^{-10}$	NA	378

Table 6.3. Gene identification and putative function of the up-regulated (FS) clones expressed in olive pistils stage 2.

Clone	Accession (Hit to NR)	Putative protein function (Organism)	E-value	Score	Size (bp)
A1	NP_176371	Postsynaptic protein-related (*Arabidopsis thaliana*)	3e-37	157	366
A2	AAK50814	Aluminium induced protein (*Avicennia marina*)	9e-41	168	390
A8	CAA17550	Receptor protein kinase-like protein (*Arabidopsis thaliana*)	2e-14	80.9	358
A11	S71562	Drought-induced protein SDi-6 – (common sunflower) (fragment)	2e-12	74.7	360
B1	CAA17550	Receptor protein kinase-like protein (*Arabidopsis thaliana*)	1e-21	104	246
B3	CAA17550	Receptor protein kinase-like protein (*Arabidopsis thaliana*)	1e-19	98.2	389
C8	BAC53928	Beta-1,3-glucanase-like protein (*Nicotiana tabacum*)	5e-26	119	277
D2	BAA74546	Thaumatin-like protein SE39b (*Nicotiana tabacum*)	4e-35	109	309
D3	Q43681	Probable non-specific lipid-transfer protein AKCS9 precursor (LTP)	2e-15	53.1	305
D10	ABK60177	Putative reverse transcriptase (*Zingiber officinale*)	4e-16	89.4	288
E8	CAO46825	Unknown, unnamed protein product (*Vitis vinifera*)	1e-33	145	223
E10	CAN65763	Unknown, hypothetical protein (*Vitis vinifera*)	3e-23	110	287
F4	Q9ATF5	60S ribosomal protein L18a (*Castanea sativa*)	1e-41	171	282
F5	AAB06586	Putative nonspecific lipid transfer; auxin induced gene	3e-22	107	544
F11	AAN65254	Photosystem II 32 kDa protein (*Populus tomentose*)	2e-19	97.4	469
F12	AAG44945	Putative delta tonoplast intrinsic protein (*Nicotiana glauca*)	4e-41	169	362
G11	CAN65763	Unknown, hypothetical protein (*Vitis vinifera*)	1e-18	95.1	159
G12	BAC23052	Putative lipid transfer protein (*Solanum tuberosum*)	1e-20	102	387
H6	ABE87816	Auxin responsive SAUR protein (*Medicago truncatula*)	8e-14	62.8	613
H10	AAR23420	Abscisic stress ripening protein (*Ginkgo biloba*)	2e-13	77.4	463
H11	AAL93619	Beta-glucosidase (*Olea europaea* subsp. *europaea*)	4e-122	425	740
H12	NP_567960	Hydrolase (*Arabidopsis thaliana*)	1e-10	57.0	364


A)
162.292
ARK1 - A. thaliana (NP-176755)
Group 1 (translation)
Group 3 (translation)
75.182
Group 2 (translation)
206.167
Arabidopsis thaliana (CAA17550)
A8 (translation)
157.146
I.trifida (U20948)
B3 (translation)
106.804
ARK2 - A. thaliana (NP-176756)
95.196
ARK3 - A. thaliana (NP-193869)
129.369
SRK - B. rapa (BAC76056)
95.338
SRK - B. napus (BAF57001)
SRK - B. oleracea (BAF91381)
B1 (translation)
50.0


B)
100.857
Olea europaea (AAK58515)
76.292
Olea europaea (CAH17549)
22.708
Olea europaea (CAH17550)
47.312
Nicotiana tabacum (BAC53928)
22.025
65.292
Ziziphus jujuba (AAZ40342)
Group 4 (translation)
31.375
62.562
Arabidopsis thaliana (AAM66024)
Group 5 (translation)
C8 (translation)
20.0

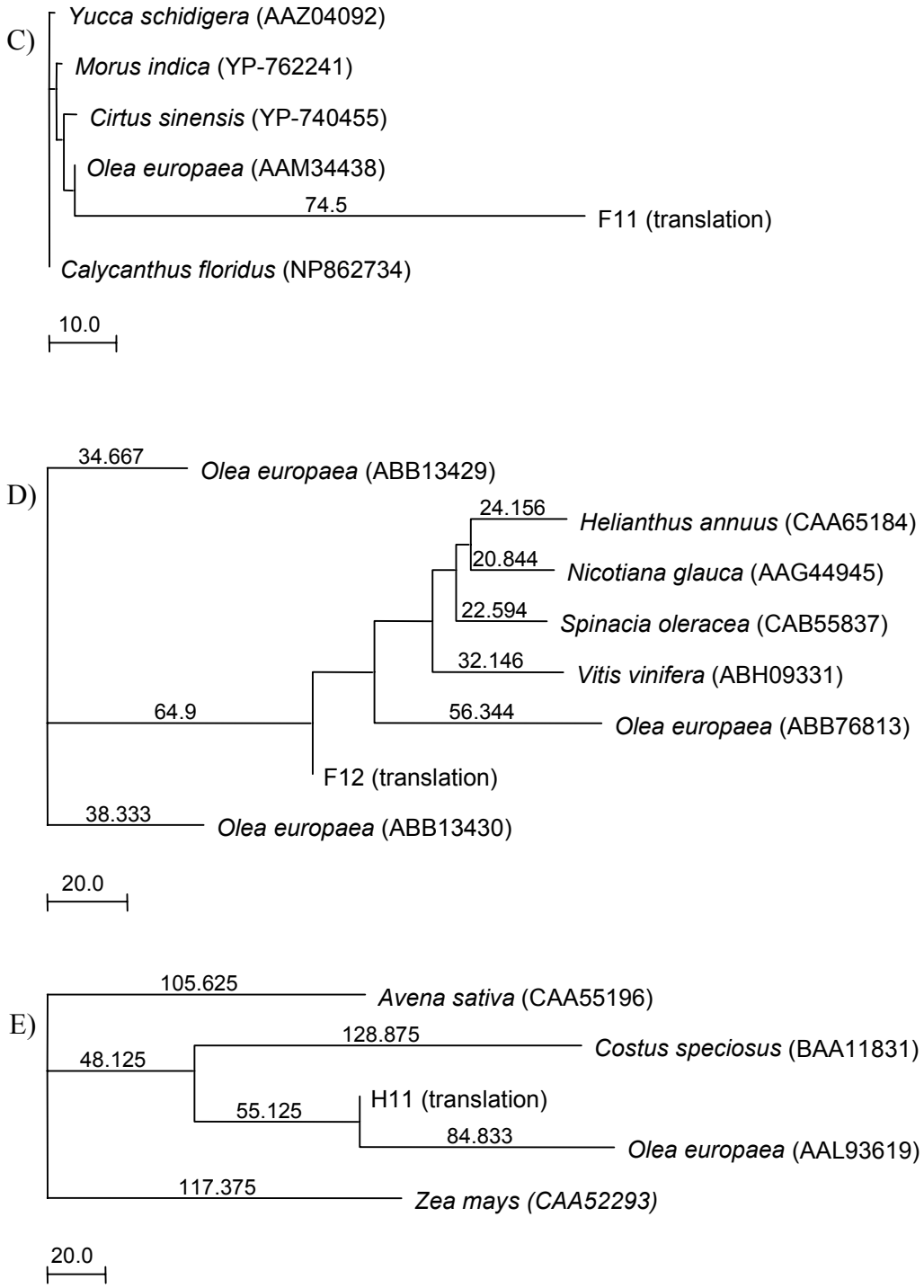


Fig. 6.15. Phylogenetic tree analysis of clustalW aligned amino acid sequences. The phylogenetic tree was calculated using the neighbour joining method and absolute identity algorithms (MacVector 9.5.4). A) Clones aligned against receptor protein kinase-like protein (groups 1-3, A8, B1, and B3). B) Clones aligned against beta-1,3-glucanase-like protein (groups 4-5, and C8). C) Clone F11 (photosystem II protein). D) Clone F12 (putative delta tonoplast intrinsic protein). E) Clone H11 (beta-glucosidase). Bars show the absolute number of differing amino acids between sequences.

Table 6.4. Pistil clones with significant homology to other proteins previously identified in olive.

Clone	Accession (Hit to NR)	Putative protein function	E-value	Score
B10	AAK58515	Beta-1,3-glucanase-like protein	4e-32	125
	CAH17549	Beta-1,3-glucanase	2e-25	103
	CAH17550	Beta-1,3-glucanase	3e-15	70.1
F11	AAM34438	Photosystem II protein D1	2e-14	67.4
F12	ABB76813	Tonoplast intrinsic protein	6e-32	124
	ABB13430	Plasma membrane intrinsic protein	3e-13	62.8
	ABB13429	Plasma membrane intrinsic protein	2e-11	56.6
H11	AAL93619	Beta-glucosidase	1e-122	425

6.4 Discussion

In the present study, differential screening techniques were used to demonstrate several classes of gene expression patterns in Kalamata olive pistils during the last stages of flower bud development. Furthermore, gene expression profiles were analysed across different tissues including leaf and petal + anther and the cultivars Frantoio, Barnea, and Pendolino. Although the function of 37 differentially expressed clones was unknown, the expression profiles provide the first glimpse to the genes that are up-regulated in olive pistils before anthesis and pollination.

In this study, 90 up-regulated cDNA clones were selected using subtractive hybridisation followed by macroarray hybridisation. All the up-regulated clones were printed on membranes in replicates of four and hybridised with both subtracted probes for the second round and unsubtracted probes to assess the reproducibility of the results. After subtracted and unsubtracted hybridisation, up-regulation was confirmed in 82 (91%) and 71 (79%) of the 90 clones, respectively. The difference between the expression patterns of these hybridisations is probably due to the higher sensitivity of the subtracted probes, in which rare sequences are retained (PCR-select differential screening kit user manual, Clontech, Catalogue K1808-1, 2001). However, screening using unsubtracted probes is recommended for confirming the results of subtracted probes. In most cases, all four replicates of a clone had the similar expression pattern; however, for unknown reasons some replicates of a particular clone did not show a

similar expression pattern across the four replicated spots on the membrane. This highlighted the importance of including colony replicates on macroarray membranes. Subsequently, the average signal of the four replicates of each clone was used to identify the overall up- and down-regulated clones.

Analysis of gene expression across different tissues of Kalamata showed that the majority of these genes (96% and 75% in pistils versus leaf and petal + anther, respectively) were pistil-specific and were not expressed in either leaf or petal + anther. The results showed that only one clone (F11) was down regulated in leaf, which was photosystem II protein, a protein involved in photosynthesis. This result is as expected given that photosynthesis occurs in the leaves and supports the veracity of the data. The only down-regulated clone in petal + anther was E10, an unknown gene.

The gene expression analysis in whole flower buds showed a high similarity in the expression pattern between Kalamata and other olive cultivars including: Frantoio (Wu et al., 2002; Mookerjee et al., 2005) and Barnea (results of Chapter 4 and 5) as two compatible cultivars, and Pendolino as an incompatible cultivar (Wu et al., 2002; Mookerjee et al., 2005). The study showed that 58%, 68%, and 71% of the clones expressed in Kalamata were also expressed equally in Frantoio, Barnea, and Pendolino, respectively. It was of interest that Pendolino, an incompatible cultivar, had a more similar expression pattern to Kalamata than the compatible cultivars. The incompatible response between Kalamata and Pendolino indicates that they probably share common genes, which are needed to recognise the incompatible pollen.

Several clones showed database matches with defence-/stress-related proteins. Seki et al. (2002) and Ozturk et al. (2002) reported more than 200 genes, induced by drought, cold, or high-salinity stress. A role in defence is clear for beta-1,3-glucanase-like protein, a PR protein, which is also found in different physiological and developmental processes in uninfected plants, including pollen germination, pollen tube growth, and fertilisation (Leubner-Metzger and Meins, 1999). Lipid transfer protein is a small hydrophilic protein that transfers membrane lipids without specificity, but it is also involved in defence against pathogens (Blein et al., 2002). The abundance of defence-/stress-related proteins in this experiment was interesting. This study attempted to reduce the stresses that may happen during sampling. The pistil sample target trees

were located less than 200 m from the laboratory. Flowering shoots were collected in the morning, when the temperature was low, transferred to the laboratory on ice, and stored with cut stems in a bucket of water in a cold room (4 °C). The pistils and other tissues were sampled immediately, frozen in liquid nitrogen, and stored at –80 °C until use. However, the samples may have faced some stress as a result of wounding shock, but the pistils from both stages were sampled in similar conditions. Some PR proteins have been reported previously to be involved in flowering processes in tobacco (Lotan et al., 1989; Kuboyama, 1998), rice (Lan et al., 2004), and barley (Liljeroth et al., 2005). Lotan et al. (1989) suggested that these proteins might be part of a larger gene family containing members with pathogen-inducible characteristics and members that are involved in flowering and reproduction. On the other hand, since exudates released from the stigma provide a favourable environment for the growth of pathogens (Lan et al., 2004), the presence of defence-related proteins may be reasonable. Alternatively, a gene may be multifunctional and act in different developmental processes.

Groups 1, 2, 3, and clones A8, B1, and B3 matched with receptor protein kinase-like protein (GeneBank accession number CAA17550). Receptor protein kinases are found in animals, microorganisms, and plants (Muschietti et al., 1998). Their main function is signal transduction, but the different members of this gene family are involved in various aspects of development and plant defence including gametophyte development, shoot apical meristem equilibrium, hormone signalling, cell morphogenesis and differentiation, and pollen-pistil interactions (Sopory and Munshi, 1998; Becraft, 2002). Protein kinases were found in *Arabidopsis* (Seki et al., 2002) and Barley (Ozturk et al., 2002), which were under drought stress. In *Brassica*, SRK has a role in pollen recognition in SSI (Nasrallah et al., 1994; Takasaki et al., 2000). Receptor protein kinases were also genetically linked to the SI locus in *Arabidopsis lyrata* (Schierup et al., 2001) and *Ipomoea trifida* (Kowyama et al., 1996). They were also expressed in rice pistils during pollination and fertilisation (Lan et al., 2004). Among the different repeats of receptor protein kinase-like protein, B7, C4, F8, and H9 were expressed equally in Kalamata and the other cultivars studied (Fig. 6.14), but some others were expressed differently. Clone B1, for instance, was expressed equally in Kalamata, Frantoio (compatible), and Barnea (compatible) but not in Pendolino (incompatible). On the other hand, E3 was expressed equally in Kalamata and

Pendolino but not in Frantoio and Barnea. Different repeats of this sequence might code for homologous genes with similar function but under different regulation.

Among the up-regulated clones, only four matched significantly with proteins in *Olea* (Table 6.4). Doveri and Baldoni (2007) reviewed 46 genes identified in olive. Previous studies focussed on genes encoding key enzymes involved in fatty acid biosynthesis, modification, and storage. Clone B10 matched with three beta-1,3-glucanases in *Olea*, which are members of the PR protein family. The first hit from the BLASTX search (GeneBank accession number AAK58515) was detected previously in pollen tissue (Huecas et al., 2001). Huecas et al. (2001) reported glucanase as a major olive pollen allergen, involved in the allergic responses of 65% of patients. The second and third hits were identified in olive roots (Calvente et al., 2004). Secchi et al. (2007) isolated three aquaporins from different tissues of olive including roots, shoots, and leaves. In this study, clone F12, expressed in pistils, matched with all three reported aquaporins, more significantly with a tonoplast intrinsic protein (TIP) (E-value 6e-32). The phylogenetic tree (Fig. 6.15, D) shows that the aquaporin found probably is a TIP. TIPs, a subfamily of plant aquaporins, are abundant in the plasma and vascular membrane and active in water transport (Alexandersson et al., 2005). Beta-glucosidase is an enzyme involved in the degradation of oleuropein, a bitter defence phenol glucoside (Mazzuca et al., 2006), and has been found in olive leaves, fruits (Soler-Rivas et al., 2000), and pistils (this study).

This study has identified a number of possible candidates for involvement in olive SI. A role in the SSI system of *Brassica* has been reported for S-locus receptor-like kinase protein (Nasrallah et al., 1994; Takasaki et al., 2000; Goring and Walker, 2004), a close relative of the receptor protein kinase-like protein found abundantly in olive pistils at stage 2, the expression of which was much lower (less than 4-fold) in olive pistils at stage 1 and also in petal + anther and leaf at stage 2. Protein kinase activity has also been detected in pollen tubes of *Nicotiana alata* (Polya et al., 1986). In the two-locus GSI system of rye (*Secale cereale* L.), the probable involvement of protein kinase has been suggested (Wehling et al., 1994). Wehling et al. (1994) treated rye stigmas using various protein kinase inhibitors followed by self-pollination and showed a strong inhibition of the SI response by Lavendustin A. In the *Papaver* GSI mechanism, p56-MAPK, a mitogen-activated protein kinase, has a role in the signalling cascade resulting

in programmed cell death about 10 min after SI induction, when the incompatible pollen tubes have been arrested (de Graaf et al., 2006; McClure and Franklin-Tong, 2006; Li et al., 2007). The major gene involved the GSI in fruit trees from the Rosaceae family (a ribonuclease called S-RNase) is a glycoprotein (Roalson and McCubbin, 2003). Two enzymes found in this study have been reported to be glycoproteins: a kind of beta glucanase (Biely et al., 1976; Villa et al., 1979; Taylor et al., 2004) and beta glucosidase (Rudick and Elbein, 1975; Grace et al., 1990). Interestingly, beta-1,3-glucanase is the main olive pollen allergen (Huecas et al., 2001), indicating high levels of this enzyme in the olive pollen. Ikeda et al. (1997) reported an aquaporin-like protein to be involved in SSI in *Brassica*. A kind of aquaporin was also found here in olive pistil stage 2 with higher expression in comparison to pistil stage 1, petal + anther stage 2, and leaf stage 2.

Identification of more novel pistil-specific genes will provide more information about the genes involved in sexual reproduction and may result in finding the SI genes. In this study, 37 clones had zero or non-significant matches when searched against the NR protein database using BLASTX and the EST database using TBLASTX. A general requirement of genes involved in SI is to have the correct tissue-specific and developmental expression patterns (de Graaf et al., 2006). Most of these novel genes were pistil-specific, the tissue in which SI process occurs, and some of them may have a role in the pollen-pistil interaction, but need more investigation.

The present study demonstrates the results of the first differential screening conducted on olive pistils. More than 80 previously identified and novel cloned genes introduced in this chapter were up-regulated in pistil. They were up-regulated in pistils just before anthesis, when they were receptive and ready to interact with compatible or incompatible pollen grains. Further analysis of these genes using real-time RT-PCR (to study the stage-specific expression patterns) and *in situ* hybridisation (to study the tissue-specific expression patterns) will help to determine whether they have any role in olive SI.

CHAPTER SEVEN

General Discussion

The olive tree originated in the eastern Mediterranean area and has been cultivated for its oil and fruit for millennia (Bertrand, 2002). Today, the demand for olive oil and table olives is increasing in traditional producing areas and elsewhere in the world. Both products have high levels of monounsaturated fatty acids and antioxidant agents and high dietetic value (de la Lastra et al., 2001). Australia consumes more olive oil and table olives than any other nation outside the traditional Mediterranean production countries (Kailis and Sweeney, 2002; Kailis and Davies, 2004), and imports of both products into Australia doubled between 1992 and 2006 (AOA-website, accessed 16 Nov 2007).

Most olive cultivars are self-incompatible and benefit from cross-pollination (Lavee, 1986; Lavee, 1990; Besnard et al., 1999; Dimassi et al., 1999; Moutier, 2002; Fabbri et al., 2004; Conner and Fereres, 2005). However, cross-incompatibility may exist between some pairs of cultivars (Griggs et al., 1975; Cuevas and Polito, 1997; Martin and Sibbett, 2005; Mookerjee et al., 2005). Climatic conditions, especially high temperatures, were found to be a major factor affecting the level of olive self- and cross-incompatibility (Griggs et al., 1975; Lavee, 1986; Androulakis and Loupassaki, 1990; Lavee et al., 2002). This may be a reason why the level of SI in some cultivars has been reported to change from year to year and from environment to environment. The implications of this previous research are, firstly, it is necessary to plant more than one cultivar in orchards; secondly, the cultivars must be cross-compatible, and thirdly, their compatibility relationship needs to be confirmed in the same or similar climate. In addition, cross-compatible cultivars must have overlapping anthesis to be able to pollinate each other efficiently. Better understanding of floral morphology and phenology is necessary for researchers to improve experimentation on olive SI and for growers to optimise cross-pollination and fruit set. This project aimed to study floral

biology and inflorescence architecture, the sexual compatibility relationships of some cultivars with widespread use in Australia, and gene expression in pistils during flowering as a tool to identify olive SI genes.

A mature olive tree produces about half a million flowers in a year under normal flowering conditions. Approximately 15-30 flowers, which are either perfect (hermaphrodite) or staminate (male), are borne on an inflorescence (Martin and Sibbett, 2005). Large variations have been reported in the number of flowers per inflorescence (Brooks, 1948; Lavee and Datt, 1978; Lavee, 1985; Cuevas et al., 1994a; Lavee, 1996; Lavee et al., 2002; Reale et al., 2006a), the percentage of perfect flowers (Badr and Hartmann, 1971; Lavee et al., 2002; Fabbri et al., 2004), and the position of perfect and staminate flowers on the inflorescence (Bouranis et al., 1999; Dimassi et al., 1999; Ateyyeh et al., 2000; Cuevas and Polito, 2004; Martin and Sibbett, 2005). The present study (Chapter 3) investigated the main sources of variation in some olive cultivars. The results showed that in Frantoio, Kalamata, and Koroneiki shoot orientation did not have any influence on the length of the inflorescence, the number of flowers per inflorescence, and the percentage of perfect flowers. The basal inflorescences on shoots were smaller, with fewer flowers, but with the same percentage of perfect flowers. There were some variations between cultivars, between trees of a cultivar, between the shoots of a tree, and between the inflorescences of a shoot in the number of flowers per inflorescence. There was also some variation between the cultivars and between the shoots of a tree in the percentage of perfect flowers but not between the trees of a cultivar or between the inflorescences of a shoot. There are some agreements and disagreements between these findings and those of previous studies. A larger sample of inflorescences, shoots, trees, cultivars, years, and environments is suggested to identify all the sources of variation.

This study also showed that in Manzanillo, Mission, and Frantoio the percentage of perfect flowers was higher at the beginning of the opening period and reduced towards the end of anthesis. Within an individual inflorescence, the position of the flower influenced opening day and gender. The first flowers to open were those located in terminal positions followed by those located on the laterals. In a cultivar with a high percentage of staminate flowers (Manzanillo and Mission), the perfect flowers were mainly in the terminal positions and the staminate flowers on the laterals. Removing the

distal half of the inflorescences, in which the flowers tend to be perfect 30 days before full bloom, decreased pistil abortion in the remaining flowers. This suggests competition between the flowers for nutrition, which may cause higher pistil abortion in poorer nurtured lateral positions.

According to the results presented here, it may be important for growers to consider the opening pattern of perfect and staminate flowers in selecting pollenisers. Early blooming cultivars, especially when they have a high percentage of staminate flowers, may not be pollinated efficiently by late blooming pollenisers, despite overlapping anthesis for some days. For example, Manzanillo in which most perfect flowers open in the first half of the opening period (Fig. 3.2) may not be pollinated efficiently by late blooming Kalamata (Fig. 2.2). Kalamata flowers start to open when most perfect flowers in Manzanillo have already opened and have probably lost their receptivity. Similarly researchers who study topics related to olive flowering must be mindful of the opening pattern of perfect and staminate flowers and their position on the inflorescence. Hand pollinations (Chapter 4) need to be conducted at early stages of bloom, and collection or sampling of pistils or flower buds with pistils (Chapter 6) must consider the opening time of the perfect flowers and their positions on the inflorescence.

To date, four different methods have been used to study pollen-incompatibility of olive cultivars: measurement of 1) fruit set and 2) pollen tube growth after controlled crossing, 3) measurement of *in vitro* pollen germination and tube growth in a culture sometimes containing the pistil extracts of other cultivars, and 4) paternity analysis using molecular markers. In this project, the second method that indicates the inhibition site of incompatibility, and the last method that is not influenced by pollen contamination and temperature changes within the isolation bags were used.

Pollen tube observation (Chapter 4) was performed on the cultivars Frantoio, Koroneiki, and Kalamata under the climatic conditions of Roseworthy, SA, Australia. The number of ovules penetrated by pollen tubes was counted under a fluorescence microscope and used to estimate the level of pollen-incompatibility. Frantoio (as a host) was found to be self-incompatible, incompatible with Koroneiki and Barnea, but partially compatible with Mission. Koroneiki, a cultivar previously reported to be self-compatible (Lavee, 1986; Lavee et al., 2002), was completely self-incompatible. It (as a

host) was incompatible with Barnea but partially compatible with Frantoio and Mission. In Kalamata, there was no significant difference between different pollen donors, and only one ovule was penetrated after open-pollination (as a potential compatible cross in an orchard with more than 50 different accessions). The experiment was repeated on Kalamata for the second year but led to similar results; therefore, it was decided to use the number of pollen tubes present in the lower style as an indication of compatibility. The results showed that Kalamata had a high level of SI in both years. It (as a host) was compatible with Barnea (in both years), incompatible with Mission and Koroneiki in 2004, but partially compatible with them in 2005. It seems that under the climatic conditions of this study seven days were not long enough for pollen tubes to reach the ovules of Kalamata. To gain a better understanding of cross-incompatibility between Kalamata (as a host) and Mission and Koroneiki, it is recommended to repeat the experiment and to sample the pistils later than seven days after pollination.

Statistical analysis showed that in incompatible crosses such as Frantoio and Koroneiki pollinated by Barnea, Frantoio, and Koroneiki there was no significant difference between the number of pollen tubes present in the upper styles, lower styles, and ovules. In other words, pollen tubes were inhibited within the stigmas and before entering the styles. This is in agreement with previous studies conducted on olive (Cuevas and Polito, 1997; Ateyyeh et al., 2000; Orlandi et al., 2005) and *Phillyrea angustifolia* another genus from the Oleaceae family (Vassiliadis et al., 2000). Both self and non-self pollen grains germinated on the stigma surface of *Phillyrea angustifolia*, but the self pollen tubes were inhibited in the stigma and did not continue to grow into the style (Vassiliadis et al., 2000).

Paternity analysis is a method to study gene flow, pollen flow, and, more recently, sexual compatibility of plants. This method (Chapter 5) was used to assess the SI of Kalamata and to identify some compatible pollenisers for this cultivar under the climatic conditions of Roseworthy, SA, Australia. Eight microsatellite markers were used for genotyping three Kalamata mother trees, 120 embryos, and all potential pollen donors. The identified alleles were analysed using FaMoz software and confirmed the high SI of Kalamata that has been previously reported (Androulakis and Loupassaki, 1990; Wu et al., 2002; Mookerjee et al., 2005). Only three Kalamata embryos were assigned to Kalamata itself, even though it was the most available pollen donor.

The alleles were also analysed using NTSYS-pc software and identified 54 potential pollen donors in the study site; however, not all of them were located within the EPD of the mother trees, which has been reported to be 30 m in olive (Ayerza and Coates, 2004; Fabbri et al., 2004; Sibbett and Osgood, 2005). According to the results of this study, Kalamata (as a host) was compatible with Barnea, Benito, and Katsourela (six Kalamata embryos assigned in each) but incompatible with Arbequina, Azapa, and Picual (zero Kalamata embryos assigned in each).

Frantoio has been reported to be compatible with Kalamata (as a host) (Wu et al., 2002; Mookerjee et al., 2005), but that does not agree with the results of this study. The disagreement may be due to different climatic conditions of the study sites and different years; however, it may also be because of the close genetic distance of Frantoio and Mission (WA) (Fig. 5.3). FaMoz showed that in 14 Kalamata embryos assigned to Mission (WA), Frantoio was the second likely pollen donor with a very close LOD score. It seems that it was difficult for FaMoz to distinguish between these two cultivars. Although, eight microsatellite markers were enough for NTSYS-pc to discriminate Frantoio and Mission (WA), they were not enough for FaMoz probably because FaMoz uses only half of the alleles to recognise the most likely pollen donor. The results suggest a larger marker suite for paternity analysis especially when they are used for a collection with a high number of potential pollen donors.

The olive SI system and its molecular mechanism are unclear. Some authors have suggested a GSI system (Ateyyeh et al., 2000; Orlandi et al., 2005). The olive has been reported to have bi-nucleate pollen grains (Wu et al., 2002; Rodriguez-Garcia et al., 2003b) and wet stigmas (Ateyyeh et al., 2000; Wu et al., 2002) although dry stigmas have also been reported (Rodriguez-Garcia et al., 2003a). Incompatible pollen tubes are inhibited after germination and inside the stigma tissue rather than on the surface of the stigma (Cuevas and Polito, 1997; Ateyyeh et al., 2000; Orlandi et al., 2005; Chapter 4). The characteristics of bi-nucleate pollen grains, wet stigma and pollen tube inhibition after germination are mostly found in plants with GSI (Heslop-Harrison and Shivanna, 1977; de Nettancourt, 1997). However, the defining characteristic determining the system of SI to be gametophytic or sporophytic is the genetic behaviour of the pollen, which is unclear in olive. The SI phenotype of germinating pollen in the gametophytic system is determined by the haploid genotype of the pollen (gametophyte) (Newbigin et

al., 1993; McCubbin and Kao, 2000; de Graaf et al., 2006) and in the sporophytic system by the diploid genotype of the pollen donor (sporophyte) (Newbigin et al., 1993; McCubbin and Kao, 2000).

Kalamata, the main cultivar investigated in this thesis, is also called Kalamon and originated in Greece (Barranco et al., 2000b). It is dual-purpose but chiefly used for producing black table olives. In Australia, Kalamata is one of the most popular table olives (Kailis and Davies, 2004). Under the conditions of this study, Kalamata had a medium number of flowers (22.3) on an inflorescence of medium length (3.2 cm). Both these morphological characters are in agreement with those previously given for this cultivar (Barranco et al., 2000b). It also had a high percent of pistil abortion (76.8) in 2004 under the climatic conditions of Roseworthy, SA. Kalamata showed a high level of SI using two methods, confirming the results of previous studies (Androulakis and Loupassaki, 1990; Wu et al., 2002; Mookerjee et al., 2005). It was self-incompatible in 2004 (assessed by paternity analysis) and 2004-2005 (assessed by pollen tube observation). This consistency of SI supports the decision that Kalamata was an appropriate cultivar in which to analyse the genes expressed in pistils during flowering, in order to search for SI genes.

Different strategies have been used to clone SI genes in plants. An S-locus-specific glycoprotein was identified in stigma tissue of *Brassica oleraceae* using differential screening techniques (Nasrallah et al., 1985). In this study (Chapter 6), subtractive and cDNA macroarray hybridisations were used to analyse gene expression in Kalamata olive pistils at two developmental stages, where SI genes were expected to be differentially expressed: 1) small green flower buds (expression of SI genes not expected) and 2) large white flower buds containing receptive pistils just prior to opening (expression of SI genes expected). The analysis resulted in the identification of 90 up-regulated cDNA cloned genes highly expressed in receptive pistils. Subtracted and unsubtracted hybridisations were used and confirmed the up-regulation of the majority of them (91% and 79%, respectively). Gene expression profiles across different tissues showed that most of the screened genes were pistil-specific and did not express in leaf and petal + anther. The expression pattern of the genes showed high similarity in Kalamata, Frantoio, Barnea, and Pendolino.

About 40% (37/90) of the up-regulated clones had zero or non-significant matches with NR protein and EST databases. Some of these novel pistil-specific genes may be involved in olive flowering and SI and deserve more analysis. Approximately the same percentage (35/90) of the up-regulated clones showed significant database match with defence-/stress-related proteins. Interestingly, such proteins have been reported previously to be involved in flowering processes in other plants such as tobacco (Lotan et al., 1989; Kuboyama, 1998), rice (Lan et al., 2004), and barley (Liljeroth et al., 2005). An explanation for their abundance in pistils may be that the exudates released from stigma tissues provide a favourable environment for the growth of pathogens (Lan et al., 2004). Another explanation is that these proteins are part of a larger gene family containing members with pathogen-inducible characteristics as well as members involved in flowering and reproduction (Lotan et al., 1989).

The most redundant and interesting up-regulated clones were those similar to a receptor protein kinase-like protein. A role in *Brassica* SSI for a kind of receptor protein kinase has been reported (Nasrallah et al., 1994; Takasaki et al., 2000; Goring and Walker, 2004). The probable involvement of protein kinase in the GSI system of *Papaver* (de Graaf et al., 2006; McClure and Franklin-Tong, 2006; Li et al., 2007) and rye (*Secale cereale* L.) (Wehling et al., 1994) has also been suggested. Further studies on the function of this gene will determine any possible role in SI.

In conclusion, several important advances have been made during this project. The morphological observations have provided a comprehensive and clear picture of inflorescence architecture in olive. Pollen tube observation showed a high level of SI in Frantoio, Kalamata, and Koroneiki under the climatic conditions of Roseworthy, SA, one of the most appropriate areas to grow olives in Australia, and identified some compatible pollenisers for them. Paternity analysis using microsatellite markers confirmed Kalamata to be self-incompatible and identified further compatible pollenisers for it. More than 80 pistil-specific cDNA sequences were identified using differential screening in Kalamata. The expression profiles provide the first glimpse to the genes up-regulated in receptive olive pistils during flowering, in which SI genes are expected to be expressed.

Appendix A

Up-regulated sequences

Except for those with poor quality. *: Sequences with zero or non-significant matches.

Clone	cDNA sequence (5′-3′)
A1	ACTTGCTTCCCACACATTGCACATACTCCTTTACTGTAAGCACATGTATGACAATACTTGGCA TCTTGATGGAGCTGCTTACAGATAATGCACTTGGTTTGTCCATAAGGTGTCCATCTGTGTTTT TTAGAGAGGAGCTTGTTCTCGTTGATCTTCCTGCCGCCGCCTTCGGTAGCGTTGTGGGCCCCT TCTTTCCATTTGTCCGGCACTATTATCTTGGACAGCTTCTTCTCGCACTTTTCGCAGACCATTG TGAGCTGGAAATTAATAACCCGATGGAAGGTGAAGGAATTTGTTGGAGAAGGGAAAGGGGG ACGATGGTGTTATTTGACGAACATACGCAGCACGCTTTACTCGCCCCCGCGT
A2	ACGCGGGGATTCCATTAGCCACTCTCTTCTTTCCAATAGTATATCCGGGTTTTGTGTGTGTTTT TTATTTTTTGGAGAGAAGATGTTGGGAGTGTTTAGCAGCTCGATAGTTTCGCCGCCGGAAGA GCTGGTGGCGGCCGGAAGTAGGACGCCGTCGCCGAAGGTGACAGCGGCTAAGCTGATGTCC AGGTTCCTGGAGACGAACTCATCGGCGGTGTCCATGCAGATTGGCGATGATGTCCAGATGGC TTACACTCACTGCGACCAGTCGCCTTTACAGCCCAGATCATTTGCAGTCAAGGACGACATGTT CTGCTTGTTTGAAGGAGCGCTCCACAACTTGGGGAGTCTGAAACAACAATACGGCCTTGCAA AGTCTGCCAACGAGGT
A3*	AGCGGGGTCGCGGCCGAGGTACACTTCCACAAAGTAGTCCCCAAACAAAGACCACAGATTA TTTAATTAAACAGAGATCTCCAAACAGAGATCTACAAGTTTATTGACTCAACCAGGCATTTG CACAAAAGCATCAAACGATTACTATCTCCTCGATGAAAGGTTCTTTGTCTGTCGCACCAGCCT TTGCCTCTGTAATTTTTGTTGGAACAGAGTCTGCAAGTGGGCAAGGTCCTTGGCAAAGACAC GGTCCATAGTCCTGAACAGCACCATGGCAAACCCAGACTTTTGCAGTATGCTTGACAAAGTT GCGGGTCACATCCTAGAGCATTCACTACAGGAGGACCACTCATAATACGATTACGTGGGCAA ATTTCTTGGCAAAGACACGGGAAACTCGGACCTTCGCAGCATGTTGGCAAACCCTGACTAGA GCAGTGTTGTTGACAAAGTTGCGGGTCACATCCAGTGGATAGGGAAACCAACAGCAGGAAG GACACCACTGCAAATGACTTCAAAGCGATCTCCATAATTGAAAGAGAGATTAAAGGCAGCCT TAGTGAAATGTGAATCCCCGCGT
A4*	AATCCTCGCTCAATGACACACTGCAATCGATCGTTCGAATGCGGCTAGCG
A5	ACGCGGGGACTGATTAGTTGCTGGAAACGGTGGAAACTCAAAGACAGACCTTCATTTCTTTG ATACTTCTATTATTTTTGAAAAAATGTTTGCCTTCACTACCTTAATGATTTGGCTCATTGCTGT GGTTTTATAAGTTGTGTATTTTTATTTCAACAGTCTCCACACGCTTTCGAACATAAGGATATC TTCATATTAGCGGGCCAAAGCAACATGGCTGGCCGAGGAGGTGTGAAGAATCGTAATTGGG ATCATATTATCCCTCCTGAATGTAGTTCAAAGCCAACGATCCTTCGGCTTACCGCAACGCTAA TGTGGGAGGAGGCACACGAACCTCTACATGAGGACATGGATCCAAATAAAACTTGTGGGGT GGGACCTGGATTAGCGTTTGCCAATTCTGCTGGGACGAAATCCTAGCATAGGGGTAATTGGT TTAGTCCCTTGTGCCTTCGGTGGATCAAGTATAGAGCAGTGGAAACGAGGCTCGAAACTATA TAATCGACTCATAAAGAGGGCTGAGGCAGCGCTACAAGATGGAGGGCAGATTCGTGCACTT CTATGGACCTG
A6*	ATTTCCAAAAAAACCCCTTCCAATTTTAAAAAAAAAAACAACCCAAAACCGGAAAAAAAAA AGGGGTACTTTTTCCCAAGGTATCCCCCAACCAAAAACTAAATTTTTTTTTAATTAAACCCATT TTAAGGGATCCCCCAATAAAATTTACATTTTTTTTTCCCCACCCCCCTTCCCAAAAAAAAAAA AAATTTTTTTGCCGGGTGTTGCACCAAACCCCCAAGCGGTTACTTTTTCTTTAAGAAAAGGCC CTTCTTTTGGGCAAATTTTTCCCCAAAAACCCCGGCCTTTAAAGGGGGAACACCCCCCCCCGG GAAAATTTTAAACCTGGCAAAATTGTAAAAAAATTTCCCCCTCCAAAGGTCCACCCCAACCA TCCCCCAAAAAAGGACAACCCTAAATACGGGAACAACCTGCTGGGCAACAACCCGAACCAA AGGGCCCGGGGCAACAGGGGGGTTTACCAAAAGGGGCCCAATTTTCCAACAAAAATCCCAT TAAATTCTTTTAACTCCACCCGGAAGACCACTCTACCAAAGACAGGAAACCACGTAGGAGGA CCTATGCAAGACCCCAAAACTCTATTGAAAAAGAAGAAGATATAGGAGCTTATGACTGTGAT CCCGTACTCGGCGCACACCTATCATATGAATCGGCCGCTGCAGTCACCATAGGAAGCTCCAC GTGAGCACTGGATTCTAGGTCCTTAATCCTGGCTACCTGGCCACGTTCCGTGATGAACGTCAG TCCACATTACGCGAGACTATGTACACTGGTCCTTAGGCAACTCATTACGTTGCAATGCTCATC GACCTGGCACTGTAGTGCACTAGAAGCTGCAATGGCCCTTCGTCGCGCCAGTTCGCTTAT
A7	ACAAGGTTTCCATTATATGACGCAGCGTTTTGTTCACTTGGACCATTCTGATTCTCGTCACCC TTTGAAGGCCACCCAGTTTCTGTCACCACCATTTTCACATCATTGAATCCTACGGCATTCATT GCTGCAAAAACTGAATCAAGTTGAGCTTCCAACAAGCTTTTATAAACAAGCCCATTATTCGG

Clone	cDNA sequence (5′-3′)
	GTCAGTTGCGCCTGTGTTATCTTTGAACAAAGCGTAATCTAAAGAGATGGTATCTGAGTTAGT AGTATATGCGAAAAATGGGTATGCATTCACCATGAGATAAGATCCAGCTTGTTTTAGGAAAC TCAATAGTGGTTTTATAACCGGTTCAACAAGGTCAGGTTTGAACGAACCGGCAGAGGGAGGA TAGGAGGTTTCGAGTGCACCTAGGGCTATTGGAGATGACACTTTGATGGACGATGCAACATT GCACCTGCCCGGT
A8	ACGCGGGGACTGATTAGTTGCTGGAAACGGTGGAAACTCAAAGACAGACCTTCATTTCTTTG ATACTTCTATTATTTTTGAAAAAAATGTTTGCCTTCACTACCTTCATGATTTTGGCTCATTGCT GTGGTTTTATAAGTTGTGTATTTTTATTTCAACAGTCTCCACACGCTTTCGAACATAAGGATA TCTTCATATTAGCGGGCCAAAGCAACATGGCTGGCCGGGGAGGTGTGAAGAATCGTAATTGG GATCATATTATCCCTCCTGAATGTAGTTCAAAGCCAACGATCCTTCGGCTTACCGCAACGCTA ATGTGGGAGGAGGCACACGAACCTCTACATGAGGACATGGATCC
A9*	ACGCGGGGACTCACATTTCACTAAAGCTTCCTATTATCTCTCTCTCTTTCTCAATTATGGAGTT CTCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAGT GGTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCACA TTTTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTCC ACGTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTT GTATACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGG TGTTCATGCGG
A10	ACAAGGTTTCCATTATATGACGCAGCGTTTTGTTCACTTGGACCATTCTGATTCTCGTCACCC TTTGAAGGCCACCCAGTTTCTGTCACCACCATTTTCACATCATTGATCCTACGGCATTCATTG CTGCAAAAACTGAATCAAGTTGAGCTTCCAACAAGCTTTTATAAACAAGCCCATTATTCGGG TCAGTTGCGCCTGTGTTATCTTTGAACAAAGCGTAATCTAAAGAGATGGTATCTGAGTTAGC AGTATATGCGAAAAATGGGTATGCATTCACCATGAGATAAGATCCAGCTTGTTTTAGGAAAC TCAATAGTGGTTTTATAACCGGTTCAACAAGGTCAGGTTTGAACGAACCGGCAGAGGGAGGA TAGGAGGTTTCGAGTGCACCTAGGGCTATTGGAGATGACACTTTGATGGACGATGCAACATT GT
A11	ACTAGCTGATGCTGCCAAAGAAGGCAGGCTTAACGATCCAAAGGTCTCTGGGGCGGCGGCG GATGTCCTTGGCGCCGCCGAGCAGTATGGTAAGCTAGATGAAAACAAGGGAATTGGACAAT ATGTTGACAAGGCCGAGGATTATCTCCGACAGCGCAGCACTACTCATTCCTCCACCGCCACC GTTAATCCTGACAAGAAATCAACTCCCACTGCCACTGAACCGCCCAAAAGCACTGAATCCGA GTCGGGTGATGGTGCTGGGGGGTTCATGAAGACGGCTGGAGATTTTTTGAAGAAGAACTGAT TTCGTTATGGGCCATAAAATCCTAGTGCTTTGTTCGTGTACCTGCCCGGGCG
A12	ACAATGTTGCATCGTCCATCAAAGTGTCATCTCCAATAGCCCTAGGTGCACTCGAAACCTCCT ATCCTCCCTCTGCCGGTTCGTTCAAACCTGACCTTGTTGAACCGGTTATAAAACCACTATTGA GTTTCCTAAAACAAGCTGGATCTTATCTCATGGTGATGCATACCCATTTTTCGTATATACTGC TAACTCAGATACCATCTCTTTAGATTACGCTTTGTTCAAAGATAACACAGGCGCAACTGACCC GAATAATGGGCTTGTTTATAAAAGCTTGTTGGAAGCTCAACTTGATTCAGTTTTTGCAGTAAT GAATGCCGTAGGATTCAATGATGTGAAAATGGTGGTGACAGAAACTGGGTGGCCTTCAAAG GGTGACGAGAATCAGAATGGTCCAAGTGAACAAAACGCTGCGTCATATAATGGAAACCTTG TA
B1	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCT
B2	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCC CACATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGATAATA TGATCCCAATTACGATTCTTCACACCTCCCCGGCCAGCCATGTTGCTTTGGCCCGCTAATATG AAGATATCCTTATGTTCGAAAGCGTGTGGAGACTGTTGAAATAAAAATACACAACTTATAAA ACCACAGCAATGAGCCAAAATCATGAAGGTAGTGAAGGCAAACATTTTTTCAAAAATAATA GAAGTATCAAAGAAATGAAGGTCTGTCTTTGAGTTTCCACCGTTTCCAGCAACTAATCAGTC CCCGCGT
B3	ACGGGGGACTGATTAGTTGCTGGAAACGGTGGAAACTCAAAGACAGACCTTCATTTCTTTGA TACTTCTATATTTTTGAAAAAATGTTTGCCTTCACTACCTTAATGATTTTGGCTCATTGCTGTG GTTTTATAAGTTGTGTATTTTTATTTCAACAGTCTCCACACGCTTTCGAACATAAGGATATCTT

Clone	cDNA sequence (5′-3′)
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B4*	AGCGTGGTTCGCGGCCGAGGTACCGGGGAAGGCACTCTTGAACCCTATATAGAGTGATACAA GGAGGGATTCATTCATTCACAGCATTTTTCCCTAAATCTTTCTCTCTCTGTGATAATATATATC TCTCATGAAAATGGAGTTCTCCATGAGGTTTCTTGCAGTGATGTCCCTCTTGCTGTTGGTTTCC ATGTCCACTGACATGGGAGGTCCTCTAATGGCGGATGCCAGCTGGGACTTGGAACTTTGTGA TGAAGGGTGCAAGCTTCTGGGTTACAAACGAGGATGTTGTGGAATTACACAAGACTGCATGT GGTGTTTTTGCTCCGATGAAGACTCGTGCCCAATCACCGAAATGGAAATAAAAATTGGCGAG AAGACTATGATGATTGCGGCAACACCCAAAAAGTCTTTCATCAAGGAGTCAGTCGCTTGATT CTACATGAATATGTATTCAAAATGAATAATCCGTCCAGAAATTGCGTGTCATTGAAAATAAA TTGTAGATCTCATCACGGAGACTATGCTGTGAAAGCCAAGCATGCATGTTTGACATGTACCT GCCCGG
B6*	ACAAAGCGAAAGCCCCAGAAGCGAGGGCTCCCATCTCTCCGAGGTGTTCCTTATGCTTATGT TCCTTCTTTTCTTTCTCATAATCATCAGTTGATTTTTCATAATCATCAGACGATTTTTCATAAT CACCACCGCCAACGGTTGTGTTGTATCTGCCACCAGCAGTCTCGGTATTATATCGGGCACCAC CAGTGGTTGTATTGTAGTCGTCACCTGCAGAATCTGTGTTGT
B7	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCC CACATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGGATAAT ATGATCCCAATTACGATTCTTCACACCTCCCCGGCCAGCCATGTTGCTTTGGCCCGCTAATAT GAAGATATCCTTATGTTCGAAAGCGTGTGGAGACTGTTG
B8*	CTTAACAAACAATATTATTAAAATTTATGCGTTATTCCAATAACTCATATATAACTTACCATT ACAACGGAGCAAAGCGAAAGATTTCATTAATTATATATATGAACTCCTTTGGCATTACTAAT TATCAATAAGCTCTCGCACACTTCGAGGCATAGGTGCAGCCATGTCGGTGAAAACAAGCGAC CTGGCGATGATTACAATTGTAGTAATTTGAACATTTGTTGCATGGAATGAAATTACTGTAGTG CAAAATATGTGGTTTCTTGCGGGTGGATTCAGGCCCCAACTTCATCTCCTCATCAACGTCACG AAATGCCCACTATTTTTAGACTCGAGGTCAACAAAATTTGTATTGTCATGTCCTGATGATGGT TGTATCAACATAACCAAGCATAGG
B10	ACAAGGTTTCCATTATATGACGCAGCGTTTTGTTCACTTGGACCATTCTGATTCTCGTCACCC TTTGAAGGCCACCCAGTTTCTGTCACCACCATTTTCACATCATTGAATCCTACGGCATTCATT GCTGCAAAAACTGAATCAAGTTGAGCTTCCAACAAGCTTTTATAAACAAGCCCATTATTCGG GTCAGTTGCGCCTGTGTTATCTTTGAACAAAGCGTAATCTAAAGAGATGGTATCTGAGTTAG CAGTATATGCGAAAAATGGGTATGCATTCACCATGAGATAAGATCCAGCTTGTTTTAGGAAA CTCAATAGTGGTTTTATAACCGGTTCAACAAGGTCAGGTTTGAACGAACCGGCAGAGGGAGG ATAGGAGGTTTCGAGTGCACCTAGGGCTATTGGAGATGACACTTTGATGGACGATGCAACAT TGTACCTGCCCGGGCGGCCGCTCGACA
B11	ACAATGTTGCATCGTCCATCAAAGTGTCATCTCCAATAGCCCTAGGTGCACTCGAAACCTCCT ATCCTCCCTCTGCCGGTTCGTTCAAACCTGACCTTGTTGAACCGGTTATAAAACCACTATTGA GTTTCCTAAAACAAGCTGGATCTTATCTCATGGTGAATGCATACCCATTTTTCGCATATACTG CTAACCCAGATACCATCTCTTTAGATTACGCTTTGTTCAAAGATAACACAGGCGCAACTGAC CCGAATAATGGGCTTGTTTATAAAAGCTTGTTGGAAGCTCAACTTGATTCAGTTTTTGCAGCA ATGAATGCCGTAGGATTCAATGATGTGAAAATGGTGGTGACAGAAACTGGGTGGCCTTCAAA GGGTGACGAGAATCAGAATGGTCCAAGTG
B12*	AGCGTGGTCGCCGGCCGAGGTACGCGGGGATCAAAGTTCCTTCTTTTTTATCTTTGTTGTGTA TGTCACTATTGACTCTCACAAAATGATTTCACGTCTGGTGTTACTTTTTCTTTTTCTCCTATGC TTGGTTATGTTGATACAACCATCATCAGGACATGACAATACAAATTTTGTTGACCTCGAGTCT AAAAATAGTGGGCATTTCGTTGACGTTGATGAGGAGATGAAGTTGGGGCCTGAATCCACCCG CAAGAAACCACATATCTTGCACTACAGTAATCTCATTCCATGCAACAAATGTTCAAATTACT ACAATTGTAATCATCGCCAGGTCGCTTGTCTTCACCGACATGGCTGCACCTATGCCTCGAAGT GTGCGAGAGCTTATTGATAATTAGTAATGCCAAAGGAGTTCATATATATAAATAATGAAA
C1	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA

Clone	cDNA sequence (5′-3′)
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C3	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACCGCTCTATACTTGATCCACCGAAGGCACAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCC CACATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGGATAAT ATGATCCCAATTACGATTCTTCACACCTCCTCGGCCAGCCATGTTGCTTTGGCCCGCTAATAT GAAGATATCCTTATGTTCGAAAGCGTGTGGAGACTGTTGAAATAAAAATACACAACTTATAA AACCACAGCAATGAGCCAAAATCATTAAGGTAGTGAAGGCAGACATTTTTTCAAAAATAATA GAAGTATCAAAGAAATGAAGGTCTGTCTTTGAGTTTCCACCGTTTCCAGCAACTAATCAGTC CCCGGTACCTGCCCGGG
C4	TCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTTGATTATATAGTTTCGAGCCTCGTTTCCACT GCTCTATACTTGATCCACCGAAGGCGCAAGGGACTAAACCAATTACCCCTATGCTAGGATTT CGTCCCAGCAGTGAATTGGCAAACGCTAATCCAGGTCCCACCCCACAAGTTTTATTTGGATC CATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCCCACATTAGCGTTGCGGTAAGCCGAAGGAT CGTTGGCTTTGAACTACATTCAGGAGGGATAATATGATCCCCGATTACGATTCTTCACACCTC CCCGGCCAGCCATGTTGCTTTGGCCCGCTAATATGAAGATATCCTTATGTTCGAAGCGTGTGG AGACTGTTG
C8	ACAGCTCCTCAAAGCACAAGGTATCACCAAGACCAAGATTTTTGACACTGACGCCACCGTGC TATCAGCTTTCTCTGGCTCAAATATTACTATCACTGTTGGGCTTCAAAACCAGCAGTTATCCG ACGCTGCTGGCAGTCAGTCATTCACCGACACCTGGGTCCAGTCAAATATCCTCCCTTATTATC CCAACACTCTCATCGATGCCATTGCTGTCGGTAATGAAATAATATTTGCGGGTCTTCAGGATT CTACCCAGCTCCTCGTACCTGCCCGG
C9*	ACGCGGGGACCCACAGTTCACTAAAGCTTCCTATTATCTCTCTCTCTTTCTCAATTATGGAGT TCTCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAG TGGTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCAC ATTTTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTC CACGTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTT GTATACAATACTGCAAGCTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGGT GTTCATGCGG
C10*	CGCGGGGACTCACAGTTCACTAAAGCTTCCTATTATCTCCCTCTCTTTCTCAATTATGGAGTT CTCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAGT GGTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCACA TTTTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTCC ACGTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTT GTATACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGG TGTTCATGCGG
C11*	CTTAACAAACAATATTATTAAAATTTATGCGTTATTCCAATAACTCATATATAACTTACCATT ACAACGGAGCAAAGCGAAAAGATTTCATTATAATTATATATATGAACCCCTTTGGCATTACT AATTATCAATAAGCTCTCGCACACTTCGAGGCATAGGTGCAGCCATGTCGGTGAGACAAGCG ACCTGGCGATGATTACAATTGTAGTAATTTGAACATTTGTTGCATGGAATGAGATTACTGTAG TGCAAAATATGTGGTTTCTTGCGGGTGGATTCAGGCCCCAACTTCATCTCCTCATCAACGTCA ACGAAATGCCCACTATTTTTAAACTCGAGGTCAACAAAATTTGTATTGTCATGTCCTGATGAT GGTTGTATCAACATAACCAAGCATAGGAGAAAAAGAAAAAGTAACACCAAACGTGAAATCA TTTTGTGAGAGTCAATAGTGACATACACAACAAAGATAAAAAAGAAGGAACTTTGGTA
C12*	CGGCCGGCGTTTGGATTGTAGTCGTCACCGAAGTATCTGTATTGTAGCGACCACCACCAGCA GTGGTATTGTAGTCGCCACCACCAGTTGAATAACCACCACCAGTAGTGGTATTATACTCGCCT CCAACACCAGTTGTCTTGTAGCTGTCACCAGAGCCATAGGTATTCTCAGAGTAGGCGGGCTT CTCCTCATCCTCATCGTCCTTGTGACGGTGGAAGAGGCCGTGGTGCTTCTTCTCTTCAGCCAT TTTACTATTAATGAAAATTACAAAGTGAATAATGATTCAGAGATGTGTCCCCGCGT
D1*	CCGGGGACTCACATTTCACTAAAGCTGCCTTTTATCTCTCTCTCTTTCTCAATTATGGAGTTCT

Clone	cDNA sequence (5′-3′)
	CTGTGAGGTCATTTGCAATGGTATCCCTCCTGCTGTTGGTTTCCATGTCCACTGATATGAGTG GTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCACATT TTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTCCAC GTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTTGTA TACAATACTGCAAGCTCAGAATTATCCAGGAGGATGCTACTCAGCCTTTAACGGCCGGTGTT CATGCGG
D2	ACTGGTTGCATCATCATATGGATAGCTGTATGATGTTGGGCAAGCTTTTTTGAACACTTGTGA ATAATTTGAAGGCTTGCATACCTGTGGATTATTGTATTCTCCACTGCAACAATATTGTGGTTC ATTGAAGGCAAGGCAGGCACTTTTACAAGCCACCGTGGCGCCATTCCCTCCGGCCACCACCA AATCTTGAGGGCAGTTCCTTGTTCAAATCCGATACGCATTTTACTTCACTACAATTTCCTGAT CCCCCCGAAGGGACGACGGAAATTGGCATGTTATATCCATCGACAAGGCTGACGTCGT
D3	ACTCACTCTTCATGAAATTGGCAATAGAGCCTTGAGAGCACAATTAACATTTGGGCGGGGAA ACCCCACAAGTGGAAGCAATTTTCTTTGCATTTGGAGAGTTAACGTATTTATTCAAATTGGGA TTCTTGAGATACTGGCAAGCAAGGCTGCTGCTCCTTGATCTTACTGCAGCAAAGTGGCGACG GTGGAGACCCCGACGAGATAGCCGACATGCAAGGGCTGAGTTCAGTTGGGTTGCAAGTTAC ACTCTCTGAAACATGTGCTCCACAGGCCAGAAGCAGTATCAGAACTGCACACACTGC
D4*	ACGCGGGGATTCACAGCATTTTTCCCTAAATCTTTCTCTCTTTCTGTGATAATATATATCTCTC ATGAAAATGGAGTTCTCCATGAGGTTTCTTGCAGTGATGTCCCTCTTGCTGTTGGTTTCCATG TCCACTGACATGGGAGGTCCTCTAATGGCGGATGCCAGCTGGGACTTGGAACTTTGTGATGA AGGGTGCAAGCTTCTGGGTTACAAACGAGGATGTTGTGGAATTACACAAGACTGCATGTGGT GTTTTTGCTCCGATGAAGACTCGTGCCCAATCACCGAAATGGAAATAAAAATTGGCGAGAAG ACTATGATGATTGCGGCAACACCCAAAAAGTCTTTCATCAAGGAGTCAGTCGCTTGATTCTA CATGAATATGTATTCAAAATGAATAATCCGTCCAGAAATTGCGTGTCATTGAAAATAAATTG TAGATCTCATCACGGAGACTATGCTGTGAAAGCCAAGCATGCATGTTTGACAAGTACCTGC
D5	CAATGTTGCATCGTCCATCAAAGTGTCATCTCCAATAGCCCTAGGTGCACTCGAAACCTCCTA TCCTCCCTCTGCCGGTTCGTTCAAACCTGACCTTGTTGAACCGGTTATAAAACCACTATTGAG TTTCCTAAAACAAGCTGGATCTTATCTCATGGTGAATGCATACCCATTTTTCGCATATACTGC TAACTCAGATACCATCTCTTTAGATTACGCTTTGTTCAAAGATAACACAGGCGCAACTGACCC GAATAATGGGCTTGTTTATAAAAGCTTGTTGGAAGCTCAACTTGATTCAGTTTTTGCAGCAAT GAATGCCGTAGGATTCAATGATGGAAAATGGTGGTGACAGAAACTGGGTGGCCTTCAAAGG GTGACGAGAATCAGAATGGTCCAAGTGAACAAAACGCTGCGTCATATAATGGAAACCTTGT
D6	ACAATGTTGCATCGTCCATCAAAGTGTCATCTCCAATAGCCCTAGGTGCACTCGAAACCTCCT ATCCTCCCTCTGCCGGTTCGTTCAAACCTGACCTTGTTGAACCGGTTATAAAACCACTATTGA GTTTCCTAAAACAAGCTGGATCTTATCTCATGGTGATGCATACCCATTTTTCGTATATACTGC TAACTCAGATACCATCTCTTTAGATTACGCTTTGTTCAAAGATAACACAGGCGCAACTGACCC GAATAATGGGCTTGTTTATAAAAGCTTGTTGGAAGCTCAACTTGATTCAGTTTTTGCAGTAAT GAATGCCGTAGGATTCAATGATGTGAAAATGGTGGTGACAGAAACTGGGTGGCCTTCAAAG GGTGACGAGAATCAGAATGGTCCAAGTGAACAAAACGCTGCGTCATATAATGGAAACCTTG TACCTGCCCGGGCA
D7*	CCTTCGGCGGCCTGGCGGCGATGCTGGGACTTATAGCTTTTGCTCTCTTGATCTTGGCCTGTT CATACTGGAAACTCTCTGACCAAACGAAGGCGCGGGAGAGGGTGAAAGAGACGTCGAGGCC GGTGATGATGATCATAAAGGCGAAAACGCCAAGACGGAGATGGCCGCGCTTCCTTTTGAAG AGAAGATTGTTGTGATTATGGCTGGTGATGTTAAGCCAACTTTCCTGGCCACTCCCATGTCGA GTAGGGATTCTTTATTTGGAGTTGGAAATGAAAAAAAAAAAAAAAAGTA
D8*	ACACGAACACTACCACCACTTGCTGTTTCGCATTCAATACCTTCTTAAATTCCAGAACTAATT TATCCTCTTTTTTGTTGTATTCATCAACAGCAAAACGAGCGACACATTCAGGGTTGTTGCCAA TTCTTTCAAACGGGGTAATCCCTCGAGAGGTGGCCTCGAGGCTTTCCTCCAATCCTTCTAAGG ACGTGGCGGCCATTCTCACAGATAACTGAATTTTTTGCTAAGAAGACTATCTCTTTCTTTTTTT TTGGTGTGTGAATAGTTGAAGGGAGTTGTAATTCCTGCACAGGTGGGCGTGGCGGCCATTCT CACAGATAACTGAA
D9*	CCGGGGATTCACATTTCACTAAAGCTGCCTTTAATCTCTCTTTCAATTATGGAGATCGCTTTG AAGTCATTTGCAGTGGTGTCCTTCCTGCTGTTGGTTTCCCTATCCACTGGATGTGACCCGCAA CTTTGTCAACAACACTGCTCTAGTCAGGGTTTGCCAACATGCTGCGAAGGTCCGAGTTCCCGT GTCTTTGCCAAGAAATTTGCCCACGTAATCGTATTATGAGTGGTCCTCCTGTAGTGAATGCTC TAGGATGTGACCCGCAACTTTGTCAAGCATACTGCAAAAGTCTGGGTTTGCCATGGTGCTGTT CAGGACTATATGGACCGTGTCTTTGCCAAGGACCTTGCCCACTTGCAGACTCTGTTCC

Clone	cDNA sequence (5′-3′)
D10	ACCATTGGTGCTGTGGTTAATTATAGTGGTGCATCTATTGAAATTTATCTATGCTAACATTGA AGGTTTGTGTTAATGTATGTATATTTATATTTTTTTTATTTCGGTTTATATTTTTAAATATACTT GTGGGCTTTTTTGTGTTCTTTTGGCTAGATTTCTTTTGAATTTCTTCTTCAATTTGTAGTTATAA TTGAAAAGTTCCAACATTTTCTTTGGCTTAATTTATTGTGCCATTCATGGATGGATTTATAGA AATGTAACTTTTGAATTTACTGATGTTATGCC
D11	CAATGTTGCATCGTCCATCAAAGTGTCATCTCCAATAGCCCTAGGTGCACTCGAAACCTCCTA TCCTCCCTCTGCCGGTTCGTTCAAACCTGACCTTGTTGAACCGGTTATAAAACCACTATTGAG TTTCCTAAAACAAGCTGGATCTTATCTCATGGTGAATGCATACCCATTTTTCGCATATACTGC TAACTCAGATACCATCTCTTTAGATTACGCTTTGTTCAAAGATAACACAGGCGCAACTGACCC GAATAATGGGCTTGTTTATAAAAGCTTGTTGGAAGCTCAACTTGATTCAGTTTTTGCAGCAAT GAATGCCGTAGGATTCAATGATGTGAAAATGGTGGTGACAGAAACTGGGTGGCCTTCAAAG GGTGACGAGAATCAGAATGGTCCAAGTGAACAAAACGCTGCGTCATATAATGGAAACCTTG T
D12	ACAATGTTGCATCGTCCATCAAAGTGTCATCTCCAATAGCCCTAGGTGCACTCGAAACCTCCT ATCCTCCCTCTGCCGGTTCGTTCAAACCTGACCTTGTTGAACCGGTTATAAAACCACTATTGA GTTTCCTAAAACAAGCTGGATCTTATCTCATGGTGAATGCATACCCATTTTTCGCATATACTG CTAACTCAGATACCATCTCTTTAGATTACGCTTTGTTCAAAGATAACACAGGCGCAACTGACC CGAATAATGGGCTTGTTTATAAAAGCTTGTTGGAAGCTCAACTTGATTCAGTTTTTGCAGCAA TGAATGCCGTAGGATTCAATGATGTGAAAATGGTGGTGACAGAAACTGGGTGGCCTTCAAAG GGTGACGAGAATCAGAATGGTCCAAGTG
E1	ACATTGTAGCGGCCGTGAGGACTCCGCAACCAACCCCAAACAGCAGCGCCCCAACCACACTC ATGATGTCCTTGGTGCGATCCCTAATCGAGCTCGAGACTGAGGAATACAATTTAACGGGCCC CTTGTATGGAATCATATCGTGTTCAAGATCGCCAGGGCGATCGTTGTAATTGACGGGACGAT GATGGTGGGGGAATCGAGAGGTGGTAGTGGTGGTGGTGAAGATGAGAGTGAGATAACGAGG CGAAGACAAGGAAGAGGGTTTTTGGAAGAGGAAATTGCCCTCGTTAGGGTTAGGGTTAGGG TTCTGGGAACGGGAGTAAGGGTGATAGCTGGTGGTTGTTGCGAAATAGAAGAGAGTTTTGCA GGGCCGGGCGGAGGCGGTGACCGCAAGGACAGAGAAAAGGAGGACAGGCAGAATCA
E3	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATTCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCC CACATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGGATAAT ATGATCCCAATTACGATTCTTCACACCTCCCCGGCCAGCCATGTTGCTTTGGCCCGCTAATAT GAAGATATCCTTATGTTCGAAAGCGTGTGGAGACTGTTGAAATAAAAATACACAACTTATAA AACCACAGCAATGAGCCAAAATCATGAAGGTAGTGAAGGCAAACATTTTTTCAAAAATAAT AGAAGTATCAAAGAAATGAAGGTCTGTCTTTGAGTTTCCACCGTTTCCAGCAACTAATCAGT CCCCGCGTACCTGCCCG
E4	ACGCGGGGACTCAACTAGGTCACATTCACTTCTCCATTCTTATAACCTCCACACCAATACAAA AATGGGAAGAACTACCCGTTTCCCTTCACTTGCCTTCTTTTTCCCCCTCCTCGAATGCTCAGCC ACACCAAACCTGAAAATCCGGGCTCGACCACAGCCATGTAAACAGCTAGTGTTTTACTTCCA TGACATTCTTTACAGCGGCCACAACTCCAAGAATGCAACTGCAGCCATTGTAGGGTCACCAG CTTGGGGAAATAGAACCATTTTAAGTGGCCAAAACCATTTTGGCAATATGGTGGTGTTTGAT GATCCCATTACACTAGATAATAATCTCCATTCAACCCCAGTTGGCCGAGCACAAGGATTCAT CTTTATG
E5	ACATTGTAGCGGCCGTGAGGACTCCGCAACCAACCCCAAACAGCAGCGCCCCAACCACACTC ATGATGTCCTTGGTGCGATCCCTAATCGAGCTCGAGACTGAGGAATACAATTTAACGGGCCC CTTGTATGGAATCATATCGTGTTCAAGATCGCCAGGGCGATCGTTGTAATTGACGGATCGAT GATGGTGGGGGAATCGAGAGGTGGTAGGTGGTGGTGAAGATGAGAGTGAGATAACGAGGTG AAGACAAGGAAGAGGGTTTTTGGAAGAGGAAATTGCCCTCGTTAGGGTTAGGGTTAGGATTC TGGGAACGGGAGTAAGGGTGATAGCTGGTGGTTGTTGCGAAATAGAGAGAGTTTTGCAGGG CCGGGCGGAGGCGGTGACCGCAAGGAGAGAGAACAGGAGGAGAGGCAGAATCGAGGTCAC CA
E6*	ACCCTCCAACGCTCGCTGCGTGATTCTTTTGACACTCTGCGTATCTCACAGTCCTAACTGATA ACGATGAATTCACTGAATTGCTCGAAGAGTCATTCCTCTTCAGCACAACTTGACGTTTTTTCA TCTCTTTGTTTCACTCTCTTTCGATCGGGGATACTTTTGAAGAATTTACAGTGCAGAATTTCAA ATTTACAGATAAGGAGATGAAGAGAAAAGAGGCAGAAGGTAGAGGGAAGGCTATGTCATTT TTGGTGCCCCCCGCGTACCTGCCCGG

Clone	cDNA sequence (5′-3′)
E7*	CGCGGGGACTCACAGTTCACTAAAGCTTCCTATTATCTCTCTCTCTTTCTCAATTATGGAGTTC TCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAGTG GTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCACATT TTGGTAAGCCAACATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTCCAC GTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTGTAT ACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGGTGTT CATGCGG
E8	GCTACTAACGGGGTCTCGGTTGATTTCCCTTCCTTTAGCTACTCAAATGTTTCAGTTCGCTAA GTTTGAAAAGTCCAAAGAGCGTAAACTAGCCACGGAGCTTGGATACGGTTTCCCGATCGGAG ATCCATGGATCACAGACGGTATCTCCCCATGGCCTTTCGCCTCTAAAAGCGTCCTTCCTTCCC AAGGCCCGGGCATCCATCCAATGCATTCCCCGCGT
E9*	ACGCGGGGACTCACAGTTCACTAAAGCTTCCTATTATCTCTCTCTCTTTCTCAATTATGGAGT TCTCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAG TGGTCCCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTGTAGAAAATATTGCGCACAT TTTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTCCA CGTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTTGT ATACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGGTG TTCATGCGG
E10	ACGCGGGGGATTCCCCTAGTAACGGCGAGCGAACCGGGAAGAGCCCAACTTGAGAATCGGA CGGCCCCGCCGTCCGAATTGTAGTCTGGAGAAGCGTCCTCAGCGGCGGACCGAGCTCAAGTC CCCTGGAAAGGGGCGCCGGAGAGGGTGAGAGCCCCGTCGCGCTCGGACCCTGCCGCACCAC GAGGCGCTGTCGGCGAGTCGGGTTGTTTGGGAATGCAGCCCCAATCGGGCGGTAAATTCCGT CCAAGGCTAAATACGGGCGAGAGACCGATAGCGAACAAGTA
E11*	CAAGAACCACGGACAAATCCAATTGTCTTGTTGAGCCTGAACTGTCTCCACCCGATAATTTTA ACGCGTTTAATAACAAGGAAATTGACTCGAAATTGATACTAGAGGATACCATTGCTGCTCCA TATTCTGCAGAGACGCAAAAAGATGATGAGAAAGAGAATGGAAAAAGCTTAATGTATGACC TTCCGCGGAAAGAATCTGGGCAGTTTAAAGCCGAATACAGATCAACTCCATCAGATAATGAC ACTGCTTCGCTGTGTGGCTCTTCCAATTACTCATCTCCTCCATCAGCAACAGATGTCCCACTT GAGGTTACAGAATCAGTCGTTTCTAGTGCAGCATCTCATTGTGGAAATAACTCTTTCAAGTCC AAGGACGATATCAATCAGACAGAGGAGGCCTTCTCTGGTA
E12*	CGGATAATTAACAAGCCATATTACTAAGGCTTGTTCTTTATTCCAACAATCATATATATAACT TACCATTACAATAAATCGAAGCTAAAATAATTCCTTATAACTGCATATATGAAATCTTTTAGC ATTACTAATTATCATAAGCTCTCGCACATCTGTTGGCCTTGGTGCAGCCACGACGGTATGCAT TAGCAATCTGGCGTTTATGACAAAGTTGTAAAAACCGTCGCGTTTGTTGCATCGGAGGTTGTC CTCCCTTAATGCGGCATAACTAATATAGCGCGATTTATACAGTTGGCGACGAGGATTCAAAG CCTAACTTCATCTCATCATCAACATCAGTGATTTGCCCGATATTTTTAAAATTAGGATCAACG GAATTTGTAATGTCATGTCCTGATGATGATTGTATCACCATGACCAAACCTAGGAAAAAAAG AAAAAGTAACACCATACGTGAAGTCATTTTGTGAGAAGCAGTAGTAGCATGCACAACAAAG ATAAACAAGGGAACCTCTTTCTTATATTTTATTTCCCCGCGTACCTG
F1*	ACGCGGGGACGGCATTTTTCCCTAAATCTTTCTCTCTTTCTGTGATAATATATATCTCTCATGA AAATGGAGTTCTCCATGAGGTTTCTTGCAGTGATGTCCCTCTTGCTGTTGGTTTCCATGTCCA CTGACATGGGAGGTCCTCTAATGGCGGATGCCAGCTGGGACTTGGAACTTTGTGATGAAGGG TGCAAGCTTCTGGGTTACAAACGAGGATGTTGTGGAATTACACAAGACTGCATGTGGTGTTT TTGCTCCGATGAAGACTCGTGCCCAATCACCGAAATGGAAATAAAAATTGGCGAGAAGACT ATGATGATTGCGGCAACACCCAAAAAGTCTTTCATCAAGGAGTCAGTCGCTTGATTCTACAT GAAAATGTATTCAAAATGAATAATCCGTCCAGAAATTGCGTGTCATTGAAAATAAATTGTAG ATCTCATCACGGAGACTATGCTGTGAAAGCCAAGCATGCATGTTTGACTG
F2*	ACCCTCCAACGCTCGCTGCGTGATTCTTTTGACACTCTGCGTATCTCACAGTCCTAACTGATA ACGATGAATTCACTGAATTGCTCGAAGAGTCATTCCTCTTCAGCACAACTTGACGTTTTTTCA TCTCTTTGTTTCACTCTCTTTCGATCGGGGATACTTTTGAAGAATTTACAGTGCAGAATTTCAA ATTTACAGATAAGGAGATGAAGAGAAAAGAGGCAGAAGGTAGAGGGAAGGCTATGTCATTT TTGGTGCCCCCGCGACCTGCCCGGGCG
F3*	CGCGGGGACTCACAGTTCACTAAAGCTTCCTATTATCTCTCTCTCTTTCTCAATTATGGAGTTC TCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAGTG GTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCACATT TTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCATGTCGTTGCACAGCAGTTTGTCCAC GTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTTGTA

Clone	cDNA sequence (5′-3′)
	TACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGGTTC ATGCGGAAAAATTTGCCCAAACGAAGAGCCTTTCTTAAGGAAATAGTAACCGCTTGATGCTT TGCTGCAACAGCAGGCAAATAAATTACTTATCTATCTGTGAAGCTGGTTGAGCTAAATAAAC TGTAGATCTCTACTTGGAGACTACATTAAACTGGCTTTAATTAAATAAACTGTAGTTTTTGCT TGGGGACTACATCGTGAAAATGTAGCCATCTTTATTTACCGGTATCGTGTTGTATACTTTATT AAACTTGGAATGAGTATATTGTGAAAT
F4	CCAGGTCGTTGGGAGAGCTCTGCCGACGGCAACGGACGAGCACCCTAAGATCTACCGAATG AAGCTATGGGCCACAAATGAGGTTCGTGCCAAGTCCAAGTTTTGGTATTTCCTGAGGAAGCT TAAGAAGGTGAAGAAGAGCAATGGCCAGATGCTTGCAATCAACGAGATATTTGAGAAAAAC CCAACTACCATCAAGAACTATGGTATATGGCTCCGTTACCAGAGTAGAACTGGCTATCACAA CATGTACGCGGGGACTCACATTTCACTAAAGCTGCC
F5	ACATAAAGAGACAATCCCAATTATCTCAATTGCAATAAAAATATTAGTACAAATCTTAAACA CCCCAACCAGTTCAGTGAGACTGAACCTATGGATATGTCAACTTATTAAATAGTGCAACTAA TAGCAAGAGAAGCAAAAGTTACACTATTATTCTCATTTAGTACTCACTCTTCATGAAATTGGC AATAGCCTTGAGAGCACAATTAACATTTGGGCGGGGAAACCCCACAAGTGGAAGCAATTTTC TTTGCATTTGGAGAGTTAACGTATTTATTCAAATTGGGATTCTTGAAATACTGGCAAAAGCAA GGCTGCTGCTCCTTGATCTTACTGCAGCAAAGTGGCGACGGGGGAAACCCCGACGAAATAGC CGACATGCAAGGGCTGAGTTCAGTTGGGTTGCAAGTTACACTCTCTGAACATGTGCTCCACA GGCCAAAAGCAGTATCAAAACTGCACACACTGCAAATTTAAATGCCTGCATTTTGCAACTTG TGGGATATGAAGGAGTTGTTATTACTTGTGTGGTTCCCCGCGACCC
F6	ACGCGGGGACTGATTAGTTGCTGGAAACGGTGGAAACTCAAAGACAGACCTTCATTTCTTTG ATACTTCTATTATTTTTGAAAAAATGTTTGCCTTCACTACCTTCATGATTTTGGCTCATTGCTG TGGTTTTATAAGTTGTGTATTTTTATTTCAACAGTCTCCACACGCTTTCGAACATAAGGATAT CTTCATATTAGCGGGCCAAAGCAACGTGGCTGGCCGGGGAGGTGTGAAGAATCGTAATTGG GATCATATTATCCCTCCTGAATGTAGTTCAAAGCCAACGATCCTTCGGCTTACCGCAACGCTA ATGTGGGAGGAGGCACACGAACCTCTACACGAGGACATGGATCCAAATAAAACTTGTGGGG TGGGACCTGGATTAGCGTTTGCCAATTCACTGCTGGGACGAAATCCTAGCATAGGGGTAATT GGTTTAGTCCCTTGCGCCTTCGGTGGATCAAGAATAGAGCAGTGGAAACGAGGCTCGAAACT ATATAATCGACTCATAAAGAGGGCTGAGGCAGCGCTACAAGATGGAGGGCAGATTCGTGCA CTTCTATGGACCTGGGCCCGGGCGGCCCGCT
F8	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGAATTGGCAAACGCTAATCCAGG TCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCCCA CATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGGATAATAT GATCCCAATTACGATTCTTCACACCTCCCCGGCCAGCCATGTTGCTTTGGCCCGCTAATATGA AGATATCCTTATGTTCGAAAGCGTGTGGAGACTGTTG
F9*	CGCGGGGATTCACATTTCACTAAAGCTGCCTTTAATCTCTCTTTCAATTATGGAGATCGCTTT GAAGTCATTTGCAGTGGTGTCCTTCCTGCTGTTGGTTTCCCTATCCACTGGATGTGACCCGCA ACTTTGTCAACAACACACTAGTCAGGGTTTGCCAACATGCTGCGAAGGTCCGAGTTTCCCGT CTTTGCCAAGAAATTTGCCCACGTAATCGTATTATGAGTGGTCCTCCTGTAGTGAATGCTCTA GGATGTGACCCGCAACTTTGTCAAGCATACTGC
F11	GTTTTTCTTCAAACCCCCAATCCGTTCTCGCTCGCCGCTACTAACGGGGTCTCGGTTGATTTCC CTTCCTTTAACTACTCAGATGTTTCAGTTCGCTAAGTTTGAAAAGTCCAAAGAGCGCAAACTA ACCACGGAGCTTGGATACGGTTTCCCGATCGGAGATCCATGGATCACAGACGGTATCTCCCC ATGGCCTTTCGCCTCTGAAAGCGTCCTTCCTTCTCAATGCCCGGGCATCCATCCAATGCATTC CCCGCGTACCTGCCCGGGCGGCCGCCCGGGCAGGTACGGGGGTAATTGATAGTCAAGGCCGT GTAATTAATACTTGGGCTGATATCATTAACCGTGCTAACCTTGGTATGGAAGTTATGCATGAA CGTAATGCTCATAACTTCCCTCTAGACCTAACTTCTATCGAAGTTCCATCTACAAATGGATAA GACCCGATCTTAGTGTATAGGAGTTATTG
F12	ACACAGTTTATGCCACAGCAGTTGACCCAAAGAAGGGCTCATTAGGCACAATTGCCCCCATT GCCATTGGCTTCATTGTTGGGGCCAACATTTTGGCCGCCGGCCCATTCTCCGGTGGCTCAATG AACCCGGCTAGGTCCTTCGGTCCGGCTGTGGCCAGCGGTGACTTTGCCGACAACTGGATTTA CTGGGTCGGTCCACCCATCGGTGGTGGTCTGGCTGGACTCGTCTATGCCTATGTGTTCATGGT CCACGAACACGTTCCTCTTGTTAGCGAGTTCTAAATTGTCTTTCGTTTTGCAATTTTCGAATCA AATTTCGAAATCTTTTTTACTTATTTTGTGGAGGTAATAAAGGTGAGG
G1*	CGCGGGGGACTCACATTTCACTAAAGCTGCCTTTTATCTCTCTCTCTTTCTCAATTATGGAGTT

Clone	cDNA sequence (5′-3′)
	CTCTGTGAGGTCATTTGCAATGGTATCCCTCCTGCTGTTGGTTTCCATGTCCACTGATATGAG TGGTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCAC ATTTTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTC CACGTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTT GTATACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGG TGTTCTGCGGAAAAATTTGCCCAAACGAAGAGCCTTTCATTAAGGAAATAGTAACCGCTTGA TGCTTTACTGCAACAGCAGGCAAATAAATTACTTATCTATCTATGAAGCTGGTTGAGCTAAAT AAACTGTAGATCTCTACTTGGAGACTACATTAAACTGGCTTTAATTAAATAAACTGTAGTTTT TGCTTGGGGACTACATCGTGAAAATGTAGCCATCTTTATTTACCGGTATCGTGTTGTATACTT TAATAAACTTGGAATGAGTATATTGTGAATCAC
G5*	CGCAGGGACTCACAGTTCACTAAAGCTTCCTATTATCTCTCTCTCTTTCTCAATTATGGAGTTC TCTGTGAGGTCATTTGCAATAGTGTCCCTCCTACTGTTGGTTTCCCTGTCGTCTGATATGAGTG GTCCTCTTGTGGTGGATGCTAAACGATGTAACTGGAATCTTTGTAGAAAATATTGCGCACATT TTGGTAAGCCAGCATGCTGCCCAGGACCATTTGGTCCGTGTCGTTGCACAGCAGCTTGTCCAC GTATTACGAGTCGTCCTCTTGTGGAGGATGCTCGAGGTCGACATTGTGACGTGGAACTTTGTA TACAATACTGCAAGCTTCAGAATTATCCAGGAGGATGCTGCTCAGCCTTTAACGGCCGGTGT TCATGCGGAAAAATTTGCCCAAACGAAGAGCCTTTCATTAAGGAAATAGTAACCGCTTGATG CTTTGCTGCAACAGCAGGC
G6*	CTAAATAAAGATGGCTACATTTTCACGATGTAGTCCCCAAGCAAAAACTACAGTTTATTTAA TTAAAGCCAGTTTAATGTAGTCTCCAAGTAAATCTACAGTTTATTTAGCTCAACCAGCTTCAC AGATAGATAAGTAATTTATTTGCCTGCTGTTGCAGCAAAGCATCAAGCGGTTACTATTTCCTT AATGAAAGGCTCTTCGTTTGGGCAAATTTTTCCGCATGAACACCGGCCGTTAAAGGCTGAGC AGCATCCTCCTGGATAATTCTGAAGCTTGCAGTATTGTATACAAAGTTCCACGTCACAATGTC GACCTCGAGCATCCTCCACAAGAGGACGACTCGTAATACGTGGACAAGCTGCTGTGCAACGA CACGGACC
G7*	ACGCGGGGATTCACATTTCACTAAAGCTGCCTTTAATCTCTCAATTATGGAGATCGCTTTGAA GTCATTTGCAGTGGTGTCCTTCCTGCTGTTGGTTTCCCTATCCACTGGATGTGACCCGCAACTT TGTCAACAACACTGCTCTAGTCAGGGTTTGCCAACATGCTGCGAAGGTCCGAGTTTCCCGTGT CTTTGCCAAGAAATTTGCCCACGTAATCGTATTATGAGTGGTCCTCCTGTAGTGAATGCTCTA GGATGTGACCCGCAACTTTGTCAAGCATACTGCAAAAGTCTGGGTTTGCCATGGTGCTGTTC AGGACTATATGGACCGTGTCTTTGCCAAGGACCTTGCCCACTTGCAGACTCTGTTCCAACAA AAATTACAGAGGCAAAGGCTGGTGCGACAGACAAAGAACCTTTCATCCAGGAGATACTAAT CGGTTGATGCTTTTGTGCAAATGCCTGGTTGAGTCAATAAACTTGTAGATCTCTGTTTGGAGA CTCTCTGTTTAATTAAATAATCTGTGGTCTTTGTTTGGGGACTACTTTGTGGAGCTGTACCTGC CCGGGCGAAAATGTAACCATCTATATTTAAGGTATCGTGTTGAATACATC
G8*	AGCAATTCAGTGAATTCATCGTTATCAGTTAGGACTGTGAGATACGCAGAGTGTCAAAAGAA TCACGCAGCGAGCGTTGGAGGGT
G9*	ACCTTTTTCTTCATCGTCGTCAGATAAAGGTCCAACTTCCAAATCCTTTTCAGTATTACCCTTC TTTTTCTTCTTCTTGTTCTTTGTCTTTGGTGATGATAATGATAGTGTCGCTGATTTTAGTTTGTG GGCGTAATGATGGCTGCGAAGAATGTGAGTAAATGCTGGCGGGGCAGACGAGAACATGGTG ATTATGATGACTGCTTTTGAGTTGTGGCGTTGAATGTGAGAGAGAAAGTAAGAGAGAAGGA GAAATTAGAATAGAAAGATAAAAGGCTCTATCTTTCAATGTTTTTCGTGCAGTTTCTTAATTT ATCTTTGATTGTTCAGAGAATTTGCTGTGT
G10	CGCGGGGACACTCAACTAGGTCACATTCACTTCTCCATTCTTATAACCTCCACACCAATACAA AAATGGGAAGAACTACCCGTTTCCCTTCACTTGCCTTCTTTTTCCTCCTCCTCGAATGCTCAGC CACACCAAACCTGAAAATCCGGGCTCGACCACAGCCATGTAAACAGCTAGTGTTTTACTTCC ATGACATTCTTTACAACGGCCACAACTCCAAGAATGCAACTGCAGCCATTGTAGGGTCACCA GCTTGGGGAAATAGAACCATTTTAAGTGGCCAAAACCATTTTGGCAATATGGTGGTGTTTGA TGATCCCATTACACTAGATAATAATCTCCATTCAACCCCAGTTGGCCGAGCACAAGGATTCT ATCTTTATGACAAAAAAGATATATTCACTGCATGGCTAGG
G11	ACTTGTTCGCTATCGGTCTCTCGCCCGTATTTAGCCTTGGACGGAATTTACCGCCCGATTGGG GCTGCATTCCCAAACAACCCGACTCGCCGACAGCGCCTCGTGGTGCGGCAGGGTCCGAGCAC GACGGGGCTCTCACCCTCTCCGCCCGCGTACCTG
G12	ACTCACTCTTCATGAAATTGGCAATAGAGCCTTGAGAGCACAATTAACATTTGGGCGGGGAA ACCCCACAAGTGGAAGCAATTTTCTTTGCATTTGGAGAGTTAACGTATTTATTCAAATTGGGA TTCTTGAGATACTGGCAAAAGCAAGGCTGCTGCTCCTTGATCTTACTGCAGCAAAGTGGCGA CGGAGGAGACCCCGACGAGATGGCCGACACGCAAGGGCTGAGTTCAGTTGGGTTGCAAGTT

Clone	cDNA sequence (5′-3′)
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H1*	GTTAAGATTTATGCGTTATTCCAATAACTCGTATATAACTTACCATTACAAAGGAGCAAAGCT AAAAGATTTCATTATAATTATATATATGAACTCCTTTGGCATTACTAATTATCAATAAGCTCT CGCACACTTCGGGGCATAGGTGCAGCCATATCGGTGAAGACAAGCGATCTGGCGATGATGA CAATGTTGTAATTTGAACATTTGTTGCATGGAATGAGATTACTGTAGTGCAAGATATGTGGTT TCTTGCGGGTGGATTCAGACCCCAACCTCATCTCCTCATCAACGTCAACGAAATGCCCACTAT TTTTAGACTCGAGGTCAACAGAATTTGTATTGTCATGTCCTGATGATGGTTGTATCAACATAA CCAAGCATAGGAGAAAAAGAAAAAGTAACACCAGACGTGAAATCATTTTGTGAGAGTCAAT AGTGACATACACAACAAAGATAAAAAGGAAGGAACTTTGATCCCCGCGCA
H2*	ACCTTTTTCTTCATCGTCGTCAGATAAAGGTCCAACTTCCAAATCCTTTTCAGTATTACCCTTC TTTTTCTTCTTCTTGTTCTTTGTCTTTGGTGATGATAATGATAGTGTCGCTGATTTTAGTTTGTG GGCGTAATGATGGCTGCGAAGAATGTGAGTAAATGCTGGCGGGGCAGACGAGAACATGGTG ATTATGATGACTGCTTTTGAGTTGTGGCGTTGAATGTGAGAGAGAAAGTAAGAGAGAAGGA GAAATTAGAATAGAAAGATAAAAGGCTCTATCTTTCAATGTTTTTCGTGCAGTTTCTTAATTT ATCTTTGATTGTTCAGAGAATTTGCTGTGTTTTTTGATAAAGAGAGAACAGACACAATGTGGC AAGAATTATTAGCATT
H3*	ACATGGTCTATGCGATGAGGGAAAAAATTATATTTTAACAAAAACGGTGTGTATACTTTTCC ATGCAAAGATTATAATATGAACTGCAGGACTTACATAGTTTTCTTTCCGTATAATTTGTGGTG ACAAAGAGAAAATGGAAATGTTTTTGTTTTTAAATACACGTCCTAATTAATTAATTTCTGGAT GGTGGATCATCTGAGTGTATATATGATGTTTCTTTCTTCTGAGTTCGACTTTGTTTCATTTTCT AAACTCCTCAGTTGCTTGCAGAATTATGTTGCGGGAAATCCTGCTTCAGTTTTGT
H4*	ACATGGTCTATGCGATGAGGGAAAAAATTATATTTTAACAAAAACGGTGTGTATACTTTTCC ATGCAAAGATTATAATATGAACTGCAGGACTTACATAGTTTTCTTTCCGTATAATTTGTGGTG ACAAAGAGAAAATGGAAATGTTTTTGTTTTTAAATACACGTCCTAATTAATTAATTTCTGGAT GGTGGATCATCTGAGTGTATATATGATGTTTCTTTCTTCTGAGTTCGACTTTGTTTCATTTTCT AAACTCCTCAGTTGCTTGCAGAATTATGTTGCGGGAAATCCTGCTTCAGTTTTGT
H5	ACCATAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCC CACATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGGATAAT ATGATCCCAATTACGATTCTTCACACCTCCCCGGCCAGCCATGTTACTTTGGCCCGCTAATAT GAAGATATCCTTATGTTCGAAAGCGTGTGGAGACTGTTG
H6	GAGGTATTAAATAAGGATACATTTATTTATTTATTTAAATATGATCTTCATAATTACAATCTA ATTTCCATGCAAGGTCGAAAGAAATCAAAATAACAAATGCAAATCTCTCTTAAAGCTGTCAG CATATTCCACAATACTAAAAAACACATTCTCATGACAAGGTAAAACAATTTTGGAATTGTTG TCAAATCCAAAACTGTTCTTGTGCCAAATGAAGCAAGAATTTGAAAAAAGGATGGTTTAATA AGCTTAAATTGACAACATATCTCTTGCAACTGTCGCCTACATATACCACAAAATGACCTTCTG GAACATCTTTTGGTACAAACTCATAATCTTCATCCAGGCTGCTTTTAGGATAATCCCATGTTA AAAGGTTGAACATTCTGGAAGCTGTTCTTTTGAACTTCCTAAGGTATATTTTGAGACAATTTT CTCTTCTCATTCTCATGCATGTAGGAGGGAGTCTCTGAAAAAGAAGCGAGCGCGCGTATATA TCTATCAACGGCCTGTTTCTCCCGCGTACCTGCCTCTTTTCTCTTCATCTCCTTATCTGTAAAT TTGAAATTCTGCACTGTAAATTCTTCAAAAGTATCCCCGATCGGAAGAG
H7*	CGCGGGGATTCACATTTCACTAAAGCTGCCTTTAATCTCTCTTTCAATTATGGAGATCGCTTT GAAGTCATTTGCAGTGGTGTCCTTCCTGCTGTTGGTTTCCCTACCCACTGGATGTGACCCGCA ACTTTGTCAACAACACTGTTCTAGTCAGGGTTTGCCAACATGCTGCGAAGGTCCGAGTTTCCC GTGTCTTTGCCAAGAAATTTGCCCACGTAATCGTATTATGAGTGGTCCTCCTGTAGTGAATGC TCTAGGATGTGACCCGCAACTTTGTCAAGCATACTGCAAAAGTCTGGGTTTGCCATGGTGCT GTTCAGGACTATATGGACCGTGTCTTTGCCAAGGACCTTGCCCACTTGCAGACTCTGTTCCAA C
H9	ACCAGAGAAGTGCACGAATCTGCCCTCCATCTTGTAGCGCTGCCTCAGCCCTCTTTATGAGTC GATTATATAGTTTCGAGCCTCGTTTCCACTGCTCTATACTTGATCCACCGAAGGCGCAAGGGA CTAAACCAATTACCCCTATGCTAGGATTTCGTCCCAGCAGTGAATTGGCAAACGCTAATCCA GGTCCCACCCCACAAGTTTTATTTGGATCCATGTCCTCATGTAGAGGTTCGTGTGCCTCCTCC CACATTAGCGTTGCGGTAAGCCGAAGGATCGTTGGCTTTGAACTACATTCAGGAGGGATAAT

Clone	cDNA sequence (5′-3′)
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H10	ACCGGATACGGTGGTGATTATGAAAAATCAACTGGTGGTGGTTATTCGACTGGTGGAGGCGA CTACAATACCACTGCTGGTGGTGGCCGCTACAACACAGATTCTGCAGGTGACGACTACAATA CAACCACTGGTGGTGCCCGATATAATACCAAGACTGCTGGTGGCAGATACAACACAACCGTT GGCGGTGGTGATTATGAAAAATCGTCTGATGATTATGAAAAATCAACTGATGATTATGAGAA AGAAAAGAAGGAACATAAGCATAAGGAACACCTCGGAGAGATGGGAGCCCTCGCTTCTGGG GCTTTCGCTTTGTACGAGAAGCACGAGTCAAAGAAAGACCCCGAGCATGCCCAAAGGCACA AGATAGAAGAAGGAGTTGCAGCAGCTGTCGGGGTTGGTTCGGGTGGATATGCTTTCCATGAG CATCACGAGAAGGAAGAGGCCAAGGATGACG
H11	ACGCGGGGGCACCAAAGTCTGCAACTTACACTACTTCAAAGAGCACCAAAGTCTGCAATGG ATATCCAAAGCAACGTCCTGACGATTACTAGCGGATCCTCACCAACTGACACTTCCTCCAAT GGTCAGGCAGCCAAATCCACCAAAGAAAGGATCAAACGCTCTGATTTTCCCAGTGACTTTGT ATTTGGCGCTGCAACTGCTTCATATCAAGTTGAAGGTGCATGGAACGAAGGAGGAAAAGGC ATGAGTAATTGGGATTACTTTACACAGAGTCAACCAGGTGGTATTTCCGACTTTAGCAATGG CACTATTGCAATTGATCACTTTAATATGTTCAAGGACGATGTTGTCGTGATGAAGAAATTGG GTTTGAAAGCATACAGATTTTCACTTTCATGGCCTAGAATCTTGCCAGGAGGAAGACTGTGT CACGGTGTATCTAAAGAAGGAGTTCAGTTCTATAACGATCTCATTGATGCACTCTTGGCAGCT GACATAGAGCCATATATAACTATCTTTCACTGGGATATTCCCCAATGTTTGCAACTAGAGTAT GGTGGCTTCCTACATGAAAGAGTTGTGCAGGATTTTATTGAGTATTCTGAGATTTGCTTCTGG GAATTTGGTGATCGGGTGAATATTGGATTACCCTTGAATGAGCCATGGTCCTTTACTGTTCAA GGATTATGTGGCTGGTGCTTTTCCGCCCAATTGTGGTGTAACTCCGAAAGATACTG
H12	ACGCGGGGGAGAGAGTGATACATATAATTTGGATGATGCGGAAAGCTAAAGAACAGCTTGA AAAGCTTGTCCAAAATGCGCGAACCGATCTCAATATGCCGTCTCTTCCGATTCTTCTAGCTTA TTGAATCAGGAGATGGTCCATTCAAAGACGAGGTTATAAAGCAACAAAAGGCGTTTAAGAT GCCTAATGTTGTAAAGGTGGATTCAAGAGGACTGGGGCTCAACAAAGATAACGTTCACTTGA ACACGGAGGCCCAGGTTCAACTGGGCAAATGGTTGGCTGATGCATATTTAAACAATTTTGCA TAAGTATTTTCACTTTTCTTGTTTTAACTCTAAACGAGGAGGAAAATTCTTATAC

Appendix B

Poster abstract

Seifi, E., Kaiser, B., Guerin, J., and Sedgley, M. 2007. Analysis of gene expression in olive pistils during flowering: the use of subtractive hybridisation to identify genes involved in olive self-incompatibility. In: Combio 2007, Sydney, NSW. 22 to 26 September, 2007. Australian Society for Biochemistry and Molecular Biology, Sydney, NSW, Australia.

Refereed publication

Seifi, E., Guerin, J., Kaiser, B., and Sedgley, M. 2008. Inflorescence architecture of olive. Scientia Horticulturae. 116: 273-279.

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