THE DETECTION OF CHERRY LEAF-ROLL NEPOVIRUS AND THE USE OF MOLECULAR MARKERS FOR GERMPLASM IDENTIFICATION IN WALNUTS (*JUGLANS REGIA* L.)

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any other university for a degree.

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SUMMARY

The aim of this study was to combine two common diagnostic tools: serological kits and genetic fingerprinting to identify cherry leaf-roll nepovirus (CLRV), and to establish a marker system to characterize walnut germplasm.

The detection of plant viruses is difficult. Restrictions are imposed for quarantine purposes on the importation of plant material from foreign countries. Modern techniques such as a PCR based screening method for CLRV are required to ensure material do not harbour viruses. A primer pair was designed to amplify a 430 bp non-coding homologous region. For the choice of primers, consensus sequences were considered and areas where the sequence data shared 98.5% homology, were chosen. The sensitivity of this detection method was 100-fold higher when compared to the ELISA. The PCR fragment was verified by nucleotide sequencing.

AFLP technology was used to identify polymorphic fragments for 6 walnut cultivars and a rootstock, and SCARs were developed from AFLP specific bands. The AFLP technique distinguished all the walnut cultivars and the rootstock. However, conversion of AFLP fragments to SCAR markers for the development of a simple robust technique for cultivar discrimination, was not successful. Using 27 AFLP primer combinations, polymorphic fragments as high as 47.8% were scored. The reason for the lack of efficient conversion was as the result of the AFLP technique. The SCAR primers were generated from sequences internal to the AFLP primers but the specificity of the markers was in the AFLP primers not the internal sequence.

In this study using AFLP, walnut cultivars were found to be closely related. The AFLP primer pairs used, provided polymorphic fragments. From these fragments, 7 SCAR markers were developed. It was expected that these SCARs derived from the AFLP markers would detect slight differences between cultivars. The Paradox SCAR marker was the only one that could divide the cultivars into two groups. When Chandler SCAR products were digested with the restriction enzyme *Rsa*I, the same banding pattern as that of Paradox SCAR products was observed.

OPSOMMING

Die doel van hierdie studie was om twee algemene opsporingstegnieke te kombineer: serologiese toetsstelle en genetiese vingerafdrukke om cherry leaf-roll nepovirus (CLRV) te eien en om 'n merkersisteem te ontwikkel wat okkerneut kiemplasma kan karakteriseer.

Die opsporing van plant virusse is baie moeilik. As gevolg van kwarantyn vereistes, word daar beperkinge geplaas word op die invoer van plant materiaal vanuit die buiteland. Moderne tegnieke soos hierdie een wat op PKR berus, word benodig om te verseker dat CLRV nie in plantmateriaal teenwoordig is nie. 'n Stel inleiers is ontwerp wat 'n 430 bp nie-koderende homoloë area amplifiseer. Hiervoor is konsensus volgordes bestudeer en slegs die volgordes wat 98,5% homologie getoon het, is gekies. In vergelyking met ELISA was die sensitiwiteit van hierdie deteksie metode 100 maal beter. DNA volgordebepaling is op die resulterende fragment gedoen om die PKR produk te verifieer.

AFLP tegnologie is gebruik om polimorfiese fragmente vir 6 okkerneut kultivars en 'n onderstok te identifiseer en SCARs is uit hierdie fragmente ontwikkel. Die AFLP tegniek kon tussen al die okkerneut kultivars en die onderstok onderskei. Die omskakeling van die AFLP fragmente in SCAR merkers om sodoende 'n eenvoudige kragtige tegniek vir kultivar onderskeiding te ontwikkel, was egter nie suksesvol nie. Met die gebruik van 27 AFLP inleier kombinasies, kon polimorfiese fragmente van so hoog as 47.8% verkry word. Die rede hoekom omskakeling onsuksesvol was lê by die

aard van die AFLP tegniek. Die SCAR inleiers is ontwikkel uit volyordes intern tot die AFLP inleiers, maar die spesifisiteit van die merkers het juis in die AFLP inleiers gelê en nie in die interne volgordes nie.

In hierdie studie, met die gebruik van AFLP, is gevind dat okkerneut kultivars baie naby verwant is. Die AFLP inleierstelle wat gebruik is, het polimorfiese fragmente gelewer. Uit hierdie fragmente is 7 SCAR merkers ontwikkel. Daar is verwag dat die SCARs wat uit die AFLP merkers ontwikkel is, klein verskille tussen kultivars sou opspoor. Dit was egter net die Paradox SCAR merker wat die kultivars in twee groepe kon verdeel. Restriksie ensiem vertering met *Rsa*I op die Chandler SCAR produkte het dieselfde bandpatrone as die van die Paradox SCAR produkte gelewer.

PREFACE

The experimental work in this study was carried out in the following institutes: Institute for Plant Biotechnology, University of Stellenbosch, under the supervision of Professor Frederik C. Botha; Agricultural Research Council Infruitec-Nietvoorbij, Plant Biotechnology Division; South African Agricultural Food and Quarantine Inspection Services, National Department of Agriculture, Stellenbosch, under the supervision of Professor E. Lucienne Mansvelt.

These studies are the original work of the author, and where use was made of the work of others, it has been duly acknowledged in the text.

The work presented here is a compilation of chapters of which chapters Three and Four are presented in the style of publication. In the text, the format of *Crop Science* was used.

Chapter One	General Introduction
Chapter Two	Literature Review
Chapter Three	Pathogen Detection
Chapter Four	Germplasm Identification
Chapter Five	Conclusions

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To the memory of my parents, Mamase J and Ndabambi J

ABBREVIATIONS

A	adenine
AFLP	amplified fragment length polymorphism
AP-PCR	arbitrary primed-PCR
APS	ammonium persulphate
ARC	Agricultural Research Council
ASAPs	allele-specific associated primers
bp	base pair
C	cytosine
CAPs	cleaved amplified polymorphic sequences
cDNA	complementary DNA
ClAA	chloroform/isoamyl alcohol
CLRV	cherry leaf-roll nepovirus
CNR	Consiglio Nazionale delle Ricerche
cpDNA	chloroplast DNA
CTAB	cetyltrimethylammonium-bromide
cv(s).	cultivar(s)
DAS-ELISA	double antibody sandwich form of ELISA
dCTP	deoxycytidine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpi	day post inoculation
dsRNA	double stranded RNA
DTT	dithioerythritol

EDTA	ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ESTs	expressed sequence tags
G	guanine
gDNA	genomic DNA
IC-RT-PCR	immunocapture reverse transcriptase-PCR
IDC	Industrial Development Corporation
INRA	Institute for National Research in Agriculture
IPB	Institute for Plant Biotechnology
IPTG	isopropanyl-β-thiogalactopyranoside
IRTA	Istitut de Recerca i Tecnologia Agroalimentaries/Institute of Research for Agriculture and Food Technology
ISSA	International Ship Suppliers Association
ISSR	inter-simple sequence repeat
K	G/T
Kb	kilobase
LB	Luria broth
M	A/C
M	molar
mM	millimolar
m/v	mass per volume
NDA	National Department of Agriculture
N	A/T/G/C
ng	nanogram
NPP	4-nitrophenyl phosphate

nt	nucleotide
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNK	T_4-polynucleotide kinase
PVP	polyvinylpyrrolidone
R	A/G
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcription
RTase	reverse transcriptase
S	G/C
SAAFQIS	South African Agricultural Food and Quarantine Inspection Services
SCARs	sequence characterized amplified regions
SDS	sodium dodecyl sulphate
SOC	complex medium for growing high-efficiency competent *E. coli*
SRFA	selective restriction fragment amplification
STE	sodium tris EDTA
STS	sequence tagged site
T	thymidine
TAE	tris, acetic acid, EDTA

TBE	tris, boric acid, EDTA
TE	tris, EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
T_m	melting temperature
Tris	tris[hydroxymethyl]aminomethane
µg	microgram
µl	microlitre
µM	micromolar
UC	University of California
US	University of Stellenbosch
USA	United States of America
USDA	United States of America Department of Agriculture
USSR	Union of Soviet Socialist Republics
UTR	untranslated region
UV	ultraviolet
V	volts
VPg	genome-linked protein
v/v	volume per volume
W	A/T
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
Y	C/T

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CHAPTER ONE

GENERAL INTRODUCTION

Plants belonging to the genus *Juglans* are forest trees, valued for both nut production and timber quality (*href*8). *Juglans* consists of approximately 21 species (Fjellstrom and Parfitt, 1994a), which include species native to North, Central, and South America, Eastern Europe and Asia (*href*8, McGranahan and Leslie, 1998). The two most important species are *Juglans regia* L., known as English or Persian walnut and *J. nigra,* known as Eastern black walnut. Several other species and hybrids in *Juglans*, notably *J. hindsii* (Northern California black walnut) and *J. hindsii* x *J. regia* (Paradox hybrid), are important rootstocks for cultivars of *J. regia* (*href*8).

Juglans regia nuts are a high-energy food, rich in oil, vitamins and minerals (McGranahan and Leslie, 1998), and are used in products for alcohol, colouring and oil. *J. nigra* is prized as a quality timber species with its wood used for veneer and furniture (FAO, 1996, Fjellstrom *et al.*, 1994a and b). Today the nut trade continues to be a well established, ordered, and structured business, and the California walnut is well known as the top quality walnut for the world (Potter *et al.*, 2002). Research programs in physiology and genetics are being carried out by national institutes from the European community such as INRA in France, IRTA in Spain, CNR and ISSA in Italy (FAO, 1996) and California in the USA (McGranahan *et al.*, 1988a and 1988b).

Cultivars commonly used for nut production are Chandler, Chico, Franquette, Hartley, Howard, Payne, Sunland, Serr, Tulare and Vina. The rootstock commonly used is Paradox, a crossbreed of *J. regia X J. hindsii*, which is a vigorous grower. *J. hindsii* is the old standard rootstock which is being phased out. *J. regia* as a rootstock is recommended where blackline disease is epidemic. This implies that cultivars are grown on their own roots (*href*9).

In the USA at least 10% of potential annual walnut production is lost to pests, diseases and environmental causes (McGranahan and Leslie, 1998). More than ten different diseases result in losses by lowering yield or quality or by requiring costly chemical control treatments (McGranahan and Leslie, 1998). Walnut blight, walnut anthracnose, deep bark canker, crown gall, walnut bunch disease, *Phytophthora* root and crown rot, and blackline disease, caused by cherry leaf-roll nepovirus (CLRV) pathogen (Mircetich and Rowhani, 1984), continue as major sources of loss in the major walnut growing regions of the world (*href*2, McGranahan and Leslie, 1998). Resistance to some of the soilborne diseases and pests is found in the vigorous rootstock Paradox. Paradox hybrid seedling rootstock presently represents the available industry standard for maximum vigour, general tolerance to lesion nematodes, and resistance to most *Phytophthora* species (*href*10). However, there is no resistance to CLRV. The pathogen infects a variety of woody and herbaceous hosts for example, cherry, peach, apple and ornamental *Prunus* species (Jones, 1985; Ogawa *et al.*, 1995). The lesion of

CLRV is located to the graft union where a blackline develops and causes necrosis of the scion. English walnut trees in orchards are significantly damaged (Mircetich *et al.*, 1985).

The commercial walnut industry in South Africa is small. Walnuts have been grown on a very small scale in South Africa near Aberdeen and Oudsthoorn for many years but the industry has not expanded much due to lack of technology and cultivars, poor codling moth control and poorly organised marketing. South Africa thus imports its total requirement of about 200 tons. Currently, walnut cultivars such as Chandler, Franquette, Hartley, Serr, Sunland and Vina are imported from a nursery in Spain. The plant material originated from California.

The National Department of Agriculture (NDA) imposes restrictions for quarantine purposes on the importation of plant material from foreign countries. CLRV has not yet been found in South Africa. The prevention of the introduction of CLRV into South Africa lies largely with international quarantine efforts. Therefore, modern techniques are required such as a PCR based screening method for CLRV. That would prevent introduction of CLRV into new places. In this study, an immunocapture reverse-transcriptase-PCR (IC-RT-PCR) method, which is an efficient, rapid and reliable procedure for detecting the viruses, was developed to effectively

index propagation material for the presence of the blackline virus in all the imported plant material (Chapter 3).

Millions of dollars are spent annually on plant research, the goal being to develop and market new plant products such as hybrids, varieties, better cultivars and to genetically engineer crops with improved traits such as disease resistance. It is generally agreed that effective protection of these new plant products is essential to provide the incentive for investment in this research (van Sambeek, 1996). Several methods are used for the identification of plant cultivars. The most commonly used method is based on morphological characters. Isozyme analysis is used to assist in identifying cultivars, but there are a limited number of isozymes, which are not sufficient to distinguish the cultivars in question. The introduction of molecular biology techniques, such as DNA-based markers, has provided a new opportunity for genetic characterization, allowing the direct comparison of different genetic material without environmental influences.

Biotechnological advances have provided better methods for identification of cultivars by observing genetic differences between cultivars. An efficient strategy to distinguish between cultivars is to limit the comparison to regions of the genome, which are known to differ frequently between individuals. The concept of genetic polymorphism is fundamental to all current methods of determining genetic identity and relatedness (Potter *et al.*, 2002).

Several previous studies have examined the genetic diversity of walnut cultivars using molecular markers. Such studies are useful for understanding the origins and relationships of germplasm and providing genetic fingerprints that can be used to test or confirm the identity of plant materials. Marker systems that have been used in previous studies of walnut cultivars include isozymes (Arulsekar *et al.*, 1986; Solar *et al.*, 1993), RFLP markers (Fjellstrom *et al.*, 1994; Staub *et al.*, 1996), and RAPD markers (Nicese *et al.*, 1998).

AFLP analysis has proven to be valuable in genotype characterization in many crop species (Vos *et al.*, 1995). In this study a DNA-based, AFLP method to distinguish walnut cultivars and rootstock authenticity was developed (Chapter 4).

The conclusions in chapter 5 clearly emphasise the successful use of the IC-RT-PCR virus detection technique and AFLP technology and its conversion to cost effective SCARs.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The walnut, *Juglans regia* has been known for thousands of years and is thought to be the oldest tree food known to man (Fisher, 2001). Early history indicates that walnuts came from ancient Persia, where they were reserved for royalty (Fisher, 2001). *J. regia* was thus commonly known as the Persian walnut. The Ancient Greeks transported the walnut around the Old World and the nuts were used as a food and medicine and for dyes. The ancient Romans associated the walnut with Jupiter, the goddess of weddings and fertility. Legend has it that walnut is one of the foods presented to Jesus by the three wise men (*href*6). In the Old Testament, King Solomon speaks with delight of visiting his walnut grove: "*I went down into the garden of nuts to see the fruit of the valley*" (Song of Solomon 6:11).

In mediaeval times walnuts were associated with English merchant sailors, as they were transported in ships around the world. Because of this, walnuts are sometimes referred to as the English walnut. Walnuts were seen in some of the earliest European trade fairs, such as Hanover. The listing for walnuts is found on cargo manifests of many ships between the Old World and New World (*href*6).

Eventually, walnuts travelled from the Mediterranean area to European countries and finally to the New World. The nuts were brought into the New World by Franciscan missionaries and cultivated on mission lands (*href*6; Potter *et al.*, 2002). The trees flourished in the Mediterranean-like climate

zones of California, and by the 1870s modern walnut production had begun with orchard plantings in southern California. In the next 70 years the centre of California's walnut production shifted with successful plantings in the central and northern parts of the state. Today the nut trade continues to be a well established, ordered, and structured business, and the California walnut is well known as the top quality walnut for the world (Potter *et al.*, 2002). Many of today's improved cultivars are descendants of those early plantings (*href*6).

2.2 The walnut tree

Walnuts are a long-lived perennial tree crop with a generation interval of 3 – 10 years (McGranahan *et al.*, 1999). Walnuts are monoecious and the male catkins are borne separately from the female flowers on one tree. The walnut is a large tree growing from 9 – 24 m high and requires deep, well-drained soil free from harmful salts. In orchards, trees are spaced depending on the growth habit and precocity of the cultivar. They have a high chilling requirement similar to pears (700 – 1 500 hours below 7°C), although some of the newer cultivars require less cold to break dormancy. The trees bear from 6 – 8 years after planting. The walnut tree life span is a century (*href*11; *href*12; *http*15; McGranahan *et al.*, 1999).

2.3 World walnut industry

Persian walnuts are grown in North and South America, Europe, Asia, USSR, and to a limited extent in Oceania and North Africa (*href*8). The four major countries that dominate world walnut exports are the USA, China, France and India. Other countries that are also role players include Italy, Turkey and Chile (*href*13). South Africa does not feature in this market.

The world walnut crop in 2001 was reported to be 545 400 metric tons (*href*12; Figure 2.1), a figure very much on the low side due to a very small production of 127 000 metric tons in China. This was caused by an ongoing dry period. The expected crop for 2002 was expected to be approximately 660 000 metric tons (*href*12). The main consumers are in China, the USA, Turkey, Italy, India and France (*href*13).

In the USA, the Central Valley of California is now the prime walnut growing region. Its mild climate and deep fertile soils provide ideal-growing conditions for the California walnut. California walnuts account for 99% of the commercial USA supply and two-thirds of world supply (*href*6). The first commercial plantings began in 1867 when Joseph Sexton, an orchardist and nurseryman in the Santa Barbara County (USA), planted English walnuts (*href*6, Potter *et al.*, 2002). For several years, walnuts were predominantly planted in the southern areas of California, accounting for 65% of all bearing acreage. Better growing areas, improved irrigation, and better pest control

methods resulted in greater yields, which gradually increased each year (Potter *et al.*, 2002).

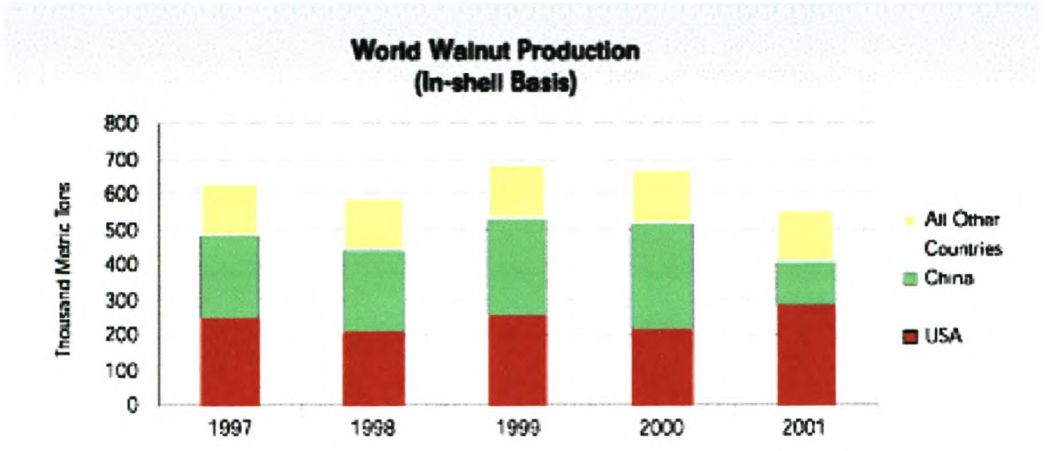

Figure 2.1 World walnut production based on the two main producing countries (USA and China) and the rest of the world. (adapted from *href*12)

2.4 South African walnut industry

Walnut growing in South Africa started in the early twentieth century (*href*15). The seedlings of the cvs. Royal and Payne, which were planted, yielded inferior nuts. During the 1980's eight cultivars with favourable horticultural characteristics were imported from California, but the failure to root cuttings in nurseries limited the expansion of the industry (*href*15).

During 1998 the Industrial Development Corporation (IDC) of South Africa identified large areas of the Eastern Cape and Free State as suitable for growing walnuts. The project intended developing 485 hectares of walnuts within five years. An annual production of 2 200 tons of walnuts is expected when the trees reach maturity by 2015. The first 32 hectares are being planted (*href*11, *href*12). The project is intended to serve as the core of a new high-value agricultural industry with the long-term objective of encouraging farmers to switch from low-value cash crops to walnuts (*href*11; *href*15).

Aliwal North (Eastern Cape Province) is set to become the walnut capital of South Africa in a R61 million development that aims to exploit a suitable climate, good soil and abundant water. The IDC of South Africa Ltd had already embarked on a large-scale commercial project of selected walnut cultivars in South Africa (*href*11; *href*15). Compared to other nuts, walnut import and consumption is very low (Table 2.1).

Table 2.1 SA tree nut imports 1996 to 1999 (metric tons). Adopted from *href*11

Type	1996	1997	1998	1999
Hazel	996	982	835	878
Cashew	377	900	791	788
Almond	568	482	505	591
Brazil	153	151	148	164
Pistachio	144	80	75	102
Walnut	52	58	64	57
TOTAL	**2 289**	**2 663**	**2 415**	**2 580**

2.5 Walnut commercial cultivars

All walnut (*Juglans* L.) species produce edible nuts, however, the English or Persian walnut is horticulturally the best developed and is widely cultivated. It is the leading producer of commercial nuts (McGranahan and Leslie, 1998). Almost all of the walnuts produced in the USA are grown in California (Table 2.2), which has approximately 74 000 bearing hectares (McGranahan and Leslie, 1998). The leading cultivars are Hartley and Payne. Hartley is popular because it consistently produces good yields of high quality kernels without intensive cultural management. Good high quality walnuts are required for commercial production in other parts of the world (*href*8). Screening for pathogens related to walnuts and characterising cultivars is very important.

2.6 Breeding programs

Breeding programs are carried out primarily for both business and research purposes. The objectives of the programs are to improve yield and to increase disease resistance. International collecting activities for Persian (or English) walnut have emphasised broadening the narrow germplasm base found in existing cultivars, and identifying sources of disease resistance (*href*8). Breeding programs are conducted at UC, Davis (USA), INRA (France), Ministry of Forestry (China), and to a smaller extent in Turkey, Morocco, India, Greece, Hungary, Romania, Ukraine and New Zealand (*href*8).

Table 2.2 List of *J. regia* cultivars used in this study.

Cultivar	[1]Origin	[2]Parentage
Chandler	University of California	Pedro X 56 - 224
Franquette	France	Franquette seedling
Hartley	California	Franquette X Mayete
[a]Milizia	Italy	?
Serr	California	Payne X PI 159568
Sunland	University of California	Lumpoc X PI 159568
Vina	California	Franquette X Payne
[b]Paradox	California	*J. hindsii* X *J. regia*

[1]Based on information reported by Nicese *et al.* (1998), [2]Based on information reported by Fjellstrom *et al.* (1994) and Potter *et al.* (2002), [a]Used by SAAFQIS, NDA for CLRV detection, [b]hybrid.

2.7 Walnut diseases

More than 10 different diseases result in losses by lowering the yield or quality, and by requiring costly chemical control treatments. In the USA, at least 10% of the production is lost each year to pests, diseases, and environmental causes (McGranahan, 1987). Few of the diseases include viral diseases (blackline and walnut ring spot), bacterial diseases (walnut blight, crown gall, deep bark canker and shallow bark canker), fungal diseases (anthracnose, *Armillaria* root rot, branch wilt, cylindrocarpon dieback, downy leaf spot, melanconium dieback, Texas root rot, *Phytophthora* crown and root

rot, *Phytophthora* trunk and branch canker and butternut canker) and nematodes (pin nematode, ring nematode, root lesion nematode, root knot nematode) (Chen and Swart, 2000; Matheron and Mircetich, 1985b; McGranahan and Leslie, 1998). Some of these losses could be avoided if genetic resistance or tolerance were available, and others would be dealt with by improving cultural practices. The most effective strategy for control of viral plant diseases is using virus-free plants (Hu *et al.*, 1995; Kölber *et al.*, 1993).

In their interactions with plants, most viruses induce important changes on plant metabolism that are usually reflected in a variety of characteristic cytological alterations (Más *et al.*, 2000; Martins, 1996). Some of these changes have been reported to be, directly or indirectly, associated with replication and accumulation of the virus (Más *et al.*, 2000).

2.7.1 The viral pathogen CLRV

2.7.1.1 Disease

Blackline disease in the USA was first discovered in Oregon in 1924 and in other parts of the country five years later (McGranahan and Leslie, 1998). It has spread through much of California and is now known to be present in most of the walnut growing regions of England, France and Hungary (McGranahan and Leslie, 1998; Mircetich *et al.*, 1985). The disease is caused by a walnut strain of the CLRV. The first symptoms result in a lethal girdle at

the graft union of Persian walnuts grafted on hypersensitive rootstocks (Mircetich and Rowhani, 1984). Later, diseased trees show dieback of terminal shoots and decline, often accompanied by profuse suckering of the rootstock. Positive diagnosis of blackline requires careful examination of the union between scion and rootstock (McGranahan and Leslie, 1998). The virus is transmitted by vectors (*Xiphinema coxi, X. americanum, X diversicaudatum* and *X. vuittenezi*), through seed, by grafting and by pollen. The hypersensitive response of the rootstock results in a band of dead cells at the union through grafting which prevents the flow of nutrients between rootstock and the scion and eventually causes the tree's death. Blackline disease is one of the most important problems facing walnut growers because of reliance in California on the hypersensitive rootstock species *J. hindsii* (McGranahan and Leslie, 1998).

Another disease incited by the CLRV pathogen is called walnut ring spot. Symptoms appear as spots on leaves and fruit, twigs have poor terminal growth which are sometimes accompanied with chlorotic, drooping leaves (Figure 2.2). Premature defoliation occurs (Mircetich and Rowhani, 1984).

Other diseases, such as walnut line pattern (Christoff, 1958) and yellow mosaic virus (Cooper and Edwards, 1980), have been described but information is vague. Apparently CLRV is also the casual organism of both diseases.

2.7.1.2 CLRV Particle Structure

CLRV belongs to the family *Comoviridae* and genus *Nepovirus* (Mas *et al.*, 2000). The nepovirus genome is composed of two separate positive-sense encapsidated RNA molecules (RNA- 1 and RNA-2) that are 3'-polyadenylated and 5'-linked to a VPg (Figure 2.3) (Brooks and Bruening, 1995a; Mayo *et al.*, 1982; Werner *et al.,* 1997). The nepoviruses have been divided into two groups based on the size of genomic RNA-2. For subgroup 1 nepoviruses, genomic RNA-2 is about half the size of RNA-1, whereas the RNA-2 of subgroup-2 nepoviruses is significantly more than half the size of RNA-1 (Borja *et al.*, 1992 and 1995; Brooks and Bruening, 1995b). Subgroup 1 nepoviruses have diverse amino acid sequences for their RNA2-encoded coat protein and for their RNA-1-encoded VPg and proteinase. The VPg (5'-terminal genome protein) has a relatively non-specific role such as protecting the 5'-end of the RNA, as has been suggested for the cap structure on molecules of other nepoviruses, e.g. tobacco ring spot virus (Köbler *et al.,* 1982; Rowhani *et al.*, 1998) or specific role, but yet undefined role such as RNA replication (Mayo *et al.,* 1982).

A B

Figure 2.2 Symptoms of English walnut ring spot caused by the CLRV pathogen. Lesions on nuts (A) and leaf spots (B).

2.7.1.3 Epidemiology

Nematodes (*Xiphinema coxi, X. americanum, X diversicaudatum* and *X. vuittenezi*) serve as an intermediate host for CLRV and infect the walnut tree with the pathogen by damaging the feeder roots. Irrespective of the virus, the nematode weakens the tree by reducing water and nutrient availability. This then reduces the vigour and yield (Topchiiska, 1993).

In surveys of disease incidence, infection levels normally range from 2.3% to 96.8% (Kölber *et al.*, 1993; Topchiiska, 1993). Upon infection, time-course analysis normally reveals that CLRV RNA accumulates in the inoculated leaf at an early stage, such as 20 hour post-inoculation. The virus accumulation reaches a peak at 8-10 day post-inoculation (dpi) and then progressively decreases. The virus RNA signal can be detected before the appearance of

symptoms. The virus invades stem vascular tissues after 3 dpi moving towards the roots before moving to the upper leaves. In systemically infected leaves, the virus appears first in the basal regions and then moves to the distal parts through the vascular system. Virus distribution in infected plants, as well as the susceptibility of the plant to systemic infection is influenced by the developmental stage of the plant. Inoculation of leaves at 95% of their final size normally results in virus replication but no systemic infection. In fully mature leaves the virus does not replicate (Mas and Pallas, 1996; Pallas *et al.*, 1992).

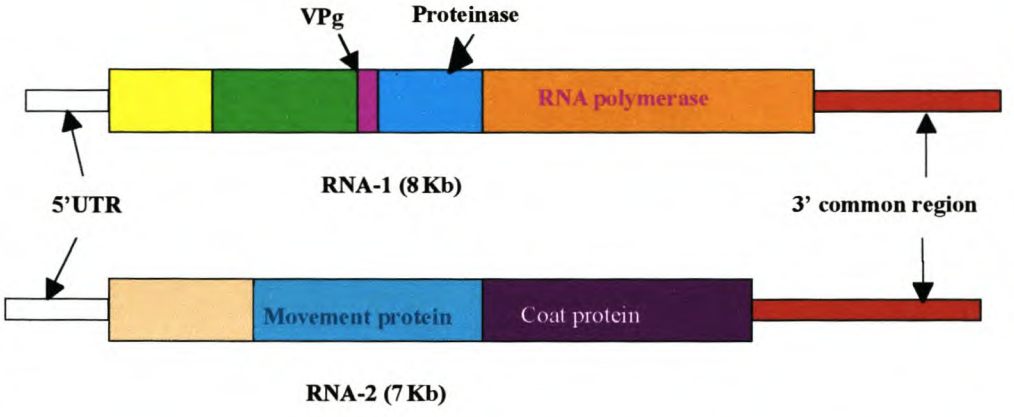

Figure 2.3 Postulated genomic organisation of the CLRV genomic RNA-1 and RNA-2, members of the subgroup-2 *Nepovirus*. The tentative gene assignments of open reading frames (large rectangles) rely on amino acid sequence similarities of nepoviruses and comoviruses as well as studies on the functions of some gene products of RNA-1 and RNA-2. Narrow rectangles represent sequences presumed not to be translated, the 5´ untranslated region (UTR) and the 3´ common region, (adapted from Brooks and Bruening, 1995b)

The distribution pattern of the virus coat protein in systemically infected leaves is parallel to that observed for the virus RNA, suggesting that CLRV requires coat protein for long-distance movement (Mas and Pallas, 1996). The structural variation in the genomic regions of the coat protein isolates at the 3'-end suggests that the coat protein gene of CLRV partly contributes to the serological distinctiveness of this strain from other CLRV strains in different parts of the world (Rowhani *et al.,* 1985). They are many different strains of CLRV (*href*14; Table 2.3). The coat protein encoding region of CLRV RNA-2 has been sequenced (Brooks and Bruening, 1995b).

2.7.1.4 Control

Many species of plant parasitic nematodes, particularly the pathogenic members pose a significant threat to agriculture. Accurate and reliable identification of these microscopic pests is fundamental to many aspects of their effective control and management (Burrows, 1990). Precautions such as quarantine measures are needed in germplasm exchange to prevent spread of the virus into new areas. Controlling nematodes can contain the spread of the virus. Once the orchard has been infected by CLRV there is no other way to eradicate the pathogen. The solution to control the virus is to increase the resistance by breeding.

Table 2.3: CLRV strains known to-date.

[a]CLRV Strains	Reference
Birch ring and line pattern virus	Cooper and Atkinson, 1975
Blackberry strains	Cropley and Tomlinson, 1971; Jones and Wood, 1978
Dogwood ring spot strain	Waterworth and Lawson, 1973
Elm mosaic strain	Jones and Murant, 1971; Varney and Moore, 1952
Golden elderberry strain	Hansen,1967; Hansen and Stace-Smith, 1971; Jones and Murant, 1971
Red elder ring spot strain	Schmelzer, 1966
Red raspberry strains	Cropley and Tomlinson, 1971; Jones and Wood, 1978
Rhubarb strain	Tomlinson and Walkey, 1967
Type (cherry) strain	Cropley, 1961
Walnut blackline virus	Cooper and Edwards, 1980; De Zoeten, 1982; Mircetich *et al.*, 1980; Rowhani *et al.*, 1985
Walnut line pattern and mosaic virus	Christoff, 1958
Walnut yellow mosaic, ring spot and yellow vein virus strains	Cooper and Edwards, 1980

[a]http://image.fs.uidaho.edu/vide/descr198.htm

2.8 Plant virus diagnostics

Detection techniques of high sensitivity are required to index trees for the presence of a virus (Vitushkina *et al.*, 1997; Wetzel *et al.*, 1992). The detection, quantitation, and control of virus infection in woody host crops may

be difficult due to low or variable titers of the virus (Clark and Adams, 1977). The ELISA is the standard diagnostic technique that is routinely used for virus detection in woody plants (Clark and Adams, 1977).

If the ELISA test is not available, soft or hardwood indexing is performed. With softwood indexing, a leaflet is removed from the young plant and inoculated onto herbaceous material known to be highly susceptible to a particular virus. The plant is observed for symptoms for up to three weeks after inoculation. Hardwood indexing is more difficult, because the material, suspected to harbour the virus, is grafted onto woody material and the symptoms take longer to develop. When the host plant shows no symptoms after several months, the plant is considered healthy (Vitushkina *et al.*, 1997).

ELISA tests are not always adequate for determining the presence of virus in symptomless trees. This is due to their lack of sensitivity and the uneven distribution of the virus within the tree. Due to the sensitivity of the tests, ELISA is being replaced by a molecular based immunocapture RT-PCR technique. DNA-based molecular methods are more reliable because of their high sensitivity that increases the virus detection threshold levels (Hu *et al.*, 1995; Wetzel *et al.*, 1992). IC-RT-PCR is a combination of techniques (Brandt and Himmler, 1995; Nolasco *et al.*, 1993; Wetzel *et al.*, 1992) that allows the detection of plant viruses in oxidising plant extracts through the capture of viroins (Borja and Ponz, 1992; Brooks and Bruening, 1995a and b; Rowhani *et al.*, 1985; Rowhani *et al.*, 1995; Tobias, 1995; Tobis *et al.*, 1995; Werner *et al.*, 1997).

2.8.1 Index host species for CLRV

Three softwood species are used for indexing CLRV. The first belongs to the genus *Chenopodium*, and the species *amaranticolor* and *quinoa* are both suitable. Chlorotic or necrotic local lesions, systemic mottle necrosis and malformation symptoms normally develop on leaves. The second is *Cucumis sativus* and CLRV symptoms are characterised as chlorotic lesions located to the inoculation site, and occasionally systemic mosaic lesions are induced. In the third set of indicator plants such as *Nicotiana rustica* and *N. tabacum* cvs. White Burley and Xanthi, symptoms are localised necrotic lesions and rings, and when systemic, necrotic or chlorotic rings develop.

2.9 Genetic analysis of crop species

The growing field of molecular biology has provided tools suitable for rapid and detailed genetic analysis of higher organisms, including agricultural species (Paterson *et al.*, 1991). The most fundamental of these tools are DNA markers, that simply detect differences in genetic information carried by two or more individuals (Melchinger, 1990; Paterson *et al.*, 1991). The potential usefulness of genetic markers as an instrument for the plant breeder was recognised more than 70 years ago (Melchinger, 1990). Advances in molecular biology during the last two decades have provided a new class of genetic markers at the level of DNA (Welsh *et al.*, 1991; Welsh and McClelland, 1990; Woeste *et al.*, 1996a). Until 30 years ago, however, its

application was largely hindered by the lack of suitable markers. The majority of markers were recessive morphological mutations that often had detrimental effects (Melchinger, 1990). The development of molecular markers promised to overcome most of the previous limitations.

2.9.1 Marker types

In general, there are three main types of markers known to date. They are morphological, biochemical and DNA-based molecular markers. Most marker systems that have been used to differentiate *J. regia* cultivars, include morphological (Malvolti *et al.*, 1994), biochemical (Aly *et al.*, 1992; Arulsekar *et al.*, 1986; Solar *et al.*, 1993) and DNA-based molecular markers (Fjellstrom *et al.*, 1994; Malvolti *et al.*, 1995; Nicese *et al.*, 1998; Potter *et al.*, 2002).

2.9.1.1 Morphological markers

Traditionally, morphological markers have long been used to identify species, families, and genera. Initially, genetic analysis of plants was based solely on the characterization of the genetic basis of naturally occurring variation in a phenotype. For this purpose, morphological markers were exploited. By definition most morphological markers developed thus far are for simply inherited traits i.e. they are based on macro-mutations of alleles with highly qualitative traits, while most agronomically important traits are thought to be

under control of qualitative trait loci (Msomi, 1998; Weising *et al.*, 1995). These markers usually have secondary pleiotropic effects on economic characters such as yield and therefore, have limited usage in crop improvement (Beckmann and Soller, 1983).

Contrary to other markers, morphological characters are often strongly influenced by the environment, and, consequently, special breeding programs and experimental designs are needed to distinguish genotypic from phenotypic variation (Weising *et al.*, 1995).

2.9.1.2 Biochemical markers

The term isozyme was first defined by Makert and Moller in 1959 (Karp *et al.*, 1998). Isozymes, the most common type of biochemical marker, are differently charged protein molecules that can be separated using electrophoretic procedures, usually starch gels (Market and Moller, 1959) or more recently polyacrylamide gels (Feldmann, 1994).

Biochemical markers have been used for studies of genetic variability. They are accepted as species-specific variants of an enzyme system according to the classification of the 'Enzyme Commission' (Muller-Stark, 1998). Isozymes originate through amino acid alterations which changes in net charge, or the spatial structure (conformation), of the enzyme molecules and also, therefore, in their electrophoretic mobility. They possess many important advantages as

gene markers (allozymes), e.g. substrate specificity of enzyme systems provides the basis for monitoring the genetic variation at specific structural gene loci, and they are usually expressed codominantly so that homozygous and heterozygous genotypes can be distinguished precisely (Muller-Stark, 1998).

2.9.1.3 DNA-based molecular markers

Due to the tremendous developments in the field of molecular genetics, a variety of different techniques to analyse genetic variation have emerged during the last few decades. These molecular markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment (Woeste *et al.*, 1996b). Therefore, none of the available techniques is superior to all others for a wide range of applications, but the key-question rather is which marker to use in which situation.

DNA markers offer many advantages over morphological markers or biochemical markers. The development of DNA markers is tedious, laborious, and expensive. However, once they are developed the progeny of newly-bred lines needs to be screened to ensure that the desired trait is present. Therefore, genetic analysis using molecular techniques, may provide new approaches to some objectives, which have proven difficult to achieve with classical techniques.

Besides being extremely useful to breeding programs, the information provided by molecular markers makes it possible to acquire knowledge about the structure and organization of plant genomes, as well as the evolution of plant genomes through phylogenetic analysis (Cervera *et al.*, 1996a; Fjellstrom and Parfitt, 1995; Ragot and Hoisington, 1993). Development of molecular markers for characterization of walnut germplasm is essential to many aspects of walnut research and industry as explained above and according to Simon and Potter (2001). The use of an accurate DNA fingerprinting method could be valuable where the differentiation of phenotypic characters is difficult (Caetano-Anolles *et al.,* 1992).

2.9.1.3.1 Restriction fragment length polymorphism analysis

RFLPs are based on the differential hybridization of cloned DNA to DNA fragments in a sample of restriction enzyme digested DNAs. RFLPs reflect differences in homologous DNA sequences that alter the length of restriction fragments obtained by digestion with type II restriction enzymes. The differences result from base pair changes or other rearrangements (e.g., transitions and inversions) at the recognition site of the restriction enzyme or from internal deletion/insertion events. The restriction fragments are separated according to their size by agarose gel electrophoresis (Fjellstrom and Parfitt, 1994a). Subsequently, a Southern blot analysis is performed: the DNA is transferred to and immobilized on a nylon membrane or nitrocellulose filter, hybridized to a labelled DNA probe, and visualized by autoradiography

or a colour reaction. They require large amounts of DNA and are relatively expensive to assay (Fjellstrom *et al.*, 1994; Fjellstrom and Parfitt, 1995).

2.9.1.3.2 Random amplified polymorphic DNA analysis

RAPDs are used to detect genetic polymorphisms by using PCR based DNA fingerprints. Short oligonucleotide sequences (9-11 base pairs) amplify fragments within a distance of between 200 and 2000 base pairs (Williams *et al.*, 1990). With some randomly chosen oligonucleotides, no sequences are amplified. With others, the same length products are generated from DNAs of different individuals, but the band patterns may differ for an individual in a population (Malvolti *et al.*, 1995).

RAPDs exhibit polymorphism and thus can be used as genetic markers. They are dominant in the sense that the presence of a RAPD band does not allow distinction between hetero- and homozygous states. Breeders should be able to identify RAPD bands closely linked to the marker they wish to transfer. Scoring individuals (or groups of individuals) for the linked RAPD marker should speed up the breeding process.

2.9.1.3.3 Amplified fragment length polymorphism analysis

AFLP is a powerful DNA fingerprinting technology (Cervera *et al.,* 1996b; Xu *et al.*, 2001) with a high multiplex ratio that was developed by Keygene (Wageningen, The Netherlands) (*href7*; Vos *et al.*, 1995). The resemblance with the RFLP technique was the basis to choose the name AFLP. This technique is based on the selective amplification of genomic restriction fragments such as SRFA and was originally developed for the detection of genetic markers in plants (Williams *et al.*, 1990). Advantage of AFLP is its high multiplexity and therefore the possibility of generating high marker densities (Barret and Kidwell, 1998). The AFLP technique uses only two primers and gives reproducible results. Many restriction fragment subsets can be amplified by changing the nucleotide extensions on the adapter sequences. High resolution is obtained because of the stringent PCR conditions. The AFLP technique works on a variety of genomic DNA samples and no prior knowledge of the genomic sequence is required (Staub *et al.*, 1996; Valsangiacomo *et al.*, 1995).

The AFLP approach provides 10–100 more markers on average than the other approaches do. AFLPs tend to be more informative than RFLPs and RAPDs (Manubens *et al.*, 1999), providing 10-50 times more informative data points per rand spent. It is an ideal tool for determining varietal identity and assessing trueness to type (Bates *et al.*, 1996). An increased understanding of walnut genetics and the ability to use markers to identify seedlings with the

best potential will enable use to be made of a much wider base of germplasm in breeding new cultivars.

2.9.1.3.4 Other DNA-based molecular markers

A number of DNA-based molecular markers not described above have been studied. This includes sequence tagged site (STS) markers, which are useful as anchoring loci between crosses. The most recently used one is the microsatellite or inter-simple sequence repeat (ISSR) marker (Potter *et al.*, 2002). Since the bases flanking the repeat are conserved, but the length of the repeat varies greatly, ISSR-specific primers can be readily designed. Each ISSR is a single locus with multiple allele sizes. Many other STS markers have been developed for use in plant species, including cleaved amplified polymorphic sequences (CAPs), sequence characterized amplified regions (SCARs), allele-specific associated primers (ASAPs), and expressed sequence tags (ESTs), a subset of sequence tagged sites (STSs) derived from cDNA (*href*3, *href*4) in different plants.

2.9.2 Utilisation of markers

Information from DNA markers serves purposes, such as forensic science, paternity testing, identifying genes responsible for genetic diseases, and inferring evolutionary relationships among organisms (Paterson *et al.,* 1991).

With DNA being the only basis of genetic differences between organisms in a population, DNA fingerprinting is presently the ultimate method of biological individualisation (Krawczak and Schmidtke, 1998). Molecular markers have been used extensively in plant species and are applied in breeding programs. Molecular markers in isogenic lines have been used to simplify breeding. In the case of plant species where back-crosses cannot be made, the molecular marker is more difficult to develop (Woeste *et al.*, 1998). Molecular marker linkage maps are valuable tools for analysing and selecting complex traits and studying individual genes that control expression of polygenic traits.

Selection of a DNA marker system for plant breeding depends on project objectives, population structure, the genomic diversity of the species under investigation, marker system availability, time required for analysis, and the cost per unit information (Staub *et al.*, 1996). Each marker system has advantages and disadvantages, and therefore it is critical to evaluate each marker system for its potential utility before use. The range of polymorphism in species also plays a role in marker selection (Potter *et al.*, 2002). So the choice of the most appropriate genetic marker will depend on e.g. the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities and know-how, time constraints, and financial limitations (Paterson *et al.*, 1991).

Properties of good DNA-based molecular markers are (i) highly polymorphic behaviour, (ii) codominant inheritance (which allows for discrimination between homozygotic and heterozygotic states in diploid organisms), (iii)

frequent occurrence in the genome, (iv) even distribution throughout the genome, (v) selectively neutral behaviour (i.e., no pleiotropic effects), (vi) easy access (e.g. fast procedures), (vii) easy and fast assay (e.g. by procedures amenable to automation), (viii) high reproducibility, and (ix) easy exchange of data between laboratories (Krawczak and Schmidtke, 1998).

2.9.3 The use of AFLPs in the identification of walnuts

The major commercial walnut cultivars are derived from superior walnut cultivars and rootstocks from Europe and the USA. Little genetic information is available for walnut crop species despite its widespread commercial use, making genetic variability measurements difficult. According to Fjellstrom *et al.* (1994) genetic relatedness among walnut genotypes is high as many genotypes share common parents. Morphological variability, although readily recorded, can be an unreliable measure of genetic diversity because of the confounding effects of environment and the unknown genetic basis of most morphological attributes (Fjellstrom *et al.*, 1994). The objective to develop markers for this study was to generate highly reliable, relatively inexpensive and reproducible SCARs. A survey of the literature showed that RAPD (Malvolti *et al.*, 1994), RFLP (Fjellstrom *et al.*, 1994; Fjellstrom and Parfitt, 1994) and ISSR (Potter *et al.*, 2002) markers were used to differentiate varieties of *J. regia.* The results supported the assumption of the narrow genetic base for walnuts.

AFLP technique is a powerful tool in saturating markers around a locus of interest (Vos *et al.*, 1995; Xu *et al.*, 1999). Yet it is difficult to employ the AFLP technique directly because of its high cost and complicated methodology. Therefore, converting AFLP markers into other types such as sequence-specific PCR-based markers is critical to expanding their usefulness. Such conversion has already been attempted (Qu *et al.*, 1998; Bradeen and Simon, 1998; Shan *et al.*, 1999). Many converted PCR-based markers have either lost their sequence specificity or their ability to amplify genomic DNA (Shan *et al.*, 1999; Xu *et al.*, 2001). However, some of the results have been encouraging, and a few AFLP markers have been successfully converted into sequence-specific PCR-based markers (Qu *et al.*, 1998; Bradeen and Simon, 1998; Shan *et al.*, 1999; Schwarz *et al.*, 1999).

Therefore, it was decided to generate a large number of mappable loci using AFLPs. This was achieved by a few amplifications. Since AFLP markers are more reproducible and highly specific, their conversion to SCARs promised higher success than other marker systems.

CHAPTER THREE

MOLECULAR DIAGNOSTICS OF CLRV IN *NICOTIANA TABACUM* AND *JUGLANS REGIA* USING IMMUNOCAPTURE REVERSE TRANSCRIPTASE-PCR

INTRODUCTION

Currently, the commercial production of walnut is limited to the Mediterranean regions of Africa, Europe, USA and South China, and to be specific, walnuts are grown in North and South America, Southern Europe, Eastern Asia, USSR, and to a limited extent in Oceania and North Africa (*href*8). The Industrial Development Corporation (IDC) has identified walnut cultivation as an agricultural priority for South Africa. The success of this initiative depends on an efficient and reliable plant propagation system, the accurate identification of germplasm and the detection of viral pathogens. At present, plants are imported from Spain. They are kept by the South African Agricultural Food and Quarantine Inspection Services (SAAFQIS), National Department of Agriculture (NDA) in quarantine for two years before being released if found free from the cherry leaf-roll nepovirus (CLRV) pathogen. CLRV, a member of the nepovirus group, infects a variety of woody and herbaceous hosts (Jones, 1985).

The walnut strain of CLRV is the causative agent of blackline disease, a fatal necrosis at the graft union of *J. regia* and *J. nigra* trees (Jones, 1985; Mircetich and Rowhani, 1984; Zhou *et al.*, 1998). CLRV is spread by nematodes, and the virus is pollen and seedborne (Mircetich and Rowhani, 1984, Rowhani *et al.*, 1995, Rowhani and Mircetich, 1988). The disease occurs in orchards ranging in age from 5 to 70 years, but its incidence is slight in trees below bearing age (Ogawa and English, 1991). On trees grafted on the hypersensitive rootstock *J. nigra*, a black line develops under the bark near the union of rootstock and scion, and when this line completely girdles the tree,

death occurs. In many cases, individual branches show leaf symptoms of chlorotic ringspot and pale yellow discolouration (Rowhani and Mircetich, 1988).

Control of viral diseases in woody crops is best accomplished by establishing new plantings from virus-tested plants (Rowhani *et al.*, 1998). The virus is usually present at very low levels in infected trees where it is unevenly distributed. The initial symptoms are leaf rolling, leaf yellowing and premature leaf loss (Tobis *et al.*, 1995), that inflicts significant damage in orchards (Klonsky and Elkins, 1988). The symptoms appear similar to walnut blight (Martins, 1996).

Detection techniques of high sensitivity are therefore required to index trees for the presence of the virus, a key step in the sanitary selection measures which are still the only effective means to control the disease (Vitushkina *et al.*, 1997; Wetzel *et al.*, 1992). As for virus screening, it is well known that there is no direct remedial treatment for viral diseases once infection becomes established in orchards or vineyards. Early detection, quantitation, and control of virus infection in woody host crops may be difficult due to low or variable titers (Clark and Adams, 1977). The ELISA is routinely used for virus detection (Clark and Adams, 1977). ELISA is a rapid, cost-effective means for detecting viruses in woody plants. ELISA tests are not always adequate for determining the presence of virus in symptomless trees. This is due to their lack of sensitivity and the uneven distribution of the virus within the tree. However, RT-PCR methods are more reliable because of their high sensitivity which increases the virus detection threshold levels (Hu *et al.*, 1995; Wetzel *et al.*, 1992). IC-RT-PCR is a combination of techniques

(Brandt and Himmler, 1995; Nolasco *et al.*, 1993; Wetzel *et al.*, 1992) that allows the detection of plant viruses in oxidising plant extracts through the capture of viroins by immobilised antibodies (Borja and Ponz, 1992; Brooks and Bruening, 1995a and b; Rowhani *et al.*, 1985; Rowhani *et al.*, 1995; Tobias, 1995; Tobis *et al.*, 1995; Werner *et al.*, 1997). The combination of sensitivity, specificity, and speed of the assay makes IC-RT-PCR a most attractive assay for plant quarantine where clean planting stock programs are to be initiated. Werner *et al.* (1997) has previously described a diagnostic method based on immunocapture-RT-PCR.

In this chapter the aim of the study was to develop a PCR based diagnostic test for the sensitive identification of the CLRV pathogen based on new primer sets. This was done by aligning published sequences of CLRV strains and designing new primers. The PCR method was combined with an immunocapture step followed by RT to develop an efficient, rapid and reliable procedure for detecting the virus. This method was used to index propagation material for the presence of the CLRV pathogen.

MATERIALS AND METHODS

3.1 Plant material

The 2-year-old infected walnut host plant (*J. regia* cv. Milizia) and 6-to-8-week-old indicator tobacco plants (*Nicotiana tabacum* cv. White Burley), obtained from the South African Agricultural Food and Quarantine Inspection Services (SAAFQIS), National Department of Agriculture (NDA) (Stellenbosch), were grown in glasshouses. The walnut plants were naturally infected with CLRV, and serve as a positive control for SAAFQIS. For inoculation, tobacco plants were moved to a growth chamber with a day length of 16 hrs, day and night temperatures of 25°C and 20°C respectively and relative humidity of 80%. Plants were inoculated with 10 ng/ml of the CLRV (SAAFQIS culture collection) by rubbing the leaves with carborundum as abrasive. Purified CLRV virus particles (positive controls) were obtained from SANOFI (France). Un-inoculated tobacco plants were used as negative control.

3.2 Sample preparation

Fresh material was cut from walnut dormant infected buds, from CLRV infected tobacco leaves and from healthy tobacco leaves on the day they were macerated. Fresh tobacco leaf (1 g) and dormant walnut buds (1 g) were used. For Enzyme Linked Immunosorbent Assay (ELISA) and the immunocapture tests, the material was ground

in phosphate buffered saline (PBS) [137 mM NaCl, 8.13 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 3.08 mM NaN_3 (pH7.4)] supplemented with 0.05% (v/v) Tween-20 and 2% (m/v) polyvinylpyrrolidone-40 (PBS-Tween-PVP) in a 1:10 (w/v) ratio and centrifuged at 20 000x g for 10 min. The supernatant was subsequently diluted in the PBS-Tween-20 at a ratio of 1:9. The undiluted and diluted samples were used directly.

3.3 ELISA

The standard direct double antibody sandwich (DAS) version method of ELISA was used. The original protocol was taken from Brandt and Himmler (1995) and Rowhani *et al.* (1998). Microtiter plates were coated with CLRV IgG (SANOFI) at a dilution of 1:100 and incubated for 3 hrs at 37°C according to the manufacturer's recommendations. Plates were washed 3 times at 3 min intervals with PBS-Tween. Plates were inoculated with the supernatant from the macerated plant samples (100 µl/well) and incubated overnight at 4°C. Alkaline phosphatase-conjugated antibodies were diluted as recommended by the manufacturer, added to the plates and incubated for 3 hrs at 37°C, and washed 3 times with PBS-Tween as before. A 1 mg/ml concentration of substrate 4-nitrophenyl phosphate (NPP) was prepared in diethanolamine and 100 µl aliquots were added per well. The optical density (OD) readings were taken at room temperature on a spectrophotometer (Multiskan Plus Lab-Systems) at 405 nm after 20, 40 and 60 min.

3.4 Immunocapture

Immunocapture was performed as described above for the standard DAS-ELISA up to where the plant macerates were added, and then performed as described by Werner *et al.* (1997). After the 100 µl aliquots supernatant from the macerated tissue were added to the CLRV IgG (SANOFI) coated plates or tubes, they were incubated overnight at 4°C and washed three times for 3 min with PBS-Tween. The remaining buffer was removed.

3.5 Oligonucleotide primer design

Sequences from the GenBank accession numbers S84124, S84125, S84126, U24694 and Z34265 (Borja *et al.*, 1995; Brooks and Bruening, 1995; Scott *et al.*, 1992) were aligned using the DNAsis program. PCR primers were designed from the consensus homologous regions on 3'-end of RNA-1 and RNA-2 (Figure 2.3, chapter 2) using primer design program Primer3 (*href*5). The Department of Biochemistry, University of Cape Town, South Africa, synthesized primers.

3.6 cDNA synthesis of immunocaptured virus

Reverse transcription (RT) was carried out in the microtiterplate or tubes in which the virus was immunocaptured without disrupting the viroins. The reverse transcriptase

(RTase) reaction was carried out in the plate by adding 25 μl of the reverse transcription mixture to each well. The RT mixture contained 1X buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 10 mM dithioerythritol (DTT), 0.2 mM dNTPs, 1 U RNaseOUT™, 5 U SuperScript™ II (Invitrogen), 1 μM CLRV-specific first strand primer (5'-**TTT GCG TCG GAA AGA TTA CG**-3') and 0.1 % (v/v) Triton X-100. The mixture was incubated for 60 min at 42°C, followed by a heat denaturation at 96°C for 5 min.

3.7 Isolation of the total RNA

Extraction of the virus RNA from infected leaf (tobacco) and bud (walnut) tissues was as described by Zhang *et al.* (1998).

3.8 cDNA synthesis from total RNA

One microlitre sample from the total RNA was added to the RT mixture described above except that the Triton X-100 was not included. The mixture was incubated as described above.

3.9 PCR amplification

Five μl aliquots of the cDNA strands derived from the immunocaptured virus as well as from the cDNA generated from the total RNA isolations, were amplified in a subsequent PCR containing 1X *Taq* polymerase buffer [40 mM Tris-HCl (pH 8.4), 50 mM KCl], 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM each of CLRV-specific first strand and second strand primers (5'-**ACT TCT GGC GAC CGT GTA AC**-3') and 1 U *Taq* polymerase (Promega) in a total volume of 25 μl. Viral cDNA was amplified in a MiniCycler™ thermocycler using the following thermal cycling scheme for 30 cycles: 30 s at 94°C for denaturing, 30 s at 55°C for primer annealing and 30 s at 72°C for elongation. Amplification products were analyzed by electrophoresis (85 V for 60 min) through 1.5% (w/v) agarose gels submerged in 0.5X TBE. The PCR products were detected by ethidium bromide staining.

3.10 Comparative sensitivity between DAS-ELISA and IC-RT-PCR

To compare the sensitivity of the DAS-ELISA to the IC-RT-PCR, sap extracts were used from fresh tobacco infected plant material. Plant material was homogenized and diluted in PBS-Tween-PVP in ten-fold serial dilutions from the 1 g original concentration of the infected tobacco. Hundred μl of each dilution were used for immunocapture. The ELISA was performed in parallel to test for the sensitivity. Ten-fold serial dilutions were used to compare ELISA to IC-RT-PCR.

3.11 DNA sequencing

PCR products were separated on a 1.5% SDS-PAGE gel. The PCR product was eluted from the gel by making an incision in the gel, the piece of gel was weighed and cleaned using the QIAGEN PCR purification kit following the manufacturer's instructions. Sequencing was performed by the University of Stellenbosch Core DNA Sequencing Facility on an ABI PRISM model 3100 sequencer (Applied Biosystems, USA) using an ABI PRISM BigDye™ terminator cycle sequencing ready reaction kit with Ampli-*Taq* DNA polymerase FS (PE Applied Biosystems, USA).

RESULTS

3.12 Selection of primer pairs

The consensus sequence used to design a set of primers was from aligned Genbank sequences (Table 3.1). Primers designed using program Primer3 were CLRV-specific first strand primer (5'-**TTT GCG TCG GAA AGA TTA CG**-3') and CLRV-specific second strand primer (5'-**ACT TCT GGC GAC CGT GTA AC**-3'), (Figure 3.1).

Table 3.1: The accession numbers of the CLRV 3'-end used to design primers.

Accession number	Strain	Host Plant	Length	Homology[a]
U24694	CLRV RNA2, 3'-end	Walnut	1565 nt	96%
Z34265	CLRV (walnut) genomic RNA	Walnut	1588 nt	96%
S84124	3'-end, RNA-1, CLRV, Genomic RNA	Birch	1743 nt	88%
S84125	3'-end, RNA-2, CLRV, Genomic RNA	Rhubarb	1805 nt	88%
S84126	3'-end, R25, CLRV, Genomic RNA	Birch and Rhubarb	1182 nt	88%

[a]The percentage homology to the SAAFQIS CLRV isolate

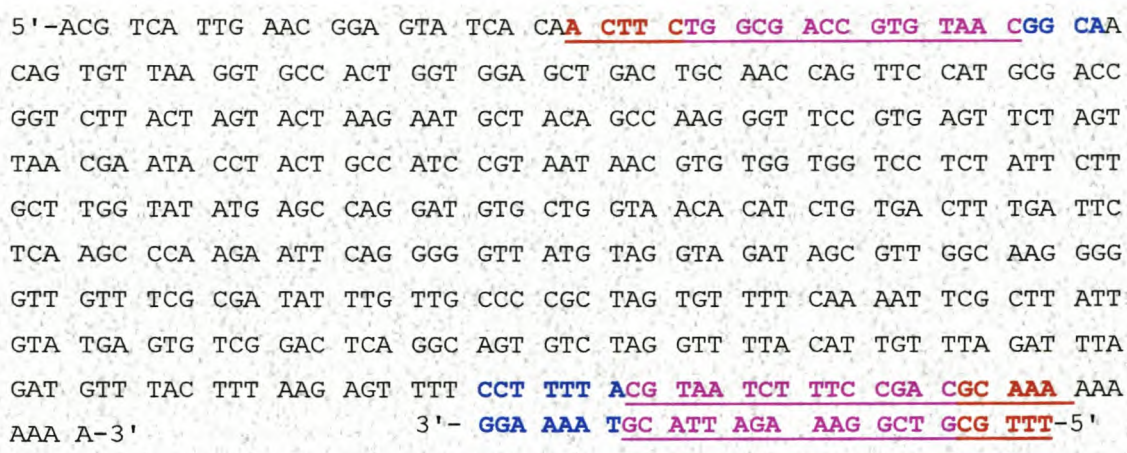

Figure 3.1: The consensus sequence used to design primers for amplification of a CLRV fragment on the RNA-1 and RNA-2 strands. The underlined nucleotides (red and pink) are the primers designed in this study. Pink (underlined) and blue nucleotides are primers according to Werner *et al.* (1997).

3.13 Diagnostic RT-PCR assay for virus detection from total RNA

The primer pair selected from the CLRV genome amplified a product of 430 bp (Figure 3.2). The RT product was amplified from total RNA preparations of infected tobacco leaves and CLRV viroin obtained from SANOFI. No amplified product was observed with tobacco plants not infected with CLRV.

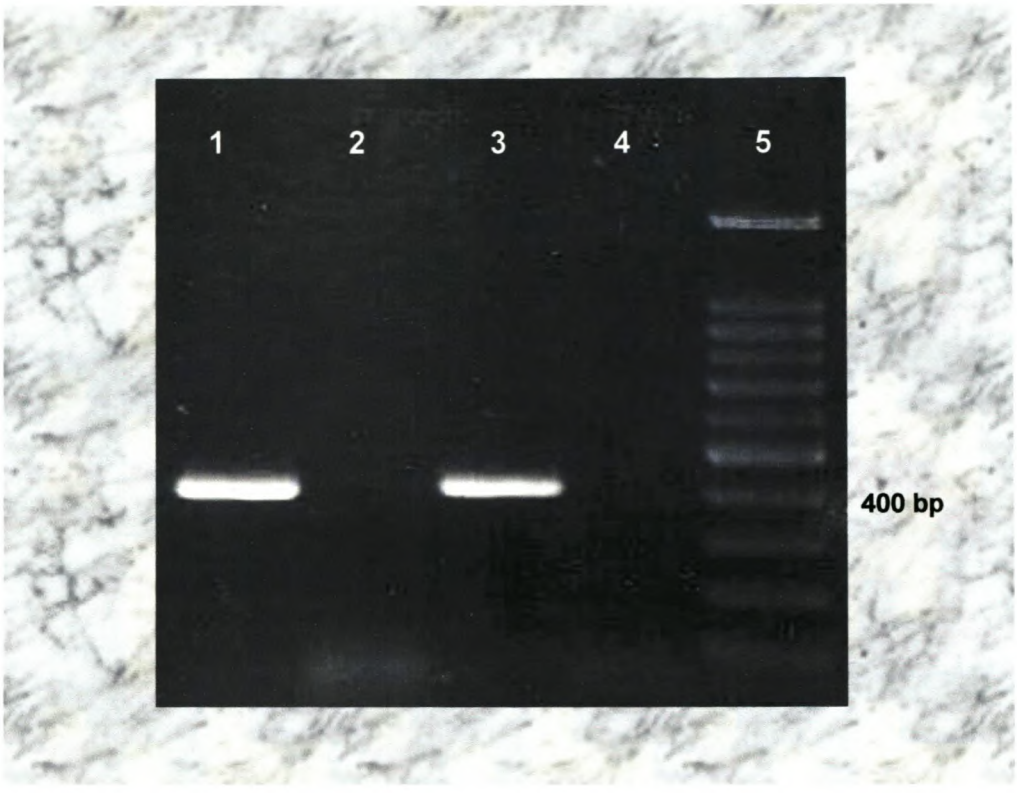

Figure 3.2: Agarose gel electrophoresis of RT-PCR products of CLRV-tobacco infected samples. The infected sample (lane 1), healthy plants (lane 2), PCR product of a CLRV virion obtained from SANOFI (France) (lane 3) and negative control (lane 4). Lane 5 is a 100 bp DNA marker (Promega).

3.14 Sensitivity of the IC-RT-PCR and DAS-ELISA for virus detection

The tenfold dilution series of CLRV infected tobacco leaf samples showed that the threshold for the IC-RT-PCR method was 10^{-4} (Figure 3.3, Table 3.2). The threshold for the ELISA was 10^{-2} (Table 3.2).

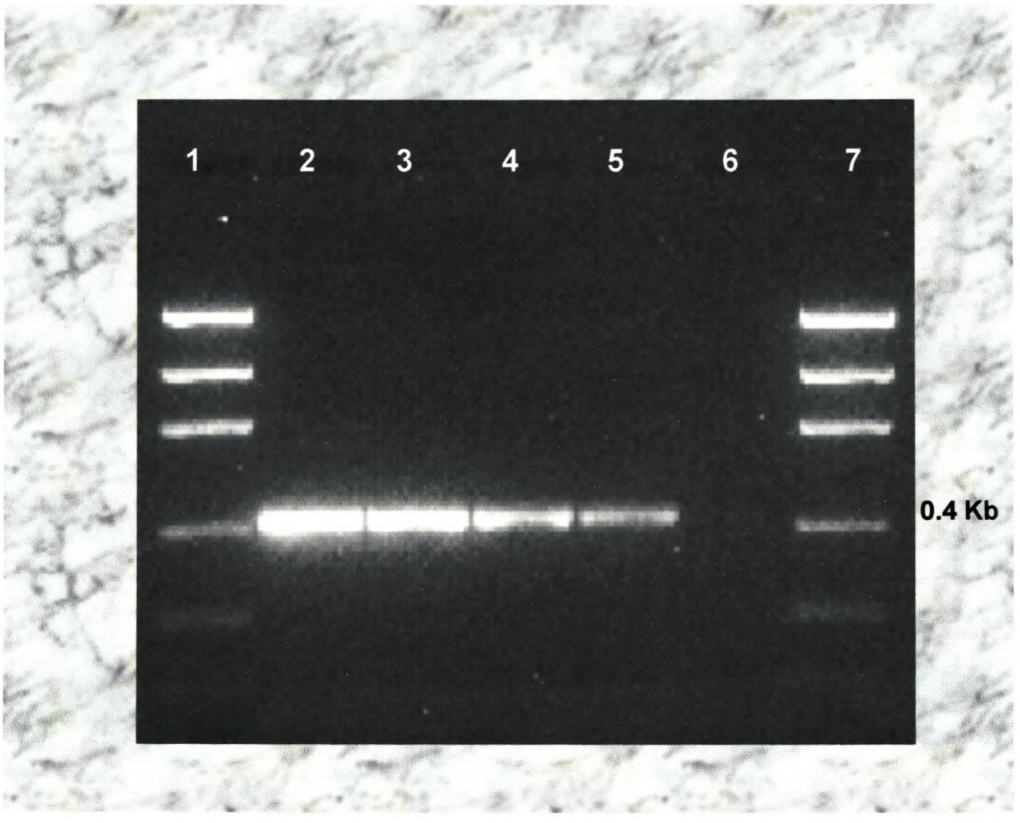

Figure 3.3: Sensitivity of CLRV detection by IC-RT-PCR in infected tobacco leaves. Plant material (100 mg) was homogenized, suspended in PBS-Tween-PVP (1 ml) and serially diluted. Lanes 1 & 7, Low Mass Ladder (Kb), lane 2, 10^{-1}dilution; lane 3, 10^{-2} dilution; lane 4, 10^{-3} dilution; lane 5, 10^{-4} dilution, and Lane 6, Negative control.

Table 3.2: Comparison of the detection range between DAS-ELISA and the IC-RT-PCR.

TEST	DILUTIONS[1]									
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
ELISA	+	+	–	–	–	–	–	–	–	–
IC-RT-PCR	+	+	+	+	–	–	–	–	–	–

[1]Suspensions of the CLRV-infected tobacco leaf samples were serially diluted. The original concentration was 100 mg infected plant material per 1 ml extraction PBS-Tween-PVP buffer. +Virus detected, -Virus not detected

3.15 Sequence analysis

The nucleotide sequences of the 430 bp fragment are presented in Figure 3.4. The sequences aligned to the 3'-untranslated region (UTR) of RNA-1 and RNA-2 shared sequence identity from 96 to 88% in search of Genbank databases (Table 3.1).

```
5'-AGG AAA CTT AAG TAA ACC TTA AAA TCA AAG AAA AAA AAC CTA AAC ACT GCC TGA GTC
CGA CAC TCA TAC AAT AAG CGA ATT TTG GAA CAC TAA CGG GGC AAC AAA TAT CGC GAA ACA
ACC CCC TAG CTA ACG CTA CCT ACC CAC ATA ACC CCC TAA ATT CTT GGG CTT AAG ATT CGA
AGT CAC AGG TGT GTT ACC AGC ACA CCC GGC TCA TAT ACC AAG CAA GAT AGA GGA CCA TCA
CAT GGA TGG CAG TAG ATA TTC GTT AAC TAA ACT AAA CTC ATG GAG CCC TTG GCT GTA GCA
TTC TTA ATA CTA CTA AGA CCG GTC GCA TGG AAC TGA TTG CAG TCA GCC CAC CAG TGT CAC
CGT AAC ACT GTT GCC GTT ACA CGG TCG CCC GGA GTA NNN NNN NNN-3'
```

Figure 3.4: 3'-end of the Cherry leaf-roll nepovirus. The nucleotide sequences of the 430 bp PCR product.

DISCUSSION

In this study, a sensitive diagnostic IC-RT-PCR was developed to detect the CLRV pathogen. A primer pair was designed to amplify a 430-bp non-coding homologous region located on the RNA-1 and RNA-2 strands of this nepovirus. The sensitivity of this detection method was 100-fold higher when compared to the DAS-ELISA, which is currently being used by the SAAFQIS, NDA.

For the choice of the primers, consensus sequences were considered. In this study, the sequence data from the walnut strain of CLRV of the RNA-1 and RNA-2 within the 1565 nucleotide 3'-terminal stretch, which share 98.5% homology (Borja *et al.*, 1995), was chosen. The sequences were aligned to other strains of CLRV isolated from walnut (Brooks and Bruening, 1995), birch (Scott *et al.*, 1992) and rhubarb (Scott *et al.*, 1992) (Table 3.1). One highly conserved primer pair was identified which amplified the 430 bp fragment (Figure 3.2). The identity of this PCR fragment was verified by sequencing of the PCR product which indicates that the PCR primer pair used in this study amplified the CLRV strain.

In a similar study, Werner *et al.* (1997) developed a method to detect CLRV in birch, beech and petunia. Likewise, they aligned the 3' UTR of RNA-1 and RNA-2. Their primers are almost similar to the ones developed in this study (Figure 3.1). Their annealing temperature is slightly lower (51°C), and the amplified fragment is 416 nucleotides.

The quarantine status of the pathogen was a limiting factor during this investigation. The non-availability of infected walnut material, necessitated that the indicator tobacco plant had to be used. Almost all the results are based on the data obtained from the tobacco indicator plant, and therefore the primer pair was not practically tested on CLRV isolates from non-related hosts. The BLAST search of the primer pair indicated that the primers would pick CLRV isolates shown in Table 3.1. The sequence data indicates that these isolates will be amplified. This makes the use of the primer pair combination more versatile for the SAAFQIS, NDA. For comparative sensitivity and specificity between DAS-ELISA and IC-RT-PCR, tobacco plants were used. Normally the RT-PCR assay is 1 000 to 10 000 times more sensitive than DAS-ELISA (Nolasco *et al.,* 1993; Werner *et al.*, 1997). Results on IC-RT-PCR compared to the DAS-ELISA show a 100-fold increase in sensitivity. The reason for the reduced sensitivity is normally ascribed to phenolic compounds in the ground samples, which inhibits the reaction to some extent. In this study the sensitivity was based on IC-RT-PCR not RT-PCR. According to Werner *et al.* (1997), the RT-PCR method increases the sensitivity because of high quality RNA used. The RT-PCR-based detection method is of special value because CLRV accumulates to low levels and is unevenly distributed in host plants (Mas and Pallas, 1996), thus not allowing isolation of purified particle preparations suitable for antibody production and development of serological methods.

The IC-RT-PCR method described in this study will in future be adopted for routine testing. The combination of the immunocapture step and the sensitivity of PCR meets the most important criteria required of a reliable diagnostic system and needs some

blind testing so as not to get false positives. Despite the decline of many thousands of walnut trees, blackline can be managed effectively, and its harmful effects mitigated in many new and existing orchards if the growers use appropriate CLRV identification technology. Beyond this, many research institutes world-wide will continue to seek solutions to this pathogen. Ultimately, this could lead to significant improvements in the quality of technique, streamlining of importation and quarantine programs, and facilitating international trade in plant materials.

CHAPTER FOUR

IDENTIFICATION OF WALNUT GERMPLASM USING SEQUENCE CHARACTERISED AMPLIFIED REGIONS FROM AMPLIFIED FRAGMENT LENGTH POLYMORPHISM ANALYSIS

INTRODUCTION

Walnut cultivars are difficult to differentiate phenotypically. The traditional methods of detecting genetic variability, based on the observation of morphological and phenotypical characters are both time consuming, and affected by the environment. According to Fjellstrom *et al.* (1994) genetic relatedness among walnut genotypes is high as many genotypes share common parents. The introduction of molecular biology techniques, such as DNA-based molecular markers, has provided a new opportunity for genetic characterization (Potter *et al.*, 2002), allowing for the direct comparison of different genetic material without environmental influences. The PCR has provided the foundation for a range of molecular marker techniques, which nowadays tend to substitute classical marker systems (Breyne *et al.*, 1997). The characterization of walnut genotypes is important both for evaluating the effects of selection over time and the development of crossing schemes in plant improvement programs (Nicese *et al.*, 1997).

AFLP analysis is based on the selective amplification of a restricted number of genomic fragments. This is achieved by digesting the DNA with two enzymes, followed by ligation of complementary adapters, and PCR amplification with primers consisting of the adapter sequences, extended with a variable number of 3' nucleotides (Breyne *et al.*, 1997). The AFLP technique represents an indigenous combination of RFLP analysis and PCR, resulting in highly

informative fingerprints (*href*1). Routinely 50 and more restriction fragments are amplified simultaneously and visualized on denaturing polyacrylamide gels (Breyne *et al.*, 1997). In principle, both dominant as well as codominant markers can be analyzed (Breyne *et al.*, 1997).

The high efficiency, reproducibility and reliability of AFLP analysis have been supported by a number of recent publications (Bradeen and Simon, 1998; Cervera *et al.,* 1996a and b; Paran and Michelmore, 1993; Qu *et al.,* 1998; Shan *et al.,* 1999; Terashima *et al.,* 2002; Valsangiacomo *et al.*, 1995; Vos and Kuiper, 1997; Xu *et al.,* 2001). AFLPs have been used in many plant species, confirming their use in plant genetic studies. They have been used to assess genetic diversity in wheat (*Triticum aesticum*) (Barrett and Kidwell, 1998; Burkhamer *et al.*, 1998), barley (*Hordeum vulgare*) (Ellis *et al.*, 1997; Shan *et al.*, 1999), maize (*Zea mays*) (Ajmone-Marsan *et al.*, 1998) and *Eucalyptus urophylle* (Gaiotto and Bramucci, 1997).

Despite the reported use of the AFLP technique in various genetic analyses, little information is available regarding the cloning of AFLP fragments for conversion to other marker types (Shan *et al.*, 1999). The value of such a conversion is that other types of marker analysis, such as sequence characterized amplified regions (SCARs), are less expensive and can be more easily employed using large populations in commercial fields (Xu *et al.*, 2001). However, information or reports on the conversion of AFLP markers into SCAR markers is limited.

A fingerprinting method for cultivar and rootstock authenticity for the imported walnut plants for the South African industry is needed. The aim of this study was to convert AFLP markers in walnuts to derivative genetic SCAR markers.

MATERIALS AND METHODS

4.1 Germplasm

Juglans regia germplasm used for this study consist of cultivars and hybrids mainly from the California walnut breeding program (Table 2.2, Chapter 2). The plant material used was purchased by the Industrial Development Corporation (IDC) from Spain. Plant material [*J. regia* cvs. Chandler, Franquette, Hartley, Serr, Sunland, Vina and the Paradox rootstock (*J. regia* X *J. hindsii*)] was kept in the ARC Infruitec-Nietvoorbij glasshouses. Young, expanding leaves were harvested from walnut plants and were stored at –80°C until needed for DNA isolation.

4.2 Isolation of genomic DNA

Total genomic DNA was isolated from leaf material following the method of Nicese *et al.* (1998) with minor modifications from Doyle and Doyle (1987). The DNA was used for AFLP amplification without further purification steps. Two grams of plant material were ground to fine powder in liquid nitrogen. The ground material was transferred to 10 ml 2% CTAB buffer [1% (m/v) PVP-40, 1% (v/v) β-Mercapto-ethanol, 0.1% Na-Bisulfite, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM Na-EDTA (pH 8.0)] preheated to 65°C, and the mixture was

incubated at 60°C for at least 30 min with intermittent shaking. The mixture was allowed to cool to room temperature, and the lysate was extracted with 10 ml chloroform/isoamyl alcohol (24:1) and centrifuged for 15 min at 13 000x g at 15°C. Twenty μg/ml of RNase was added to the lysate and incubated for 30 min at 37°C. In order to precipitate the nucleic acids, the aqueous fraction was mixed with a two-third volume of cold isopropanol and the precipitate was pelleted by spinning at 13 000x g for 10 – 15 min at 4°C. The nucleic acid precipitate was washed with 76% ethanol (wash buffer) with 10 mM ammonium acetate and air-dried overnight before being resuspended in 1 ml TE buffer. The concentration of extracted DNA was determined using a spectrophotometer at 260 nm.

4.3 Restriction digestion of genomic DNA

Restriction fragments for amplification were generated by cutting the genomic DNA with two restriction enzymes, *Pst*I (rare-cutting enzyme) and *Mse*I (frequent cutter). A total volume of 25 μl, containing 300 ng genomic DNA, 2.5 U of each restriction enzyme (*Pst*I and *Mse*I), 5 μl restriction-ligation buffer [final concentration of 5 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 50 mM K-acetate] was mixed briefly and incubated overnight at 37°C.

4.4 Restriction – Ligation reaction

A 50 µl cocktail was made up of the following reagents: 23 µl of the adapter solution [1 µl of *Pst*I adapter (5 pmol), 1 µl of *Mse*I adapter (50 pmol), 0.4 mM ATP, 5 µl 5x reaction buffer and 15 µl AFLP grade water] and 2 µl Invitrogen T4 DNA ligase were added to each of the tubes containing 25 µl restriction reaction. The mixture was gently mixed, briefly centrifuged and incubated overnight at 20°C. Five microlitres of the restriction-ligation reaction genomic DNA products was visualized on a 1.5% (m/v) agarose gel with 0.5x TBE and stained with ethidium bromide. A 10-fold dilution [1:10 in 1x $TE_{0.1}$ (10 mM Tris-HCl pH 8.0; 0.1 mM EDTA)] of the ligation mixture was used for subsequent pre-amplification using the AFLP primers.

4.5 Pre-amplification

The PCR reaction was carried out in a total volume of 25 µl. Ten microlitre of mix 1 [0.25 µM of each primer (*Pst*I and *Mse*I), 0.2 mM of each dNTP (Roche) (dCTP, dGTP, dATP, and dTTP) and AFLP grade water], 12.5 µl of mix 2 (1.5 mM $MgCl_2$, 1x PCR buffer, 0.02 U Invitrogen *Taq* DNA polymerase and AFLP grade water) and 2.5 µl from the 10-fold diluted restriction-ligation genomic DNA products described above were added together. The mixture was gently mixed and briefly centrifuged. The PCR cycling parameters were 30 s at 94°C for

(denaturing), 60 s at 56°C for (annealing) and 60 s at 72°C for (elongation) for 30 cycles. Ten microlitre of the pre-amplification products were separated on a 1.5% (m/v) agarose gel with 0.5x TBE and stained with ethidium bromide.

4.6 Primer labelling

Primer labelling was performed by phosphorylating the 5'-end of the AFLP primers with Invitrogen T_4-polynucleotide kinase (PNK) and γ^{33}P-ATP. In a cocktail, an AFLP *Pst*I forward extension (0.05 µl of 50 ng/µl) (Table 4.1) was labelled with 0.025 µl PNK buffer (10x) and 0.01 µl Invitrogen PNK (10 U/µl), 0.05 µl γ^{33}P-ATP (10 µCi/µl) and AFLP grade water for a total volume of 0.25 µl. The mixture was incubated at 37°C for 2 h and then the microfuge tubes were transferred to 65°C for 10 min to denature PNK.

Table 4.1 AFLP primers used in this study for scoring polymorphisms.

Type	Primers	AFLP primer character	Selective nucleotides	bp	2+/3+
Pst I	P_{02}	5'- AGA CTG CGT ACA TGC A	GC- 3'	18	2+
	P_{03}	5'- AGA CTG CGT ACA TGC A	GG- 3'	18	2+
	P_{15}	5' - GAC TGC GTA CAT GCA G	CA- 3'	18	2+
	P_{19}	5' - GAC TGC GTA CAT GCA G	GA- 3'	18	2+
	P_{20}	5' - GAC TGC GTA CAT GCA G	GC - 3'	18	2+
	P_{22}	5' - GAC TGC GTA CAT GCA G	GT - 3'	18	2+
	P_{75}	5' - GAC TGC GTA CAT GCA G	GTA - 3'	19	3+
Mse I	M_{12}	5' - GAT GAG TCC TGA GTA A	AC- 3'	18	2+
	M_{13}	5' - GAT GAG TCC TGA GTA A	AG- 3'	18	2+
	M_{14}	5' - GAT GAG TCC TGA GTA A	AT- 3'	18	2+
	M_{15}	5' - GAT GAG TCC TGA GTA A	CA- 3'	18	2+
	M_{20}	5' - GAT GAG TCC TGA GTA A	GC - 3'	18	2+
	M_{47}	5' - GAT GAG TCC TGA GTA A	CAA - 3'	19	3+
	M_{48}	5' - GAT GAG TCC TGA GTA A	CAC- 3'	19	3+
	M_{49}	5' - GAT GAG TCC TGA GTA A	CAG- 3'	19	3+
	M_{50}	5' - GAT GAG TCC TGA GTA A	CAT- 3'	19	3+
	M_{61}	5' - GAT GAG TCC TGA GTA A	CTG - 3'	19	3+
	M_{65}	5' - GAT GAG TCC TGA GTA A	GAG - 3'	19	3+
	M_{66}	5' - GAT GAG TCC TGA GTA A	GAT - 3'	19	3+

4.7 Selective amplification

Using a 10-fold diluted pre-amplification mixture, selective amplification was performed in 0.2 ml PCR reaction tubes. For a total volume of 10 µl, 3.5 µl of mix 3 [0.3 µl unlabelled *Mse*I primer (50 ng/µl), 0.4 µl of each dNTP (5 mM) (dCTP, dGTP, dATP, and dTTP), and AFLP grade water], 4 µl of mix 4 [0.25 µl labelled *Pst*I primer, 1 µl PCR buffer (10x), 0.3 µl $MgCl_2$ (50 mM), 0.04 µl Invitrogen *Taq* DNA polymerase (5 U/µl), and AFLP grade water] and 2.5 µl diluted pre-amplification mixture were mixed.

The PCR thermal cycling parameters were modified from the described one by increasing the annealing temperature initially to 65°C, for the first cycle, then lowering it by 0.7°C decrements for 11 cycles until 56°C was attained. The following 22 cycles the annealing temperature was at 56°C.

Ten microlitre of formamide loading buffer [80% (v/v) formamide, 10 mM EDTA (pH 8.0), 1.83 mM xylene cyanol and 1.6 mM bromophenol blue] was added to each microfuge tube. AFLP PCR products were denatured for 4 min at 94°C.

4.8 Polyacrylamide gel electrophoresis and autoradiography

The gel was made up with 5% (v/v) polyacrylamide (40%), 1x TBE and 7 M urea, 250 µl ammonium persulphate (APS) (10%) and 50 µl TEMED. The 36 cm gel was pre-run at 80 W (40 – 50 v/cm) for 30 min. Five microlitres of each selective amplification product was loaded on the 5% (v/v) polyacrylamide gel. Bromo-phenol blue (1 mg/ml) and xylene cyanol (1 mg/ml) were used as tracking dyes. After loading the samples, the gel was run for 1 h 40 min at constant voltage at normal temperatures between 42 – 45°C. The gel was dried onto filter paper (Whatman 3MM) for 1.5 to 2 h at 80°C, using a standard slab gel drier.

The gel was exposed to standard X-ray film overnight without intensifying screens, or to BioMax MR film (Kodak) with the exposure time of 48 – 72 h. The gel was soaked in a shallow plastic tray containing a silver staining solution (1.0 g/l silver nitrate and 0.75 ml/l ultra-pure formaldehyde) and agitated for 3 min. It was then agitated for 3 min in the developing solution (30 g/l sodium carbonate, 0.75 ultra-pure formaldehyde and 2 mg/l sodium thiosulphate) and finally for 3 min in the stop solution (10% acetic acid). The gel was washed (twice) with distilled water to remove excess sodium carbonate, dried overnight at room temperature and photographed.

4.9 Isolation of AFLP fragment from 5% polyacrylamide gel

Fragments of interest were excised from the silver-stained 5% (v/v) polyacrylamide gel and placed into 30 µl of distilled, autoclaved water in 0.2 ml PCR tubes. The tubes were incubated at 95°C for 20 min. The tubes containing the fragments were stored at -20°C until further use.

4.10 Re-amplification of excised fragment

Re-amplification was performed according to Habu *et al.* (1997). Amplification reactions were performed in 20 µl volumes containing 1 µl of a 10-fold dilution containing excised fragment, 50 ng of each AFLP primer (*Mse*I and *Pst*I), 100 µM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1.5 mM $MgCl_2$, 1x PCR buffer [50 mM KCL, 10 mM Tris-HCL (pH 8.3)] and 1 U Bioline *Taq* DNA polymerase reagents. The amplification reactions were run for 30 cycles as described in section 4.5. Amplified products were checked on a 1.5% (m/v) agarose gel with 0.5x TBE and stained with ethidium bromide.

4.11 Ligations

The re-amplified fragment was cloned into pGEM®-T (Figure 4.1) using the pGEM®-T Easy Cloning Vector kit (Invitrogen Corporation, USA) according to the manufacturer's instructions. The reaction procedure was as shown in Table 4.2. For maximum transformation, the reaction volumes were thoroughly mixed by pipetting and incubated overnight at 4°C.

Table 4.2: Ligations using the pGEM®-T Easy Vector kit.

	AFLP fragment	Positive control	Background control
2X Rapid Ligation Buffer, T4 DNA Ligase	5 µl	5 µl	5 µl
pGEM®-T Easy vector (50 ng)	1 µl	1 µl	1 µl
PCR product (12 ng/µl)	3 µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase (3 Weis U/µl)	1 µl	1 µl	1 µl
Deionized water	-	1 µl	3 µl
TOTAL	10 µl	10 µl	10 µl

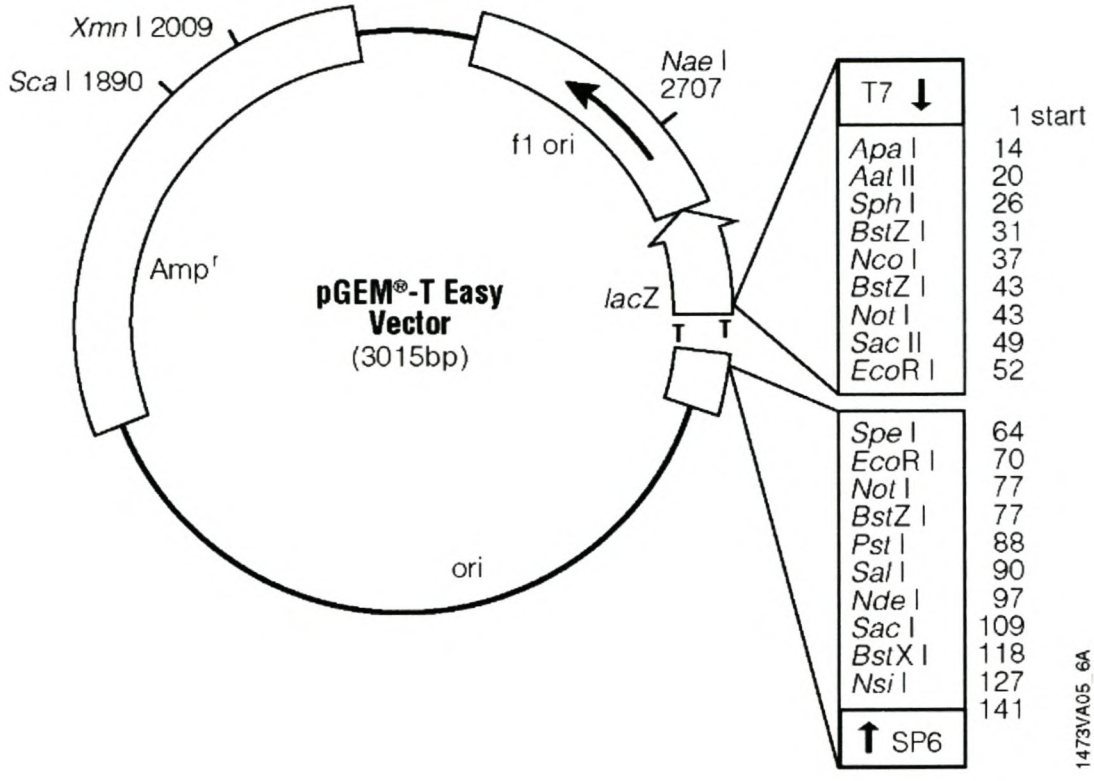

Figure 4.1: pGEM®-T Easy Vector map. PCR products are cloned into the multiple cloning site of the pGEM®-T Easy Vector. The *EcoR*I restriction site has 3'-T overhangs that improve the efficiency of ligation of the PCR product. T7 and SP6 RNA polymerase promoters flank a multiple cloning region within the α-peptide coding region for β -galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening on indicator plates. Adapted from *href*4.

4.12 Transformations using the pGEM®-T Easy Vector ligation reactions

The transformation procedure was performed according to the manufacturer's instruction (Invitrogen Corporation, USA). LB plates [1.0% (m/v) bacto-tryptone, 0.5% (m/v) yeast extract, 0.5% (m/v) NaCl] with ampicillin (10 μg/ml) were made. X-Gal (20 mg/ml) and IPTG (200 mg/ml) were added to LB plates with ampicillin. DH10B High Efficiency Competent Cells (Invitrogen Corporation, USA) were used. Four hundred and fifty μl SOC medium at room temperature was used. After 1.5 h incubation at 37°C with shaking (150 rpm), cells were centrifuged at room temperature in a benchtop microfuge at 25 000x g for 60 s and resuspended in a 100 μl SOC medium.

4.13 Amplification of clones

Recombinant colonies (white clones) were picked up using toothpicks and submerged in 10 μl double distilled water in PCR tubes. PCR total volumes of 25 μl reactions were made with 0.4 μM of both plasmid primers (T7 and SP6), 100 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1x PCR buffer [50 mM KCL, 10 mM Tris-HCL (pH 8.3)], 1.5 mM $MgCl_2$ and 0.05 U Bioline *Taq* DNA polymerase. The amplification reactions were run for 30 cycles using the same cycling parameters as described in section 4.5. Amplified PCR products were run on a 1.5% (m/v) agarose gel with 0.5x TBE and stained with ethidium bromide.

4.14 DNA sequencing

For each AFLP marker, colonies containing a cloned fragment of the same size as that of the original AFLP marker were selected for sequencing. Amplified PCR products were purified using the QIAGEN PCR purification kit following the manufacturer's instructions. The purified products were sequenced using both T7 and SP6 amplification plasmid primers. Sequencing was performed by the University of Stellenbosch Core DNA Sequencing Facility on an ABI PRISM model 3100 sequencer (Applied Biosystems, USA) using an ABI PRISM BigDye™ terminator cycle sequencing ready reaction kit with Ampli-*Taq* DNA polymerase FS (PE Applied Biosystems, USA).

4.15 Primer design

DNA sequences of clones corresponding to a specific AFLP marker were chosen and aligned using DNAsis program. AFLP PCR sequenced products each with two or three selective nucleotides (Table 4.1) and the internal sequences from both ends of the AFLP marker were used to design candidate SCAR primers using *Primer3* (*href*5). Different base pairs lengths and different temperatures were considered. Primers were synthesized by Integrated DNA Technologies, INC.

4.16 Amplification of SCARs

PCR reactions were performed in 20 μl volumes containing 16 ng of genomic DNA, 0.3 μM of each SCAR primer, 200 μM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1x PCR buffer, 1.5 mM $MgCl_2$ and 0.1 U Invitrogen *Taq* DNA polymerase. Amplified products were run on a 1.5% (m/v) agarose gel with 0.5x TBE and stained with ethidium bromide.

4.17 Restriction of SCAR products

AFLP marker sequences, used for SCAR primer design, were used to predict putative restriction sites. Several different or similar restriction enzymes predicted to cut SCAR product sequences giving different sized (base pairs) products (Table 4.6) were selected. Amplified SCAR products were digested with five different endonucleases (*Acc*I, *Alu*I, *Bgl*I, *Hae*III, *Hinf*I, *Rsa*I) with different base-pair recognition sequences.

4.18 Sequencing of 'restriction sites'

Cultivars were characterized based on restriction analysis information. When ambiguities were found after restriction reaction, PCR-SCAR products were sequenced.

4.19 Characterization of cultivars

Complete digestion was obtained of the walnut genomic DNA with the restriction enzymes *Pst*I and *Mse*I (Figures 4.2A). Pre-amplification products were reproducibly generated in the AFLP procedure (Figure 4.2B).

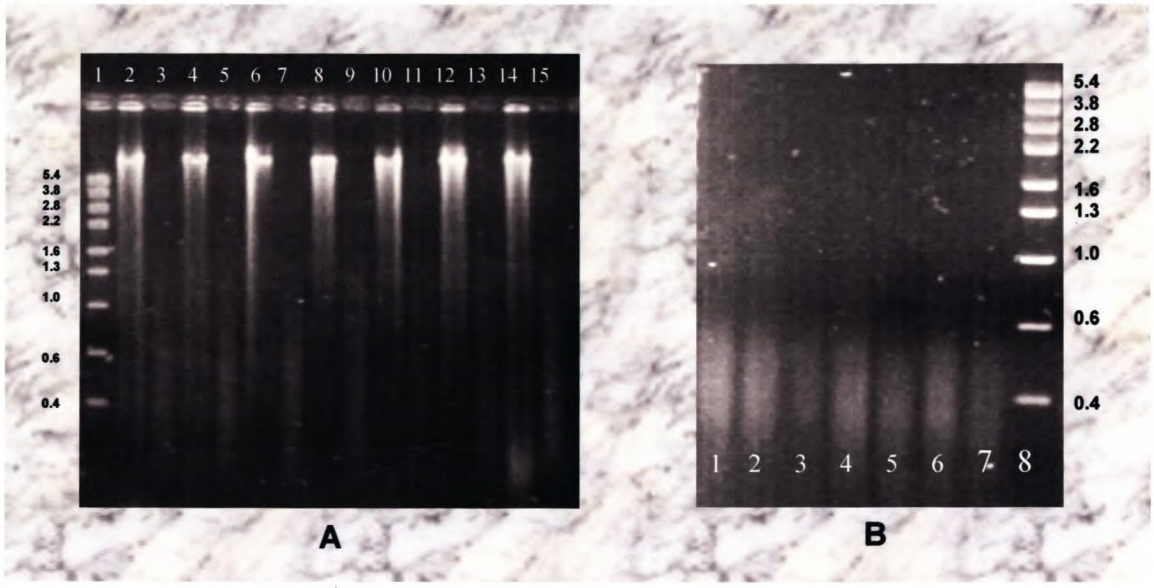

Figure 4.2: Agarose gel electrophoresis of walnut genomic DNA. **A**: Lane 1, EL DNA Marker (Kb) (IPB, US). The even numbered lanes represent undigested genomic DNA (300 ng) of cvs. Chandler, Franquette, Hartley, Serr, Sunland and Vina, and the Paradox rootstock respectively. The odd numbered lanes are *Pst*I/*Mse*I digested genomic DNA (300 ng). **B**: Pre-amplified DNA of the *Pst*I/*Mse*I digest loaded in the same order as A (lanes 1–7), and lane 8, EL DNA Marker (IPB, US).

Using *Pst*I and *Mse*I primer combinations (Table 4.1), typical polymorphic fragments were detected. All the 27 primer combinations revealed some polymorphisms among the six cultivars and the rootstock examined (Table 4.3). The number of bands per lane ranged between 62 (P_{15}/M_{20}) and 132 (P_{75}/M_{14}); the numbers of polymorphic bands per primer combination were between 4 (P_{02}/M_{48}) and 44 (P_{22}/M_{66}) (Table 4.3). Of these combinations, the polymorphic fragments of primer combination P_{22}/M_{66} (47.8%) and P_{75}/M_{49} (19.8%) were selected for further analysis. The cultivars and Paradox had unique fingerprints when only the primer combination of P_{22}/M_{66} was used to generate AFLP markers (Figure 4.3).

Table 4.3: Summary of AFLP profiles for six walnut cultivars and one rootstock.

Primer combination	Total number of bands	Polymorphic fragments	% Polymorphism
P_{02}/M_{13}	65	6	9.2
P_{02}/M_{20}	64	8	12.5
P_{02}/M_{48}	63	4	6.3
P_{03}/M_{13}	86	8	9.3
P_{15}/M_{13}	65	5	7.7
P_{15}/M_{20}	62	6	9.7
P_{15}/M_{48}	70	8	11.4
P_{19}/M_{12}	68	7	10.3
P_{19}/M_{13}	90	6	6.7
P_{19}/M_{14}	98	7	7.1
P_{19}/M_{15}	69	6	8.7
P_{20}/M_{61}	85	13	15.3
P_{20}/M_{65}	78	11	14.1
P_{20}/M_{66}	104	26	25.0
P_{22}/M_{61}	62	21	33.9
P_{22}/M_{65}	109	29	26.6
P_{22}/M_{66}	92	44	47.8
P_{75}/M_{12}	104	30	28.8
P_{75}/M_{13}	80	6	7.5
P_{75}/M_{14}	132	18	13.6
P_{75}/M_{15}	88	20	22.7
P_{75}/M_{20}	70	16	22.9
P_{75}/M_{47}	64	14	21.9
P_{75}/M_{48}	120	6	5.0
P_{75}/M_{49}	86	17	19.8
P_{75}/M_{50}	64	19	29.7
P_{75}/M_{13}	110	20	18.18

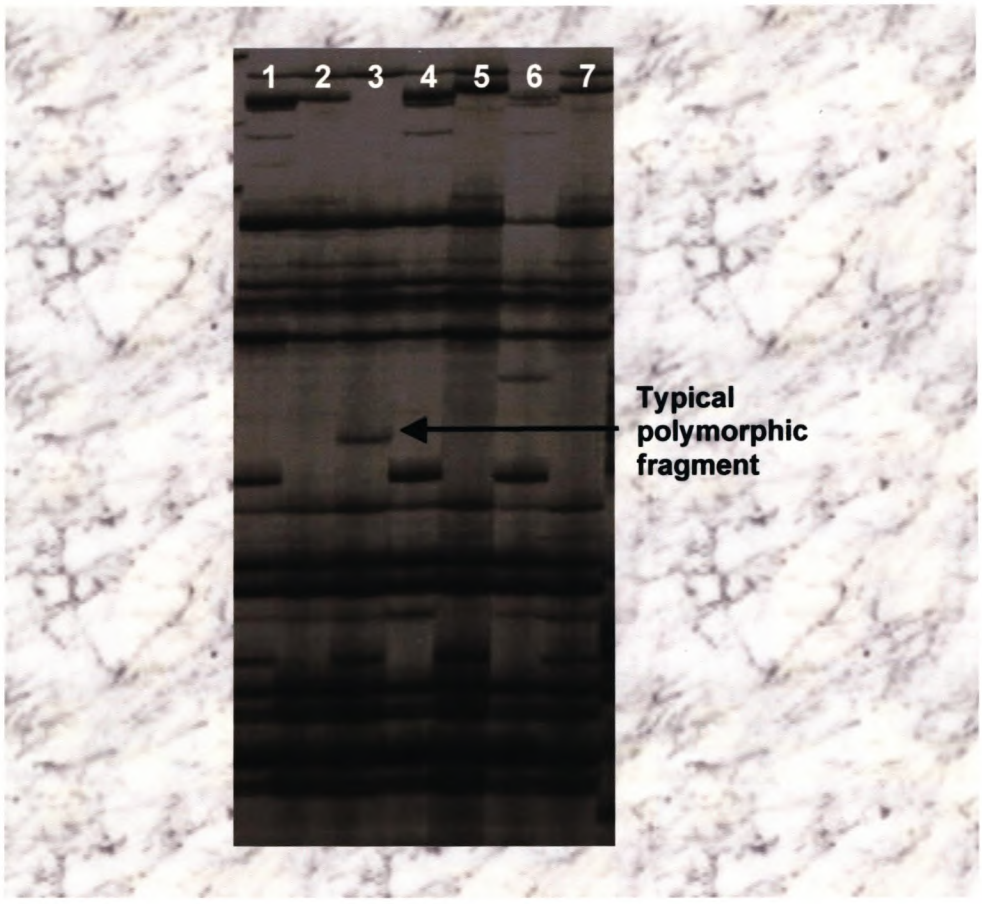

Figure 4.3: 5% Polyacrylamide gel electrophoresis of AFLP markers from six *J. regia* cvs. and *J. hindsii* X *J. regia* (Paradox) hybrid. Lane 1 – Chandler, 2 – Franquette, 3 – Hartley, 4 – Serr, 5 – Sunland, 6 – Vina and 7 – Paradox. The AFLP fragment indicated with the arrow was used for further characterization.

4.20 Sequencing and design of SCAR primers

For each AFLP marker, 10 colonies were selected for further analysis. By using the labelled AFLP primer, almost all colony-PCR products were similar in size and corresponded to the original AFLP fragment excised from the gel (Figure 4.4).

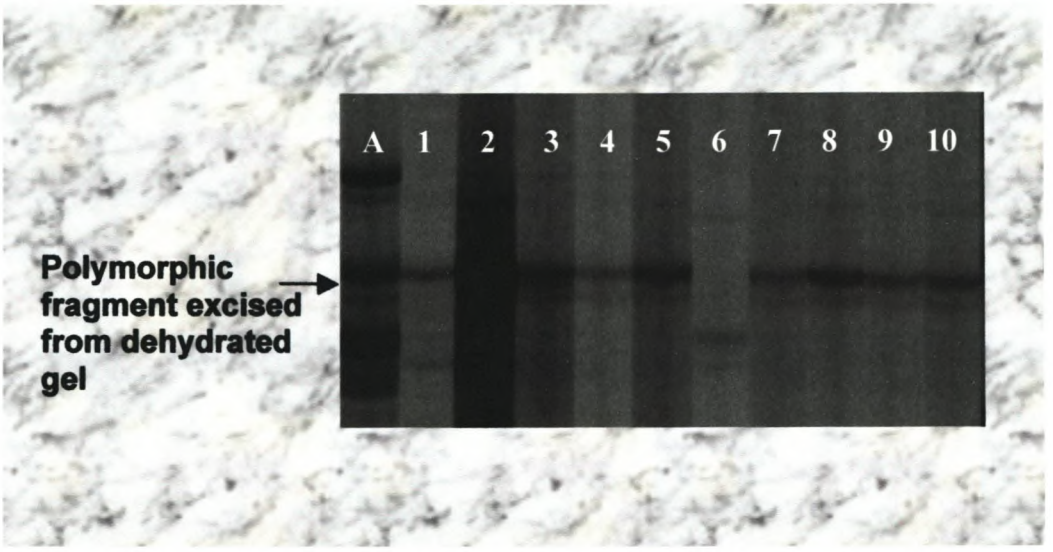

Figure 4.4: 5% Polyacrylamide gel electrophoresis of a cloned AFLP marker. A – Hartley AFLP original fragment, 1 – 10 PCR products from the inserts corresponding to the original AFLP. Lane 6 shows a smaller PCR product compared to the original AFLP fragment (A) and other PCR products of clones.

The cloned AFLP fragments which were sequenced, confirmed the size of the AFLP marker (Table 4.4). From the consensus sequence of the cloned fragments SCAR primers were designed (Figure 4.5). This revealed the sizes of PCR-SCARs that could be expected (Table 4.4).

Table 4.4: Sizes of the AFLP markers and the sizes of the SCAR markers derived from the fragments generated by different AFLP primer combinations.

Cultivar	AFLP primers	Sizes of the excised fragment determined by sequence analysis	Expected sizes from PCR-SCARs
Chandler	P_{20}/M_{61}	231 bp	186 bp
Franquette	P_{75}/M_{49}	259 bp	187 bp
Hartley	P_{22}/M_{66}	291 bp	230 bp
Serr	P_{75}/M_{49}	211 bp	200 bp
Sunland	P_{75}/M_{49}	308 bp	298 bp
Vina	P_{75}/M_{49}	307 bp	285 bp
Paradox	P_{22}/M_{66}	306 bp	263 bp

5'- **GAT GAG TCC TGA GTA AGA T**AC TCA AAT CAA TTG AAT GGG TTG
AAT TTC GGA CTT TGA GAT TCA TAT GAT GGA GCT ATT GAA GTG TGG
CTC TTT GGG CAA TGT AGG ACA ATT CTT TTA CAT CAC AAT AAC CCT
AAA GCT AAC TGA ATC CAT GTT GAC AGT TCA AAC TCA AAC ATG ATG
ATA ATA TAC ATT TTT TTG TAA CTA CAG AAT CTG ATC CCT TAG GAA
ACT ACA TAG AAA TCT GGC AGA AAT TTG AAG AAA GAT CAG ATT TCA
TGC ACA ACT GAG AGA GAG AGA GAG AGA GAG AGA GAG AGA GAG ATT
ATT TTT GG**A CCT GCA TGT ACG CAG TC** – 3'

Figure 4.5: Consensus sequence (306 bp) of 3 sequenced clones from Paradox. New specific SCAR primers, namely 22-mer (blue, 5' end) and 24-mer (pink, 3' end) primer pair were designed in an attempt to amplify a candidate PCR-SCAR marker of 263 bp from walnut genomic DNA. The AFLP *Mse*I primer sequence is in green (3' end, bold), the AFLP *Pst*I primer is in red (5' end, bold).

4.21 Development of SCAR markers

SCAR primer pairs designed are as shown in Table 4.5. The candidate SCAR primer pair, designed to amplify the fragment of a specific cultivar was screened against the genomic DNA of the other 6 cultivars and Paradox. Figure 4.6 shows that the PCR-SCAR products were generated in all the 6 cultivars with Franquette and Hartley SCAR primers.

Table 4.5: SCAR primers designed for the identification of cultivars.

Name	Primer sequences in a 5' – 3' orientation	Bp	Temperature
Chandler-forward	5' – TTG GAG TTT TTA TGC TTG AAT TG – 3'	23	52°C
Chandler-reverse	5' – TAA TTT CCA AGA ATT GCC CAA AC – 3'	23	
Franquette-forward	5' – CTT GGT TTT GAA GTG AGT ATG ACG –3'	24	56°C
Franquette-reverse	5' – CAC TAG TAG ACA CAC ACA CCC – 3'	21	
Hartley-forward	5' – GAT TCT GAC ATT TTG CGA GGC CCC – 3'	24	62°C
Hartley-reverse	5' – CCC TCC CGC GCA TGA TAA AGT TAT TA – 3'	26	
Serr-forward	5' – GTC TCT TTG ACC TTT TAG GTT TGC – 3'	24	56°C
Serr-reverse	5' – ACT GTA GCA ATC TCT ATC CGA AGG – 3'	24	
Sunland-forward	5' – ATA CTG TGG CCT TCT GTT AGG AAG – 3'	24	56°C
Sunland-reverse	5' – AGT TTC CCA CTT TAC CTG GAA TG – 3'	23	
Vina-forward	5' – GGT TGA ACT CAC GCA AAT AAA TTA C – 3'	25	56°C
Vina-reverse	5' – ACT CAA CCA ATG TTG ACC TCG T – 3'	22	
Paradox-forward	5' – CAA ATC AAT TGA ATG GGT TGA A – 3'	22	56°C
Paradox-reverse	5' – CTC TCT CTC AGT TGT GCA TGA AAT – 3'	24	

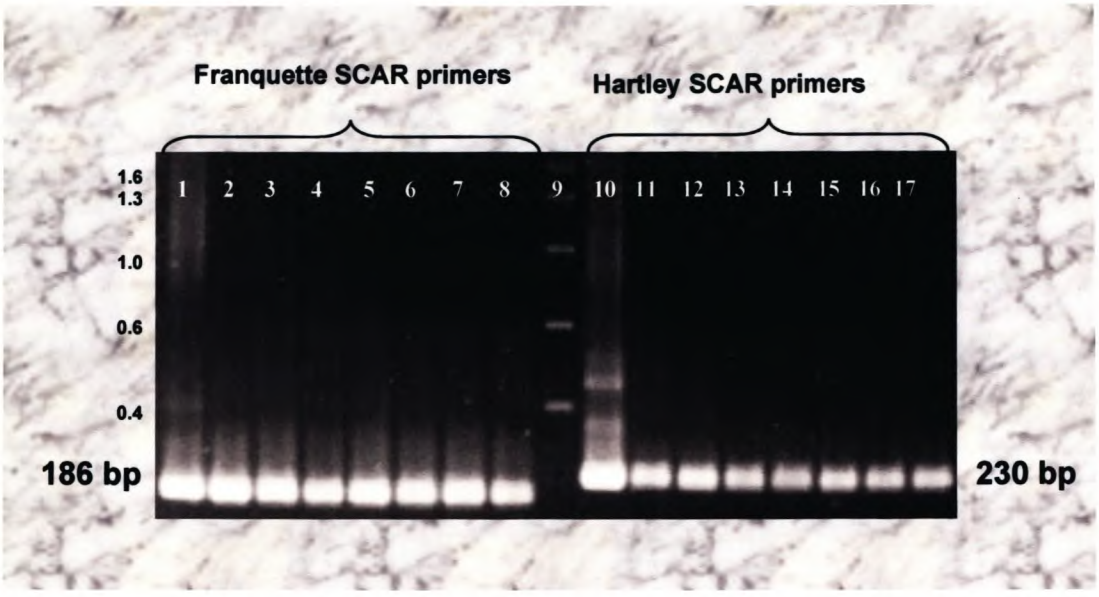

Figure 4.6: PCR fragments generated from 6 walnut cultivars and a rootstock amplified with Franquette and Hartley SCAR primers. Lane 1, Franquette SCAR product generated from cloned fragment. Lanes 2 – 8, 186 bp SCAR fragments amplified from the genomic DNA from all cultivars using Franquette SCAR primers, lane 9, EL Marker (Kb) (IPB, US), lane 10, Hartley SCAR product generated from cloned fragment, lanes 11 – 17, 230 bp SCAR fragments amplified from the genomic DNA from all cultivars using Hartley SCAR primers.

The PCR-SCAR primers derived from Paradox, which was the only rootstock included, discriminated some of the cultivars (Figure 4.7). The cultivars, Serr and Franquette and Paradox rootstock grouped together but no SCAR products were found for the other cultivars.

Figure 4.7: PCR fragments generated from 6 walnut cultivars and a rootstock amplified with Paradox-SCAR primers. Lane 1, EL Marker (Kb) (IPB, US), Lanes 2 – 8, 264 bp SCAR fragments amplified from the genomic DNA from all cultivars. Paradox-SCAR primers did not amplify Chandler (lane 2), Hartley (lane 4), Sunland (lane 6) and Vina (lane 7).

4.22 Restriction analysis strategy

SCAR products from each individual candidate SCAR primer pair were digested using selected restriction enzymes (Table 4.6). For example, Figure 4.8 shows Chandler SCAR primer products digested with *Rsa*I. The sizes of the bands released, showed partial digestion for Franquette (lane 9), Serr (lane 11) and Paradox (lane 14).

Table 4.6: Restriction enzymes predicted to digest individual SCARs.

Enzyme Name	SCAR products from individual candidate SCAR primer pairs						
	Chandler	Franquette	Hartley	Serr	Sunland	Vina	Paradox
	186 bp	187 bp	230 bp	200 bp	298 bp	285 bp	264 bp
	Numbers correspond with products (base pair) of the restriction enzymes from SCARs						
*Acc*I	N[a]	8; 179	N	N	N	N	N
*Alu*I	N	N	68; 162	N	108; 190	29; 37; 248; 256	52; 115; 149; 212
*Bgl*I	N	N	34; 196	N	N	N	N
*Hae*III	N	N	21; 80; 150; 209	23; 177	10; 98; 200; 288	N	N
*Rsa*I	58; 128	N	N	N	141; 157	75; 82; 127; 158; 203; 210	N

[a]N = no restriction site

In Figure 4.9 the sequences between Vina and Paradox (amplified by the Chandler SCAR primers) differed by single nucleotide base changes but not in the *Rsa*I site. Likewise, differences were found between Serr and Sunland using Hartley SCAR primers (Figure 4.9), but not when Franquette SCAR primers were used.

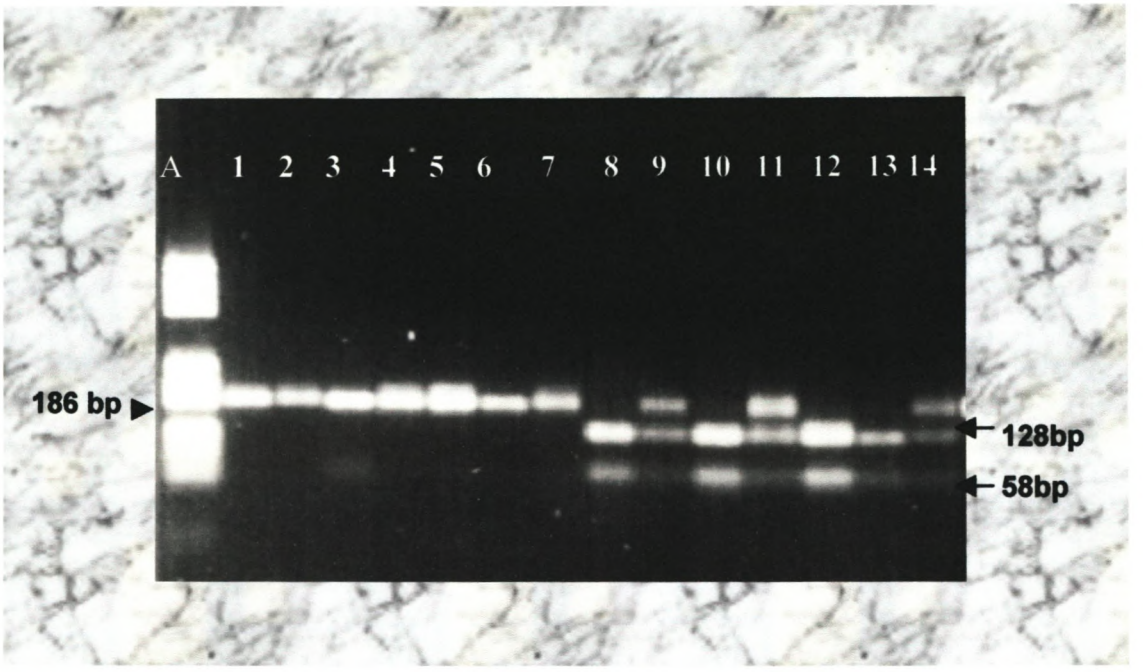

Figure 4.8: Chandler primer SCAR products (186 bp) (lanes 1-7) on a 2% (m/v) agarose gel. Lane A, Marker V, lanes 1 and 8, Chandler; lanes 2 and 9, Franquette; lanes 3 and 10, Hartley; lanes 4 and 11, Serr; lanes 5 and 12, Sunland; lanes 6 and 13, Vina and lanes 7 and 14, Paradox.

A. Vina and Paradox sequences using Chandler SCAR primers

TTGGAGTTTTTATGCTTGAATTGCTAACATCMAAGCGACCRATTGAGAGAGGGAATGTA
CATCGTCCGTCAGGTGAGAACARCRATGAACAAGGAYGACGAAGAGCACTATGGTTTGT
GGGATATGATTGATTCRTCCATTAGAGACACACCAAATCTTATAGGGTTTGGGCAATTC
TTGGAAATTAA

B. Serr and Sunland sequences using Hartley SCAR primers

CCCTCCCGCGCATGATAAAGTTATTAGTATCGCCYTACTGGCTAGAACCCCAGGCAGTA
ACCGCCATCCTTGTYGCCCTGCCTTCCCGCACGCCCACGCGGTATWATCGATTTTCAGA
GTTCCCCTCTGAGGTYTTGAATCAAGCATGGCCGGRGCGCAAGCTGAAGACATAGCCTT
CACCAAGCATCCCTATATCGAGGACGTGGGGCCTCGCAAAATGTCAGAATCA

C. Serr and Sunland sequences using Franquette SCAR primers

CACTAGTAGACACACACACCCACAAAAATGATTCTATGGATGAAAACAAATAACTTGGG
TAAAAATATACACCAATGTTTTCATCTCAATGCAGTTATCATTTTGAGGTTACTATTTC
TCAAATTTGTAAGAATCTCATATTGCATTTTCTCTATTTGTAAAACGTCATACTCACTT
CAAAACCAAGA

Figure 4.9: Consensus sequences of clones from different walnut cultivars and a rootstock amplified with SCAR primers. The SCAR primers produced the expected sized PCR products of 186 bp (A), 230 bp (B) and 187 bp (C). **A**. Vina and Paradox sequences using Chandler SCAR primers. Nucleotides in blue are *Rsa*I recognition site, and nucleotides in pink (underlined) are *Mun*I recognition site, R = A/G. W = A/T, Y = C/T, **B**. Serr and Sunland sequences using Hartley SCAR primers (lanes 14 and 15 in Figure 4.6). Nucleotides in red are for differences from each cultivar, and the rest of the sequence is highly homologous. **C**. Serr and Sunland sequences using Franquette SCAR primers.

DISCUSSION

AFLPs distinguished all the walnut cultivars and the rootstock included in this study. The fingerprints generated illustrate that *J. regia* has high levels of polymorphism. However, the objective of the study which was to convert AFLP fragments to SCAR markers for the development of a simple robust technique for cultivar discrimination, was not achieved.

For closely related individuals, AFLP can be used successfully to detect low levels of polymorphism. Manubens *et al.* (1999) established that one primer pair was needed to differentiate between 14 peach and nectarine cultivars, whereas Goedhart (1993) used 8 primers revealing 12 RAPD polymorphisms in order to discriminate 13 peach cultivars. Walnuts have high degrees of polymorphism (this study; Potter *et al.*, 2002; Nicese *et al.*, 1998). For fingerprinting 48 walnut cultivars, Potter *et al.* (2002) detected polymorphism levels as high as 57% using ISSR markers. When RAPD markers were used to differentiate 19 walnut cultivars, the scorable polymorphic fragments were only 25% (Nicese *et al.*, 1998). In both studies, one or two primers were necessary to distinguish the walnut cultivars. In this study, using the 27 AFLP primer pair combinations, polymorphic bands as high as 44 out of 92 (47.8%; Table 4.3) were scored. Using AFLP markers, one primer pair combination can distinguish between six walnut cultivars and one rootstock (this study). For discriminating among the

walnut cultivars the AFLPs were used even though technology is more complex, time-consuming and expensive.

The presence or absence of scorable bands was the sole criterion for recording results. To characterize an AFLP marker, polymorphic fragments clearly separated from other fragments were chosen as candidates for excision and subsequent cloning. The fragments were excised from silver-stained gels since this procedure stains both DNA bands. This was done to reduce the chances of cloning invisible contaminating AFLP bands. When fragments are detected with radioactive (^{33}P or ^{32}P) or fluorescent labeling, a single DNA strand with labeled *Pst*I primer can be observed in an AFLP analysis, while the position of its invisible complementary DNA strand often significantly shifts in mobility (Vos *et al.*, 1995).

It has been reported that only a few AFLP markers have been successfully converted into sequence-specific PCR-based markers in barley (Schwarz *et al.*, 1999), carrot (Bradeen and Simon, 1998) and in wheat (Qu *et al.,* 1998; Shan *et al.*, 1999). In this study, the AFLP markers converted to the SCAR markers did not retain their specificity. The reason for the lack of efficient conversion may lie with the nature of the AFLP primers. The SCAR primers were generated from sequences internal to the AFLP primers and as a result the specificity was lost. As suggested by Xu *et al.* (2001), when testing candidate SCAR markers pre- and selective-amplification products should be used as template DNA. It is

known that pre-amplification products in an AFLP analysis are a subgroup of amplified *Pst*I or *Eco*RI/*Mse*I fragments, and that selective-amplification products observed on an AFLP film are composed of about 50 to 100 fragments. Therefore, using pre-amplification products as template DNA will reduce the influence of the genomic DNA background to a minimum, especially when highly homologous regions, involving AFLP markers are present.

Restriction enzyme analysis allows searching for restriction site differences and to separate fragments into smaller pieces in order to facilitate the detection of small fragment length differences (Table 4.6). Using the Chandler SCAR primer pair combination to amplify genomic DNA, and cutting the SCAR products with *Rsa*I restriction enzyme, the digested products of Chandler, Hartley, Sunland and Vina grouped separately from Franquette, Vina and the Paradox hybrid (Figure 4.8). This grouping pattern was retained with the Paradox SCAR markers. It is not clear why these groups were found on the two independent occasions. The sequence analysis of two Chandler SCAR products derived from Vina and Paradox clones, revealed that the *Rsa*I restriction site was retained, but a few ambiguities were found (Figure 4.9A). AFLP markers are co-dominant markers, therefore, it is possible to detect heterozygosity. Walnuts are tetraploids, and it is possible that one of the alleles had a site of point mutation, it could be a nucleotide mismatch, or may be a PCR artifact. Another possibility is a partial digestion using *Rsa*I endonuclease.

The aim of developing identification techniques should be continued. A project for developing microsatellite markers for walnut is being conducted by the USDA in California (Potter *et al.*, 2002). Once this technology has been developed, it will probably replace most other methods developed.

CHAPTER FIVE

CONCLUSIONS

The research presented in this thesis had two objectives: (i) to combine two common diagnostic tools: serological kits and genetic fingerprinting to identify cherry leaf-roll nepovirus (Chapter 3), and (ii) to establish a marker system to characterize walnut germplasm. The first objective was fully met, even though some of the tests were not performed on *Juglans regia* cultivars, and *Nicotiana tabacum* (an indicator plant) had to be used. To fulfill the second objective, AFLP technology was used to identify polymorphisms, and SCAR markers were developed from them. Success was hampered by the lack of polymorphisms in the SCAR marker bands, but in spite of this promising preliminary results were obtained (Chapter 4).

The results on the detection of CLRV presented in this study were reproducible and demonstrate the utility of IC-RT-PCR for detection of nepoviruses from naturally infected, dormant walnut tissues, suggesting that this technique can be applied for all members of this group for which specific antibodies are available. Due to the fact that the specificity of an immunoaffinity capture step, reverse transcription and the sensitivity of PCR are combined, the technique meets the most important criteria required of a reliable diagnostic system. When compared to DAS-ELISA, the method proved to be at least 4 times more sensitive. As the entire procedure can be performed in one single tube, it is technically relatively simple and the risk of sample contamination is minimal.

Walnut cultivars were bred from germplasm originating from diverse geographic regions, and several studies examined the genetic diversity of walnut cultivars using molecular markers. For the purpose of finding new cost-effective SCAR markers, it was decided to use a more advanced DNA technology, namely AFLPs, as the main means of developing new DNA markers. Previous studies showed that AFLPs could produce more informative polymorphisms in a shorter period of time than other methods, e.g. RAPDs (Bates *et al.*, 1996).

In this study, walnuts were found to be closely related. The AFLP primer pairs used provided polymorphic DNA fragments. From these fragments, 7 SCAR markers were developed. It was expected that these SCARs derived from the AFLP markers would detect slight differences between cultivars. However, 6 out of the 7 generated identical fragment sizes respectively. The Paradox SCAR marker was found to be the only one that could discriminate the cultivars into 2 groups. The close-relatedness of the cultivars was confirmed when the SCAR products of Chandler were digested with the restriction enzyme *Rsa*I. The fingerprints generated, clustered into the same banding pattern as that of Paradox SCAR products.

In conclusion, improvement and optimisation of the IC-RT-PCR technique and the development of SCAR markers derived from AFLPs for walnut has been the first project of its kind in South Africa. This information is invaluable for importation and commercialisation of walnuts. Firstly, material can now be

accurately screened and certified as CLRV free. Secondly, for the first time the current germplasm in South Africa can be accurately identified using AFLPs. The SCAR markers developed in this study was not so useful and need to be improved. In the long term, the knowledge gained from this project will be applied in establishment of commercial orchards for the South African walnut industry.

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