Identification of molecular markers linked to woolly apple aphid (*Eriosoma lanigerum*) (Hausmann) resistance in apple

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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 university for a degree.

SUMMARY

Apple (*Malus x domestica* Borkh.) is an important horticultural crop worldwide and in the Western Cape. The income generated from apple and other deciduous fruit production amounts to approximately 25% of the gross total value of horticultural production in the Western Cape. Unfortunately diseases and pests adversely affect fruit production in this region.

Woolly apple aphids (*Eriosoma lanigerum* L. (Hausmann)) have a significant effect on the apple industry in the Western Cape. Damage caused is two-fold, occurring aerially and terrestrially. Insects colonise the plants, feeding off the phloem sap. Aphid infestation around the root system results in repeated infestation of the foliage as it serves as a reservoir of aphids. In extreme cases, the apple cores are also infested, thus affecting the sale of apples. In 1962, Northern Spy was identified as a woolly apple aphid resistant rootstock and has since then formed the basis for traditional rootstock breeding programmes. The Er_1 gene in Northern Spy confers resistance. According to one report, the natural resistance of Northern Spy was overcome in South Africa in 1968, but this was not confirmed in an independent study.

The main objectives of this study was to firstly identify molecular markers more closely linked to the woolly apple aphid resistance gene, Er_1, than existing markers, by applying AFLP technology to selected seedlings, identified to be resistant by conventional phenotyping. If identified, these markers can be incorporated into existing breeding programmes. Secondly, previously identified RAPD and SCAR markers were tested to determine their applicability in local populations for use in breeding programmes. Ultimately the segregation of the Er_1 gene in South African populations can be determined if tightly linked markers are identified.

Three families were derived from crosses of each of three resistant genotypes, namely Northern Spy, Rootstock 5 and Russian Seedling and a susceptible cultivar, Braeburn. For the three successive years of the study, each resistant genotype was allowed to cross-pollinate in isolation with the susceptible parent. Two hand-pollinated families, Russian Seedling x Liberty and Russian Seedling x Northern Spy, were also included in the study. The amplified fragment length polymorphism (AFLP) technique was used in an attempt to identify markers in the resistant and susceptible seedlings. No markers were identified using this technique. Known sequence characterised amplified regions (SCAR) and random

amplified polymorphic DNA (RAPD) markers were used due to their suitability in marker-assisted selection for woolly apple aphid resistance. Varying results were obtained with these markers and no conclusive information was acquired with regard to the segregation of the Er_1 gene in any of these rootstocks and crosses. This underlines the need for the development of markers that can readily be applied in local breeding programmes. The identification and integration of such markers will greatly benefit the local and world wide apple industries.

OPSOMMING

Appels (*Malus x domestica* Borkh.) is wêreldwyd en in die Wes-Kaap 'n belangrike landbougewas. Inkomste gegenereer deur appels en ander sagtevrugte vorm bykans 25% van die bruto inkomste uit vrugte in die Wes-Kaap. Siektes en insekpeste verlaag egter die produksie van vrugte in hierdie streek.

Appelbloedluise (*Eriosoma lanigerum* L. (Hausmann)) het 'n groot invloed op appelproduksie in die Wes-Kaap. Skade word bogronds en ondergronds aangerig. Insekte koloniseer die plant en leef op floeëmsap. Besmetting van die wortels lei tot herhaalde besmetting van bogrondse dele aangesien die insekte aanteel op die wortels. In uiterste gevalle word die vrugte geaffekteer, wat vrug-verkope beïnvloed. 'Northern Spy' is in 1962 geïdentifiseer as 'n onderstam met natuurlike weerstand teen appelbloedluis en het vir lank die basis gevorm vir tradisionele telingsprogramme. Weerstand word verleen deur die Er_1 geen. Volgens een verslag is die natuurlike weerstand van Northern Spy egter in 1968 in Suid-Afrika oorkom, maar dit is nog nie in 'n onafhanklike studie bevestig word nie.

Die hoof doelstellings van hierdie studie was om eerstens deur middel van die AFLP tegniek molekulêre merkers te identifiseer wat nouer gekoppel is aan die appelbloedluis weerstandsgeen, Er_1, as bestaande merkers. Hierdie tegniek is toegepas op saailinge wat deur konvensionele fenotipering geselekteer is. Indien merkers suksesvol geïdentifiseer is, kan dit in bestaande telingsprogramme geïntegreer word. Tweedens is bestaande RAPD en SCAR merkers ook getoets om hul toepaslikheid te bepaal vir gebruik in plaaslike teelprogramme. Oplaas sal die segregasie van die Er_1 geen in Suid-Afrikaanse populasies ook deur middel van nou gekoppelde merkers bepaal kan word.

Kruisings van elk van die drie weerstandbiedende genotipes, naamlik 'Northern Spy', 'Rootstock 5' en 'Russian Seedling', en die vatbare kultivar, 'Braeburn', het drie families daargestel. Elke weerstandbiedende genotipe is toegelaat om in isolasie te kruisbestuif met die vatbare ouer. Twee hand-bestuifde families, 'Russian Seedling' x 'Liberty' en 'Russian Seedling' x 'Northern Spy', is in 'n latere stadium van die studie ingesluit. Die AFLP tegniek is gebruik vir die identifikasie van polimorfiese merkers tussen vatbare en weerstandbiedende populasies. Geen merkers is egter geïdentifiseer nie. Bestaande SCAR en RAPD merkers is vervolgens gebruik om te bepaal of hulle geskik is vir gebruik in merker-bemiddelde seleksie vir appelbloedluis weerstand. Wisselende resultate is verkry ten opsigte van amplifikasie, herhaalbaarheid van resultate was swak en geen onweerlegbare bewyse oor die segregasie van die Er_1 geen is bekom nie. Dit beklemtoon die noodsaaklikheid om

merkers wat geredelik in plaaslike teelprogramme toegepas kan word, te ontwikkel. Die identifikasie en integrasie van sulke merkers sal die plaaslike en wêreld-wye appel industrieë aansienlik bevoordeel.

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ABBREVIATIONS

A	adenine
AFLP	amplified fragment length polymorphism
ASAP	allele-specific associated primer
ATP	adenine 5'-triphosphate
bp	base pair
°C	degrees centigrade
C	cytosine
cM	centimorgan
CTAB	cetyltrimethylammoniumbromide
ddH_2O	double distilled water
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ds	double strand
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
et al.	et alibi
EtOH	ethanol
G (in terms of dNTPs)	guanine
G (in PCR profile)	gradient
g	gram
h	hour
HCl	hydrochloric acid
i.e.	that is (to say)
kb	kilobase
l	litre
M	molar
MAS	marker-assisted selection
mg	milligram
$MgCl_2$	magnesium chloride

min	minutes
ml	millilitre
mM	millimolar
NaCl	sodium chloride
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PVP	polyvinylpyrrolidone
R	Ramp speed
RAPD	random amplified polymorphic DNA
RNAse	ribonuclease
rpm	revolutions per minute
SCAR	sequenced characterised amplified region
s	second
T	thymidine
TBE	tris, boric acid, EDTA
TE	tris, EDTA
TEMED	N, N, N', N', -Tetramethyl-Ethylenediamine
Tris	tris [hydroxy] aminomethane
µl	microlitre
µM	micromolar
UV	ultraviolet
V	volts
v/v	volume per volume
WAA	Woolly apple aphid
w/v	weight per volume

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CHAPTER 1
INTRODUCTION

1.1 *Malus* x *domestica* (Borkhuis)

1.1.1 The evolution of *Malus*

1.1.1.1 Introduction to *Malus*

The apple is one of the most important and ubiquitous fruit crops of the colder and temperate parts of the world. Evidence for the collection of apples from the wild can be found in the Neolithic (11200 bp) and Bronze Age (*c.* 4500 pc) sites throughout Europe (Zohary and Hopf, 1994). Evidence for the cultivation of apples can be traced as far as 12 centuries BC in Egypt and in Greece 600 BC. Evidence of apple cultivation can also be found in the Bible (Ryugo, 1988).

To trace the origin of apples it is necessary to investigate the entire *Malus* genus as little is known about the possible time and place of apple domestication, except that in Graeco-Roman times it was already widely grown in the Old World. Numerous wild species are distributed throughout Europe, Asia and the North American continent and these are in most cases infertile and not viable for commercial cultivation (Watkins, 1995).

1.1.1.2 Pre-history of *Malus*

One of the oldest trading routes is the Old Silk Road that crossed from the Black Sea/Caspian region, through Tashkent, Samarkand and Alma Ata (Almaten), to western China. These parts were traversed by travellers near the beginning of the Neolithic period and were well established by the time the Bronze Age started. Ruminants, horses, donkeys and mules ate apples and as the seeds passed through their alimentary canals undamaged, they greatly contributed to the distribution of seeds along the Old Silk Roads. This way of distribution also provides an explanation for the hybridisation between geographically isolated species resulting in new species (Way *et al.*, 1990). The hybridisation of these remote species would not have occurred naturally if ruminants and other animals have not ingested these fruit and distributed seeds to other regions.

1.1.1.3 The natural distribution of the genus *Malus*

The primary centre for diversity of both *Malus* (apple) and *Pyrus* (pear) is within the region that includes Asia Minor, the Caucasus, Central Asia, Himalayan India, Pakistan and the western provinces of China (Zhang *et al.*, 1993). Rehder (1940) identified twenty-five native species of *Malus* arranged in five sections. In the absence of detailed phylogenetic information, thirty-three species can be recognised occurring in three geographical groups, namely Chinese, European and American.

1.1.1.4 The evolution of cultivated apple and grafting techniques

The origin of cultivated apple is highly controversial despite the importance of the crop. Currently it is suggested that *Malus sieversii* (Lindl.) Roem., which has a distribution near the middle and also somewhat towards the Chinese (eastern) end of the Old Silk Road, played a seminal role in the origin of domesticated apples. This species is diverse and wild trees bearing the full range of forms, colours and tastes are found in Kazakhstan and other independent countries of Central Asia formed from the break up of the Soviet Union and especially around Alma Ata (Father of Apples). The traits of *M. sieversii* fruit range from inedible crab apples to those not dissimilar to modern varieties, and collection trips to Asia has verified that *M. sieversii* has all the qualities present in *M. domestica* (Janick *et al.*, 1996). To the east *M. sieversii* would probably have hybridised with other Chinese species e.g. *M. sieboldii*. Westward hybrids would have occurred from species such as *M. turkmenorum* Juz., and *M. sylvestris* (L.). The introduction of grafting technology resulted in cultivars derived from random hybridisation as various cultivars were grafted on the basis of different horticultural characteristics. The origin of grafting has not been established clearly, but it is possible that the Chinese or Persians were responsible for the development of these techniques (Watkins, 1995; Bultitude, 1983).

1.1.1.5 The historical period

The Greeks were familiar with the art of grafting (Href 7). Roman horticulturists were also knowledgeable of budding, grafting and rootstocks and were responsible for the introduction of selected apple cultivars into Britain. The survival of these cultivars through the Dark Ages is, however, debatable. Cultivars were brought back to Britain after the Norman Conquest and included 'Court Pendu Plat', 'Pearman' and Pomme d'Api (Bultitude, 1983). It is unlikely that the native British crab,

M. sylvestris, has produced hybrids of any commercial value even though apple growing spread widely in Britain after the Middle Ages. The influence of *M. sylvestris* is likely to have been mainly on cider apples (Bultitude, 1983).

The climate on the British Isles was agreeable for the propagation of alien apple cultivars. Consequently these cultivars hybridised, principally amongst themselves, and developed to such a degree and proliferated to the point where at the close of the nineteenth century virtually every village and town in central/southern England could claim an apple tree (Morgan, 1983).

1.1.2 Current production of new cultivars and rootstocks in the apple industry

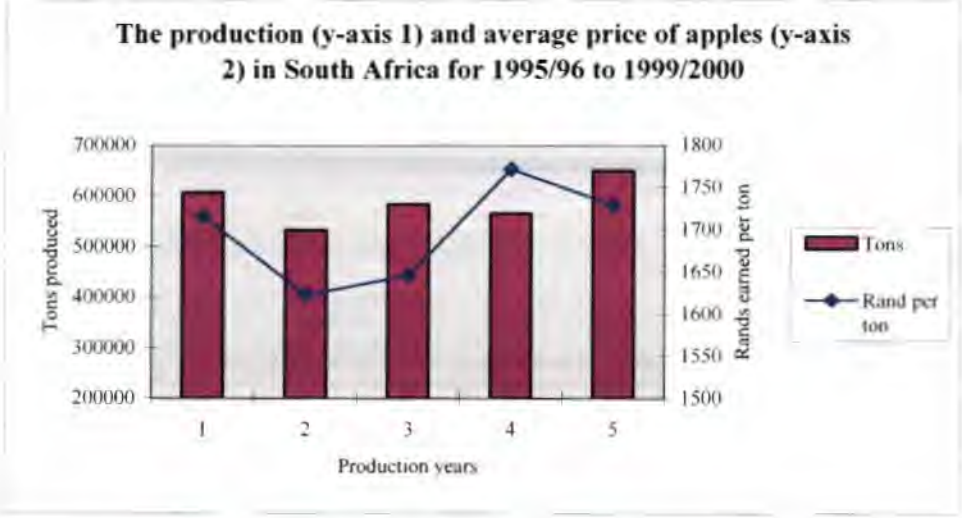
Since the start of the 20th century, selection has become scientifically based with lesser input from traditional practices. Research institutions now apply modern genetic principles to aid in breeding programs. Breeders are aiming to improve the quality of tree growth and fruit. Diseases can also have a debilitating effect on the apple industry. Genes conferring resistance to diseases are being researched and breeding programs could incorporate such genes into new resistant cultivars and rootstocks.

An easy way to expand the commercial improvement is the selection of improved clones of a variety. This would ensure that plants used in cultivation all stem from a single parent with good commercial and horticultural characteristics. Recently mutations have been induced in scion material by γ-radiation and other methods of radiation (Bultitude, 1983). The use of ionisation radiation to induce mutagenesis in apple has been reported to enhance self-incompatibility in 'Cox's Orange Pippin' (Campbell and Lacey, 1982). A number of clones of 'Bramley's Seedling' with compact growth have been obtained following gamma radiation, some of which have been reported to be stable (Lacey, 1982).

1.1.3 Apple and other deciduous fruit production in South Africa

The main fruit producing areas of South Africa are the Western, Southern and the Eastern Cape. Warm, dry summers and cold winters prevail in these areas making it suitable for deciduous fruit cultivation. For the 2000 season, the estimated area of deciduous fresh fruit cultivation was 60 000 hectares and the area for producing fruit for canning and dried fruit purposes was 15 000 hectares (Href 8).

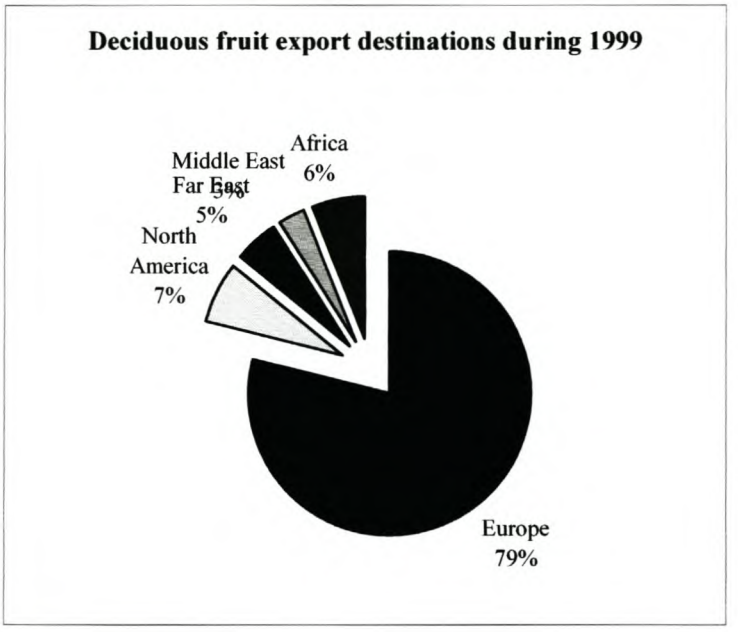
It is estimated that there are 2500 producers of fruit for fresh and dried fruit consumption, and about 1000 for canning purposes. The production of deciduous fruit for 1999/2000 was estimated at 1 461 054 tons, which was an increase of more than 5.5 % on the 1998/1999 season. In 1999, plums and apricots showed a decrease in production of 15 and 12 %, respectively. Contrary to the decrease in production for some fruits, apple production increased by 15 % for that season. For the period 1995/1996 to 1999/2000, the deciduous fruit industry showed an annual average growth of 4.6%. Within the same period, apple production varied between 530 000 and 650 000 tons of fruit per year (Href 8). The apple production data is depicted in graph 1.1 below.



Graph 1.1 The production and the average price of apples in South Africa for the time period 1995/96 to 1999/2000. Production figures are depicted, in terms of tons produced, (in maroon) on the y-axis 1. The average earnings are indicated, in terms of Rand earned per ton, on the y-axis 2 (in blue). Data provided by www.nda.agric.za/docs/Trends2000/horticulture/htm

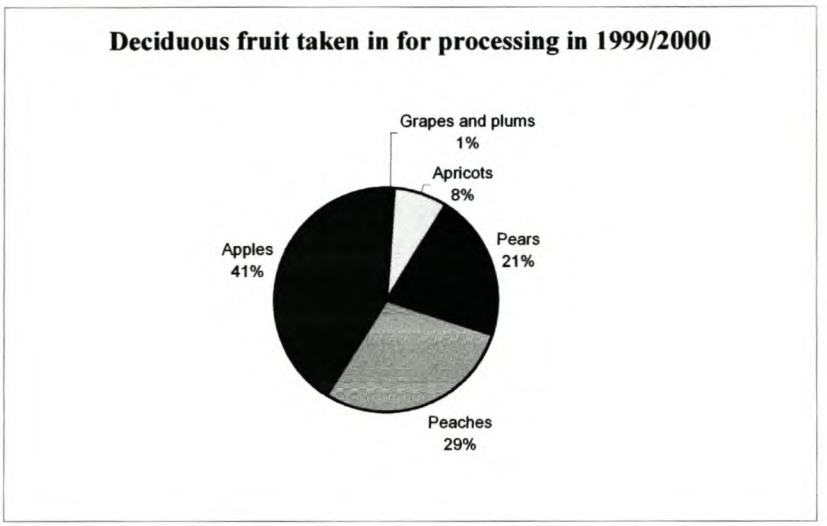
The majority of deciduous fruit produced in South Africa is destined for the export market, which requires compliance with certain quality standards as well as the procurement of a Perishable Products Export Control Board (PPECB) certificate. The deregulation of the industry in the 1990s meant that single channel marketing for export was abolished. Presently there are 50 exporters competing to sell South African produce overseas. Therefore, South African products are in direct competition with each other and this has had an adverse effect on the price of goods. During the 1999/2000 season, approximately 357 319 tons of deciduous fruit was sold locally, an increase of more than 23 000 tons from the previous year. In comparison, 635 816 tons were exported in the same period, which is

2higher than in the 1998/1999 season. 64% of produced fruit was thus exported and 86 % of the gross value from deciduous fruit was earned in foreign exchange (Href 8). Deciduous fruit contributes approximately 25 % of the gross value of horticultural production. The deciduous fruit export destinations in 1999 are given in graph 1.2.



Graph 1.2 South African deciduous fruit export destinations for 1999. Data provided by www.nda.agric.za/docs/Trends2000/horticulture.htm.

For the time period 1995/1996 to 1999/2000 the average price of apples on the 16 local fresh produce markets averaged between R1623/ton to R1772/ton (see graph 1.1). Thirty-nine percent of deciduous fruit produced in 1999/2000 were processed (see graph 1.3). Apples formed 41 % of this bulk. Apples are mainly used for juices and in 1999/2000, 96 % of processed apples were used for juices and 4 % for canning purposes. Producers receive on average R599 and R333 per ton for apples used for canning and juices, respectively (Href 8).



Graph 1.3 Deciduous fruit processed in South Africa in 1999/2000. Data provided by www.nda.agric.za/docs/Trends2000/horticulture.htm.

From the abovementioned data it can be concluded that apple is a very important crop in South Africa, whether destined for the export or local markets. If an effective control measure can be implemented to eradicate or minimise the effects of woolly apple aphids on apple as well as other disease and pests, increased financial gain from this growing industry can be reaped in future.

1.1.4 The taxonomy of apple (*Malus* x *domestica* Borkh.)

Apples are part of the rose family, *Rosaceae*. The subfamily *Maloideae* is one of the four subfamilies of *Rosaceae*, and includes important fruit trees such as apples, crab apples, pear, woody ornamental plants and many members of the natural plant communities. These plants are most diverse in the Northern Temperate Zone although there are a few subtropical species (Href 1).

The flowers of the subfamily *Maloideae* have 5 sepals, 5 petals, many stamens, a hypanthium, and (typically) a syncarpous gynoecium of 2-5 connate carpels with an inferior ovary. The fruit of this subfamily is by definition, pomes. The outer flesh, exterior to the "core", comes from the hypanthium tissue, while everything inside the "core" comes from the ovary tissue (Href 1).

Compared to the other subfamilies *Prunoideae*, *Spiraeoideae* and *Rosoideae*, which have a haploid chromosome number of 8, 9 and 7, respectively, the haploid chromosome number of the *Maloideae* is

unusually high (n = 17) (Href 2). Challice (1974), however, proposed that the basic chromosome number of *Maloideae* is seven and that the member having haploid numbers of eight or nine may have arisen from duplication of individual chromosomes. Most apples cultivars are functional diploids (2n = 34) but approximately 10% are triploids (2n = 51) (North, 1979). These triploids are more vigorous than diploid cultivars and tend to have larger fruit, which proved to be a selective advantage. Triploids do, however, produce poor pollen, leading to pollination problems. A diploid is required to pollinate a triploid to produce good seeds. Seeds produced are sufficiently fertile to produce good fruit if properly fertilised. If these plants are, however, selfed they produce poor seeds, which result in aneuploid seedlings that are weak and seldom develop into a tree (Sanford, 1983).

Tetraploids (2n = 68) may also have arisen. Depending on the nature of the chimera, flowers may develop from diploid or tetraploid tissue. Tetraploids can be formed as the result of pollination of an unreduced egg cell in a triploid (3n = 51) by normal pollen (n = 17) from a diploid. The number of seeds formed is usually very small and are also aneuploid. The nuclear DNA content of apple (*Malus x domestica*) has been determined and found to be 11,54-1,65 pg/2C and 743-796 ~Mbp/1C (Href 3).

1.2 Woolly apple aphids (*Eriosoma lanigerum* L. Hausmann)

1.2.1 Background

The three aphid species that are considered to be the most important apple pests are: the apple aphid, *Aphis pomi*; rosy apple aphids, *Dysaphis plantaginea* (Passerini) and the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) (Morgan and Madsen, 1970).

Woolly apple aphids are insects that are found primarily on cultivated apple (*Malus x domestica*) and wild apple trees. Pears, quince and other trees may also serve as secondary hosts (Href 1). Aphids are autoecious, implying that they are capable of living on one or a few species of a particular genus of plants (Eastop, 1973). Ten percent of aphids are found to be heteroecious, which means that these insects spend autumn, winter and spring on the primary hosts, and summer on the secondary host. The secondary host is rarely related to the primary host.

Four thousand species of aphids have been described thus far, and of these, most are found in temperate regions of the world. Aphids differ from most other groups of insects by showing a relationship

1.2.3 Biology of Woolly Apple Aphids

1.2.3.1 Appearance of woolly apple aphids

Apterae are wingless aphids, they are small to medium-sized. Their colour varies from purple, red or brown and they are covered with a thick white flocculent wax, occurring on roots, trunk or branches of the host plant as can be seen in Figure 1.1. Alatae are winged aphids and have a reddish-brown abdomen with woolly white wax posterior. The size of apterae ranges from 1.2 –2.6 mm and for alatae from 1.8- 2.3 mm (Blackman and Eastop, 2000).

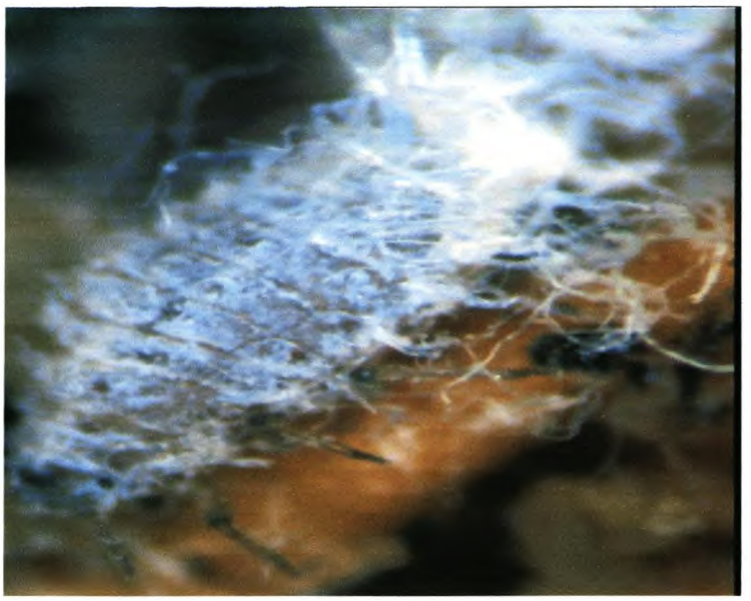


Figure 1.1 An adult woolly apple aphid showing its waxy threads

1.2.3.2 Life and season cycle

In the Eastern United States and Canada, *E. lanigerum* displays holocyclic (monocyclic) behaviour, occurring only on the American elm, hatching in spring to produce a stem mother. In the rest of the world, aphids display anholocyclic behaviour (Blackman and Eastop, 2000).

Expanding buds are attacked by aphids, resulting in the formation of a rosette of leaves instead of a normal shoot. The stem mother produces a generation of winged (also known as alatae) females, which

fly to another apple tree or summer host giving rise to wingless (also known as apterae) females. Several generations are produced each season and in autumn two forms occur: (i) a wingless female similar to summer broods that remain on the apple all winter, and (ii) winged females that migrate to the elm and deposit sexual males and females. Each sexual female produces a winter egg. There are thus two distinct aphid life cycles, one where the aphids remain on the apple all year and the other, which alternates between apple and elm (Crane *et al.*, 1936). In parts where the American elm does not occur, such as South Africa, the aphid cycle remains on the apple tree. In America, it has been reported that many young aphids move up from the roots in the spring and the young aphids hatched in early summer migrate to the roots (Baker, 1915). A similar migration pattern has been reported in South Africa (Nel, 1983).

Aphids reproduce by cyclical parthenogenesis, which is the alternation of a phase of sexual reproduction and a phase of parthenogenetic reproduction in the life cycle of one species. This process consists of two basic functions, namely sexual reproduction and increase of biomass. Sexual reproduction involves gene recombination and segregation to produce an array of new genotypes. Within *Eriosomatinae*, the first parthenogenetic generation, the fundamatrix, is a relatively large, vivaparous, highly fecund, gall-forming plant parasite. The female of the sexual generation is small and non-feeding, producing one egg as large as itself (Blackman and Eastop, 2000).

In *Aphididdae*, parthenogenesis is combined with viviparity. Viviparity is significant in the development of the divergence of the sexual and the multiplicative functions of aphids. Aphid development is very rapid, so that embryos could start to develop even before birth of the mother and are ready for existence soon after the mother hatches and becomes an adult. The shortening of the generation time and the overlapping of generations are measures that ensure a dramatic increase in aphid populations, especially during their parthenogenetic phase (Blackman and Eastop, 2000).

1.2.4 *Eriosoma lanigerum* biotypes

A biotype can be defined as a population or an individual distinguished from other populations or individuals of its species by a non-morphological trait. This definition does not imply any reproductive barrier, morphological, cytological or biochemical differentiation, or geographic isolation between such populations. Biotype has been used to describe insect populations that differ in diurnal or seasonal

activity pattern, size, shape, colour, insecticide resistance, migration or dispersal tendency and the ability to utilise a particular plant (Eastop, 1973).

Biotypes are the natural mechanism for survival for the perpetuation of insect species. They typically develop as a result of selection from parent populations in response to exposure to resistant cultivars or insecticide pressure. It has been concluded that biotypes are more likely to develop on plants with monogenic resistance rather than plants having polygenic resistance. Development of biotypes capable of surviving on resistant plants limits the use of these plants. New biotypes in insect pests are comparatively infrequent due to an insect's own complex physiology and pest resistance in host plants. This can often be related to host-finding behaviour of insects (Eastop, 1973).

Two woolly apple aphid biotypes have been identified in Australia by Sengupta and Miles (1975) and in the United States of America (USA) (Young *et al.*, 1982). In South Africa biotypes have also been identified that are capable of colonising cultivars with Northern Spy-type resistance (Giliomee *et al.*, 1968). This study suggested a new mutant strain of *Eriosoma lanigerum* that have evolved in the Elgin region, which has overcome the inherent factor of resistance in Northern Spy, Merton and Malling-Merton rootstocks. As the factor of aphid resistance have been obtained from a common parent, it is not surprising that they became susceptible at the same time.

1.2.5 Specific responses due to damage caused by *Eriosoma lanigerum*

E. lanigerum is a bark feeder, infesting roots, tender areas on the trunk and branches, new lateral growth and where damage has occurred, either accidentally, during pruning or by hail (Carnegie, 1963). Aphid feeding affects the cambium and cortex (Mani, 1964). Krebs galls (tumours) are caused by *E. lanigerum* infestations. These are characterised by irregular, cracked swellings that arise on branches and less frequently on crowns and aerial parts of roots. According to Staniland (1924), the formation of galls is caused by the insect's saliva on the cambium. This stimulates abnormal meristematic activity, thus producing unusually large parenchymatous cells that suppress the differentiation of xylem and sclerenchyma cells. Woody elements are scattered throughout the relatively undifferentiated parenchyma and continued production of the latter causes build-up of the poorly lignified tissue to form galls. This results in the eventual collapse into a pulpy mass.

Previous studies concluded that resistance is due to a hypersensitive reaction on the part of the plant resulting in necrotic regions. This prevents the insect's stylets from penetrating deep into the plant, and the biochemical effects of this action influence the insect's feeding (Sengupta and Miles, 1975). In studies conducted by Miles (1968), attention was paid to the nutritional value of tissues as determined by their total content of α-amino nitrogen and the content of potentially toxic plant materials as measured by their total content of phenolic compounds. According to Sengupta and Miles (1975), *E. lanigerum* shows greater preference for cultivars with higher amounts of soluble nitrogenous compounds in comparable tissues. Insects were, however, found not to feed at sites within trees that had the highest nitrogen content (Sengupta and Miles, 1975). Preferences for feeding sites between and within trees can be attributed to the selection of tissues with low ratios of phenolic to nitrogenous water-soluble contents. This suggests that the phenolic content of tissues of apple trees in some measure is toxic to woolly apple aphids. These insects have adapted to deal with the salivary phenol-phenolase system. Apple phenolics have been found not to inhibit feeding, but limit feeding to a rate required for detoxification of ingestion. The higher the phenolic content, the greater the concentration of nitrogenous nutrients needed to compensate for the lower feeding rate (Sengupta and Miles, 1975).

Further results from experiments conducted by Sengupta and Miles (1975) show that within apple varieties, an increased nitrogen content of tissue was usually associated with increasing susceptibility. The inverse relationship was, however, true for the tips and bases of shoots. Studies have also proven that within varieties, increased phenolic content in tissue was also associated with decreasing susceptibility, but not when shoots were compared with roots. The ratio of phenolics to nitrogen was more consistent in its inverse (reverse) relation with susceptibility than either factor alone, which translates to the following: the less phenolics determined at a given titre, the greater the susceptibility of those varieties to be attacked by woolly apple aphids. Variation of phenolic content between varieties had no significant association with susceptibility, but the ratio of phenolics to amino nitrogen appears to have a consistent inverse relationship with susceptibility.

This information provides evidence that woolly apple aphids can cope with tissues containing widely varying contents of nitrogen in various plants. One of the factors described involving woolly apple aphid feeding and development, involves the nitrogen content. Miles (1969) found that the toxicity of ingested materials, such as phenolics, is dependent on the nutritional value of the tissue. High toxic content can be tolerated, as the tissue is high in nutrients and small quantities of toxicant is ingested with sufficient quantities of food.

1.2.6 Mechanism of damage by *Eriosoma lanigerum* (Hausmann)

Indirect damage caused by aphids weakens the tree when feeding by aphids occurs on the bark and roots, thereby reducing tree health. Aphids also inhibit the healing of scar tissue and promote the transmission of perennial apple cankers caused by fungi invasion in these sites (Crane *et al.*, 1936). Direct infestation can be observed when the fruit cores are infected, but this only happens in exceptional cases.

Damage to trees is twofold, aerial and terrestrial (Figure 1.2). Aerial damage can be observed most commonly. Stem mothers feed at the base of leaf buds and cause the leaves to curl and thicken once the development starts. Aphids are particularly drawn to open wounds; pruning scars as well as on one- or two-year old shoots. On one year-old shoots aphids seem to attack the young buds in the leaf axils. On pruning wounds, aphids can appear earlier than on older wood. The presence of aphids serves as an indicator early in the season for the presence of woolly apple aphids (Nel, 1983).

Feeding in aphids is achieved by means of piercing and sucking mouthparts, thereby enabling these insects to feed on shoots, stems and sometimes roots. The mouthparts are so constructed that it can circumvent impenetrable tissue, in search of tissues suitable for feeding. Galls are formed at the feeding sites if feeding occurs on the branches or twigs. The size of galls varies depending on the severity of infestation. Another manifestation in severe cases is the 'Christmas tree effect' whereby young shoots and buds are entirely covered by woolly apple aphids and have a white appearance (Nel, 1983).

If control measures are not applied early, aphids will eventually move to the fruit. Fruit in the vicinity of the infested wood will become sticky as it is covered in honeydew, which is excreted by aphids. A black sooty mould grows on the honeydew forming a black layer on the fruit rendering it to be unsuitable for sale. Although this damage is in fact indirect, it is nonetheless undesirable (Nel, 1983; Href 5; Href 6). Sooty mould affects the rate of photosynthesis and respiration when occurring on leaves (Georgala, 1953). However, sooty mould affects apple production to a lesser extent in South Africa than in other apple producing regions.

2 (a) 2 (b)

Figure 1.2 Damage as inflicted by *Eriosoma lanigerum* L. (Hausmann) in apples, (a) aerially and (b) terrestrially. Gall-formation is also clearly evident.

Terrestrial damage is the second type of damage that can be inflicted on the plant. It is also the most devastating to the plant, especially since it goes unnoticed at first. Gall-formation also occurs underground. Furthermore, roots provide a reservoir for aphids and can lead to re-infection of the aerial parts. Aphids feed in the same manner underground as they do above-ground, resulting in the malformation of the infested roots. Sometimes galls and nodules formed on the shallower roots are visible, as the white fluffy appearance of the aphids is apparent. Root damage reduces plant vigour, especially in younger plants (Href 5; Href 6; Nel, 1983).

1.3 Current control measures in agriculture

1.3.1 Control of aerial populations

1.3.1.1 Biological control

To date, the most important parasitoid in the biological control of woolly apple aphid is *Alphelinus mali* (Haldeman) (Hymenoptera: Aphididae) (Lundie, 1924). *Alphelinus mali* is a wasp that lays its eggs in aphids and also feeds directly on the aphids. *A. mali* was first introduced in South Africa as a control mechanism against woolly apple aphids, in 1920 (Lundie 1939). The level of control of *E.*

lanigerum was so effective in aerial parts, that no spraying against the woolly apple aphids was necessary. Various factors such as weather may impair the activity of the parasitoid, thus affecting the wasp's ability to control of woolly apple aphids. The level of parasitism by *A. mali* has also been found to be higher on trunks and branches where less pruning occurred and also in the presence of a cover crop (El –Haidari, *et al.*, 1978). Pruned sites provide aphids with feeding sites and will encourage colonisations as the healing of these sites is inhibited (Crane *et al.*, 1936). Cover crops protect aphids against possible spraying that may take place in orchards. On the other hand, pruning has the advantage of decreasing the prevalence of infestation as over wintering colonies is removed from broken galls (Greenslade, 1936).

The interaction between woolly apple aphids and *A. mali* is affected by season. Usually there is little activity of *A. mali* in the southern hemisphere during September, with a rising percentage of parasatised aphids observed in October. The seasonal shift of the percentage of parasitism is dependent on the density of the aphid population. An erratic distribution of aphid colonies is normal, except in the case of heavy infestations where large numbers of aphids are observed. Fluctuations in aphid incidence hampers effective parasitism by *A. mali*. The parasitoid spreads slowly from tree to tree and from one part of the tree to another. The females of both the aphids and *A. mali* produce an average of 100 eggs. Approximately half of the *A. mali* progeny of each female is male whereas all the progeny of the aphids are female. Therefore aphids increase the population at twice the rate of their parasitoid and this would explain the increase in infestation at the start of spring. It has also been discovered that over-wintering individuals of the two insects mature and commence reproduction at the same time in spring. *A. mali* is scarce after April, and it is probable that the increase in aphid populations can be attributed to this factor.

Several other factors may also be responsible for initial more rapid increase of aphids compared to *A. mali*. Temperature is a major factor in biological control of woolly apple aphids by *A. mali* as the aphids have different developmental rates to that of parasitoids. Both insects are known to survive low winter temperatures. In warmer regions *A. mali* has substantial to complete control but in colder regions control is less effective (DeBach, 1964). *E. lanigerum* has a higher developmental rate than *A. mali*, which implies that by developing slower than aphids, these parasitoids will have a continued availability of hosts and thereby ensuring their own survival (Campbell *et al.*, 1974). It was found that at low and intermediate temperatures *E lanigerum* matures more rapidly than its parasitoid (Walker, 1988). This allows aphids to escape biological control during cool periods. Lung *et al.* (1960) found

that when *A.mali* appeared during spring they could not find sufficient hosts suitable for oviposition, which can reduce the *E. lanigerum* population's levels. Early season spraying against other apple pest such as codling moth can further reduce *A. mali* levels. Low humidity may also responsible for a decrease in parasitoid productions, but this does not occur in all the regions where aphids are found. Extreme high temperatures are also conducive to lowered aphids population numbers (Dumbleton and Jeffreys, 1938) as the threshold temperatures for development for *A. mali* is higher than that of *E. lanigerum*.

Another means of biological control has been identified, namely pathogen control. The fungal pathogen *Verticillium lecanii* has the potential to be used as a microbial insecticide. According to earlier reports, *V. lecanii* infected both scale insects and aphids and it is speculated that a single strain of this fungus could successfully control both pests. This fungus can be utilised by using a fungus-based preparation as a myco-insecticide (Asante, 1999).

Pruning may also reduce the levels of *E. lanigerum* by the removal of many over wintering colonies in broken galls on one year old wood, and also by keeping the tree open to wind and sun (Greenslade 1936). In South Africa, larvae of syrphid flies, and adults and larvae of the coccinellid, *Exochomus flavipes* (Thnb.), have also been reported to feed on *E. lanigerum* (Carnegie, 1963). The activity of natural enemies can be enhanced by encouraging the growth of plants rich in nectar, such as *Phacelia* spp (Van den Bosch and Telford, 1964). These insects feed on the nectar produced by these plants and would in turn attract more natural enemies of woolly apple aphids, thereby controlling the presence of these insects in orchards.

1.3.1.2 Chemical control

The introduction of synthetic pesticides resulted in an added measure to control *E. lanigerum* (Smit, 1964). Initial treatments against *E. lanigerum* included sprays consisting of lie-sulphur and winter oil at the end of August. Poor chemical control of woolly apple aphids can be attributed to a legion of causes, including a degree of tolerance to registered insecticides; under-spraying and under-dosing; concentrate spraying; branch and foliar density of trees, and incorrect time of spraying (Swart *et al.*, 1990).

Vamidothion (Kilval) provides very effective control of *E. lanigerum* since the middle sixties. It has been used as a pre- and post-harvest spray (Swart *et al.*, 1990). This insecticide's efficiency is vastly increased when used in conjunction with either a pre-harvest spray such as fenitrothion (Folithion) or a post-harvest spray such as methidathion (Ultracide, Suprathion, and Untrathion). Effective woolly apple aphid control has also been reported after the use of endosulfan (Thiodon, Agrisulfan) (Swart *et al.*, 1990).

In the Elgin area, problems arose when it became evident that the aphid has become resistant to Vamidothion (Pringle *et al.*, 1994). Routine sprays do not provide effective control as aphids are protected by a tangled mass of wax threads. Aphids tend to aggregate, protecting some individuals from sprays. *E. lanigerum* also tends to feed in wounds that provide them protection against sprays. Aphids reproduce parthogenetically, thus rendering a single female capable of starting a colony, thereby reducing the efficiency of chemical control (Greenslade, 1936). The ideal chemical should control aphids in aerial parts of the tree and also prevent their upward migration, providing a high rate of initial death with very few residual effects (Madsen and Hoyt, 1957).

1.3.1.3 Cultural control

Wandering aphids can be captured on grease bands, but unless there is a definite migration upward, this would prove ineffective (Le Pelley, 1927). Irrigation can also contribute to reduced migration into trees, especially in areas of heavy soil as it would prevent cracking and therefore reduce root infestation (Greenslade, 1936). These techniques are, however, outdated and are not used on a large-scale, especially in the commercial production of apples.

1.3.1.4 Integrated control

No single method is effective in treating the effects of *E. lanigerum*. The integration of different methods will contribute to the effective control of *E. lanigerum* populations. Biological control can be enhanced by the use of chemicals that are fairly non-toxic to *A. mali*. Prolonged control of woolly apple aphids by *A. mali* can be achieved by means of spraying during the inactive period in the parasite's lifecycle, which would ensure the reduction of these pests. These spraying procedures would be effective as it would not affect the reproductive potential and pest control ability of *A. mali*, and

thereby help to curb aphid populations. The long-term use of Vamidothion resulted in the disappearance of *A. mali*, thereby necessitating repeated applications (Cohen *et al.*, 1996).

1.3.2 Control of subterranean aphid populations

Thus far there is no effective method of control, but studies are being conducted in the use of fungi to control these populations (Pringle, pers. comm. 2001). Indications are that an added means of control is needed to effectively combat woolly apple aphid infestations.

1.3.2.1 Future control measures

Molecular study in search of markers linked to effective resistance genes should aid in the optimisation of breeding programmes to develop resistant rootstocks. The use of rootstocks resistant to woolly apple aphids should provide facilitates an integrated control of this pest, and has significant financial advantages for the apple industry. Molecular markers will enable an effective screening procedure that can readily be applied to young seedlings, thus avoiding the costs incurred to grow these plants for extended periods.

1.4 Resistance to aphids

Host plant resistance is a broad concept that has not been defined clearly as a result of the complexity of this phenomenon. A plant is no longer considered to be a host when it is immune to attack by a parasite. Plant resistance is heritable and controlled by one or more genes. Resistance is relative and can only be measured by comparison to other genotypes. Resistance is also variable and can be altered by physical, chemical and biological factors (Auclair, 1989). Painter (1951) defined resistance to insect attack as the relative amount of heritable qualities the plant possesses, which reduces the ultimate damage done by the insect.

1.4.1 Types of resistance

There are six types of resistance according to Singh (1986). They include

- Resistance based on the mode of inheritance, e.g. dominant, co-dominant or recessive inheritance;
- Resistance based on the effect of genes and gene products;

- Resistance based on growth stages of the host plant;
- Resistance in epidemiological terms;
- Cytoplasm resistance;
- Resistance based on defence mechanisms.

The type of resistance that is applicable for this study can be defined in terms of epidemiology. Resistance in this class can be defined as vertical or horizontal. Vertical resistance (VR) can be described as when a variety is more resistant to some races of a pathogen than to others. This represents differential interaction between varieties of the host and races of the pathogen. VR is easy to manipulate in a breeding program. It is liable to break down when the pathogen produces a new virulent pathotype. Time of breakdown and its agricultural importance vary greatly depending on the nature of the disease under consideration (Robinson, 1971).

Horizontal resistance (HR) is when resistance is evenly spread against all races of the pathogen. The stability is possible due to its polygenic inheritance. Horizontal resistance also acts to reduce the effectiveness of one or more components of parasite fitness through amassing resistance genes, which act quantitatively in the host (Van der Plank, 1968). Nelson (1978) redefined HR as resistance that reduces the apparent infection rate. Aggressiveness is the capacity of a parasite to invade and grow in its host plant and to reproduce on or in it. A parasite's aggressiveness or the inability to attack can be described as counterparts of horizontal susceptibility and resistance in the host (Van der Plank, 1975b).

1.4.2 Genetics of Resistance

Assessment of resistance is based on plant injury, symptoms of the insect attack and on the reaction of insects to plants. Pest-host interactions are scored on a scale based on the reaction of a plant to insects. Reactions of the parents, F1, F2 and F3 progenies are used to study the dominant/recessive or incomplete dominant as well as the qualitative or quantitative nature of the mode of inheritance.

1.4.3 Degrees of resistance

Resistance is a term descriptive of a condition that is modelled between two extremes namely immunity and high susceptibility. Immune plants would represent non-hosts; and any degree of

reaction less than immunity would be resistance or susceptibility depending on the severity of the reaction. Any condition exceeding immunity is impossible.

Painter (1951) has defined the following scale for the degrees of resistance:

- **Immunity**: An immune cultivar is never consumed or attacked by a specific insect;
- **High resistance**: A variety with high resistance possesses a quality (-ies) resulting in small damage by a specific insect under a given set of conditions;
- **Low resistance**: Possession of quality (-ies), which cause a variety to show slightly less than average damage or infection for the crop under consideration;
- **Susceptibility**: Shows average or more than average infestation or damage by an insect;
- **High susceptibility**: The insect under consideration does more than average damage.

1.4.4 Mechanisms of plant resistance

Painter (1951) has described the mechanisms involved with plant resistance to insects as follows:

- Non-preference (also known by the terms non-acceptance and antixenosis). This makes plants unsuitable or unattractive for colonisation or oviposition by the insect;
- Antibiosis, which adversely affects the life cycle of the insect by reducing growth, survival and reproduction whenever the insect uses a resistant host for food;
- Tolerance enables the host plant to grow and reproduce itself or to repair injury to an extent;
- A fourth type of resistance, pest avoidance (Russel, 1978) has also been described, which suggests a tendency to escape infestation. The host plant might not be at a susceptible stage when the pest population is peaking.

Escapes can also be considered a form of functional resistance as it can be described as the lack of infection to the host plant due to transitory circumstances such as incomplete infestation (Herber, 1979). Escapes can however also be the result of a temporary, environmental situation.

1.4.5 Breeding (host) plants resistant to insects

Due to genetic heterogeneity and natural biological control, epidemics are very rarely observed in natural plant populations and wild species. Modern agricultural practices introduced important changes to traditional agriculture. The changes are as follows: (i) these changes presented a narrowed genetic

base of cultivars, thereby altering the dynamic balance between host and parasites, thus leading to epidemics; (ii) changes introduced also generated continuously distributed populations, thereby changing the whole ecosystem, creating habitats profoundly altered for the host and parasite (Singh, 1968).

Cultivating plants that are resistant to aphids does not differ essentially from similar breeding practices for other plant characteristics. Methods suitable for this venture depend largely on the breeding system of the host plant concerned. Factors to consider include whether the plant is cross- or open-pollinated or self-pollinated, as well as the sources of resistance that are available.

In cross-pollinated crops, individual plants are selected for their resistant qualities and cannot form the basis of resistance in a variety unless this particular trait is propagated on a large scale. Cross-pollinated plants are self-incompatible and thus cannot be selfed. If plants are self-pollinated, inbreeding results in a decrease in yielding quality. The effect of inbreeding does not have the same significant influence on vigour and yield in self-pollinating as in other crops. The choice of method to develop new cultivars also depends on the objectives of breeding programmes; reproductive characteristics of plants and inheritance of resistance to be incorporated. Most commonly used methods or aspects for effective breeding programs include (i) mass selection (Harvey *et al.*, 1972); (ii) backcrossing; (iii) pedigree selection; (iv) single seed descent; (v) early-generation testing and (vi) recurrent selection (Maxwell and Jennings, 1980; Niles, 1980). Basic plant characteristics imparting resistance or susceptibility to insects may be morphological or biochemical in nature. The introduction of an integrated pest management system allows the use of insect-resistant plants in combination with other control measures providing the most convenient and economical method of pest control (Auclair, 1989).

Various problems are associated with breeding plants resistant to insects (Singh, 1968):

- Intimate knowledge of the biology and feeding habits of insects is important;
- Insect variability; biotype differences within species shows different behaviour in laboratory-reared and natural colonies;
- Natural variability in growth, fecundity and other developmental of insects parameters;
- The development of an effective and reliable seedling-screening technique for mass selection;
- Year to year variation in host response;

- Environmental factors affecting infestation (temperature and humidity);
- Growth factors affecting the host behaviour, such as the influence of hormones, environment, etc;
- Maintenance of insect populations for accurate assessment of infectious studies and pre-screening;
- Whole year availability of insects and plants.

1.4.6 Assessment of host plant resistance

A descriptor scale for the evaluation of apple pests and diseases have also been proposed by the International Board of Plant Genetic Resources (IBPGR) and EC Committee on Disease Resistance Breeding and the Use of Gene Banks (Lateur and Populer, 1994). Screening techniques include the following (i) greenhouse screening; (ii) nursery screening and (iii) mini-cage studies, but these are not widely practised (Cummins and Aldwinkle, 1983).

An accurate assay of resistance depends on the severity of the infection. A rapid, repeatable scale must be developed that is related to (i) the development of the insect or (ii), to the damage done by the insect. Sometimes both can be used as criteria in screening plant genotypes. The effects of the insect upon the plant can be assayed in terms of a damage-rating scale or a recovery-rating scale (Jenkins, 1981). Knight *et al.* (1962) has developed a scale for the evaluation of aerial woolly apple aphid infestations, which is widely used in breeding programmes. According to this classification, classes 1-3 are considered resistant and classes 4-6 susceptible. Group 1 represents immune seedlings; group 2 seedlings with very slight infection that are not permanent. Group 3 has seedlings with small aphid colonies and small galls. For group 4, the whole seedling is attacked and infestations are severe, but not until late in the season. For group 5, the whole seedling is attacked as well and severe infestations are apparent as early as August in the northern hemisphere. Group 6 seedlings are severely infested and killed due to the severity of the attack (Knight *et al.*, 1962).

An evaluation scale has also been established by Cummins *et al.* (1981), based on visual ratings of infestations of woolly apple aphids. In similar studies conducted in New Zealand, a scale for the classification of seedlings corresponding to the one used for phenotypic screening in this study, has been developed ranging from zero to five, indicating extreme resistance and extreme susceptibility, respectively. For marker analysis, the rating 0 was considered to be resistant and ratings 1 to 5, ranging from low resistance to high susceptibility (Bus *et al.*, 1999).

The pictorial standard of damage may be used at the initial stages to identify rapidly infested material and to differentiate intermediate and susceptible. It is more effective scoring the plants individually than scoring whole plots of infested plants. Later evaluations should involve the level and expression of resistance, indicating insect counts. It is important that plants of the same growth stage or maturity level should be evaluated. Seedling stage and adult plant stage resistance may be correlated (Singh, 1968).

Aiding in the identification of resistance and appropriate scales, Parlevliet (1981), classified disease symptoms in three categories, which proved useful for resistance, studies, namely:

- Proportion of plant units infested or the percentage of diseased plants;
- Proportion of total area of plant tissue affected by the infestation (James, 1974);
- Detailed evaluations measuring number and size of successful infections for each plant.

1.4.7. Woolly apple aphid resistance in *Malus* spp

The varieties Northern Spy and Winter Majetin are considered to have been resistant to woolly apple aphids for more than 100 years (Painter, 1951). In 1967, Knight *et al.* identified 18 varieties that are immune or highly resistant to woolly apple aphids. In the resistant cultivars, Northern Spy has been used as a breeding parent in the development of commercial rootstocks, for example the Malling (M) and Malling-Merton (MM) series in the 1950's (Preston, 1955). Propagation of these rootstocks in different regions e.g. Australia, South Africa and North America are now common practice. However, infestations of the above-mentioned rootstocks in these regions have provided evidence of the existence of resistant-breaking biotypes in different parts of the world.

Painter (1951) and Knight *et al.* (1962) identified a single dominant gene for resistance to *Eriosoma lanigerum*, namely Er_1. According to Knight *et al.* (1962) this gene is closely linked to the self-incompatibility gene, *S*. The presence of this incompatibility gene can contribute to the segregation distortion observed in previous studies. Studies conducted in New Zealand identified the Er_1 gene in Northern Spy as well as another major gene, Er_3, in *M. sieboldii* 'Aotea' (Bus *et al.*, 1999).

1.5 Molecular assessment study

Classic strategies for evaluating genetic variability and the structure of components related to resistance and susceptibility are increasingly being complemented by molecular techniques. These techniques include the analysis of chemical components and also the characterisation of macromolecules. The identification of markers, DNA or non-DNA based, are essential for the increased success of combining traditional breeding with modern molecular techniques.

A variety of markers have been developed for use in breeding programmes. These include phenotypic or morphological and molecular markers. The scarcity of simply inherited morphological characters in apple is widely attributed to its allopolyploid origin. Mendelian segregation has been reported for numerous loci linked to, in particular pest and disease resistance, as well as fruit quality components (Alston and Batlle, 1992). Distinctive effects due to the presence or absence of genes have been observed, but intermediate types are also observed which can be attributed to accompanying minor genes or modifiers. Studies of morphological markers are limited and have hardly been done beyond the F1. Morphological markers linked to genes of agronomic importance have been identified and include the following: *Smh* (hypersensitivity to *Dysaphis plantaginea*); *Co* (columnar habit); *Rt* (anthocyanin pigmentation in all tissues and *l* (pale green lethal) (Alston and Batlle, 1992).

Molecular markers are based on polymorphisms found in proteins and DNA. These markers facilitate research in fields such as taxonomy, phylogeny, ecology, genetics and plant breeding. Early molecular studies identified isozymes as the most convenient markers since they can be determined at the whole plant, tissue or cellular level using straightforward laboratory techniques. Isoenzymic markers that have become well established in studies include the following: *GOT-1* (glutamate oxaloacetate transaminase) (linked to the S incompatibility locus); *ENP-1* (endophosphatase) and *ACP-1* (acid phosphatase) linked to *l,* the pale green lethal locus (Alston and Batlle, 1992). However, isozyme loci in apples have been found to be limited, as there is insufficient polymorphism amongst the loci of cultivated apples and selections used in breeding programmes. Despite the high level of polymorphism in *Malus,* cultivars are usually limited to one or two alleles at each enzyme locus. *GOT,* is an exception as it has six alleles distributed amongst apple cultivars (Manganaris and Alston, 1987).

The markers used in current research are DNA based, as they are easy to apply in modern-day breeding programmes. In plants and animals there are three potential sources of DNA: chloroplast (cpDNA),

mitochondrial (mtDNA) and nuclear (nDNA) genomes. DNA markers can be identified using different molecular techniques to detect polymorphism. Most DNA-based markers fall into one of three categories, depending whether markers used are hybridisation based or polymerase chain reaction (PCR)-based (Karp and Edwards, 1997). These categories are as follows: *Category 1*- hybridisation based (non PCR-based); *Category 2*- arbitrarily primed PCR and other PCR-based multi-locus profiling techniques; and *Category 3*- sequence targeted and single locus PCR. Some of these techniques are also derivatives or combinations of other methodologies.

Category 1 includes techniques such as restriction fragment length polymorphism (RFLP) analysis in which probes are hybridised to filters containing DNA, which have been digested with restriction enzymes. Resultant fragments are separated by gel electrophoresis and transferred onto filters by Southern Blot (Southern, 1975). Hybridisation is also possible with probes for minisatellite or microsatellite sequences to give variable number of tandem repeats (VNTR) and oligonucleotide fingerprinting.

Category 2 is the result of the advent of PCR, which removed the need for probe hybridisation steps. These techniques also do not require sequence information of the genome under investigation. The ranges of approaches also differ in length and sequence of the primers used, the stringency of the PCR conditions, and the method of fragment separation and detection (Karp and Edwards, 1997).

Within one subgroup of this category, single arbitrarily chosen primers are used in PCR and conditions in which primers will initiate synthesis despite an imperfect match with the template. Such techniques are known as multiple arbitrary amplicon profiling (MAAP) and arbitrarily amplified DNA (AAD) (Shierwater, 1995). Each product amplified will be derived from a region of the genome containing two segments that share sequence similarity to the single primer and are on opposite strands and sufficiently close together for successful amplification (Karp and Edwards, 1997).

A technique developed from the above-mentioned approach is random amplified polymorphic DNA (RAPD) analysis (Williams *et al.*, 1990). Amplified products are separated on agarose gels in the presence of ethidium bromide and visualised by means of ultraviolet light. Arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990) and DNA amplification fingerprinting (DAF) (Caetano-Anollès *et al.*, 1991) amplification products are separated and visualised on poly-acrylamide gels.

Modifications in primer design and PCR conditions for DAF reactions have been developed and include the use of mini-hairpin primers in DAF reactions (mhpDAF) and template cleavage involving the restriction of template DNA with restriction enzymes prior to amplification with one or more arbitrary primers. Arbitrary signature from amplification profiles (ASAP) (Caetano-Anolles and Gresshoff, 1996) and DAF products are compared using mini-hairpin or primers complementary to interspersed repeat sequences.

In the second subgroup the primers are semi-arbitrary, which means they are based on restriction enzyme sites/sequences interspersed in the genome such as repetitive elements, transposons and microsatellites. The use of primers based on restriction sites is the basis of selective restriction fragment amplification (SRFA) or amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993).

Due to the high mutability of simple sequence repeats (SSR) (Litt and Luty, 1989; Tautz, 1989) there are many versions of methodologies making use of 'microsatellites' as primers in micro-satellite (repeat)-primed PCR (MP-PCR) (Nelson *et al.*, 1989; Sinnert *et al.*, 1990). The first of these makes use of unanchored SSR primer amplification reactions (SPAR) (Gupta *et al.*, 1994) and is similar to RAPD, but the primers are SSR-based. Polymorphisms are, however, not SSR-based. The second technique uses inter SSR amplification (ISA or inter ISSR PCR) (Zietkowicz *et al.*, 1994) where two primers anchored to either the 5' or 3' end are used. Randomly amplified microsatellite polymorphism (RAMP) (Wu *et al.*, 1994) makes use of the random distribution of nucleotide sequences immediately flanking a SSR and is performed between a 5' unanchored mono-, di- or trirepeat and an arbitrary decamer primer. This approach also reflects the variation in SSR. Related techniques include randomly amplified macrosatellite polymorphisms (RAMPO) and randomly amplified microsatellite sequences (RAMS) (Richardson *et al.*, 1991; Cifarelli *et al.*, 1991). Arbitrary primers can be used to generate a SSR that can be hybridised to PCR products. The fourth approach is a SSR based modification of AFLP, namely selective amplification of microsatellite polymorphic loci (SAMPL) (Vogel and Morgante, 1995). This is performed using one labelled SSR primer and one unlabeled adaptor primer. Combining arbitrary or semi-arbitrary primed PCR with denaturing gradient gel electrophoresis (DGGE) (Myers *et al.*, 1987) can also attain increased polymorphism detection.

In category 3, the lack of allelic information, in terms of dominance as well as the assignment of alleles for arbitrarily amplified DNA can be avoided by PCR directed to specific single locus targets. A

prerequisite for this is knowledge of the sequence of the target region. Sequencing is, however, labour and resource intensive and often insufficient polymorphisms can be detected. Where polymorphic sequences have been detected, a number of simple PCR based assays have been established. "Normal" SSR analysis involves two primers flanking the SSR at a specific repeat (Litt and Luty, 1989). CAPS (cleaved amplified polymorphic sequence) analysis (Williams *et al.*, 1991) (also known as PCR-RFLP) involves the digestion of PCR products with restriction enzymes to reveal restriction site polymorphisms. If markers are required for highly sensitive detection of specific variants of a single locus, a number of techniques can be used such as allele specific oligonucleotide (ASO) hybridisation (Saiki *et al.*, 1989), amplification refractory mutation system (ARMS) (Newton *et al.*, 1989) and oligonucleotide ligation assays (OLA) (Nickerson *et al.*, 1990).

Properties of molecular markers, including DNA markers that make them suitable for use in research studies include the following (Weising *et al.*, 1995). All markers do not share properties mentioned below:

- They display highly polymorphic behaviour;
- They are co-dominantly inherited, allowing the distinction between homozygotic and heterozygotic states in diploid organisms;
- Their occurrence is frequent throughout the genome;
- They are evenly distributed throughout the genome;
- They portray selectively neutral behaviour, meaning that no pleiotropic effects are displayed;
- They are easily accessible;
- They are relatively easy to use and are amenable to automation making these markers a very fast, effective method of testing;
- They are highly reproducible;
- Information can easily be exchanged between laboratories.

1.5.1 Molecular marker technology in agriculture for effective resistance breeding

1.5.1.1 Why are markers needed for agricultural purposes?

The need for molecular intervention arose from the fact that traditional breeding and farm practices were not entirely effective in the eradication of pests and diseases afflicting crops. Intervention by

means of the incorporation of markers linked to disease resistance with commercially viable characteristics can lead to the cultivation of rootstock and cultivars able to withstand attacks from various diseases and pests. Marker technology has enabled breeders to produce economically viable crops without the time and possible financial constraints that formed part of early research to combat disease and other pests.

In apples the following limitations linked to conventional breeding have to be overcome in order to propagate this crop successfully. The sexual hybridisation process for the improvement of the apple is slow and lengthy. The juvenile period for apple seedlings range from six to eight years per generation cycle combined with another major deterrent namely a high level of self-incompatibility and the highly heterozygous nature of the genome. To transfer important characteristics of apple such as disease and insect resistance, cold hardiness and other desirable horticultural characteristics, requires several generations of crosses and involves large number of seedling populations in order to recover the trait along with other important traits of cultivated apple. If a major gene(s) controls traits, this process will be relatively shorter than if several minor genes controlled the trait. Despite being controlled by single genes e.g. *Vf* gene for apple scab, the time period for the introduction of the gene and the recovery of tree and fruit quality characteristics, may take up to 25-30 years. Self-incompatibility generally dictates that for each generation cycle of hybridisation, a new parent should be used for crossing with the selected progeny. The potential of *Malus* germplasm remains under-exploited due to limitations of conventional breeding. The implementation of biotechnological techniques for genetic improvement of apple is essential for overcoming the hindrances of the sexual hybridisation system.

1.5.1.2 The application of markers in marker-assisted selection (MAS)

Marker-assisted selection (MAS) is based upon the principle that if a gene(s) conferring a trait of interest is linked to an easily identifiable molecular marker, it may be more efficient to select in a breeding programme for the marker than for the trait (Lande and Thompson, 1990; Melchinger, 1990). MAS can be used with traditional breeding methods for the incorporation of either disease resistance genes or good horticultural qualities into commercially viable lines. Progenies can be screened and those containing the markers linked to the gene of interest can be identified if molecular markers on both sides of the gene are assayed. These markers can also be used in fine mapping around the gene, which forms the basis of positional cloning. Map segregation in highly heterozygous plant species can make use of informative genetic markers that segregate in a 1:1 fashion for the presence or the absence

of a DNA fragment in the F1 progeny. A parameter that affects the efficiency of MAS is the population size, as it controls the power of detection of marker-trait associations (Moreau *et al.*, 1998). Recent advances have also made it possible to produce near-isogenic lines and gene pyramids for disease and pest resistance but this is, however, not applicable to apples (Jones *et al.*, 1995). The introduction of PCR-based genetic marker systems led to dramatic changes in genetic studies in various crops. These techniques have resulted in reduced costs of identifying genetic markers and allow large-scale molecular marker studies that can be applied to genotyping of individuals at many loci.

Three different strategies can be followed when performing gene targeting by using molecular markers. One of these strategies involves the use of maps. The introduction of molecular markers has enabled researchers to construct dense maps in a short time-span. The first molecular marker map to be constructed for a crop plant was that of tomato (Bernazky and Tanksley, 1986). Map construction is a relatively straightforward procedure that can be done with the help of several software packages. Problems faced when constructing maps are the following: (i) there are four possible segregation ratios [1:1; 1:2:1; 3:1 and 1:1:1:1]; (ii) the linkage phase of the markers are not known in the parents and must be inferred from segregation of each pair of loci; (iii) linkage can only be estimated between pairs of loci that are heterozygous in the test plant (Tsai and Oka, 1966). Markers in heterozygous parents can be used as anchor points that allow the establishment of homologies between two maps. If a gene of interest is located close to a marker (5cM or less) or flanked by two markers (within an interval of 15-20cM), these markers can be used for selection. A pre-requisite for use in selection is the conversion of molecular markers to PCR markers enabling easy use in commercial breeding projects.

1.5.2 Maps and markers for apples

Mapping projects for apple were initiated almost a decade ago when a collaborative Apple Genome Mapping Project was launched in 1989 at the Horticultural Research International at East Malling. Partners in this project include Italy, Greece, France, Germany, the Netherlands and Switzerland. The project is based on four progenies and the mapping is conducted with phenotypic/morphological markers that are determined by single genes (King *et al.*, 1991). This project yielded a relational database and a patented application, APPLE-STORE, which can be used for the combined management of genotypic and phenotypic data. Studies are also being carried out to quantify the sensory properties and preferences for apples and to establish a link with genetic analyses. Related studies have also been

started to quantify the economic and social effects of apple production, aiming to provide breeders with a range of decision-making tools for effective cultivar selection (King, 1996).

The first linkage maps for apple were established for White Angel and Rome Beauty using isozymes and DNA polymorphisms, in a segregating population produced from a Rome Beauty x White Angel cross (Hemmat *et al.*, 1994). Linkage maps for 'Prima' and 'Fiesta' were also constructed using RFLP, RAPD, isozyme, AFLP, SCAR and microsatellite markers in 'Prima x Fiesta' progeny (Maliepaard *et al.*, 1998). In apple, the construction of testcross or backcross populations is not possible due to self-incompatibility. Inbred lines are not available for this crop either. The pseudo-testcross design is used in which the variety of interest is crossed to a standard variety known not to segregate for the traits being investigated. The segregation ratio for single gene traits is 1:1 in such populations and should make genetic analysis relatively simple (Hemmat *et al.*, 1994). The pseudo-testcross format represents the basic experimental design used in most breeding programmes.

Today, extensive genetic linkage maps and numerous molecular markers have been developed for apples. These include markers for resistance to different diseases such as apple scab (*Venturia inaequalis*), powdery mildew (*Podosphaera leucotricha*) and many more (Hemmat *et al.*, 1994; Weeden *et al.*, 1994; Gardiner *et al.*, 1994; Yang and Krüger, 1994). The types of markers identified for apple scab and powdery mildew include RFLPs, SCARs and RAPDs. Thus far Gardiner *et al.* (1999) have isolated two SCAR and three RAPD (Gardiner pers.comm., 2001) markers linked to the gene, Er_1, conferring resistance to woolly apple aphids. The SCAR markers are, however, fairly distant from the gene and more closely linked markers are envisaged. The SCAR marker, GS327, maps 11.9cM from the Er_1 gene and the RAPD marker, OPC20, 7.9cM from the gene, respectively. The possibility exists that recombination can occur thus rendering these markers ineffective for the identification of putative positive seedlings, containing the *Er1* gene. Gardiner *et al.* (2000a, 2000b) has also identified the Er_3, Er_{lon} and Er_{MIS} genes for woolly apple aphid resistance. Er_3 was identified in *M. sieboldii* 'Aotea', Er_{lon} in 'Prima x Longfield' and Er_{MIS} in 'Fiji' x MIS o.p., respectively.

Other genes have also been identified that are linked to other diseases and pest and are proving useful in breeding programmes. Genes conferring resistance to apple scab include V_f, V_m, V_{MIS}, V_{h2} and V_{h4}, while Pl_1, Pl_w *and* Pl_{MIS} provide resistance to powdery mildew and Sd_1 to rosy apple aphid (Gardiner *et al.*, 2000b).

1.5.3 Molecular markers used in the study

1.5.3.1 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism, or AFLP, is a PCR-based technique based upon selective restriction fragment amplification, developed and described by Zabeau and Vos in 1993.

AFLP can be used effectively for the detection and evaluation of genetic variation in germplasm collections and screening biodiversity. Sets of restriction fragments can be visualised by PCR without previous knowledge of the nucleotide sequence. This method also allows for the co-migration of a large number of restriction fragments (Vos *et al.*, 1995). AFLP can overcome all the problems experienced with techniques such as RFLPs. It combines the reliability of the RFLPs and the power of the PCR technique. The first phase in the AFLP technique involves the digestion of genomic DNA with two restriction enzymes, namely a six-base pair (hexa or rare cutter) and a four-base pair cutter (tetra or frequent cutter). The six-base pair restriction enzyme can either be *Eco*RI (Vos *et al.*, 1995), *Hind*III (Meksem *et al.*, 1995), *Pst*I (Milbourne *et al.*, 1997, Sharma *et al.*, 1996) or *Sse*I (Donini *et al.*, 1997) with *Mse*I as the four-base-pair restriction enzyme (Hartl and Seefelder, 1998). Methylation problems can be overcome by the use of methylation insensitive enzymes such as *Sse*I, which has successfully been used in *Triticum aestivum* (Donini *et al.*, 1997).

The main reasons for using AFLPs instead of other techniques are as follows:

- This technique can detect a relatively high level of polymorphism;
- This technique does not require prior sequence information;
- This technique allows many markers can be analysed in a short time (Zhu *et al.*, 1998 and Hartl *et al.*, 1998);
- This technique is fast and less laborious than other techniques (Maughan *et al.*, 1996; Castiglioni *et al.*, 1998);
- This technique provides more information per single experiment than other techniques (Maughan *et al.*, 1996; Castiglioni *et al.*, 1998).

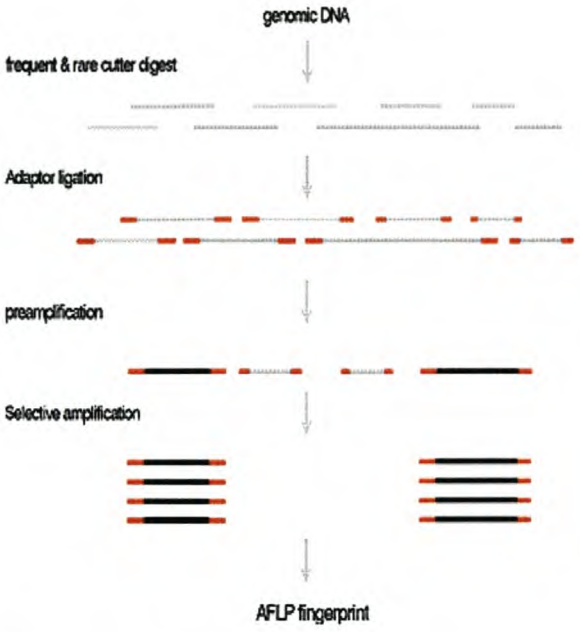


Figure 1.3

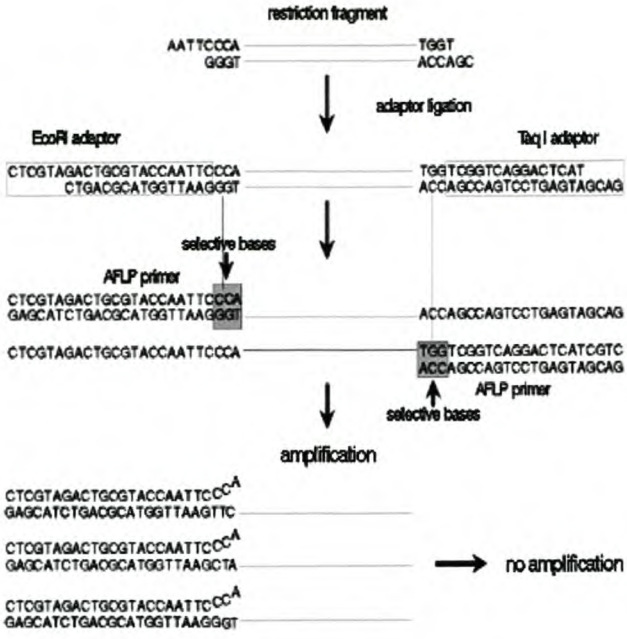


Figure 1.4

Figures 1.3 and 1.4 The AFLP steps are indicated in the above-mentioned illustration. The first part of the process entails the digestion of DNA using a rare and a frequent cutter restriction enzyme. In the second phase, adaptors are ligated to the ends of the restricted fragments. Amplification of a subset of the restriction fragments are performed using two primers complementary to the adaptor and restriction sequences, and extended at their 3' ends by selective nucleotides (adapted from www.keygene.com/html/aflp.htm)

The complexity of the banding patterns can easily be tailored by varying the number of selective base pairs at the 3` termini. This technique has the ability to detect more than 50 independent loci in a single PCR reaction (Maughan *et al.*, 1996). The AFLP fragment of interest can be excised from the gel, cloned, sequenced and transformed into a SCAR, as is also the case with other techniques such as RAPDs (Weising *et al.*, 1995).

Although the AFLP technique is supposed to yield highly repeatable results, AFLP profiles done on different organs of the same specimen can result in different profiles. Observed differences can possibly be attributed to differential DNA methylation levels in tissues. It has been established that DNA methylation levels differ temporally (different stages of plant growth and development) and spatially (different tissues). Preliminary studies have shown that the lower the incidence of methylation in the tissue, the higher the complexity of the AFLP profile. It is thus of vital importance

that the same plant tissue is used in phylogenetic studies and for identification purposes (Donini *et al.*, 1997).

Polymorphisms can be detected by the differences in length of the amplified fragments by poly-acrylamide gel electrophoresis (Daly *et al.*, 1998). Small differences, not only in size, but also in nucleotide composition, will change the mobility of a marker in a poly-acrylamide gel. Specific experiments have generated on average number of 101 fragments identified per AFLP reaction (ranging from 50 to 500 nucleotides), and an approximate number of 3500 different band positions per gel. From this data, we can estimate that per assay the probability of co-migration arising by chance is 0.03 (101/3500) (Rouppe van der Voort, *et al.*, 1997).

AFLPs do, however, not provide the highest level of polymorphism, but this is compensated by this technique's capacity to reveal many polymorphic bands per lane (Russell *et al.*, 1997). When compared to RFLPs, Simple Sequence Repeats (SSRs) and RAPDs, AFLPs have the highest marker index or diversity index (Mueller and Wolfenbarger, 1999). This is a reflection of the superior efficiency of AFLPs to simultaneously analyse large number of bands rather than the level of polymorphism detected (Russel *et al.*, 1997). A non-random distribution of AFLP markers can occur and this is usually due to clustering of markers in certain regions. Clustering may be due to reduced recombination rates caused by the presence of unrelated genetic material in one of the parents. Recombination is suppressed in regions where the genotype is heterozygous for the chromosomal segments originating from distantly related species (Rouppe van der Voort, *et al.*, 1997).

Polymorphic fragments produced by the AFLP technique may be considered as putative markers. Segregation analysis and linkage studies indicate that the AFLP markers are inherited in a Mendelian manner and that they segregate independently from each other. Nearly all the AFLP markers that have been examined appeared to display dominant segregation patterns where DNA fragments of the same length are amplified from one individual, but not from another. This has significant consequences for the alignment of genetic maps of different genotypes especially for plant species like *Solanum* that have high levels of intra-specific variation (Rouppe van der Voort *et al.*, 1997).

Despite the fact that segregation analysis of AFLP markers cannot be used to distinguish between heterozygotes and homozygotes, dominant markers have been commonly used in the analysis of taxonomic relationships, genetic diversity, genomic fingerprinting, and for the construction of linkage

maps (Angiolillo *et al.*, 1999; Hartl and Seefelfder, 1998; Lerceteau and Szmidt, 1999). Co-dominant AFLP markers may also occur where DNA fragments of differing lengths are amplified from the same locus (Maughan *et al.*, 1996). AFLP have been used to generate markers that can be used as SCARs for application in breeding programmes (Xu *et al.*, 2001).

1.5.3.2 Randomly amplified polymorphic DNAs (RAPDs)

RAPD is a modified PCR technique that requires no prior knowledge of the template DNA sequences (Williams *et al.*, 1990). This technique has also previously been referred to as blind analysis of DNA (Haymer, 1994).

Single short oligonucleotides of randomly chosen DNA sequences are combined with template genomic DNA, dNTPS and thermostable polymerase. This reaction mixture is subjected to PCR amplification under relaxed conditions. The annealing temperature usually varies from 35°C to 37°C. Amplification is restricted to the regions where the particular DNA sequences (complementary to the primer sequence) and the reverse primer sequence is found in the genome. A pre-requisite, however, is that sequences should be in a size range that can successfully be amplified by PCR. A range of DNA fragments are generated which can be visualised by gel electrophoresis, thereby making it possible to detect size differences in the sequences obtained for individuals, if it exists (Williams *et al.*, 1990). Sequence similarity or differences can only be verified by means of sequence analysis. Different primers generate different amplified bands that are randomly spread throughout the genome.

Minor adaptations in reaction conditions can significantly affect the number and intensity of amplified products. This is a major hindrance for obtaining reproducible results (Halldén *et al.*, 1996). An error rate of 5%-10% for RAPDs compromises the value of this technique for marker-assisted selection (Gu *et al.*, 1995). Reproducibility studies have been conducted for various molecular experiments, including RAPDs, wherein reproducibility and robustness of techniques were compared between different laboratories (Jones *et al.*, 1997). RAPDs were proved to be one of the more difficult techniques to reproduce.

The outcome of RAPD assays is greatly determined by the competition for priming sites (Williams *et al.*, 1993). It is possible to detect one allele at a locus during assays. This allele gives rise to an amplified product. RAPDs appear to display a dominant behaviour in most plant species and can thus

only be scored for presence or absence of a specific DNA fragment. As a direct result the information content of the data obtained is much lower than with other assay methods (Reiter *et al.*, 1992; Williams *et al.*, 1990). RAPDs have identified dominant markers in the tomato genome (Kawchuk *et al.*, 1994) as well as co-dominant markers in *Brassica nigra* (Quiros *et al.*, 1995). The scarcity of co-dominant markers can be attributed to the lack of sequence homology between the primers and target DNA (Rafalski and Tingey, 1993). When alleles of different sizes are amplified, heteroduplex formation can occur. This is observed when RAPD bands are amplified in the progeny and are present in the parental samples. However, this is not a common occurrence as heteroduplex formation rarely occurs in RAPD assays (Riedy *et al.*, 1992). Various studies have also identified RAPD markers in apple and many of these markers are linked to scab resistance (Yang and Krüger, 1994; Yang *et al.*, 1997; Bus *et al.*, 1999). RAPDs have found application in variety identification (Demeke *et al.*, 1993), pedigree analysis (Paran and Michelmore, 1993) and mapping in different populations (Quiros *et al.*, 1993).

1.5.3.3 Sequenced characterised amplified regions (SCARs)

A SCAR is a genomic DNA fragment at a single genetically defined locus that is produced by PCR amplification using a pair of specific primers (Paran *et al.*, 1993). SCARs, like other DNA-based markers are developmentally stable, detectable in all tissues, and unaffected by environmental conditions. These characteristics make them particularly well suited for conducting linkage analysis and chromosome mapping (Lu *et al.*, 1998).

The SCAR procedure is very useful for MAS as it illustrates the ability to examine many genotypes under stringent conditions that would eliminate the influence of contaminating DNA from unrelated organisms. These stringent measures are due to the fact that long primers complementary to specific genomic loci are used. SCARs can readily be applied to commercial breeding programs, as they do not require the use of radioactive isotopes (Paran *et al.*, 1993; Kawchuk *et al.*, 1998). SCARs are also helpful in molecular taxonomy studies as they only detect a single locus when examined across different species (Paran *et al.*, 1993). This type of marker is useful in map-based cloning as it can be used to screen pooled genomic libraries by PCR, unlike RAPDs that may contain interspersed repetitive sequences making it unlikely candidates for probing. SCAR markers are also an essential tool for mapping studies as co-dominant SCARs are much more informative for genetic mapping than dominant RAPDs.

SCAR markers can be derived following cloning and sequencing of AFLP or RAPD fragments (Van de Wiel *et al.*, 1998). Alleles/markers can be co-dominant and allele-specific, thus enabling the identification of susceptible, heterozygous resistant and homozygous resistant genotypes. Therefore linkage distance between the genetic markers and the locus of interest can be determined efficiently with high resolution. The use of SCARs also eliminates the need for a marker in the repulsion phase (Kawchuk *et al.*, 1998).

SCARS are primarily defined genetically; this makes them useful as physical landmarks and well as genetic markers. SCARs can also contain repetitive DNA sequences within the amplified region as they are analysed by PCR only. The uniqueness of these SCARs are determined by the sequence and spacing of the primers rather than hybridisation alone (Paran *et al.*, 1998). A further advantage of SCAR is that very little DNA is needed as compared with other techniques such as RFLPs (Perry *et al.*, 1998). SCARs also allow better evaluation of a genotype at a specific locus and an estimation of the contribution of the locus to the genotype variation between lines. It is also possible to reveal polymorphisms by the digestion of amplification products with restriction enzymes (Lahoque *et al.*, 1998). SCARs have been developed for the use in breeding programmes for apples (Bus *et al.*, 1999; Xu *et al.*, 2001).

A variation on SCARs is known as ASAPs (allele-specific associated primers). With these markers stringent annealing temperatures for the primers only generate single DNA fragments and only in those individuals possessing the appropriate allele (Gu *et al.*, 1995). This approach eliminates the need to separate the amplified fragments by electrophoresis. Direct staining of DNA with ethidium bromide identifies samples.

SCARs are similar to sequence-tagged sites (STSs) in construction and amplification, as both can be used as DNA landmarks in physical maps. A STS is a short single-copy DNA sequence amplified by PCR from genomic DNA using specific primers. STSs allow the easy exchange of information between independent laboratories. Known STS sequences also eliminate the need for the storage and distribution of large numbers of clones (Olson *et al.*, 1989; Lanham *et al.*, 1997; Paran *et al.*, 1998). STS markers have been developed for the V_m gene that confers resistance to apple scab (Cheng *et al.*, 1998).

AIMS OF THE STUDY

Apples are one of the important agronomic crops in the Western Cape and insect infestations and diseases related to their cultivation have dire financial implications for the industry. The damage caused by the woolly apple aphid, *Eriosoma lanigerum* (Hausmann) is a major concern to all producers in this region. The most feasible control of this problem would be the introduction insect resistant rootstocks. This control method would reduce the need for extensive use of pesticides and insecticides, and traditional control procedures that have also proven not to be as effective in a changing environment. Aphids have been able to acquire resistance to the known resistant cultivar; Northern Spy, and an alternative source of resistance has to be found. When the study was initiated, Er_1, was the only gene studied for which two SCAR markers have been identified. These markers have been proven to segregate in New Zealand cultivars derived from Northern Spy and are linked to woolly apple aphid resistance. No studies have been conducted to verify the segregation of this gene and its identified markers in South African cultivars and rootstocks. This gene, if pyramided with other genes for woolly apple aphid resistance, could provide effective control against various aphid subtypes known to affect apples.

Without the use of DNA-based molecular markers, the breeding of rootstocks and cultivars resistant to woolly apple aphids can be tedious and unreliable due to environmental influences. DNA-based markers have the advantage that they are stable, detectable in all tissues, and unaffected by environmental conditions or epistatic interactions. Expanding on existing methodology for the production of DNA-based molecular markers in apple would prove to be a great advance in breeding programmes.

The main aims of this study were firstly to identify molecular markers more closely linked to the woolly apple aphid resistance gene, Er_1, than existing markers, by applying AFLP technology to selected seedlings, identified to be resistant by conventional phenotyping. If successful, these markers can be incorporated into existing breeding programmes. Secondly, previously identified RAPD and SCAR markers were tested to determine their applicability in local populations for use in breeding programmes. Ultimately, the segregation of the Er_1 gene in South African populations can be monitored if tightly linked markers are identified.

Specific aims of this study were:

- Assessment of plant material
  - This included the inoculation of seedlings on aerial and terrestrial level.
  - Conventional screening of insect progress after successful inoculation.
- Preparation of plant material for DNA marker analysis
- Evaluation of various genomic DNA isolation procedures
- Evaluation of the AFLP and RAPD techniques, for identifying new markers tightly linked to the Er_1 gene and optimisation of these techniques for production of reproducible profiles for application in molecular screening.
- Testing the applicability of previously identified SCAR and RAPD markers in local population

CHAPTER 2
MATERIALS AND METHODS

2.1. Plant material used for the study

2.1.1 Seedlings developed from open-pollinated crosses

The Agricultural Research Council (ARC) Infruitec-Nietvoorbij Breeding and Evaluation Division supplied seedlings from 17 open-pollinated families. Information on only one parent of these families was known. From these 17 families, three were selected for molecular analysis i.e. Northern Spy (open-pollinated family 2); Rootstock 5 (open-pollinated family 6) and Russian Seedling (open-pollinated family 12) as in Table 2.1. Seedlings from Braeburn (open-pollinated family 15) were selected as the susceptible control.

The seedlings of each family were initially planted in 96-well seedling trays. After the first evaluation of the seedlings, they were replanted into individual bags, where they remained for the duration of the study. Plants were housed in a greenhouse with an air temperature of 27°C (± 5°C). The greenhouse temperature was regulated by means of a wet wall and a heater. Plants were subjected to winter rest, and housed in a room with an air temperature of 4°C. The humidity of this room varied from 40% to 70%. For this room also, temperature regulation was dependent on an air conditioner and a heater.

Table 2.1 List of open-pollinated seedlings included in the study

Open-pollinated seedlings	
Family Number	**Maternal genotype**
2	Northern Spy
6	Rootstock 5
11	Russian Seedling
15	Braeburn

2.1.2 Seedlings developed from hand-pollinated crosses

Hand-pollinated controlled crosses, for woolly apple aphid resistance, using six different genotypes (from which some of the the 17 open-pollinated families derive) were attempted in 1999 and 2000 (see Table 2.2). Three genotypes, from the original group of 17, selected for molecular analysis were included: Northern Spy, Rootstock 5 and Russian Seedling. Crosses resulted in seedlings of which both parents were known. Unsuccessful crosses of 1999 were repeated in 2000 but were not indicated if failed.

During a latter phase of the study (2001), two additional crosses were included for molecular analysis, namely Northern Spy x Russian Seedling and Northern Spy x Liberty. These seedlings resulted from cross 1, made in 1999 and cross 4, repeated in 2000, as indicated in Table 2.2.

Table 2.2 Crosses performed in 1999(ϒ) and 2000(ψ)

Crosses	# of seedlings (+ available seeds
1. Russian Seedling x Libertyϒ	500 (+300)
2. Northern Spy x Libertyϒ	100
3. Northern Spy x Russian Seedlingϒ	70
4. Mildew Resistant x Russian Seedlingϒ	None
5. Mildew Resistant x Golden Hornetϒ	450
6. Golden Hornet x Golden Deliciousϒ	250
7. Mildew Resistant x Russian Seedlingψ*	Data not available
8. Northern Spy x Russian Seedlingψ	Data not available

(* Repeat of 1999 crosses)

2.1.3 Infestation of seedlings

Two methods of infestation were applied in this study, namely aerial and terrestrial. In seasons 1999 and 2000 only aerial infestations were used. In the later stages of 2001, terrestrial infestations were also included to be able to compare the efficiency of the infestation methods used in the study, as well as to establish an effective method for the evaluation of subterranean aphid populations.

2.1.3.1 The protocol for aerial infestations

Two techniques were used for aerial infestations. During 1998/1999 and 1999/2000, dusting of seedlings was performed. Dusting techniques have previously been used successfully in infestation procedures (Dr K. Pringle pers. comm., 2000; Lyth and Watkins, 1981). For infestations, nymphs were shaken from the host plant onto the seedlings. This technique is effective and efficient when screening and infesting large numbers of seedlings. Infested plant material was obtained from the ARC Elgin Experimental Farm.

During 1999/2000 and 2000/2001 infested seedlings were attached to and planted next to test seedlings. This infestation allowed for re-testing or secondary screening. This procedure is illustrated in Figure 2.1.

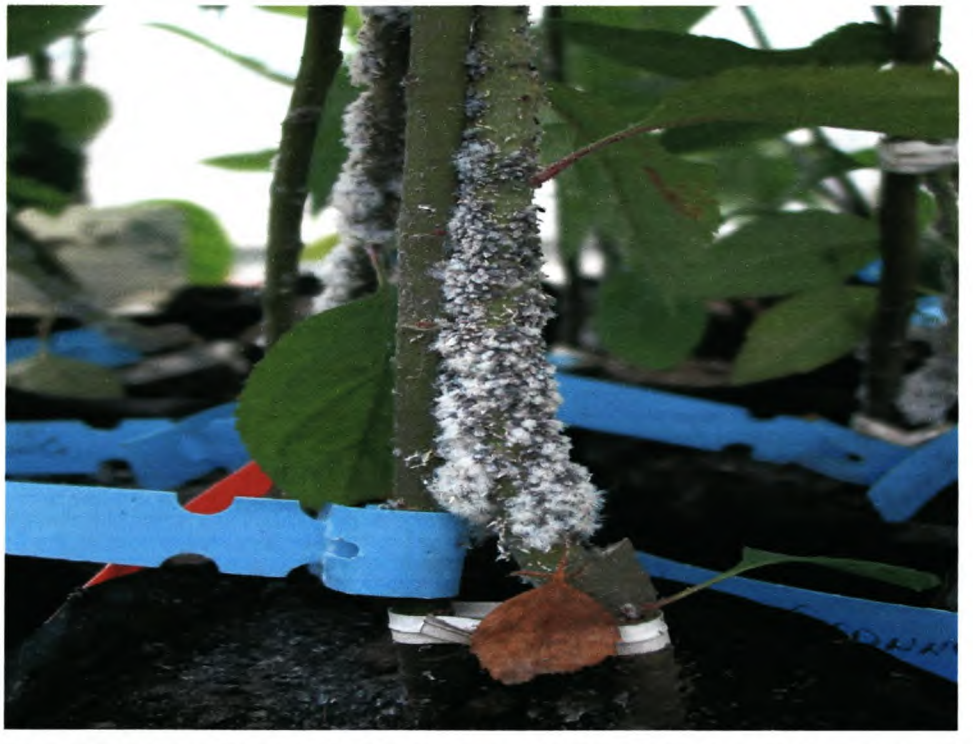


Figure 2.1 The seedling with the blue tag on the left, is infested by attaching and planting an infested seedling, on the right, directly next to each plant.

2.1.3.2 The protocol for terrestrial infestations

Infested roots were collected from the ARC Experimental Farm at Elgin. A pre-requisite for selection of roots was that all the material used had to be at least 2cm underground as this would ensure that no

aphid parasites, *Aphelinus mali*, were present. Roots were cut as near as possible to 1cm x 1cm. Root pieces were placed directly next to the root system of individual seedlings, planted in plastic bags, to ensure that aphids were able to access the roots of the seedlings. This piece of root material, in an opening in the soil, was covered with waterproof paper cups to ensure that aphids were not drowned, thus ensuring undisturbed migration. Root evaluations were performed after 6-8 weeks.

2.1.4 Evaluation of seedlings

2.1.4.1 Aerial evaluations

Plants were included in the study on the basis of phenotypic evaluation of seedlings. The following criteria were used for the selection of seedlings namely:

- Gall formation;
- Effective aphid infestation (% infected axils);
- Percentage infection (Infected leaf axils/Total axils x 100%) (Swart and Flight, 1990).

By phenotypic evaluation five classes of seedlings were defined ranging from extremely resistant to extremely susceptible. The classes were as follows: zero = clean/resistant; one = uninfected with regard to aphid infestation; two = medium infested and gall formation; three = highly infested, while seedlings were still alive (also known as "high, alive class") and four = highly infested but seedlings died because of infestation with WAA.

One of the measures of classification is the extent to which aphids have colonized the plant. Colonization was evaluated and also quantified on stems and roots. The criteria can be defined as follows: percentage infection is determined by taking into account the number of infested axils of each seedling and dividing it by the total number of axils on the plant. This provides a ratio that is expressed as a percentage. These percentages were used as additional criteria for the designation of classes. The infestation and the extent of colonisation are directly correlated to each other. A high level of colonisation leads to an increased disease level in plants and also enables aphids to spread in a very efficient manner within a controlled environment.

For the molecular study, twenty plants from the resistant (class zero) and highly susceptible (class three) categories were selected, respectively. These plants were chosen on the basis of percentage infection displayed in each individual seedling. The seedlings falling in the infection range of 0%-10% were selected for the resistant class and seedlings falling in the range 20%-100% were selected for the susceptible class. Plants with a higher percentage infection rate, higher than 80%, were preferably selected for the susceptible group of seedlings. This criterion had to be applied, as all the plants could not be included in the molecular study and it resulted in a dramatic decrease in the number of plants for molecular analysis. Bulk segregant analysis (BSA) consisting of three sets of bulks, including a resistant and susceptible bulk for each set was chosen as evaluation criteria. This would also result in decreased costs for molecular purposes. Classes one and two were discarded when bulks were decided upon, as only extreme classes were to be included.

2.1.4.2 Terrestrial evaluations

Terrestrial evaluations of infested seedlings were performed at the Department of Entomology, University of Stellenbosch. This procedure entailed the removal of aphids from roots by spraying and sieving the aphids and other debris three times. Finally the aphids collected were sieved through a 0.25-micron sieve and aphids were collected in a saturated sugar solution. The sugar solution is used, as aphids can easily be collected as they float on the surface. Aphids were categorized as: alive, dead and dry. Dry aphids were discarded for evaluation purposes (Damavandian, 1999) as it could not be determined whether these aphids died during the collection process of before. By including these aphids, skewed results would be obtained for that level of infestation in selected seedlings.

Ten susceptible and ten resistant seedlings were randomly selected for root analysis and evaluation of gall formation and aphid distribution. Dr K. Pringle from the Department of Entomology at the University of Stellenbosch performed this evaluation during February 2001.

2.2 Genomic DNA preparation

Suppliers of chemicals and reagents used are given in Table 2.3, while buffers and stock solutions are given in Appendix A.

Table 2.3 Chemicals and reagents used in DNA isolation procedures

Reagents	Manufacturer (if applicable)
Extraction buffer	See Appendix A
Beta-mercapto-ethanol	Merck, Germany
Polyvinylpyrrolidone (PVP-40)	Sigma, St Louis, USA
Tris-HCl	See extraction buffer and TE buffer
NaCl	Merck, Germany
Na_2EDTA	See Appendix A
CTAB	Merck, Germany
Chloroform:octanol	
RNAse A	Boehringer Mannheim (Roche)
Proteinase K (Sigma, St Louis, USA)	Sigma, St Louis, USA
TE Buffer (See Appendix A)	See Appendix A
Phenol	Merck, Germany
Agarose powder	FMC BioProducts, Rocklands, ME
Ethidium bromide	Sigma, St Louis, USA
1x Tris-borate EDTA buffer	See Appendix A
Low Mass DNA ladder	Life Technologies Ltd/GibcoBRL™, Paisley, UK
Bromophenol Blue	BPH Chemicals Ltd, England
Xylene cyanol	Boehringer Mannheim (Roche)
Sucrose	Merck, Germany

2.2.1 Genomic DNA isolation protocols evaluated

Various genomic extraction methods were evaluated to determine the optimal isolation technique, providing good yield and quality DNA. Isolation protocols included the following:

1. An SDS-based extraction protocol used specifically for apple (Gardiner *et al.*, 1996). The methodology is as follows:

Leaves (1-2 g) were ground in 2 ml extraction buffer (140 mM sorbitol; 220 mM Tris-HCl pH 8.0; 20 mM Na_2EDTA; 0.8 M NaCl; 0.8 % CTAB; 1% (w/v) N-lauroylsarcosine; 1 % (w/v) polyvinlylpyrrolidone). A 1.6 ml sample of the pulp was extracted using 0.4 ml of chloroform:octanol (24:1) (v/v) at 65°C for 30 minutes, whereafter it was centrifuged at 14000g for 10 minutes. DNA was

precipitated from the aqueous layer by adding 1 ml ice-cold isopropanol. The pellet was recovered after 5 minutes of centrifugation at 12000 g, washed twice with 70% ethanol, dried under vacuum, and resuspended in 0.1 ml of sterile water.

2. A CTAB-based large-scale plant genomic DNA isolation (Lavi *et al.*, 1987; Murray & Thompson, 1980). The methodology is as follows:

Leaf material (1-2 g) was ground in 10 ml of warm CTAB (2-5%) extraction buffer, 1% (v/v) 2-mercapto-ethanol. The mortar and pestles were rinsed with a further 2 ml 2% CTAB solution and transferred to the rest of the sample. The samples were incubated at 60°C for an hour with intermittent shaking. The specimens were allowed to cool to room temperature. Three chloroform:isoamyl alcohol (24:1) extractions were performed. The aqueous layer, between each extraction, was collected by centrifugation at 9000 rpm (8512 g) for 30 minutes at room temperature. The DNA from final aqueous layer collected, was precipitated 2.5x volume 1% CTAB extraction buffer and 1% 2-mercapto-ethanol. The suspension was gently mixed and precipitated overnight at room temperature. Centrifuging at 9000 rpm (8512 g) for 25 minutes pelleted the DNA. The supernatant was removed and the pellet was resuspended in 500 µl to 2 ml 1M CsCl, depending on the amount of DNA obtained. The DNA was precipitated with 2x volume of absolute ethanol for 30 minutes at -20°C. DNA pellets were obtained by centrifuging at 9000 rpm (8512 g) for 20 minutes at room temperature. The pellets were washed with 70% ethanol by a gentle swirling action, ethanol discarded and the pellets allowed to air-dry. Pellets were resuspended in 200 µl to 1 ml of TE buffer, depending on the size of the pellet.

3. A CTAB modified protocol (Murray and Thompson, 1980; Saghai-Maroof, 1994). The methodology is as follows:

At room temperature leaves (1-2 g) were ground to a fine powder. Five ml extraction buffer (2-5%) was added to the powder and samples were incubated at 55°C for 60 minutes with occasional mixing. An equal volume of chloroform/isoamyl alcohol (24:1) was added to each sample and mixed gently and thoroughly. The suspension was centrifuged at 5000 g for 30 minutes at 20°C. After centrifugation the upper aqueous layer was removed and transferred to a new tube, whereafter 2.5 volumes of ice-cold ethanol was added to each tube and gently mixed until the DNA precipitated. The sample was centrifuged at 1000g for 5 minutes. The supernatant was gently decanted, the tube inverted and the pellet allowed to air-dry for 5 minutes. The pellet was resuspended in 1 ml 0.5 M NaCl and precipitated in 2.5 volumes of ice-cold alcohol while mixing gently. The DNA was removed using a

pipette and rinsed in 10 ml 75% ethanol containing 10 mM ammonium acetate. The samples were incubated at room temperature for 20 minutes, with occasional swirling. The DNA was collected on the side of a 1.5 ml microfuge tube and allowed to air-dry. The DNA was dissolved in 200-800 µl sterile TE buffer, depending on the size of the pellet.

4. A CTAB-based protocol to investigate the suitability of repetitive DNA for cultivar identification in grapevine (Thomas *et al.*, 1993). The methodology is as follows:

Leaves (1-2g) were frozen in liquid nitrogen and ground to a fine powder, thawed and resuspended in 25 ml of Buffer A (0.25 M NaCl; 0.2 M Tris-Cl pH 8.0; 50 mm EDTA; 0.1% v/v 2-mercapto-ethanol; 2.5% w/v polyvinylpyrrolidone). The crude pellet obtained was centrifuged at 4000g for 10 minutes at 4°C. The pellet was resuspended in 5 ml of extraction buffer B (0.5 M NaCl; 0.2 M Tris-Cl pH 8.0; 50 mM EDTA; 1% v/v 2-mercapto-ethanol; 2.5% w/v polyvinylpyrrolidone; 3% sarkosyl; 20% ethanol). The suspension was incubated at 37°C for 30 minutes with occasional shaking. An equal volume of chloroform/isoamyl alcohol (24:1) was then mixed in by brief vortex, and the phases were separated by centrifugation at 16000 g for 10 minutes. The aqueous layer was collected, and 0.54 volume of isopropanol was added to the precipitate the DNA. The pellet obtained was resuspended in 600 µl TE containing 30 µg of RNAse A for 15 minutes and transferred to an Eppendorf tube. Protein was removed by the addition of a half volume of 7.5 M ammonium acetate, followed by centrifugation and transfer of the supernatant to a new tube where the DNA was resuspended with a 0.54 volume of isopropanol. DNA was resuspened in 200 µl of sterile water.

5. The use of polyethylene glycol for the purification of DNA from leaf tissue of woody plants (Rowland and Nguyen, 1993). The methodology is as follows:

This protocol is primarily based on the method of Doyle and Doyle (1987). Modification comprised of the addition of more CTAB after the chloroform extraction step and includes a final precipitation using polyethylene glycol (PEG). One-fifth volume of 5% CTAB (5% CTAB; 0.7 M NaCl) was added to the chloroform extraction step, followed by the precipitation with isopropanol. After the precipitation with ammonium acetate and ethanol, the pellets were resuspended in 0.5 ml TE, transferring the DNA to 1.5 ml eppendorf tubes, and precipitating with PEG. This involves the addition of 0.125 ml 4 M NaCl and 0.625 ml 13 % PEG and then incubating on ice for 1 hour. DNA was collected by centrifuging at maximum speed in a microcentrifuge for 10 minutes at 4°C. The pellets were washed in 70% ethanol and resuspended in a minimum volume of TE.

6. A CTAB-based extraction protocol for grapevine (adapted for apple) using lithium chloride precipitation (J.T. Burger, pers.comm. 1999). The methodology is as follows:

Two punched leaf discs and a pinch of carborandum (180 grid) were added to a 1.5 ml microfuge tube and ground to a paste. 100 µl of extraction buffer (0.5 M Tris-Cl, pH 8.0; 1.4 M NaCl; 3 % CTAB; 20 mM EDTA; 0.5 % 2-mercapto-ethanol) was added to each tube and ground further. After completing the grinding process, 400 µl of extraction buffer was added and the suspension mixed very well. The samples were incubated at 60°C for 15 minutes and allowed to cool to room temperature before adding 500 µl chloroform:isoamyl alcohol (24:1) and mixing by inversion. The samples were centrigufed at 12000 rpm (12879 g) for 15 minutes at 4°C. The supernatant was transferred to a new tube. One-third volume of 8 M LiCl was added and incubated overnight on ice. The samples were centrifuged at 12000 rpm (12879g) for 30 minutes at 4°C and the supernatant transferred to a clean tube. The DNA was precipitated by adding 0.8 volumes isopropanol and centrifuged at 8000 rpm (5724 g) for 10 minutes. 500 µl of 70% ethanol was added to the pellet and centrifuged at 8000 rpm (5724 g) for 5 minutes. The ethanol was discarded and the pellets briefly dried in a SpeedyVac. The pellet was resuspended in 50 µl sterile water.

7. A modified CTAB extraction protocol for extracting DNA from plants with high polyphenol and polysaccharide levels (Porebski *et al.*, 1997). The methodology is as follows:

Half a gram of leaf material was ground to a fine powder in liquid nitrogen, using a mortar and pestle. The homogenate was then transferred to 50 ml polypropylene centrifuge tubes. The extraction buffer (see Appendix A) was heated to 60°C and 5 ml added to the ground material in each centrifuge tube. β-mercapto-ethanol (0.3% v/v) was also added to each sample. For the removal of polyphenols and polysaccharides, 50 mg PVP-40 was added to the leaf material/CTAB solution in each tube. The suspension was mixed by inversion and incubated in a 60°C shaking water bath for an hour. The tubes were removed from the water bath and the suspension was cooled to room temperature for 4-6 minutes.

Next, 6 ml chloroform:octanol [24:1 (v/v)] was added and once again the tubes were mixed by inversion to form an emulsion. After mixing, the emulsion was centrifuged at 1000 g (3000 rpm) for 20 minutes in a tabletop centrifuge (Beckman Avanti™ 30 centrifuge) at room temperature. After centrifugation, the aqueous layer was removed and transferred to a new polypropylene tube using a wide-bore pipette tip. The chloroform:octanol extraction was repeated at least once more with the

aqueous phase to ensure that all contaminants were removed from the mixture. The equivalent of half the recovered volume of aqueous solution 5 M NaCl (see Appendix A) was added to each sample. The solution was mixed thoroughly whereafter two volumes of cold (-20°C) 95% (v/v) ethanol were added to each tube. The solution was once again mixed by inversion. To accentuate precipitation, the mixture was incubated at –20°C for 10 minutes. The precipitate was centrifuged at 1000 g (3000 rpm) for 6 minutes, followed by the removal of the supernatant. The pellet was then washed with cold 70% (v/v) ethanol, dried in a 37°C oven for 1 hour, resuspended in 300 µl TE buffer (see Appendix A) and allowed to dissolve overnight at 4°C.

The next day, the suspension was transferred to 1.5 ml Eppendorf tubes. Following the transfer, three microlitres of RNAse A (0.5 mg/ml) were added to each tube and incubated in a water bath at 37°C for an hour. Proteins were removed by the addition of 0.03 mg Proteinase K (10 mg/ml) (Sigma, St Louis, USA) and the tubes containing the treated solutions were incubated in a 37°C water bath for 30 minutes. Adding 150 µl phenol and 150 µl chloroform to each tube served to extract phenols. The tubes were vortexed briefly to ensure homogeneity, and then centrifuged (Beckman GS-15R) at 16300 g (14000 rpm) for 15 minutes. The upper layer from each sample was removed and collected in a new 1.5-ml tube. An additional 50 µl TE buffer was added to the phenol (bottom) phase. The tubes were once again vortexed and centrifuged at 16300 g (14000 rpm) for 15 minutes whereafter the upper layer was removed and added to the previously collected sample. By the addition of one-tenth ($1/10^{th}$) volume 3 M sodium acetate (see Appendix A) and two volumes absolute ethanol, then followed by gentle mixing, precipitation of DNA was achieved. The mixture was incubated overnight at -80°C to ensure thorough precipitation. Tubes were then centrifuged at 16300 g (14000 rpm) for 15 minutes to pellet the DNA. Contaminants were removed from each pellet by adding 70% (v/v) ethanol after which the pellets, facing outward, were centrifuged at 16300 g (14000 rpm) for 15 minutes. The supernatant was removed and the pellets vacuum-dried. Each of the pellets was resuspended in 50 µl TE buffer.

2.2.2 Electrophoresis protocol for determination of DNA integrity and concentration

The integrity and concentrations of the DNA samples were determined on 1.8% (w/v) agarose gels containing 0.5 µg/ml ethidium bromide, along with a Low Mass DNA ladder. One microlitre of DNA was mixed with five microlitres loading buffer consisting of 0.25% Bromophenol Blue, 0.25% Xylene Cyanol and 40% (w/v) sucrose. Visualization of DNA was achieved by the addition of ethidium

bromide to a final concentration of 0.5mg/ml to each gel. Gels were electrophoresed in 1X TBE (see Appendix A) running buffer at 80V for 1½ hour. The bands were observed on an UV-illuminator (UVO Inc. San Gabriel, CA). For future reference, images were captured with a Mitsubishi video copy processor, Printer Model P68B or the Biorad GelDoc System.

2.3 AFLP procedure

Table 2.4 Chemicals and reagents used for the AFLP procedure

Restriction digestion	**Manufacturer (if applicable)**
Genomic DNA	
Pst I	Boehringer Mannheim (Roche)
Mse I	New England Biolabs, Inc. Beverly, USA
Bovine serum albumin (BSA)	New England Biolabs, Inc. Beverly, USA
All-phor-one buffer	USB
Sterile water	Adcock Ingram, SA
Restriction-digestion-adaptor reaction	
Mse adaptor	IDT Technologies
Pst adaptor	IDT Technologies
All-phor-one buffer	USB
ATP	Amersham Pharmacia Biotech
T4 DNA ligase	USB
Sterile water	Adcock Ingram, SA

Table 2.4 (continued)	
Pre-selective amplification	
Mix 1	
Poo primer	IDT Technologies
Moo Primer	IDT Technologies
DNTPs	Promega, Madison, WI, USA
Sterile water	Adcock Ingram, SA
Mix 2	
Taq polymerase	Bioline, London, UK
NH_4 Buffer	Bioline, London, UK
Magnesium chloride	Bioline, London, UK
Sterile water	Adcock Ingram, SA
Agarose electrophoresis	
Agarose powder	FMC Bioproducts, Rocklands, ME
Tris-borate EDTA buffer	See Appendix A
Ethidium bromide	Sigma, St Louis, USA
1Kb-ladder	Promega, Madison, WI, USA
Primer labelling	
All-phor-one buffer	USB
T4 DNA polymucleotide kinase	Amersham Pharmacia Biotech
Pst primer	IDT Technologies
[γ-^{33}P] ATP	NEN™, Boston, USA
Sterile water	Adcock Ingram, SA

Table 2.4 (continued)	
Selective amplification	
Mix 3	
Mse primer	IDT Technologies
dNTPs	Promega, Madison, WI, USA
Sterile water	Adcock Ingram, SA
Mix 4	
Taq polymerase	Bioline, London, UK
10x PCR buffer	Bioline, London, UK
Magnesium chloride	Bioline London, UK
[γ-^{33}P] labeled Pst primer	
Sterile water	Adcock Ingram, SA
Denaturation and electrophoresis	
Deionised formamide	Sigma, St. Louis, USA
EDTA	Merck, Germany
Bromophenol Blue	BPH Chemicals Ltd, England
Xylene cyanol	Boehringer Mannheim (Roche)
Urea	Promega, Madison, WI, USA
Acrylamide/bisacrylamide solution (19:1)	Promega, Madison, WI, USA
10x TBE	See Appendix A
TEMED	Life Technologies/GibcoBRL Paisley, UK
Ammonium persulphate	Life Technologies/GibcoBRL Paisley, UK
Chromatographic paper	Merck, Germany
Kodak Biomax MR Film	Sigma, St Louis, USA
Development of autoradiographs	
Developing solution	Protea Medical Services
Water	
Fixing solution	Protea Medical Services

2.3.1 Restriction digestion, ligation and primer combinations

AFLP analysis as developed by Zabeau and Vos (Vos *et al.*, 1993) is based upon selective amplification of restriction fragments and combines the strengths of various marker systems. The first step of AFLP analysis involved the restriction digestion of template DNA with *Mse*I and *Pst*I . The components of the restriction digestion are listed in Table 2.5.

Table 2.5 Reagents of the restriction digest reaction

Reagents	Quantity /Volume per reaction
Genomic DNA (gDNA)	500 ng
Pst I (10U/µl)	5U (0.5 µl)
Mse I (4U/µl)	5U (1.25µl)
BSA (10mg/ml)	0.1µg/µl (0.4 µl)
All-Phor-One buffer (10X)	1X (4 µl)
Sterile water	Varies according to DNA input (Total volume = 40 µl)

Genomic DNA digestions were performed at 37°C for 3 hours. The digestion of 500 ng of genomic DNA was followed by the sticky end ligation of *Mse* and *Pst* double-stranded adaptors. The sequences of the adaptors and primers used in the AFLP analysis are listed in Appendix B. The components of the ligation reaction are listed in Table 2.6. The sequence of the adaptors and the adjacent recognition sites served as primer binding sites for the subsequent amplification of the restriction fragments.

Table 2.6 The reagents of the restriction-digestion-adapter reaction

Reagents	Quantity/Volume per reaction
Mse adaptor (50 pmol/µl)	50 pmol (1 µl)
Pst adaptor (5 pmol/µl)	5 pmol (1 µl)
All-Phor-One buffer (10X)	1X (1 µl)
ATP (10mM)	1 mM (1 µl)
T4 DNA ligase (7.5U/µl)	1 U (0.13 µl)
Sterile water	5.87 µl
Restriction-digestion mixture (total volume)	10 µl

Ligation-adapter reactions (total volume 50 µl) were incubated overnight at 37°C. Five microlitres of each reaction were kept for testing while the remaining 45 µl of the restriction-ligation mixtures were diluted in 1X $TE_{0.1}$ (10mM Tris-HCl, 0.1mM EDTA, pH 8.0).

2.3.2 Pre-selective amplification (Cold amplification)

Each reaction was carried out in a total volume of 50µl. The reactions were composed as follows:

13µl of the 10X diluted restriction-ligation (± 13ng of the template)

12µl of Mix 1 (Table 2.7)

25µl of Mix 2 (Table (2.8)

Table 2.7 Reagents of Mix 1

Reagent	Quantity/Volume per reaction
Poo primer (50ng/µl)	75 ng (1.5 µl)
Moo primer (50ng/µl)	75 ng (1.5 µl)
dNTPs (5 mM)	200 µM (2 µl)
Sterile water	7µl (to a final volume of 12 µl)

Table 2.8 Reagents of Mix 2

Reagents	Quantity/Volume per reaction
Taq polymerase (5U/µl)	1U (0.2 µl)
NH_4 buffer (10X)	1X (5 µl)
$MgCl_2$ (50mM)	1.5 mM (1.5 µl)
Sterile water	18.3 µl (to final volume of 25µl)

The AFLP Cold PCR profile was as follows:

72° for 5 min

94°C for 30s
56°C for 1 min
72°C for 1 min
} x30 cycles

72°C for 5 min

2.3.2.1 Verification of effective restriction-ligation and pre-selective amplification

Five microlitres of each reaction mixture were kept aside for verification testing. The remaining mixtures were diluted 1:9 with 1X $TE_{0.1}$. Five microlitres of loading buffer containing 0.25 % Bromophenol Blue, 0.25 % Xylene cyanol and 40 % (w/v) sucrose were added to each restriction-ligation and pre-selective amplification product. Samples were loaded onto a 1.8 % (w/v) agarose gel containing 0.5 mg/ml ethidium bromide. A 1kb-ladder served as a molecular size marker. Gels were electrophoresed in 1X TBE for 2 hours at 80V. Visualization of products was on a UV-illuminator (UVO Inc. San Gabriel, CA). For future reference, images were captured with a Mitsubishi video copy processor, Printer Model P68B or the Biorad GelDoc System

2.3.3 Primers and Primer labelling

2.3.3.1 Primers

The AFLP amplification employed two sets of oligonucleotide primers, e.g. one set corresponds to the frequent cutter (*Mse*I) RE ends and the other set to the rare cutter (*Pst*I) RE ends. The AFLP primers

consisted of three parts, namely a core sequence, an enzyme specific sequence (ENZ) and a selective extension (EXT) (Vos *et al.*, 1995). All the primers used are described in Appendix B.

2.3.3.2 Primer labelling

The *Pst* primer was radioactively labelled in each selective reaction, ensuring that only rare-cutter restriction fragments were displayed on autoradiographs. Primers were end-labelled using [γ-^{33}P] ATP and T4 polynucleotide kinase. The reagents used in the labelling reaction are presented in Table 2.9.

Table 2.9 The reagents used in the labelling reaction (sufficient for 20 reactions)

Reagent	Quantity per reaction /Volume per 20 reactions
All-Phor-One buffer (10X)	1X (0.5 µl)
T4 polynucleotide kinase (6U/µl)	0.05U (0.17 µl)
Pst primer (50ng/µl)	2.5 ng (1 µl)
[γ-^{33}P] ATP (10mCi/ml)	0.5 µCi (1 µl)
ddH_2O	2.33 µl

The labelling solution was incubated at 37°C for one hour and then inactivated at 65°C for 10 minutes. The solution was used immediately or stored at -4°C until needed.

2.3.4 Selective amplification (Hot amplification)

Selective amplification reactions were performed using radioactively labelled primers, following pre-selective PCR. These reactions were performed in a 20µl reaction mixture.
Each PCR consisted of the following:

- 8µl Mix 3 (Table 2.10)
- 7µl Mix 4 (Table 2.11)
- 5µl of 10x dilution of pre-selective PCR product

Table 2.10 Reagents used in Mix 3

Reagent	Quantity/Volume per reaction
Mse primer (50ng/µl)	30 ng (0.6 µl)
dNTPs (5 mM)	200 µM of each dNTP (0.8 µl)
Sterile water	6.6 µl (To a final volume of 8 µl)

Table 2.11 Reagents used in Mix 4

Reagent	Quantity/Volume per reaction
Taq Polymerase (5U/µl)	0.025 U/µl (0.1 µl)
10x PCR buffer	1X (2 µl)
Magnesium chloride (50mM)	1.5 mM (0.6 µl)
[γ-^{33}P] labelled primer	2.5 ng (0.5µl)
Sterile water	3.8 µl (To a final volume of 7 µl)

All amplification reactions were performed in an Eppendorf Gradient Multicycler. Once PCR reactions were completed, 10µl of loading buffer was added to each reaction. The loading buffer consisted of 98% deionised formamide, 0.2mM EDTA, 0.01% (v/v) Bromophenol Blue and 0.01% (v/v) Xylene Cyanol.

The AFLP Hot PCR conditions were as follows:

95°C for 30 s
65°C for 30 s
0.7°C
R= 3.0°C /s
G= 0°C
72°C for 1 min
} x 13 cycles

94°C for 30 s
56°C for 30 s
72°C for 1 min
} x 23 cycles

2.3.5 AFLP Electrophoresis protocol

The resulting PCR mixtures (containing loading buffer) were denatured at 94°C for 5 minutes and quickly cooled on ice. Four microlitres of each sample were loaded on a 6% denaturing (sequencing) poly-acrylamide gel. A gel mix of 75ml consisted of 11.25ml (40% (v/v)) acrylamide/bisacrylamide solution, 7.5ml 10X TBE (see Appendix A), 30g of Urea, 40µl 99% TEMED and 400µl of 10% (w/v) ammonium persulphate. The gels were cast and placed in a Model S2 Sequencing Gel Electrophoresis Apparatus (Life Technologies Ltd, Paisley, UK). 1X TBE was used as running buffer (200ml 10X TBE added to 1800ml dH_2O). Before AFLP amplifications were loaded, the gel was equilibrated for 30 minutes @ 80W. Electrophoresis of the loaded gels was continued at constant power of 80W for about 2 hours.

Once electrophoresis was completed, the gel was removed from the glass plates onto 3MM blotting chromatographic paper. The gel was dried using a gel dryer set at 80°C for 2 hours. Once the gel was dry it was placed in a cassette with a Kodak Biomax MR film covering it. The length of time of exposure was dependent on the readings obtained from the Geiger-Muller counter. The exposure time varied from 1 to 7 days. When signals were higher than 20K, the film was only exposed for one day. Autoradiographs that signalled between 10-20K were exposed for 3-4 days and all other autoradiographs with lower signals were exposed for up to 7 days.

2.3.6 Development of autoradiographs

In a dark room, pour 2 l of developing solution in tray. Place the autoradiograph in the solution and agitated the tray slightly, to distribute the solution evenly across the film, until banding patterns appears. After the appearance of banding patterns on the film, rinse the film in water, removing excess developing solution. Add 2 l of fixing solution to another tray, and place the rinsed film in the bowl. Allow fixation for approximately 2 minutes. Remove the film and rinse in clean water, removing excess fixing solution. Allow film to dry.

2.4 Verification screening

Table 2.12 Chemicals and reagents used for verification screening

Reagents	Manufacturer (if applicable)
Primers as specified below	IDT Technologies
Genomic DNA	
Magnesium chloride	Bioline, London, UK
DNTPs	Promega, Madison, WI, USA
Taq polymerase	Bioline, Madison, WI, USA
PCR reaction buffer	Bioline, Madison, WI, USA
Sterile water	Adcock Ingram, SA
Bromophenol Blue	BPH Chemicals, London, England
Xylene cyanol	Boehringer Mannheim (Roche)
Sucrose	Merck, Germany
Agarose powder	FMC Bioproducts, Rocklands, ME
Ethidium Bromide	Sigma, St Louis, USA
1kb-ladder	Promega, Madison, WI, USA

Table 2.13 List of primers used for screening

Marker	Linked Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
GS327	Er_1	GCCAAGCTTCAATGTCG GAGTAGAT	CAAGCTTCCCCTAAGGC TATTGCCA
OPC20	Er_1	ACTTCGGCAC	CCCAGTCACTGGCAAG AGAAATTAC
OPO05aSCAR	Er_1	CCCAGTCACTAACATAA TTGGCACA	CCCAGTCACTGGCAAG AGAAATTAC
OPO05bSCAR	Er_1	AACGTCATGTCAATAT	CCCAGTCACTGGCAAG AGAAATTAC
OPC20aSCAR	Er_1	TCTCTAACTCAATAACT CCCAAGAC	GCCTACATGAATCAG
OPC20bSCAR	Er_1	CTACATTAAGCTTTG	GCCTACATGAATCAG

To determine the optimum PCR conditions for each of the sets of primers, genomic DNA, primer and magnesium chloride ($MgCl_2$) concentrations were varied until the best results were obtained for each of the individual primer sets. Initially temperature gradients were performed to determine the optimal temperature range for each of the primers as published annealing temperatures did not yield optimal results. Final magnesium chloride concentrations were varied from 1-3 mM and genomic template concentrations varied from 10-60 ng. Primer concentrations varied from 5-10 pmol/µl. Optimal PCR conditions for the primers are given in Table 2.14.

The 20µl reaction volume for the GS327 SCAR marker consisted of the following: 40 ng of the template DNA, 10 pmol each of the forward and reverse primers. In addition 200 µM of each dNTP (Promega, Madison, WI), 0.5U units of Bioline *Taq* polymerase, 1/10 (v/v) PCR reaction buffer [1mM Tris-Cl (pH8.3), 5mM KCl], 2 mM of $MgCl_2$ and sterile water were also included. PCR conditions for the other markers differed only in the amount of DNA used as well as magnesium chloride concentrations. PCR was performed on a Perkin-Elmer GeneAmp PCR system 2400 or a Perkin-Elmer GeneAmp PCR system 9700.

Table 2.14 Optimal PCR conditions for the primers were as follows:

Primer set	gDNA (ng)	[$MgCl_2$]	Annealing temperature
GS327S SCAR	40 ng	2 mM	69°C 10x cycles, 67°C 30x cycles
OPC 20	40 ng	2 mM	34°C
OPO 5a SCAR	20 ng	2 mM	55°C 10x cycles, 52°C 30x cycles
OPO 5b SCAR	20 ng	1.5 mM	48°C 10x cycles, 45°C 30x cycles
OPC 20a SCAR	20 ng	2 mM	55°C 10x cycles, 52°C 30x cycles
OPC 20b SCAR	No optimal conditions	No optimal conditions	No optimal conditions

The PCR profile for the GS327 SCAR marker was the following:

94°C for 4 min

94°C for 30 s
69°C for 1 min
72°C for 2 min
} x 10 cycles

94°C for 30 s
67°C for 1 min
72°C for 2 min
} x 30 cycles

72°C for 10 min

The PCR profiles for OPO5a and OPC20a were as follows:

94°C for 4 min

94°C for 30 s
55°C for 30 s
72°C for 1:40 min
} 10x cycles

94°C for 30 s
52°C for 30 s
72°C for 1:40 min
} 25x cycles

72°C for 7 min

The PCR profile for OPO5b was as follows:

94°C for 4 min

94°C for 30 s
48°C for 30 s
72°C for 1:40 min
} 10x cycles

94°C for 30 s
45°C for 30 s
72°C for 1:40 min
} 25x cycles

72°C for 7 min

On completion of PCR amplifications, 5 µl of the PCR product were mixed with 5 µl of loading buffer. The loading buffer consisted of 0.25% Bromophenol Blue, 0.25% Xylene Cyanol and 40% (w/v) sucrose. Ten microlitres of the resulting mixture were loaded onto 1.8% (w/v) agarose gels, stained with ethidium bromide, and electrophoresed in 1X TBE buffer for 2 hours at 80V. A 1kb-ladder was used as a molecular size marker. The amplified products were observed on a ultra-violet transilluminator (UVO Inc. San Gabriel, CA) and for future reference; photos were taken with a Mitsubishi video copy processor, Printer Model P68B or the Biorad GelDoc System.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Plant material

3.1.1 Selection of plant material

The initial trial was performed on seedlings developed from open-pollinated varieties where one parent was known. Of the seventeen open-pollinated families included for evaluation three families were selected for molecular analysis. These families were: Northern Spy, Rootstock 5 and Russian Seedling. The susceptible control included in the study was an open-pollinated Braeburn family.

Subsequent crosses were performed to obtain seedlings with both parents known for more extensive molecular analysis. Final selection for molecular analysis was based upon the seedlings' reaction to woolly apple aphid infestation. A summary of the data according to the percentage infection of the three families selected for molecular analysis is indicated in Table 3.1. The ratio resistant to susceptible seedlings was determined from the number of infected axils per total number of axils, and expressed as a percentage.

Table 3.1 Summary of the data on the segregation for resistance to *Eriosoma lanigerum* (Hausmann) in progenies of Northern Spy, Rootstock 5 and Russian Seedling.

Family	Evaluation date	Binomial distribution (Snedecor and Cochran) (95% confidence interval) Resistant seedlings		Probable ratio	Chi square for 1 degree of freedom (Total samples)	P value
		Number /Total	%			
Northern Spy	February 1999	26/57	46 (37-66%)	1:1	0.438 (57)	0.508
	May 2000	23/45	51 (32-61%)	1:1	0.011 (45)	0.916
	February 2000	25/58	43 (36-44%)	1:1	1.793 (58)	0.180
Rootstock 5	February 1999	32/61	52 (50-77%)	1:1	0.148 (61)	0.700
	May 2000	31/57	54 (47-75%)	1:1	0.438 (57)	0.508
	February 2000	29/53	55 (43-72%)	1:1	0.472 (53)	0.492
Russian Seedling	February 1999	29/64	45 (43-72%)	1:1	0.563 (64)	0.453
	May 2000	23/57	40 (32-61%)	1:1	2.122 (57)	0.145
	February 2000	25/59	42 (36-64%)	1:1	1.373 (59)	0.241

Northern Spy had displayed resistance to woolly apple aphids in past trials and this has also been documented in literature (Painter, 1951 and Knight *et al.*, 1962). A 1:1 segregation pattern was described, which indicates the influence of a single dominant resistance gene. Similar results were obtained in the present study. After open pollination, the resistant to susceptible progeny from the Northern Spy segregated approximately 1:1. This segregation ratio was confirmed by using the Chi square test and the binomial distribution according to Snedecor and Cochran (1967) (Table 3.1). According to the estimated 95% confidence intervals of the binomial distribution, Northern Spy fits a 1:1 ratio. For each of the seasons, the percentage resistant seedlings fall within the percentage range as indicated in brackets, showing that it meets the criteria of a 1:1 segregation pattern. The P values obtained from the Chi square test, for each progeny for the total samples indicated in brackets, for the three seasons, also confirm a probable ratio of 1:1. This data may, however, not be conclusive because the cross was derived from an open pollination where the male parent could also have contributed to resistance. Furthermore, the class 1 and class 4 ratings were not considered in the calculation, as described in Materials and Methods (Chapter 2).

Rootstock 5 and Russian Seedling have also shown resistance to woolly apple aphids and 1:1 resistant to susceptible segregation ratios were also observed (Table 3.1). Although not established on a molecular level, it is speculated that Rootstock 5 could have Northern Spy as one of its parents (Dr I. Labuschagné, pers. comm., 1999). From the segregation patterns observed, it is possible that the 1:1 segregation ratio for the Rootstock 5 progeny could be the result of the same gene responsible for resistance in Northern Spy.

Russian Seedling has never been known for its resistance to woolly apple aphid but has been included here on the basis of the seedling screening results. A segregation pattern ($\approx$ 1:1) similar to that in the other two populations was shown (Table 3.1). It is possible that the resistance in Russian Seedling originates from a gene(s) other than Er_1. In recent studies, the presence of the other genes for woolly apple aphid resistance has been described in *Malus robustus* and MIS (Mildew Immune Selection) (Bus *et al.*, 1999). Braeburn was selected as the susceptible control as it had previously shown susceptibility to aphid infestations (Dr I. Labuschagné, pers.comm., 1999).

Crosses were performed in both 1999 and 2000 to obtain plants of known parentage to perform molecular analysis. In 2001, plant material was obtained from successful crosses performed in 2000

and could be included in the molecular study. Two populations from the hand-pollinated crosses were included in the study for molecular analysis (see Table 2.2 in Chapter 2).

3.1.1.1 Classification of seedlings

A high level of infestation and colonization lead to an increased disease level in seedlings and also enabled the efficient spread of aphids. Plants in class zero were considered to be resistant, as they were not afflicted by aphid infestations. These plants were healthy and also of normal height. The undefined class one included plants that showed stunted growth, but appeared normal otherwise. Stunted growth could be attributed to physiological and growth factors. Plants in class two were seedlings displaying medium levels of infestation. No visible colonization had taken place but there was a high incidence of single aphids. Gall formation was an additional factor contributing to classification within class two. Plants in class three suffered from severe aphid infestation but were still viable. Extensive colonization was visible on these plants and it extended across the entire plant. Class four included dead plants, irrespective of whether the seedlings death was the result of aphid infestation or other causes, e.g. pathogenic infections.

For the molecular study, twenty plants from the resistant (class zero) and highly susceptible (class three) categories were selected, respectively. These plants were chosen on the basis of percentage infection displayed in each individual seedling. The seedlings falling in the infection range of 0%-10% were selected for the resistant class and seedlings falling in the range 20%-100% were selected for the susceptible class. Plants with a higher percentage infection rate, higher than 80%, were preferably selected for the susceptible group of seedlings. This criterion had to be applied, as all the plants could not be included in the molecular study and it resulted in a dramatic decrease in the number of plants for molecular analysis. Bulk segregant analysis (BSA) consisting of three sets of bulks, including a resistant and susceptible bulk for each set was chosen as evaluation criteria. This would also result in decreased costs for molecular purposes. Classes one and two were discarded when bulks were decided upon, as only extreme classes were to be included.

3.1.2 Infestation procedures

3.1.2.1 Aerial infestations

This technique, used from the second season (2000) onward, comprised the attachment of an infested shoot to individual seedlings (Knight *et al.*, 1962). This method is used routinely to re-test resistance against seedlings pre-selected for resistance by means of the "dusting" technique. Direct migration of aphids from the infested shoot to the selected seedling minimizes the change for seedlings to escape infestation.

3.1.2.2 Terrestrial infestations

A trial using terrestrial infestation was performed to test the association between aerial and terrestrial infestation methods. According to literature, a seasonal migration pattern on the tree has not been observed for woolly apple aphids in South Africa (Giliomee *et al.*, 1968). Placing the infested roots directly next to the seedling's root system facilitates migration of aphids and should therefore result in higher infestation levels. This method of infestation would also ensure that an underground population of aphids would be established for each seedling, if this had not occurred yet. Eight weeks had to pass after the direct infestations, to allow for the aphids to settle on the plants, and to make provision for possible migration.

3.1.3 Evaluation of infestations

3.1.3.1 Aerial evaluations

Evaluation of seedlings was based on percentage infection and galling. Over a three-year period some variation was observed in the classification of individual plants. This implies, for example, that seedlings classified as resistant in season one, could have shown a susceptible phenotype in following seasons. Such plants can be described as escapes.

Infestation results for each of the selected populations for a three-year period are given in Tables A-F in Appendix C. It should be noted that infestation data for November 2000 is not available for all seedlings because this infestation was applied as a test to verify results of the pre-selection. Randomly

selected seedlings were scored. Many of the seedlings that do not have complete infestation data for some of the seasons have been omitted accidentally when evaluations were performed. All the seedlings as listed in Table 2.1 (Chapter 2) were housed in the same greenhouse, all positioned at random.

The percentage infection (as indicated in Appendix C) was determined for each seedling in the resistant and the susceptible classes. Information derived over the three-year period gives an indication of whether the initial classification of seedlings was indeed correct. In some instances, in both susceptible and resistant pools, seedlings were identified that were initially incorrectly classified during pre-selection.

The infestation data for Northern Spy are summarised in Tables A and B (in Appendix C). A total of 26 plants were consistently classified as resistant over a three-year period. These seedlings had infestation levels of 10% or less for three seasons. The abovementioned data corresponds well with documented literature that indicates resistance in Northern Spy that is mediated by a single dominant gene in the heterozygous state (Crane *et al.*, 1936). It was postulated that the reaction of seedlings due to aphids feeding, but not consuming the plant, could possibly be attributed to antibiosis. Antibiosis is a biochemical reaction of the plant to prevent aphids from establishing colonies. The classification of fewer plants within a specific group can also be the result of a cross between a heterozygous immune parent and a homozygous susceptible parent, as a single dominant gene mediates the trait of resistance (Daubeny, 1983). One plant from this group, namely 2.11(0), died during this study.

In the susceptible seedling group of the Northern Spy population, a total number of 31 plants were selected. Sixteen of the selected seedlings displayed consistent levels of infestation over a three-year period that would justify their classification within this group. Variable resistance were observed for the remainder of the seedlings. Some of the seedling data were not available due to death during evaluation. Two escapes have also been identified within this group, namely 2.20 (3) and 2.27 (3), as they have shown no susceptibility to woolly apple aphids in two seasons.

Overall, the majority of Northern Spy seedlings appear to be correctly classified. The segregation ratios obtained indicate the trend of a 1:1 segregation between the resistant and susceptible seedlings. Misclassification has occurred but it was expected, as the data for the three-year period would confirm the correct classes for the seedlings, and infestation pattern would be evident.

The infestation data for Rootstock 5 is summarised in Tables C and D in Appendix C. A total of 32 plants were selected in the resistant group and 29 in the susceptible group. Twenty plants showed resistance over a three-year period, thereby confirming their resistant status. Two escapes have been classified within the resistant pool, namely 6.19 (0) and 6.21 (0). These seedlings appeared highly resistant in three evaluations, including November 2000, but the last evaluation in February 2001 indicated high levels of infestation. Of the susceptible plants selected for Rootstock 5, 16 of the 19 were infested over a three-year period. Five susceptible plants died as a result of either woolly apple aphid infestation or other reasons, e.g. pathogen infection. It is evident from the data that no incorrect classification was made. Most of the seedlings reflected an infection percentage of 100% at the last evaluation for this study performed during 2001, indicating that most seedlings were initially correctly classified. Segregation patterns for this population also fit the 1:1 ratio, which is indicative of resistance, mediated by a single dominant resistance gene.

The data for Russian Seedling is summarised in Tables E and F in Appendix C. Twenty-one seedlings were selected for the resistant group of which 17 showed resistance behaviour for the duration of the study. Some escapes can be identified in this group; six plants showed resistance when first classified, but further analysis have proven them to be susceptible to woolly apple aphid attack.

The susceptible group of seedlings for Russian Seedling comprised of 35 plants. The bulk of the group, namely 26 seedlings, were consistently scored as susceptible for three seasons. Two escapes have also been identified within this group. The levels of infestation recorded for each evaluation varied significantly.

Literature searches have yielded no evidence of similar data for Rootstock 5 and Russian Seedling. No conclusive evidence is available to compare the response to woolly apple aphids in the above-mentioned rootstocks. The information obtained can however, form the basis of future studies, in search of suitable markers and genes linked to woolly apple aphid resistance.

3.1.3.2 Terrestrial evaluations

For subterranean testing, an evaluation was performed on randomly selected samples. Gall formation and number of aphids on roots of each seedling were determined. Gall formation was only observed on

susceptible plants, thus implying that these plants cannot resist attack by woolly apple aphids. Evaluation procedures were followed as set out by Damavandian (1999) and summarized in Section 2.1.4.2. Data obtained from this evaluation can be seen in Tables 3.2 and 3.3.

From the data below in Table 3.2, it can be concluded that woolly apple aphid root infestation is very low in the resistant populations. These evaluations were only performed on Northern Spy populations (see Chapter 2, Table 2.1). The number of aphids in the 10 plants included in the study varied from seven to 81. No defined colonies could be identified on the seedlings. It appears as though the aphids could not effectively establish themselves on the root systems of these seedlings. This is possibly due to antibiosis described previously (Knight *et al.*, 1962).

Table 3.2 Root evaluations for randomly selected resistant seedlings in 2001.

Seedling number	Number of aphids	Galls present
Resistant		
2.1.0	56	0
2.7.0	52	0
4.2.0	6	0
4.8.0	7	0
4.16.0	31	0
4.17.0	81	0
5.4.0	45	0
5.6.0	52	0
5.7.0	11	0
5.9.0	19	0

No gall formation was evident in any of the resistant seedlings, in this particular evaluation. Gall formation is partially caused by feeding of aphids on the seedlings (Staniland, 1924). Galls are formed by the insects' saliva, thereby stimulating abnormal meristematic activity producing large parenchymatous cells that suppress differentiation of xylem and sclerenchyma cells. The aphids present on the resistant seedlings cannot feed effectively on these plants, thus the absence of galls.

Table 3.3 Root evaluations for randomly selected susceptible seedlings in 2001.

Seedling number	Number of aphids	Galls present
Susceptible		
2.2.3	413	1
2.7.3	260	3
2.23.3	88	0
4.22.3	1237	3
4.23.3	136	2
4.24.3	383	0
4.31.3	112	3
4.36.3	563	0
4.37.3	618	3
4.84.3	741	3

In direct contrast with the results obtained for the resistant Northern Spy seedlings, high numbers of aphids are evident on all the susceptible seedlings included for evaluation. The number of aphids varied from 88 to 1237. Half of the seedlings displayed aphid numbers in excess of 400 per plant. This is a sign of successful colonization by the insects. In all seedlings except three, galling was also observed. This is consistent with observations of aphid feeding on susceptible plants.

During the evaluation procedure aphids of all developmental stages were counted. Aphids were classified as alive, dead or dry. It was assumed that dead aphids that were still in a good condition were killed during sampling, transporting or washing. Therefore, alive and dead aphids were counted for analysis purposes. Dry aphids were assumed to have been dead prior to sampling and were excluded from analysis (Damavandian, 1999).

For more conclusive results, a larger population should be studied over an extended period of at least three seasons. There is, however, a very clear difference or trend in the results when comparing the two tables, one representing resistant and the other susceptible seedlings and terrestrial evaluation results seem to be in accordance with aerial evaluation results.

3.2 DNA preparation

3.2.1 Genomic DNA isolation protocols evaluated

The isolation of high-quality plant DNA is widely known to be difficult due to the presence of contaminating polysaccharides. DNA extraction from woody plants is especially difficult due to the presence of polyphenolic compounds and their quinone oxidation products, as well as carbohydrate polymers present (Murray and Thompson, 1980). These compounds become oxidised after cell disruption and irreversibly react with nucleic acids (Hughes and Galau, 1988).

Some polysaccharide-like contaminants of crude plant DNA are difficult to detect by non-degradative techniques, interfere with quantification of nucleic acids, cause shifts in mobility during electrophoresis which leads to misinterpretation of fragment differences among genotypes and also inhibit the activity of most restriction and other DNA modifying enzymes (Murray and Thompson, 1980). The presence of these contaminants renders DNA preparations viscous, thus making it indigestible in restriction digestion reactions and unamplifiable in PCR (Lodhi *et al.*, 1994). For most purposes DNA extracted by the CTAB method would require further purification, typically caesium chloride gradient centrifugation, which is slow and costly. Alternative purification methods for DNA include differential solvent precipitation, but this is highly toxic to the users due to the solvents required (Gilmore *et al*, 1993). The abovementioned were taken into account when an appropriate DNA isolation method was selected.

Various protocols were tested to find one aimed at the high quality and large quantity of DNA required for AFLP analysis. Obtaining high yields of DNA was further hampered by the availability of plant material, as large quantities were required by some of the protocols tested. We had to identify a protocol that would yield high quality DNA in large quantities. Most of the protocols investigated were based on the use of CTAB, as it is effective in the lysis of the cell and nuclear membrane, despite its drawback described earlier. The results obtained with the different protocols listed in Chapter two, are as follows:

Protocol 1: This is a CTAB-based protocol established for the use on apple material (Gardiner *et al.*, 1996). Important components of the extraction protocol are CTAB and PVP. CTAB is effective in the

lysis of cellular membranes of cells. NaCl has been proved to remove polysaccharides (Lodhi *et al.*, 1994) and PVP is effective in the removal of polyphenolic components. RNA was detected in the samples, as no RNAse treatment was included in the protocol. This would be important, as pure DNA was required for AFLP analysis, where effective restriction digestion of samples was essential. The yields obtained from these samples were also quite low, and did not meet with the required amount of 500 ng per reaction as needed for AFLP analysis.

Protocol 2: The protocol used for this isolation procedure is a combination of Murray and Thompson (1980) and Lavi *et al.* (1991). This protocol was initially selected as the isolation protocol for this study based on its previous success with apple material (Morris, 2000). An important component of this protocol is the inclusion of CTAB for effective cell lysis. At first good yields and intact DNA was obtained. After all the samples were selected for the study and tested for yield and intactness, it was evident that most of the samples had very low DNA yields. A factor that could attribute to the difference in results between previous successes (Morris, 2000) and this study could be the physical condition of the leaves. In this study extractions were performed on mature leaves in most cases. Some of the plants were also afflicted by powdery mildew infections, which could have affected DNA yields obtained. Older leaves have higher levels of polysaccharides and polyphenols that could influence molecular manipulation. No provision was made for the removal of these components from the extracts. The initial success could not be repeated using this protocol and therefore required additional optimisation of the DNA isolation process.

Protocol 3: This is also a CTAB-based protocol, which is a combination of previously described procedures (Murray and Thomspon, 1980; Saghai-Maroof *et al.*, 1984), and yielded variable results. The use of CTAB in this purification procedure was originally described for a procedure that involved precipitation of DNA by lowering the NaCl concentration in the presence of CTAB (Saghai-Maroof *et al.*, 1984). This precipitation method presumably removes many polysaccharides which are still soluble at the lower NaCl concentration. The original procedure, which included a cesium chloride ultracentrifugation step, was shortened to a more rapid, inexpensive protocol that can be used for processing many samples simultaneously (Rogers and Bendich, 1985). The same CTAB extraction buffer was used by Saghai-Maroof *et al.*, with the only difference being that DNA was precipitated only once by the addition of isopropanol. For our study however, a high DNA yield was not obtained. Limited plant material was also available for DNA isolation and extractions could thus not be repeated. In some cases only enough material was available for a single extraction.

Protocol 4: The protocol used was specifically designed for extraction in grapevine and other *Vitis* species, which also poses the same problems of excess polyphenols and polysaccharides. Two buffers were used in this procedure, both containing PVP that is helpful in the removal of excess polysaccharides. Buffer B also contains SDS, which is effective in cell membrane lysis to release cell nucleus contents. Excess proteins were also removed by means of a chloroform extraction. In addition to a chloroform extraction, remaining proteins were isolated by means of ammonium acetate precipitations. DNA precipitation was achieved with isopropanol. An RNAse step was also included for the removal of RNA. Despite all the above-mentioned precautionary measures for yielding good quality DNA, none could be isolated. A factor that possibly influenced the extraction procedure is that older leaf material was used. It has been reported previously that mature leaf material yields very little DNA (Kidwell and Osborn, 1992).

Protocol 5: This method is primarily based on Doyle and Doyle (1990) but the purification and concentration steps are according to Sambrook *et al.* (1989). A major drawback of the Doyle and Doyle protocol and other similar protocols, which are CTAB-based, is the occasional isolation of partially degraded DNA that cannot be digested completely by common restriction enzymes (Khanuja *et al.*, 1999). In the protocol used, this was overcome by the addition of more CTAB after the chloroform extraction step as well as a final precipitation using PEG (Rowland and Nguyen, 1993). PEG precipitation is used in extraction protocols to circumvent the use of caesium chloride gradients. Despite these additional precipitation steps, no DNA was obtained. It is possible that the leaf material used in the method was too old to yield any DNA and that proper lysis of cells has not occurred. In the protocol originally described this method was used on young leaf material of several blueberry species as well as apple, pear, peach and cherry varieties (Rowland and Nguyen, 1993). Thus far this protocol has been the only one to make use of PEG for plant genomic DNA precipitation and no other procedures have been documented using this.

Protocol 6: This protocol was adapted from a method previously described for grapevine (Dr J.T. Burger, pers. comm., 1999). The extraction buffer specified for this protocol is CTAB-based. It has been adapted for the isolation of RNA as well as DNA. It is a small-scale protocol that can easily be modified for large-scale extraction of DNA or RNA. Instead of grinding the samples with liquid nitrogen, grinding is achieved by using carborandum of grit 1800. Other carborandums used were found to be too granular for effective pulverisation. Chloroform extractions successfully removed the

soluble proteins. An overnight incubation step with lithium chloride enhanced the RNA precipitation. DNA was precipitated using isopropanol. Initially a large quantity of RNA was isolated using this method. The RNA bands were clearly visible on agarose gels, with insignificant amounts of DNA being present. The amount of RNA obtained in the extraction procedure would also warrant the implementation of an RNAse treatment step. The yield of DNA was consistently low and not adequate to be used in AFLP procedures.

Protocol 7: This protocol was described by Porebski *et al.*, (1997) and was developed for use on strawberries and other plants containing high concentrations of phenolic compounds and polysaccharides. Essential components included in the extraction process were 2-mercapto-ethanol and PVP, as these components are essential in suppressing the oxidation of phenolic compounds in the initial extraction process. CTAB and sodium chloride were also included to reduce polysaccharide contamination in the early steps. This DNA extraction protocol is relatively fast since a lengthy ultra centrifugation is not required as in some other PVP protocols (Maliyakal, 1992). PVP is a solid polymer that has high molecular weight and is water-soluble and chemically inert and was shown to remove polyphenols, while maintaining a higher yield than polyvinylpolypyrrolidone (PVPP), an insoluble cross-linking polymer. PVP forms a complex with polyphenols through hydrogen bonding, allowing them to be separated from the DNA, thereby reducing the levels of polyphenol in the product (Maliyakal, 1992). Although PVPP is useful in improving stability of enzymes by removing phenolic impurities, it yields significantly less DNA than PVP from fully mature leaves (Sigma Chemical Company, 1993). This protocol has the potential to be effective for use on mature leaf material as acceptable results were consistently obtained using this protocol (Porebski *et al.*, 1997).

Good yields and high quality of DNA was obtained using this procedure. Electrophoresis indicated that no RNA was present in the samples, which was important for effective AFLP analysis. In addition, a proteinase K step formed part of the extraction process thus providing further measures to eradicate contaminants. Varying amounts of PVP were added to test the efficacy of the removal of phenols and polysaccharides. The protocol recommends that 5% PVP is to be added to each extraction sample; 10% PVP was also added and this resulted in no significant difference in results in terms of DNA quality or yield (see Figure 3.1). Five percent PVP was therefore used in subsequent extractions, as suggested in the protocol.

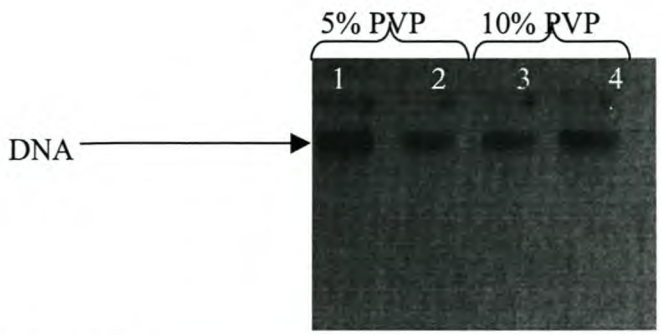


Figure 3.1 DNA extracted according to the Porebski method (protocol 7) using 5% and 10% PVP. Lanes 1-4 represent Northern Spy samples.

Further advantages of this protocol are:

- Less leaf material is required than in most of the protocols tested;
- Homogenizing with liquid nitrogen eased the grinding process, compared to using compounds such as carborandum;
- PVP was effectively used for the removal of excess polyphenols and polysaccharides;
- Additional salt precipitations could be performed with sodium acetate.

A major factor that influenced the quality of DNA obtained was the maturity of the leaves used for extraction. For the bulk of extractions performed the leaves were not necessarily the youngest obtainable from the plants. The apical or auxiliary meristems are the ideal leaves to use but were not available for picking. Previous studies have shown that the highest yields of DNA were obtained when the youngest leaves were used for extraction (Kidwell and Osborn, 1992). The methods used for storage of leaves also affect the yield and quality. If leaves are freshly picked, stored on ice, lyophilized and then stored at -80°C, there will be no significant loss in DNA yield or quality. Leaves used in this study were picked and then stored at -20°C, which could have affected the DNA yield and quality obtained.

The use of CTAB in plant DNA purification was originally described for a procedure that involved precipitating DNA by lowering the concentration of NaCl in the presence of CTAB (Murray and Thompson, 1980). The precipitation method presumably removes many polysaccharides, which are still soluble at the lower NaCl concentration. Two problems should be considered in relation to the extraction of DNA from "difficult" species, notable those with small genome size, high levels of polyphenolic compounds and much polymeric carbohydrates. The first problem relates to achieving

adequate yield of crude CTAB/nucleic acid to permit successful purification of DNA. Yields obtained with CTAB is highly variable from sample to sample within and between species dependent on tissue age, tissue type, developmental phase and physiological conditions as well as on post-harvest preservation and storage. The second problem relates to average fragment size of DNA preparations. Plant DNA extracted for general purposes should be in the order of 50 kb in length (Murray and Thompson, 1980). Aside from the problems introduced by leaf preservation and cell disruption, shearing inevitably accompanies efforts to extract intact DNA.

3.2.2 Electrophoresis and quantification of DNA

The quality and quantity of DNA obtained with every method was compared using electrophoresis. Procedures one and seven proved to be the best for DNA extraction. However, protocol one was not consistent in yield. DNA was not obtained with every extraction performed and yields were also very low. For protocol seven, DNA was obtained with almost every extraction performed. The yields were also consistently higher than with any of the other isolation methods used. DNA obtained using protocol seven also showed no RNA contamination. DNA was also consistently intact and could therefore be used for restriction digestion, ligation and AFLPs. Typical results are shown in Figure 3.2.

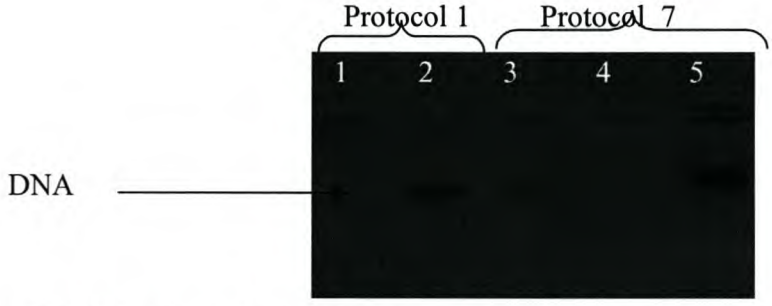


Figure 3.2 DNA extracted according to protocols 1 and 7. Lanes 1 to 5 are Northern Spy samples selected for genomic DNA isolation optimisation.

Initially DNA concentrations were determined by means of spectrophotometer readings. However, these proved to be inaccurate when used in restrictions and PCR amplification analysis. When results obtained from the spectrophotometer were compared to gel quantification, it differed significantly. On gels (0.8% (w/v) agarose), DNA quantities were estimated by comparing the intensities of the DNA bands to that of the Low Mass Ladder (Promega) standard (see Fig 3.3). The gel readings were significantly lower than estimates previously obtained. On the spectrophotometer in the Department of

Genetics, yields were estimated to be as high as 100 µg per extraction for 0.5 g leaf material. Readings were performed on three spectrophotometers and all the resulting values differed from each other. No correlation could even be established between the results obtained from identical spectrophotometers. Readings from the spectrophotometer of the Institute of Plant Biotechnology were much lower than that of the Department of Genetics. The results obtained with the spectrophotometers were disregarded and all further quantifications were performed on agarose gels using the low mass standard. When compared with gel results, the highest yields were approximately 20 µg/0.5g leaf material.

Each of the fragments of the Low Mass Ladder corresponds with a specified quantity of DNA depending on the quantity of ladder loaded per well. For the determination of DNA concentrations, four micro litres of ladder was loaded per gel, which corresponded with the following values for each of the individual fragments: 200ng/µl for the 2000bp band; 120ng/µl for the 1200bp; 80ng/µl for the 800bp; 40ng/µl for 400bp; 20ng/µl for 200bp; and 10ng/µl for the 100bp band. When comparing the intensities of the DNA bands obtained with that of the ladder it can be concluded that most of the samples' concentrations ranged between 40 and 80ng/µl sample. From the figure below (Figure 3.3), the intactness of the genomic DNA samples is evident. None of the samples visualised on agarose gels appeared to be degraded, as would be indicated by smears on the gels.

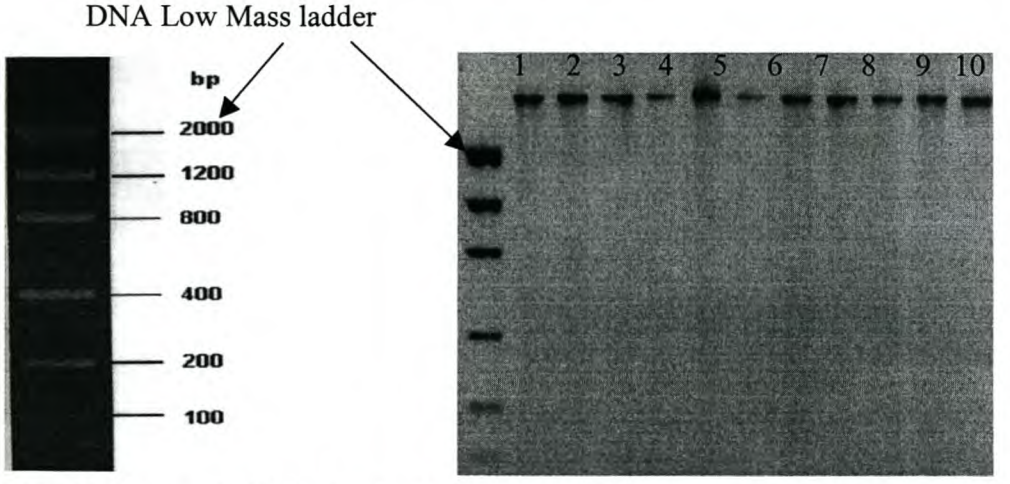


Figure 3.3 Purified genomic DNA obtained using protocol 7, compared with the DNA Low Mass Ladder and its corresponding values. Two thousand bp corresponds to 200ng, 1200bp to 120ng, 800bp to 80ng, 400bp to 40ng, 200bp to 20ng and 100bp to 10ng of DNA per microliter. Lanes 1 –10 are the resistant Northern Spy samples selected for molecular analysis.

In future, it would be advisable to use electrophoresis in combination with another technique, such as fluorometry or Genequant quantification, to establish the integrity of the DNA samples. The Low Mass Ladder seems to provide a good measure for the amount of DNA extracted, and can also be recommended. A small amount of DNA is needed for the analysis, and in our hands provided a more accurate measure of DNA concentration than spectrophotometry. A drawback of fluorometry is that the dye used for the quantification is very costly. The GeneQuant has an added feature; the DNA can be retrieved again for future use especially if low quantities of DNA have been isolated.

3.3 AFLP

3.3.1 DNA used in the AFLP procedures

DNA used for AFLP reactions were samples obtained using protocol seven. These included samples from Northern Spy, Rootstock 5 and Russian Seedling families. DNA and restriction-ligation mixes were also obtained from Ishaam Morris (ARC-Infruitec) for control purposes. The ARC also provided one sample of each of the following: Golden Delicious x Priscilla (resistant to apple scab), Priscilla x Anna (resistant to apple scab); and Prima x Anna (susceptible to apple scab). An additional sample, maize sample 110, obtained from the Small Grain Institute (SGI), was also included in the trials for control purposes.

3.3.2 Reproducibility profiles

A problem of AFLP and other arbitrarily primed PCR procedures is the ability to reproduce data profiles. Reproducibility is essential for scientific research, as work conducted in one laboratory should be reproducible at other laboratories. Consistent and reproducible results are obtainable when standardised assays and techniques are employed. Standard techniques enable the direct collation and comparison of data.

For this particular study, reproducible profiles had to be achieved on every level of investigation, namely DNA extraction, restriction-ligations, pre-selective amplification and selective amplification, as well as electrophoresis. A representation of a test of reproducibility in the present study is given in the following scheme. A single DNA sample, obtained using protocol seven, was used on which two

restriction digests were performed, using standard digest conditions. Each of the restriction reactions was split into two, where after two ligation reactions were performed on the four restriction samples (i.e. split samples). Each of the ligation products were once again split into two, and a pre-selective amplification ("cold" amplification) was performed for each of the ligated samples (i.e. split samples). Each pre-selective product in turn, was split into two and a selective amplification ("hot" amplification) performed on each of the samples. Selective amplification products were electrophoresed on denaturing polyacrylamide gels. A schematic representation of the procedures is given in Figure 3.4.

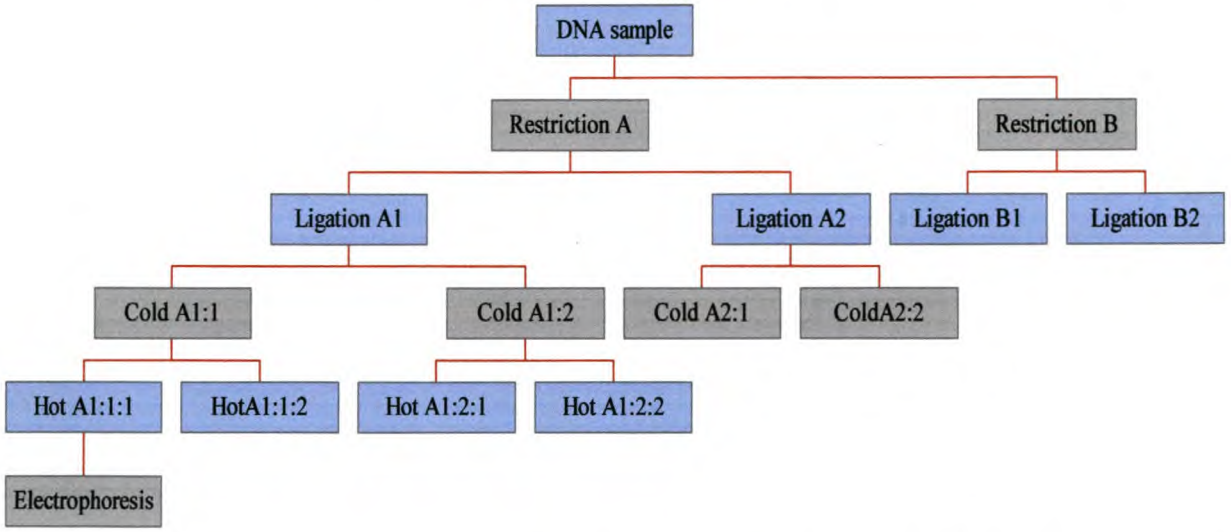


Figure 3.4 A representation of the procedures to be followed for the parallel testing of reproducibility in the AFLP process.

The outcomes of the reproducibility experiment are described in subsections 3.3.2.1 through 3.3.2.7.

3.3.2.1 Input DNA

Samples from the same seedlings were extracted on different days, for a period from December 1999 to February 2000. Very few of the samples that were finally selected were extracted on the same day. It is therefore possible that extraction on different days could have contributed to differences in terms of DNA quality and quantity. This problem could not be circumvented due to the availability of equipment needed for extractions. Only limited number of mortars and pestles, centrifuge tubes and appropriate centrifuges were available for use per day. A maximum of eight samples could be extracted per day using the available resources. In some of the cases all the leaf material for a specific

seedling was used in the first extraction and no additional DNA isolations could be performed to increase the yield or quality of DNA needed for AFLP analysis. Unfortunately, the study had to continue with the DNA available at that stage.

3.3.2.2 Restriction digestions

Digests were repeated using 500ng of DNA, as originally stated in the John Innes Centre protocol (Dr R. Prins, pers.comm., 1999). Restrictions were also done with less DNA namely 200ng DNA. The success of the digestion reactions could only be judged after the ligations and pre-selective amplifications were performed (see sections 3.3.2.3 and 3.3.2.4). Restrictions were not checked by electrophoresis as ligation was performed directly after the digestion was complete. The success of restriction is determined after pre-selective amplification when restriction-ligation products are compared to pre-selective amplification products by means of agarose electrophoresis.

3.3.2.3 Ligations

The initial protocol that was followed stated that ligations should take place at 20°C. In practice however, a much higher success rate was achieved by incubating these reactions at 37°C (Dr R. Prins, pers. comm., 1999). This part of the protocol was amended accordingly. The increased temperature yielded an improved profile compared to the lower temperature. A ligation temperature of 37°C is used more commonly than 20°C and the results obtained in other studies could imply an increased efficiency as restrictions are also performed at 37°C (Dr R. Prins. Pers.comm.., 1999).

3.3.2.4 Comparison of restriction-ligations with pre-selective amplification products

With this reaction it is anticipated that the ideal size range for products formed should be approximately 500bp (Figure 3.4). This was obtained initially, but in subsequent reactions the size range decreased to as little as 100bp, if not less. Restriction-ligation products usually appear to be very faint and are not always distinct. The pre-selective products were more clearly visible and some of the smears appeared to be very large. Large smears can possibly be attributed to too high concentrations of DNA present. With the smaller size of the pre-selective product obtained, problems are experienced with AFLP analysis.

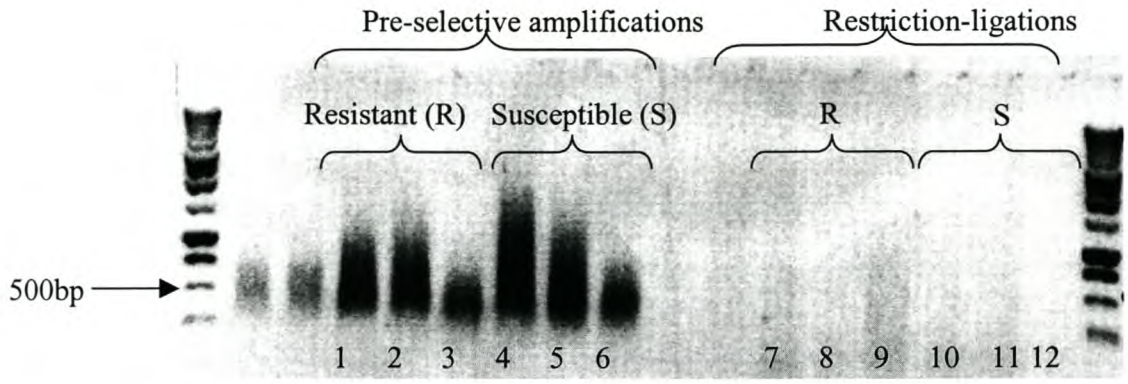


Figure 3.4 Comparison of restriction-ligation and pre-selective amplification products. Lanes 1-6 contain the pre-selective amplification products and lanes 7-12 the restriction-ligation products. Lane 1= 2.17(0), 2=2.21(0), 3=2.24(0) (same samples in lanes 7-9); and Lanes 4=2.12(3), 5=2.13(3), 6=2.20(3) (same samples in lanes 10-12).

In later stages of the study, it was found that the products for both the restriction-ligation and the pre-selective amplification were significantly smaller than 200bp. This possibly explains the erratic results obtained in the AFLP profiles. If smaller fragments are amplified in pre-selective amplification, it is possible that these bands would provide enough information about possible polymorphisms, as regions rich in polymorphisms might not have been successfully amplified. The lack of amplification of pre-selective products necessitated that optimisation measures had to be incorporated into this study. These measures included the re-annealing of adaptors, variation of DNA input into restriction digestions and the comparison of different gel composition.

3.3.2.5 Comparison of re-annealed adaptors

From the results obtained for AFLP analysis, it was evident that the primers were effective and that the problem with amplification was not due to insufficient/unsuccessful annealing of adaptor sequences.

Due to problems experienced with the cold amplification, denaturation and re-annealing of the adaptors were done to test the working of adaptors and the efficiency of the ligation procedures. Denaturation took place at 95°C and re-annealing at 65°C, in a waterbath, for 10 minutes before samples were allowed to cool to room temperature. As an additional control measure, identical adaptors were obtained from other laboratories and tested along with adaptors initially used in this project. When the

pre-selective reactions were performed the profiles obtained were similar, suggesting that the ligation step was successful as seen in Figure 3.5 below.

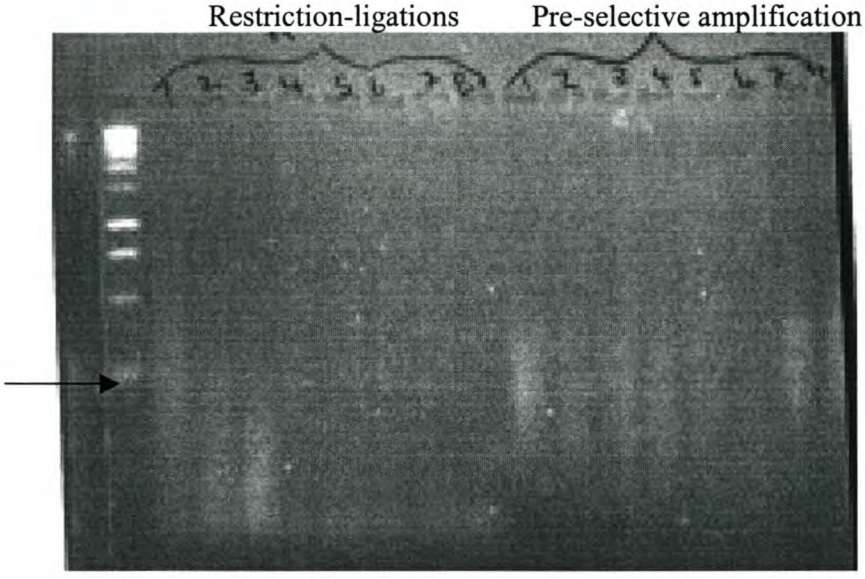


Figure 3.5 Restriction-ligation and pre-selective amplification products after re-annealing of adaptors. The size marker is the 1kb ladder from Promega. The arrow indicates the 100bp size fragment of the ladder.

The adaptors could therefore be used in follow-up experiments. In each of the experiments performed, effective annealing of the adaptors to the restricted DNA occurred. This was confirmed by slight smears visible on gels. This is, however, not a normal procedure as only the restriction-ligation mix and pre-selective amplifications are visualised on agarose to establish the success of the reaction. After the effective working of the adaptors was established, this step was omitted from the experimental procedure.

3.3.2.6 Comparison of pre-selective amplifications

Using varying quantities of restriction-ligation products pre-selective amplifications could be compared. According to the original protocol, 500ng of genomic DNA had to be digested and used in subsequent reactions. However, an experiment to compare the use of less DNA per reaction was first performed. Reactions using 200ng, 300ng and 400ng were thus included (Figure 3.6). Reactions were performed in duplicate and the results obtained, for each sample, were similar in terms of electrophoresis patterns.

When using 200ng of the restriction-ligation mix, similar profiles were obtained for each of the samples. Although the smears were not as pronounced as with the higher DNA concentrations, it could be identified clearly. When using 300ng and 400ng of DNA, similar smears were observed on the gel. Each amplification reaction of sample 4 consistently produced a smaller smear than the amplification reactions of sample 1. The size of the smears appears identical for sample 4 at 200ng and 300ng of DNA, but the sample appears slightly darker when used in the 400ng reaction. The dark smear obtained for sample 1 at 400ng DNA input can possibly be attributed to a higher concentration of DNA used than was intended.

None of the samples tested in the above-mentioned procedure were used for further AFLP analysis, i.e. pre-selective amplification, selective amplification and electrophoresis. It would have given a clear indication of whether variation in input DNA resulted in a significant difference in the electrophoresis patterns observed on autoradiographs. The input DNA per sample used in the study was 500ng as suggested by Vos *et al.* (1993) as well as the protocol followed for this study. No corresponding data are available where specifically the DNA input for AFLP studies have been investigated, and to which the above-mentioned experiment could be compared.

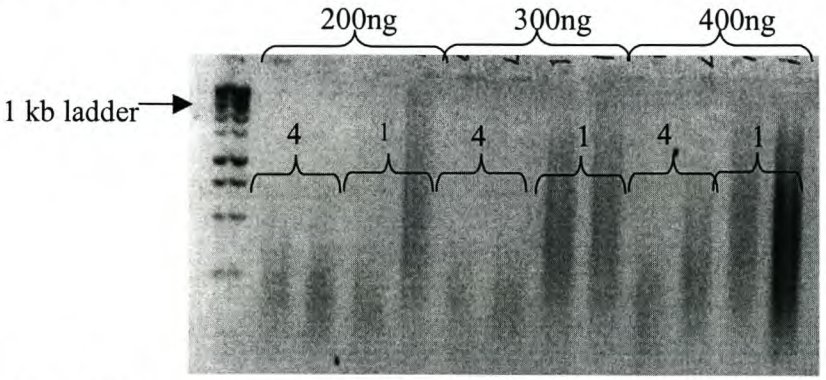


Figure.3.6 Comparison of varying amounts DNA used in pre-selective amplification reactions. Sample 4=2.24(0) and sample 1=Russian Seedling parental sample. Marker used is the 1kb ladder from Promega.

A comparison was also done using the pre-selective primers, Moo and Poo, from different laboratories. The same restriction-ligation products were used for each of the reactions. Three sets of primers were used on samples that have previously been used successfully in pre-selective PCR reactions. Initial results indicated that the reactions had not worked as no smears could be observed. A restriction-ligation reaction mixture prepared from DNA obtained from Infruitec was used as a control along with my own samples in order to test the pre-selective primers in a follow-up study. The resultant reactions

showed a smear. From this information it can be concluded that all the primers were functional and therefore the problem was not at the pre-selective reaction level.

3.3.2.7 Comparison of different gel compositions

A comparative study was initiated to compare the results obtained for the same set of samples when using different polyacrylamide gel compositions. It would be possible to test whether there was a difference in results when using polyacrylamide gel prepared from individual acrylamide and bisacrylamide ingredients, as was done to this point, or a gel made of pre-mixed ingredients.

The gels used for comparison, in Figures 3.7 and 3.8, were both prepared from pre-made gel mixes. The one sequencing gel consisted of 6% acrylamide gel mix containing 7 M urea, and the second sequencing gel mix was a 4% acrylamide gel mix containing 4 M urea. The samples loaded for this experiment were prepared from the same batch of pre-selective and selective amplification products. The same samples could, however, not be loaded on both gels due to insufficient quantities of sample available. The one autoradiograph contained 2+ *Mse* primers (Figure 3.6) and the other 3+ *Mse* primers (Figure 3.7).

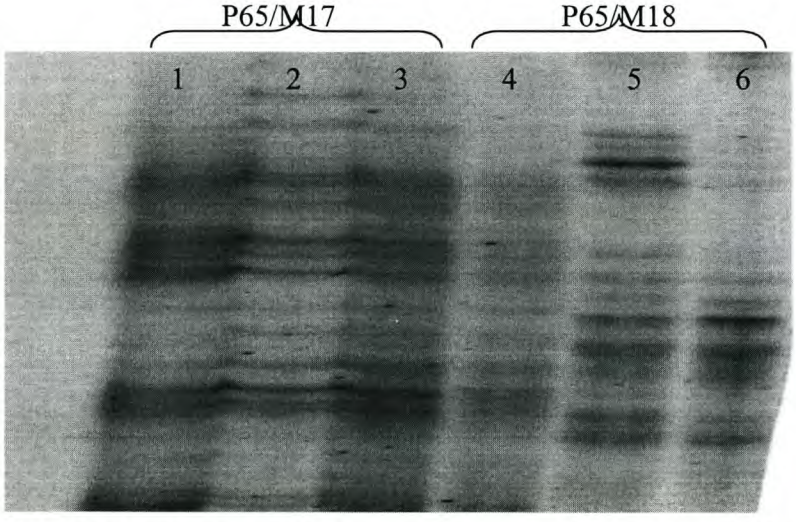


Figure 3.7 Banding profile obtained using a 4% denaturing sequencing gel containing 4M urea. Lanes 1=2.12(0), 2=2.8(0), 3=2.23(0), 4=2.3(3), 5=2.4(3), 6=2.17(3).

In the section of the autoradiograph shown above (Figure 3.7), slightly fuzzy banding patterns were observed on the gel. However, these bands were more distinct than the banding profiles observed when using individual mixtures acrylamide and bisacrylamide. Similar banding profiles could be seen

between the different samples. The resolution is sufficient when using individual mixtures of acrylamide and bisacrylamide to detect, similarities and differences in banding profiles between the samples loaded in the different lanes. A probable polymorphic fragment was observed on this gel, but the result is inconclusive as only six samples were compared. From this figure, it can be concluded that the PCR amplification has been sufficient and the problem of resolution could be due to gel composition.

On the section of the autoradiograph shown in Figure 3.8, three samples were loaded per primer pair. The brackets in Figure 3.8 indicate the grouping of samples on the gel, where each group include different samples using the same primer combination. Similar banding patterns were observed between samples using a specific primer set. Although it has not been confirmed, possible polymorphic fragments could be identified. The composition of the sequencing gels apparently had a significant influence on the results in terms of band resolution, as seen on the autoradiographs. Much more sharper defined bands were obtained using the 6% sequencing gels with a higher urea content.

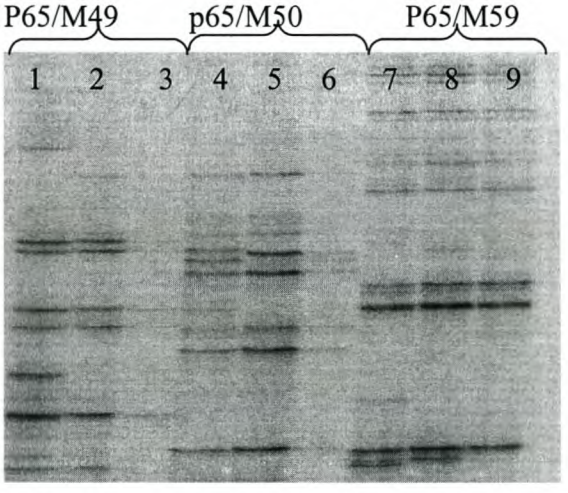


Figure. 3.8 Section of autoradiograph of gel consisting of 6% acrylamide and 7 M urea. Lanes 1, 4, 7=2.14(0), lanes 2, 5, 8=2.17(0) and lanes 3, 6, 9 = 2.21(0).

The section of the autoradiograph shown in Figure 3.9 represents samples that had previously been loaded onto gels made by mixing the separate gel components. From the resolution on gels presented in Figures 3.7-3.9, it can be concluded that polymerisation of the gels were better when using premixes rather than the individual gel components. Banding patterns were also much improved when using premixes. Less background was apparent and fragments could be clearly distinguished allowing for

the effective analysis of gels, and the identification of polymorphic fragments. Banding intensity was not always satisfactory, but this could be improved by extending the period of exposure. Pre-made gel mixes were therefore used in subsequent analyses.

In some instances autoradiographs had a black spotted appearance (as in Fig 3.9), which was also evident in autoradiographs obtained in earlier experiments. Other laboratories in our department have reported a similar problem. The dots did not affect the interpretation of gels and were ignored. It is possible that the dots on the autoradiographs are the result of powder from gloves.

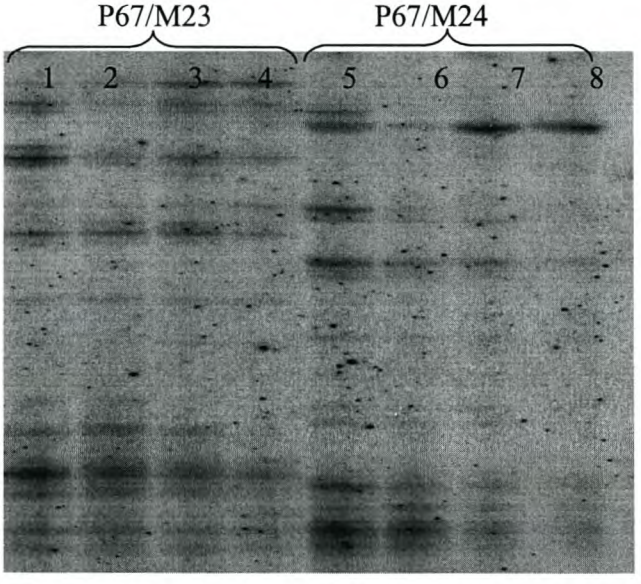


Figure 3.9 Section of autoradiograph using pre-made gel components. Lanes 1, 5=2.4(0), 2, 6=2.6(0), 3, 7= 2.14(0) and 4, 8=2.24(0).

3.3.2.8 AFLP profiles obtained

AFLP profiles were obtained utilising combinations of various primers. The primers used in this study are given in Appendix B. The selectivity of these primers is dependent on the base pair overhangs on the 3` end of each primer. In this study, primers were used that varied from no selectivity (i.e. no 3` overhangs) to highly selective (i.e. three base pair overhang on 3` region of primer). The selective characteristic of these primers is illustrated when it is used in different primer combinations. A combination of non-selective primers should theoretically yield a high number of fragments per lane for AFLP analysis. This is due to the fact that ubiquitous fragments in the genome are detected. When the selective primers are combined or non-selective and selective primers are used together, the number

of fragments amplified decrease due to the selective nature of the primers. Ultimately it is anticipated that the whole genome of any organism can be examined in this manner and that commonly occurring regions can be avoided and more polymorphic or gene-rich coding regions identified.

We have observed that primers used in a 2:1 selective combination produced more bands. If bands on autoradiographs are all identical for all the individuals, it serves to indicate the absence of polymorphism using the specific primer combination and test conditions. Some of the primer combinations used in this study did not yield any polymorphic fragments. Non-differentiating profiles were also obtained in an attempt to identify apple cultivars using the AFLP technique (Tignon and Kettmann, 2000). This highlights the need to test as many primer combinations as possible for the identification of polymorphisms. Comparing profiles obtained between cultivars and within cultivars can test the usefulness and reliability of the AFLP technique.

The profile shown in Figure 3.10 has fewer bands than in Figure 3.9. This is despite the fact that a 2:1 selective ratio also applied in terms of primer selectivity. A varying number of bands were amplified for each of the samples used. The first three lanes were amplified using the primer combination of P19/M04. The other lanes' results are in sharp contrast to this. Less bands are evident for the other samples amplified. No bands could be identified that segregate only within the resistant or susceptible populations. For this study no predetermined AFLP systems were used, but primer combinations were selected from the primers as listed in Appendix B. A previously documented AFLP analysis performed on apples used the AFLP Analysis System (Gibco BRL) (Ndabambi *et al.*, 2000). This system uses predetermined primer combinations for AFLP profiling. This system enabled easier identification of fragments in apples. In contrast to this, AFLP analyses yielding no markers have also been reported when using the above-mentioned system (Morris, 2000). This system could present an alternative to radioactive AFLP analysis, but variable results should also be expected.

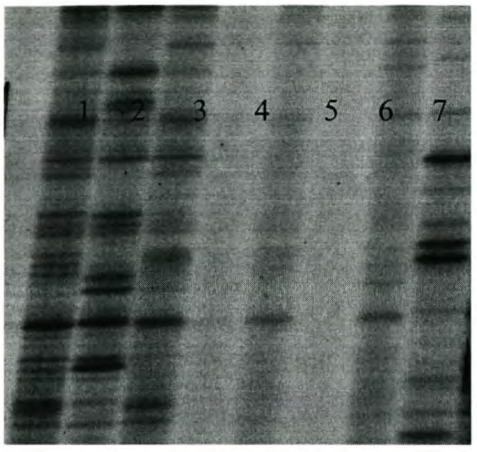


Figure 3.10 AFLP banding profile obtained from the primer combination, P19/M04. Lanes 1=2.1(0), 2=2.4(0), 3=2.6(0), 4=2.9(0), 5=2.14(0), 6=2.17(0) and 7=2.24(0)

One of the major problems encountered was the reproducibility of profiles. In Figure 3.11 the non-reproducibility of some profiles obtained in this study is shown. The same pre-selective products were used for two sets of selective amplification reactions. These reactions were performed on different days. The sections of the autoradiographs include the same samples, and variation between the patterns can be observed for the two sections. The conditions for all the reactions performed were the same except that it was performed on different days. It is thus unlikely that the conditions for all reactions were identical although it was attempted to keep it that way.

We expected a highly reproducible pattern between the two sets of reactions but it is not so in this case. There are very few similar patterns that could be identified or scored between the samples. There are also very few consistent bands appearing between the two sets either. However, no generalisation about the reproducibility of the AFLP technique can be made on the basis of the small samples cited here.

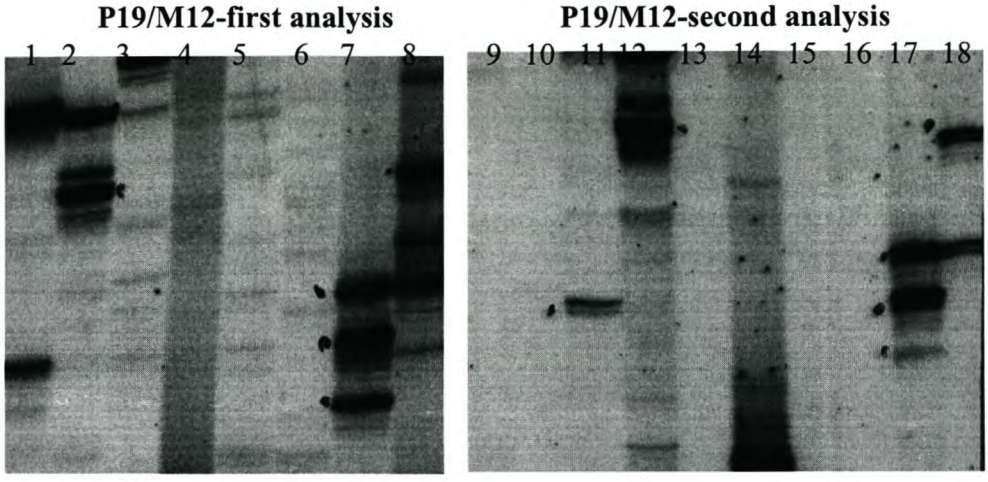


Figure 3.11 AFLP banding profile obtained for the same set of samples, originating from the same pre-selective amplification product. Lanes 1=11, 2 = 12, 3 = 13, 4 = 14, 5 = 15, 6 = 16, 7 = 17 and 8 = 18.

The section of the autoradiograph shown in Figure 3.12 indicates a possible linked polymorphic fragment that occurs in the repulsion phase as it has been identified in the susceptible seedlings. This fragment is, however, not present in the susceptible control (Braeburn) parent, as would be expected if marker had close linkage to the resistance locus. Braeburn was not a pollinating parent for all the open-pollinated crosses in the study. It is therefore possible that the fragment would occur in a different susceptible or heterozygous resistant parent that served as a pollinator for the Northern Spy population. The possible polymorphic fragment also appears in one or two of the resistant samples. The polymorphism thus has to be confirmed before further marker development is attempted. AFLPs are time-consuming and expensive and if reproducibility is a problem, it presents a major problem for implementation in MAS.

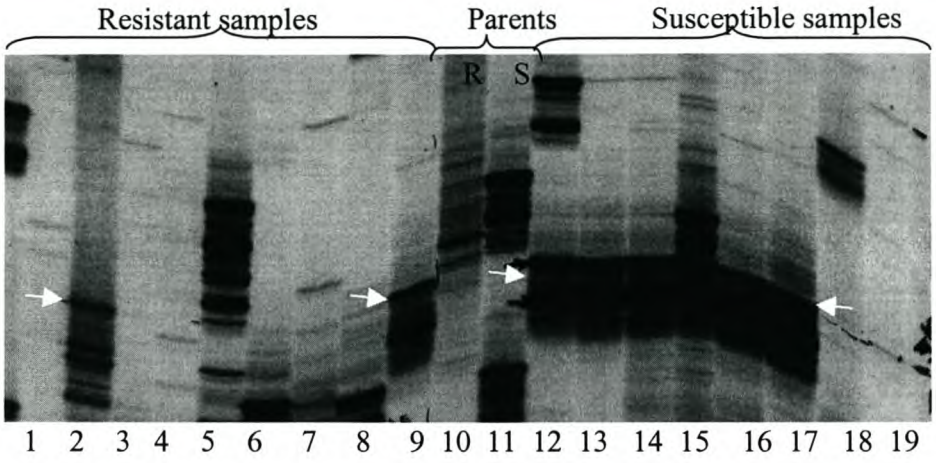


Figure 3.12 AFLP banding profile obtained with primer combination P19/M20. Resistant and susceptible samples are selections from the open-pollinated Northern Spy population, the resistant sample is Northern Spy and susceptible sample is Braeburn. The arrows indicate that presence of a fragment that appears in at least six susceptible samples and two resistant samples. If proven to be a polymorphism, linked to resistance, it appears to be in repulsion phase.

Initial results appear to be similar to the reproducibility patterns obtained for four apple cultivars in another study (Broothaerts, *et al*, 2000). In this study profiles could not be reproduced repeatedly. No defined primer profiles could be determined for primer combinations used. A selective ratio of 2:2 for primer combinations provided the best evidence of genetic diversity in apple. A primer combination of 2:1 produced profiles where many similar bands were detected. Changes in the selective ratio of primer combinations result in vast differences between resistant and susceptible samples, but also within groups.

AFLP, however, has also been used successfully for the identification of apple cultivars and mutants (Tignon and Kettman, 2000). The technique provided markers that could identify all the cultivars used in their study. It was concluded that primers with three selective bases could distinguish all the cultivars. It is a generally accepted fact that the number of bands obtained with AFLP decreases with the use of primers having three or more selective bases. The profiles obtained in this study disprove this statement (Tignon and Kettman, 2000). The number of bands obtained varied greatly for the primer combinations used. This was also evident in my experiments. A possible explanation for this is that apples are very diverse in terms of their genetic composition. When making use of *Mse* primers

with one selective base, numerous bands were obtained making it difficult to identify possible polymorphic bands.

It has been documented that the method of DNA extraction used, physiological stage of the samples and the different organs used did not influence the patterns obtained in SSR. In AFLP, however, this is not the case as the restriction digestion of DNA constituted an additional source of experimental fluctuation. It has been observed by others that partial digestions could lead to erroneous profiles (Arnau *et al.*, 2001).

3.4 SCAR verification

SCAR and RAPD markers for the detection of Er_1 (Gardiner *et al.*, 1999) were included in the study to provide a means of verifying the accuracy of the initial classification of resistance phenotypes in Northern Spy and to test for it in local populations. It could also be determined whether these markers co-segregate with resistance genes in the other cultivars tested.

Verification screening was performed on both open- and cross-pollinated seedlings. Populations included in the study were not all derived from Northern Spy from which the markers originated, but also from Rootstock 5 and Russian Seedling. The parentage of Rootstock 5 has not been established but it is possible that it could derive from a Northern Spy cross. Russian Seedling was included based on the phenotypic assessment data that showed good resistance reaction to woolly apple aphids (refer to Appendix C). It was hoped that using cultivars other than Northern Spy, for which resistance has been established, additional sources of resistance to woolly apple aphid resistance could be found.

The SCAR GS327 marker is expected to amplify a 1.6kb fragment (Figure 3.13). Initial amplification of the marker yielded the desired band, but in some instances the product appeared to have a doublet appearance. This doublet did not appear in all the samples and was not limited to either resistant or susceptible plants. The doublet bands were sequenced and this indicated that these bands are almost identical in sequence. The formation of this doublet could be the result of slippage by *Taq* polymerase enzyme, which accounts for the formation of stutter bands for SSR. It is also possible that the band is a duplication of a region within the genome.

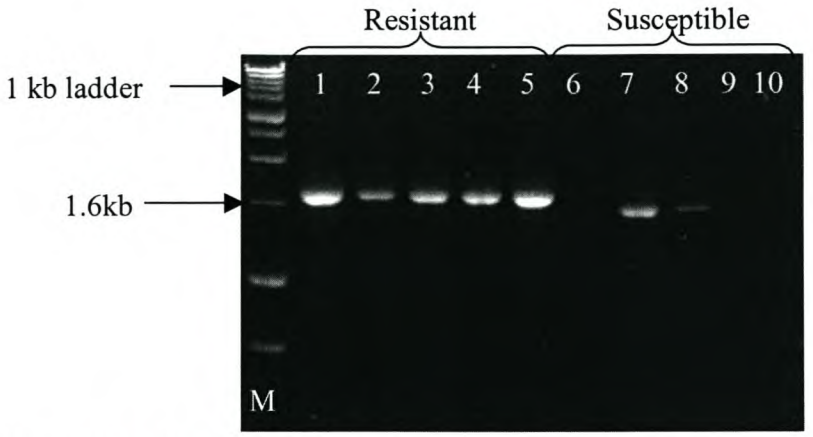


Figure 3.13 Amplification of GS327 SCAR marker in resistant and susceptible Rootstock 5 samples. Marker (M) used is a 1kb ladder (Promega).

A comparison was also drawn between using this marker with DNA obtained from the Porebski *et al.* (1997) method and the combined procedure of Lavi *et al* (1987) and Murray and Thompson (1980). Resistant and susceptible plants from each of the progeny populations were used, Northern Spy, Rootstock 5 and Russian Seedling. The fragment was amplified in all the DNA samples extracted according to the Porebski method. The fragment also amplified in Rootstock 5 and Russian Seedling samples. This strengthens the assumption that Northern Spy could have been used in grafting operations. The origin of Rootstock 5 has not been established by means of molecular analyses. The origin of Russian Seedling is unknown, but it is likely that the resistance gene could have been transferred to the progeny. Previous molecular work has not been done on Russian Seedling. It appears as if the quality of DNA obtained with the Porebski *et al.* (1997) protocol, was better for the amplification reactions. The results of this reproducibility test can be seen in the Figure 3.14. Amplification was achieved in all the samples extracted by means of the Porebski method, but not with DNA extracted using the Lavi/Murray and Thompson method.

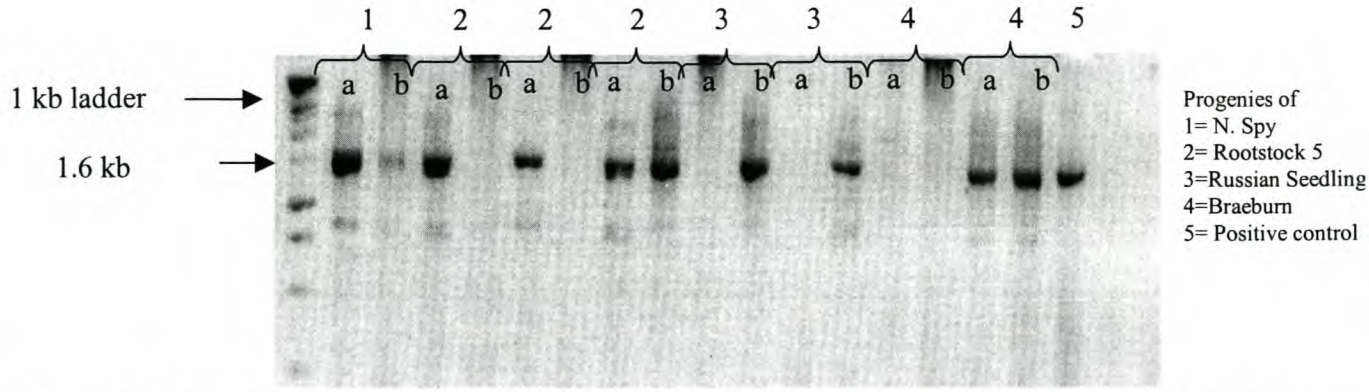


Figure 3.14 Depiction of the reproducibility test for the amplification of SCAR GS327 in new (a) and old (b) samples of the same seedling. (a) Depicts DNA samples that were extracted using the Porebski method and (b) is DNA extracted using the Lavi method. The size marker is the 1 kb ladder from Promega.

However, in subsequent runs, unreliable amplification of this fragment was seen in Northern Spy, Rootstock 5 and Russian Seedling individuals. For all of these samples amplification was not limited to the resistant plants. Samples (a) and (b) were the same seedling and from the results it can be concluded that the procedure or its execution is suspect since at times (b) amplifies but not (a) and vice versa. A control that has previously repeatedly been amplified would give a more accurate assessment of the success of this procedure.

When Northern Spy was tested initially, 16 of the 20 resistant samples amplified the desired fragment. In subsequent amplifications only 8 of 14 samples yielded a positive result (Figure 3.14a). For the susceptible plants 11 out of 16 samples the fragment was amplified (Figure 3.14b). These results are in sharp contrast with the assumption that was made initially that this marker could distinguish between resistant and susceptible seedlings. There is a considerable error factor in the measurement of resistance to woolly apple aphids. When infestations were done there was no control over the number of aphids that have originally colonised the seedlings. Evaluations, however, was largely based on the number of aphids on the plant at a later stage, which might indicate that some of the resistance classifications may be wrong. The diagnostic band may also occur in the susceptible varieties. In any cross intended for MAS, it needs to be determined beforehand, which alleles occurred in the parents. This makes the present data unreliable for verification and selection procedures. The results for each of these populations are reflected in the following Figures 3.15a and 3.15b.

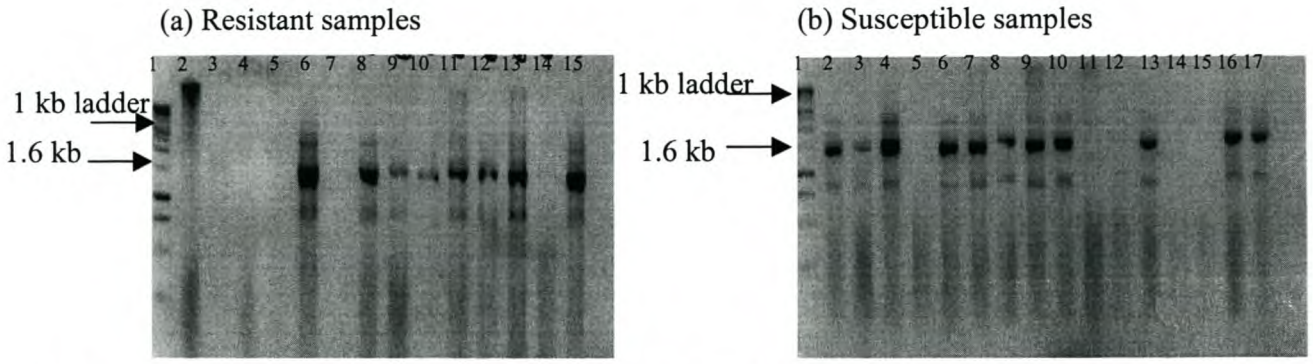


Figure 3.15a and 3.15b. The amplification of marker GS327 in resistant and susceptible Northern Spy open-pollinated progeny. In both gels, lane 1 indicates the size marker, namely the 1 kb ladder from Promega.

Inconsistent PCR amplification results were obtained for Rootstock 5 (Figure 3.16). Eleven of the 14 resistant samples tested, amplified the desired fragment (Figure 3.16a). The bands appeared to be uniform in size. Among the susceptible plants 13 of the 17 samples amplified a fragment of varying size (Figure 3.16. Not all of the amplified fragments were 1.6kb in size and therefore not the expected fragment. Most of the bands were larger than 1.6kb. The larger fragments could possibly be ascribed to insertions in the target sequence, thereby yielding fragments in excess of 1.6kb.

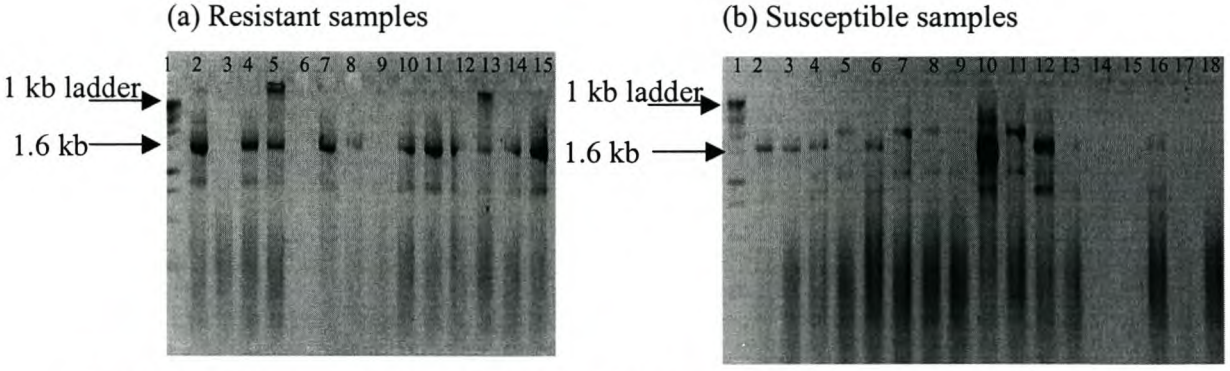


Figure 3.16 The amplification of marker GS327 in resistant (a) and susceptible (b) in individuals of the open-pollinated Rootstock 5 progeny. For both gels, lane 1 contains the size marker, the 1 kb ladder from Promega.

Despite the unknown origin of Russian Seedling, its inclusion in the study was based on its apparent resistance to woolly apple aphids. Six of the 15 resistant samples amplified a fragment (Figure 3.17a), although not the 1.6kb fragment. Only three of the samples amplified the 1.6kb fragment, the others were all larger in size. Once again larger fragments are probably due to insertions. For the susceptible plants only six out of 21 samples amplified a fragment (Figure 3.17). Three of the samples were 1.6kb

in size, and the other three were larger than 1.6kb. The amplification of this fragment confirms that it does not occur exclusively in the Northern Spy cultivar. The expected fragment also amplifies in both resistant and susceptible samples, which is not suitable for MAS.

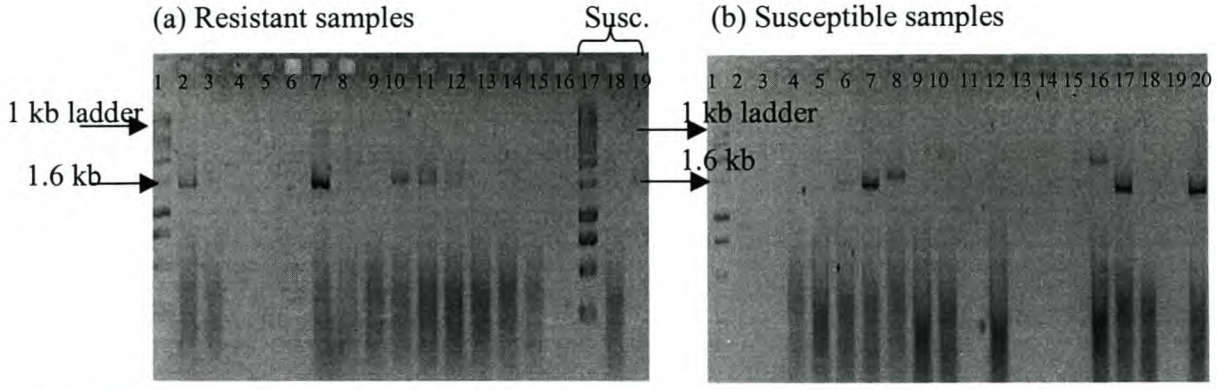


Figure 3.17a and 3.17b The amplification of a marker, GS327, in resistant (a) and susceptible (b), Russian Seedling open-pollinated progeny. Lanes 1 (both gels) and lane 17 (gel 1) indicates the size marker, which is the 1 kb ladder from Promega.

The study has shown that the diagnostic fragment appeared in both the resistant and the susceptible classes. The presence of the fragments in the susceptible progenies could be due to misclassification or recombination. This had bearing on the decision to work with bulks in the AFLP study. If bulks were used as proposed, the classification for phenotypic as well as molecular screening had to correspond in order to confirm the resistant and susceptible nature of individuals. This was not the case in this study; therefore bulking had to be abandoned as unreliable results were obtained. From the results obtained it can be concluded that it should have been confirmed beforehand whether the resistant parent had the diagnostic band. It had, however, not been determined whether the male parent lacked the diagnostic band and was in actual fact susceptible to woolly apple aphid infestation. A controlled cross would have yielded better results as the parentage of such crosses are known, and it would be easy to determine the segregation of markers used for screening. Without knowledge of the parents and the linkage association of the marker to resistance, no deductions can be made with regard to the segregation of markers linked to resistance in specified progenies. Even if individuals rather than bulks were tested, no added information would have been gained unless it was known what the genotypes and linkage associations of the parents were.

The RAPD marker, OPC20 (Gardiner *et al.*, 1999), was also one of the published markers obtained for verification purposes. Initial PCR reactions yielded no results following the specified reaction conditions. The estimated size of the product of this marker is 2.2kb. This rendered it necessary to

perform a temperature gradient to re-determine the optimum annealing temperature for this primer. The initial temperature was 37°C, but the newly determined temperature was 39.1°C (Figure 3.18). When testing of the samples commenced no amplification was obtained at the higher temperature. The annealing temperature was gradually lowered and finally results were obtained at 34°C (Figure 3.19). Further complications arose when the bands could not be resolved clearly with agarose gel electrophoresis. One of the alternatives that was tested was a Mighty Small (Hoefer) polyacrylamide gel. This yielded an improved result, but a disadvantage was the fact that additional bands were amplified due to the lowered annealing temperature. The difficulty in reproducing the marker made for it to be excluded from the verification procedures.

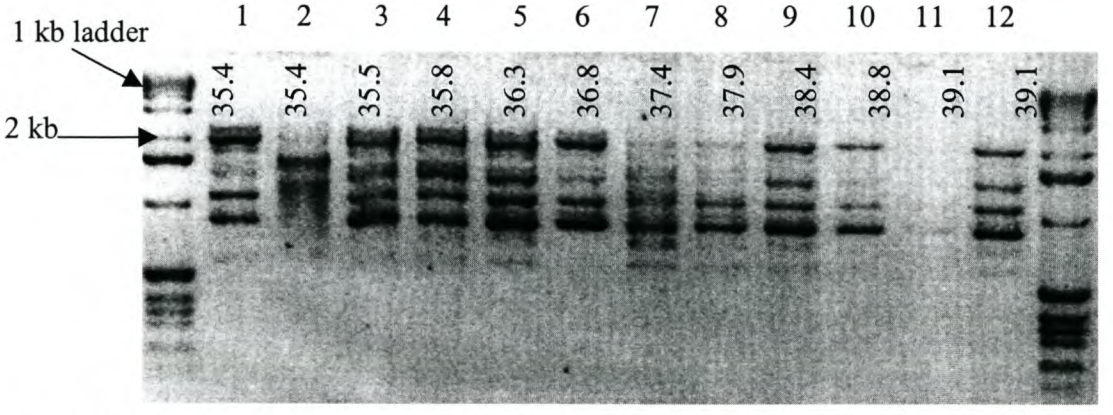


Figure 3.18. The temperature gradient used to determine the optimal annealing temperature for primer OPC20, using a single Northern Spy sample. Lane 12 indicates 39.1°C, which was established as the optimal temperature. The range of temperatures used is indicated above the lanes. The size marker used is the 1 kb ladder from Promega.

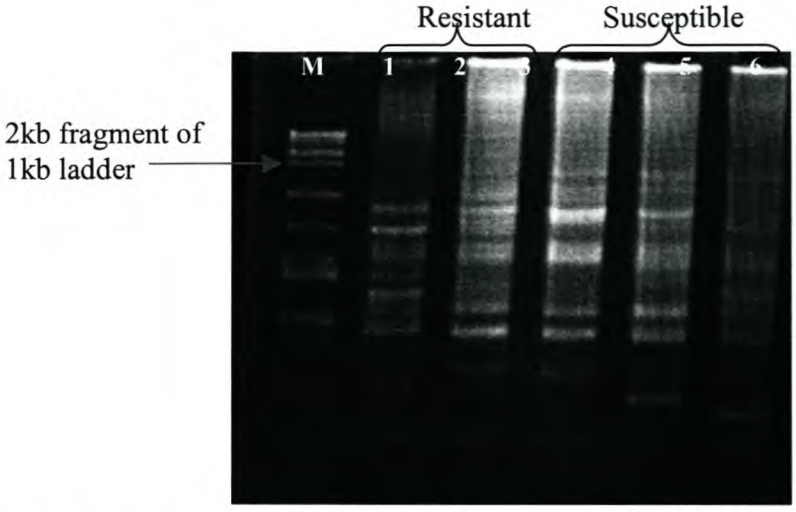


Figure 3. 19 The amplified product at 34°C for OPC20 on Mighty Small 6% polyacrylamide gel. The samples used are Northern Spy samples. The ladder used is the 1 kb marker of Promega.

Additional markers were obtained from the Gardiner research group in New Zealand in an attempt to find a further means of verification of the resistance/susceptibility status of individual plants. These markers are linked to the self-incompatibility locus in apples (Knight *et al.*, 1962). There are two sets of SCAR derived from each of the two RAPD primers, namely OPO05 (a) and (b); as well as OPC20 (a) and (b). The second primer was developed to allow a switch between genetic backgrounds. If the (a) set of primers failed to distinguish the resistant and the susceptible plants, the (b) primer set should provide a further means of distinction between the seedlings. At this stage of the study, extra populations were introduced in the verification study, which had originated from the crosses made in earlier seasons. These crosses will provide an accurate assessment of the presence of the presence of the proposed markers.

Problems were experienced with the amplification of the additional markers. OPO5a and OPO5b did not give reproducible results. Optimisation procedures had to be implemented which included, the addition of DMSO, formamide and glycerol. Other variables that were also changed included magnesium chloride concentration and change of annealing temperature. The use of DMSO and formamide are, however, not standard practices when doing PCR, and are only used when problems are experienced with amplification. A new PCR reaction mixture was established for primer 5a, which yielded the correct profile, but this could not be repeated (Figure 3.21). A temperature gradient to establish the annealing temperature was then performed. The optimum temperature was established at

54.4°C but a combined cycle proved to be the best option at 51,6°C and 48°C (Figure 3.22). Successive amplification of the DNA samples could however not be achieved.

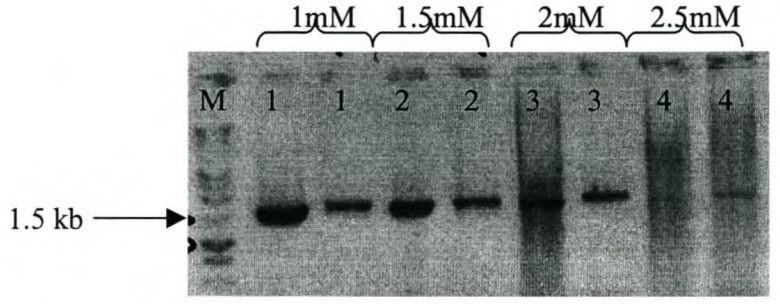


Figure 3.20 Amplified OPO5a product at 60°C at varying magnesium concentration gradients using duplicate Northern Spy samples. M=1kb ladder (Promega). The DNA sample used was 2.20(0).

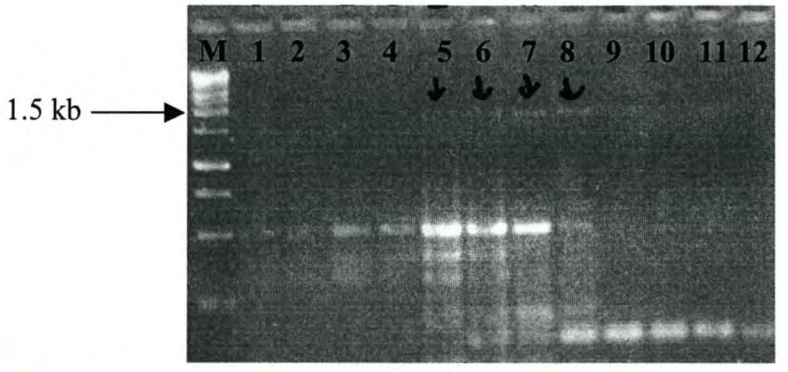


Figure 3.21 The temperature gradient established for OPO5a between 40°C and 60°C. The successful amplification is indicated with the arrows. Optimal product formed at 54.4°C (lane 8, indicated by the last arrow). The sample used for optimisation was Northern Spy, 2.20(0). The size marker used was the 1 kb ladder from Promega.

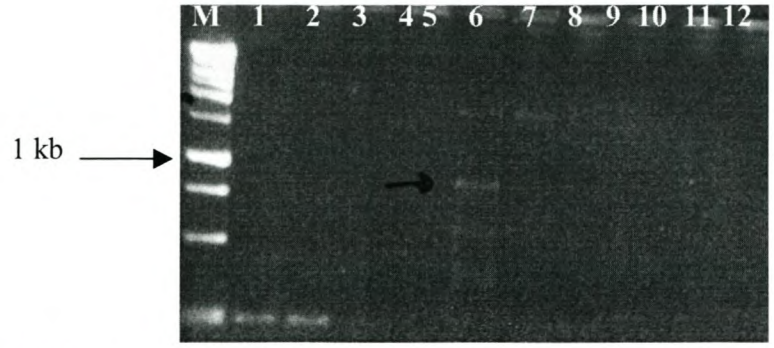


Figure 3.22 The temperature gradient established for OPO5b between 40°C and 60°C. The optimal annealing temperature was 45.9°C (lane 6). The arrow indicates the desired fragment of 880bp. The sample used was Northern Spy, 2.20(0). The size marker used is the 1 kb ladder from Promega.

Primer OPO5b presented the same problems as OPO5a. The same optimisation procedures were applied as with OPO5a. The same erratic and irreproducible results were obtained. The size of the fragment to be amplified was 880bp. The final annealing temperatures that were determined are 48°C (10 cycles), 45°C (25 cycles) (Figure 3.22).

All the SCAR markers tested failed to give reproducible results, which does not indicate a good correlation between the presence of the markers and woolly apple aphid resistance. Therefore, these markers are not ideal for use in local MAS breeding programmes.

CHAPTER 4
CONCLUSION

The impact of the woolly apple aphid on the apple industry is significant and the control of this pest should be addressed to ensure sustained competitiveness of the industry. For the long term, the preferred control option for this problem would be the development of woolly apple aphid (WAA) resistant rootstocks. Currently in South Africa, no commercially viable rootstock with good horticultural characteristics and WAA resistance is available. No useful markers for the resistance genes in currently known resistant material, such as Northern Spy and others, are available for use in marker-assisted selection (MAS) breeding programmes.

The use of markers tightly linked to a single resistance gene as identified in Northern Spy (Knight *et al.*, 1962), should in effect half the number of seedlings that need to be planted for further horticultural evaluation. The development of rootstocks that have durable resistance to various diseases, including WAA, will be beneficial to the industry. Commercial apple rootstocks combining good horticultural qualities and resistance to disease, that are adapted to South African climatic conditions will help to increase the industry's competitiveness on overseas markets. This will also be beneficial to consumers as there is an increased demand for elimination of the excessive use of pesticides. Lowered input costs will result from decreased pesticide usage.

The aim of this study was to identify molecular markers linked to the Er_1 gene in Northern Spy and resistance genes in other cultivars. These markers would be essential to accelerate breeding programmes aimed at combining WAA resistance with commercially desirable characteristics of current rootstocks.

Two inoculation procedures for the aerial evaluations were applied. The first method employed was dusting, a technique proven to be useful for pre-screening. The second method was adopted for older seedlings for the verification of pre-screening. In addition, a terrestrial infestation method was also investigated to compare the efficacy and the effects of the two methods. Results of the two methods were found to correspond. Phenotypic data pertaining to WAA infestation and evaluation in the three progeny populations, namely Northern Spy, Rootstock 5 and Russian Seedling, were collected for a period of three seasons. Classifications of seedlings into their respective classes were based on the

phenotypic data observed during the first season's evaluations (pre-selection). Follow-up inoculations were performed on these populations for the duration of the study in order to verify the initial seedling classifications. This data included the percentage infection for individual seedlings, as well as inoculation data. Percentage infection served as the main criterion for the classification of seedlings in their respective classes. The ordinal nature of the classification system creates limitations for genetic analyses; therefore percentage infection was applied as a quantification method during re-testing of pre-selected seedlings. Re-testing of seedlings identified misclassified seedlings. Only specific samples of which data were consisted over seasons were used in the molecular analyses therefore suggesting that the use of a larger population and long-term study will significantly increase the knowledge of the dynamics of subterranean WAA populations in South Africa.

The statistical analysis of infestation data confirmed a proposed 1:1 segregation pattern in Northern Spy. Similarly, this ratio was also obtained for Rootstock 5 and Russian Seedling, which indicate the influence of a single dominant resistance gene for WAA. Though not necessarily the Er_1 gene identified in Northern Spy, this data provides a basis for further investigation in other cultivars exhibiting WAA resistance.

Before AFLP analysis could be initiated in this study, various problems were encountered of which the lack of sufficient quantities of high molecular weight DNA was a major stumbling block. Apples, like grapevine, strawberries and other plants, have a high level of polyphenolic compounds and polysaccharides. These compounds adversely affect the extent to which molecular manipulation, such as restriction digestions, can be applied to DNA obtained from these materials. It was determined that a CTAB-based extraction protocol that included PVP, was effective in yielding good quality DNA for AFLP analysis (Porebski *et al.*, 1997).

Experiments were designed and performed to test the effectiveness and reproducibility of the initial phases of the analyses namely restriction digestions, adaptor ligations and pre-selective amplification. Results indicated that these steps were effectively performed. Other problems such as high background on the autoradiographs were resolved. Despite optimisation of the initial phases of the technique, no AFLP markers could be identified.

A number of SCAR markers , previously reported to be linked to the *Er1* gene (Gardiner *et a*l., 1999; Gardiner *et al.*, 2000a and Gardiner *et al.*, 2000b), were tested in local populations. The SCAR markers were initially successfully amplified, but problems were experienced with the reproducibility of these results. The GS327 marker, linked to the *Er1* resistance gene, was also found in a relatively high percentage of susceptible seedlings. Presence of this marker in the susceptible progeny can be the result of recombination. Another possible explanation is phenotypic misclassification. Despite various attempts, no reproducible profile could be obtained for any of the RAPD markers tested in this study. This in not entirely unexpected as this is a major drawback of this technique (Jones *et al.*, 1997). A marker used in a previous study (Gardiner *et al.*, 2000b) to detect *Er3*, was found to also associate with *Er1* in Northern Spy. This raises the question of possible allelism of these genes and would warrant further investigation (Bus *et al.*, 2000).

This attempt to identify molecular markers linked to woolly apple aphid resistance was the first project of its kind in South Africa. Although no markers could be identified, an aerial and terrestrial evaluation procedure was succesfully applied and relevent data in this regard were obtained for rootstocks used in South African. Previously identified SCAR and RAPD markers tested in the study did not yield any useful results for discriminating between resistant and susceptible individuals. The reproducibility of these markers presented the biggest hurdle. This underlines the need for the development of markers that can readily be applied in local breeding programmes. The identification and integration of such markers will greatly benefit the local and world wide apple industries. Ultimately, the use of pesticides and insecticides in agricultural practice may be reduced and the productivity and good fruit quality increased.

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APPENDIX A

BUFFERS AND STOCK SOLUTIONS

DNA extraction buffer – make up to a specified volume

- 100mM Tris-HCl (pH 8.0) (using a 1M stock solution)
- 1.4 M NaCl (using a 5M stock solution)
- 20mM Na_2EDTA (pH 8.0) (using a 0.5M stock solution)
- 2% (v/v) CTAB (using a 10% CTAB stock solution)
- Adjust the volume to the specified volume with H_2O.

5M NaCl – 1 liter

- Dissolve 292.2 g sodium chloride (NaCl, MW = 58.44) in 800 ml H_2O.
- Adjust the volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

3M NaOAc – 1 liter

- Dissolve 408,24 g sodium acetate–$3H_2O$ (NaOAc–$3H_2O$, MW = 136.08) in 800 ml H_2O
- Adjust to pH 5.2 with glacial acetic acid or to pH 7.0 with diluted acetic acid.
- Adjust the volume to 1 liter with H_2O.
- Sterilize by autoclaving and store at room temperature.

1M Tris – 1 liter

- Dissolve 121.14 g Tris (hydroxymethyl) aminomethane (Tris, MW = 121.14) in 800 ml H_2O.
- Adjust pH to the desired value by adding concentrated HCl:

  pH 7.4: ~ 70 ml

  pH 7.6: ~ 60 ml

  pH 8.0: ~ 42 ml
- Adjust the volume to 1 liter with H_2O
- Sterilize by autoclaving and store at room temperature

1x TE – 1 liter

- Add 10 ml 1 M Tris (pH 8.0, 7.6 or 7.4) and 2 ml 0.5 M Na_2EDTA (pH 8.0) to 800 ml H_20.
- Mix and adjust the volume to 1 liter with H_2O
- Sterilize by autoclaving
- Store at room temperature

0.5 M Na_2EDTA – 1 liter

- Dissolve 186.12 g disodium ethylenediaminetetraacetate–$2H_2O$ (Na_2EDTA–$2H_2O$, MW = 372.24) in 800 ml H_2O; stir vigorously on a magnetic stirrer.
- Adjust pH to 8.0 with NaOH (~ 20g NaOH pellets) and adjust the volume to 1 liter with H_2O.
- Divide into aliquots and sterilize by autoclaving.
- Store at room temperature

10 x TBE (Tris-borate) – 1 liter

- Dissolve 108 g Tris and 55 g Boric acid in 900 ml H_2O.
- Add 40ml 0.5 M Na_2EDTA (pH 8.0) and adjust volume to 1 liter with H_2O.
- Store at room temperature.

Developing solution

- Add 500 ml of solution A, 500 ml of solution B and 50 ml of solution C to 1400 ml H_20.
- Mix thoroughly and adjust the volume to 2 liters with H_2O.
- Store in a dark place at room temperature.

Fixative solution

- Add 500 ml of solution A, 500 ml of solution B to 1400 ml H_2O.
- Mix thoroughly and adjust the volume to 2 liters with H_2O.
- Store in a dark place at room temperature.

APPENDIX B

LIST OF PRIMERS USED IN AFLP ANALYSIS

Primer name	Sequence
Pst/Sse adaptor 1	5' CTCGTAGACTGCGTACATGCA 3'
Pst/Sse adaptor 2	3' CATCTGACGCATGT 5'
MseI adaptor 1	5' GACGATGAGTCCTGAG 3'
MseI adaptor 2	3' TACTCAGGACTCAT 5'
POOL	5' GTAGACTGCGTACATGCAG 3'
PO3L/SOO	5' AGACTGCGTACATGCAGG 3'
P19/SO1	5' GACTGCGTACATGCAGGA 3'
P20/S02	5' GACTGCGTACATGCAGGC 3'
P21/S03	5' GACTGCGTACATGCAGGG 3'
P22/S04	5' GACTGCGTACATGCAGGT 3'
P63/S11	5' GACTGCGTACATGCAGGAA 3'
P64/S12	5' GACTGCGTACATGCAGGAC 3'
P65/S13	5' GACTGCGTACATGCAGGAG 3'
P66/S14	5' GACTGCGTACATGCAGGAT 3'
P67/S15	5' GACTGCGTACATGCAGGCA 3'
P68/S16	5' GACTGCGTACATGCAGGCC 3'
P69/S17	5' GACTGCGTACATGCAGGCG 3'
P70/S18	5' GACTGCGTACATGCAGGCT 3'
P71/S19	5' GACTGCGTACATGCAGGGA 3'
P72/S20	5' GACTGCGTACATGCAGGGC 3'
P73/S21	5' GACTGCGTACATGCAGGGG 3'
P74/S22	5' GACTGCGTACATGCAGGGT 3'
P75/S23	5' GACTGCGTACATGCAGGTA 3'
P76/S24	5' GACTGCGTACATGCAGGTC 3'
P77/S25	5' GACTGCGTACATGCAGGTG 3'
P78/S26	5' GACTGCGTACATGCAGGTT 3'
MOO	5' GATGAGTCCTGAGTAA 3'
MO1	5' GATGAGTCCTGAGTAAA 3'
MO2	5' GATGAGTCCTGAGTAAC 3'

Appendix B continued	
MO3	5' GATGAGTCCTGAGTAAG 3'
MO4	5' GATGAGTCCTGAGTAAT 3'
M11	5' GATGAGTCCTGAGTAAAA 3'
M12	5' GATGAGTCCTGAGTAAAC 3'
M13	5' GATGAGTCCTGAGTAAAG 3'
M14	5' GATGAGTCCTGAGTAAAT 3'
M15	5' GATGAGTCCTGAGTAACA 3'
M16	5' GATGAGTCCTGAGTAACC 3'
M17	5' GATGAGTCCTGAGTAACG 3'
M18	5' GATGAGTCCTGAGTAACT 3'
M19	5' GATGAGTCCTGAGTAAGA 3'
M20	5' GATGAGTCCTGAGTAAGC 3'
M21	5' GATGAGTCCTGAGTAAGG 3'
M22	5' GATGAGTCCTGAGTAAGT 3'
M23	5' GATGAGTCCTGAGTAATA 3'
M24	5' GATGAGTCCTGAGTAATC 3'
M25	5' GATGAGTCCTGAGTAATG 3'
M26	5' GATGAGTCCTGAGTAATT 3'
M47	5' GATGAGTCCTGAGTAACAA 3'
M48	5' GATGAGTCCTGAGTAACAC 3'
M49	5' GATGAGTCCTGAGTAACAG 3'
M50	5' GATGAGTCCTGAGTAACAT 3'
M59	5' GATGAGTCCTGAGTAACTA 3'
M60	5' GATGAGTCCTGAGTAACTC 3'
M61	5' GATGAGTCCTGAGTAACTG 3'
M62	5' GATGAGTCCTGAGTAACTT 3'
M67	5' GATGAGTCCTGAGTAAGCA 3'
M68	5' GATGAGTCCTGAGTAAGCC 3'
M69	5' GATGAGTCCTGAGTAAG CG 3'
M70	5' GATGAGTCCTGAGTAAGCT 3'

APPENDIX C

Table A. Woolly apple aphid selection for molecular analysis for resistant seedlings in Northern Spy, open-pollinated population 2.

Seedling number	Initial Class 1999	% infection February 1999	% infection May 2000	% infection * November 2000	% infection February 2001
2.3	0	0	10	Ω	100
2.4	0	0	0	Ω	0
2.5	0	0	15	Ω	0
2.6	0	0	11	100	100
2.7	0	6	0	Ω	0
2.8	0	0	0	0	0
2.9	0	4	0	Ω	0
2.10	0	0	Ω	Ω	15
2.11	0	0	0	dead	dead
2.12	0	3	Ω	0	0
2.13	0	0	0	0	0
2.14	0	8	0	Ω	0
2.15	0	0	Ω	Ω	0
2.16	0	0	0	0	0
2.17	0	0	0	0	0
2.18	0	0	0	0	8
2.19	0	0	4	0	0
2.20	0	0	0	Ω	100
2.21	0	0	0	0	0
2.25	0	6	0	Ω	5
2.(22)	0	5	0	Ω	0
2.(23)	0	7	0	Ω	11
2.(24)	0	6	3	Ω	0
2.(3)	0	0	10	Ω	100
2.26(?)	0	8	4	Ω	0

Ω - Data is not available for these samples.

* Evaluation on performed on selected seedlings to determine woolly apple aphid activity before onset of last infestation.

Table B. Woolly apple aphid selection for molecular analysis for susceptible seedlings in Northern Spy, open-pollinated population 2.

Seedling number	Initial Class 1999	% infection February 1999	% infection May 2000	% infection* November 2000	% infection February 2001
2.1	3	70	53.00	Ω	100
2.2	3	70	31.00	100	100
2.3	3	43	44.00	7.00	100
2.4	3	67	80	Ω	100
2.5	3	79.00	0	17.00	100
2.6	3	29.00	19.00	Ω	100
2.7	3	29.00	12.00	Ω	100
2.8	3	47.00	11.00	dead	dead
2.9	3	33.00	dead	dead	Dead
2.10	3	67.00	0	Ω	100
2.11	3	60	dead	dead	Dead
2.12	3	38.00	12.00	Ω	100
2.13	3	75.00	dead	dead	dead
2.14	3	100	dead	dead	dead
2.15	3	71.00	15.00	Ω	100
2.16	3	53.00	36.00	Ω	100
2.17	3	22.00	57.00	50	100
2.18	3	47.00	0	Ω	100
2.19	3	100	dead	dead	dead
2.20	3	100	0	Ω	0
2.21	3	54.00	0	13.00	100
2.22	3	30	75.00	16.00	14.00
2.23	3	33.00	100	Ω	100
2.24	3	100	dead	dead	dead
2.25	3	68.00	26.00	0	100
2.26	3	58.00	19.00	41.00	100
2.27	3	56.00	0	0	0
2.28	3	64.00	Ω	Ω	100
2.29	3	73.00	Ω	Ω	100
2.30	3	57.00	dead	dead	dead
2.31	3	56.00	0	Ω	100

Ω - Data not available for these samples.

* Evaluation on performed on selected seedlings to determine woolly apple aphid activity before onset of last infestation.

Table C. Woolly apple aphid selection for molecular analysis for resistant seedlings in Rootstock 5, open-pollinated population 6.

Seedling number	Initial Class 1999	% infection February 1999	% infection May 2000	% infection* November 2000	% infection February 2001
6.1	0	0	0	Ω	0
6.2	0	0	0	Ω	0
6.3	0	0	36.00	Ω	0
6.4	0	0	0	0	0
6.5	0	0	0	Ω	0
6.6	0	0	0	0	0
6.7	0	0	0	Ω	0
6.8	0	0	dead	dead	dead
6.9	0	0	5.00	Ω	0
6.10	0	0	0	0	0
6.11	0	0	0	0	0
6.12	0	0	0	Ω	0
6.13	0	0	26.00	0	0
6.14	0	0	0	0	0
6.15	0	0	0	Ω	0
6.16	0	0	0	0	0
6.17	0	0	0	Ω	0
6.18	0	0	0	0	0
6.19	0	0	0	0	57.00
6.20	0	0	0	Ω	0
6.21	0	0	0	0	100
6.22	0	4.00	0	0	0
6.23	0	0	0	dead	dead
6.24	0	0	0	Ω	0
6.25	0	0	0	0	0
6.26	0	0	0	0	0
6.27	0	0	9.00	0	0
6.28	0	0	0	Ω	0
6.29	0	0	0	0	0
6.30	0	0	dead	dead	dead
6.31	0	0	0	0	0
6.32	0	0	7.00	0	0

Ω - Data not available for these samples.

* Evaluation on performed on selected seedlings to determine woolly apple aphid activity before onset of last infestation.

Table D. Woolly apple aphid selection for molecular analysis for susceptible seedlings in Rootstock 5, open-pollinated population 6.

Seedling number	Initial Class 1999	% infection February 1999	% infection May 2000	% infection* November 2000	% infection February 2001
6.1	3	57.00	25.00	Ω	100
6.2	3	74.00	40	Ω	100
6.3	3	50	dead	dead	dead
6.4	3	43.00	33.00	dead	dead
6.5	3	40	23.00	0	100
6.6	3	29.00	0	100	100
6.7	3	40	29.00	5.00	100
6.8	3	70	18.00	20	100
6.9	3	0	dead	dead	dead
6.10	3	100	13.00	0	100
6.11	3	57.00	0	Ω	100
6.12	3	58.00	20	0	100
6.13	3	58.00	25.00	0	100
6.14	3	42.00	16.00	Ω	100
6.15	3	73.00	0	Ω	100
6.16	3	49.00	0	0	13.00
6.17	3	64.00	0	Ω	100
6.18	3	30	55.00	33.00	100
6.19	3	52.00	33.00	Ω	100
6.20	3	46.00	12.00	Ω	100
6.21	3	25.00	0	dead	dead
6.22	3	25.00	0	Ω	33.00
6.23	3	31.00	12.00	28.00	100
6.24	3	32.00	0	Ω	100
6.25	3	67.00	Ω	Ω	100
6.26	3	30	10	Ω	100
6.27	3	28.00	26.00	41.00	100
6.28	3	48.00	0	dead	dead
6.29	3	69.00	0	Ω	100

Ω - Data not available these samples.

* Evaluation on performed on selected seedlings to determine woolly apple aphid activity before onset of last infestation.

Table E. Woolly apple aphid selection for molecular analysis for resistant seedlings in Russian Seedling, open-pollinated population 11.

Seedling number	Initial Class 1999	% infection February 1999	% infection May 2000	% infection * November 2000	% infection February 2001
11.1	0	0	0	0	0
11.2	0	0	0	0	0
11.3	0	0	0	Ω	0
11.4	0	0	Ω	0	0
11.5	0	0	7.00	0	0
11.6	0	0	0	0	0
11.7	0	0	0	Ω	0
11.8	0	0	0	Ω	25.00
11.9	0	0	24.00	Ω	100
11.10	0	0	15.00	0	0
11.11	0	0	0	Ω	0
11.12	0	0	0	Ω	0
11.13	0	0	0	Ω	0
11.14	0	0	0	0	0
11.15	0	0	Ω	0	dead
11.16	0	0	22.00	100	100
11.17	0	0	0	0	0
11.18	0	0	Ω	0	8.00
11.19	0	0	0	Ω	0
11.20	0	0	0	Ω	100
11.21	0	0	11.00	50	100
11.22	0	0	0	0	0
11.23	0	0	0	0	0
11.24	0	0	13.00	0	100
11.25	0	0	0	0	0
11.26	0	0	0	0	0
11.27	0	0	0	0	0
11.28	0	0	dead	dead	dead
11.29	0	0	dead	dead	dead
11.30	0	0	dead	dead	dead
11.31	0	0	4.00	Ω	0

Ω - Data not available * Evaulation on peformed on selected seedlings to determine woolly apple aphid activity before onset of last infestation.

Table F. Woolly apple aphid selection for molecular analysis for susceptible seedlings in Russian Seedling, open-pollinated population 11.

Seedling number	Initial Class 1999	% infection February 1999	% infection May 2000	% infection * November 2000	% infection February 2001
11.1	3	23.00	30	42.00	100
11.2	3	50	50	Ω	100
11.3	3	32.00	0	Ω	100
11.4	3	36.00	20	16.00	100
11.5	3	63.00	0	Ω	100
11.6	3	27.00	11.00	Ω	100
11.7	3	38.00	33.00	13.00	100
11.8	3	0	14.00	23.00	100
11.9	3	17.00	0	Ω	100
11.10	3	22.00	42.00	Ω	100
11.11	3	53.00	20	Ω	100
11.12	3	50	0	33.00	100
11.13	3	59.00	37.00	Ω	100
11.14	3	57.00	13.00	100	100
11.15	3	32.00	50	100	100
11.16	3	20	50	Ω	100
11.17	3	43.00	17.00	dead	dead
11.18	3	39.00	4.00	Ω	100
11.19	3	17.00	29.00	0	100
11.20	3	0	11.00	100	100
11.21	3	22.00	42.00	22.00	100
11.22	3	22.00	0	12.00	100
11.23	3	0	0	Ω	7.00
11.24	3	50	53.00	100	100
11.25	3	39.00	31.00	Ω	44.00
11.26	3	50	Ω	22.00	100
11.27	3	0	20	Ω	0
11.28	3	0	43.00	Ω	100
11.29	3	24.00	60	Ω	100
11.30	3	0	29.00	0	100
11.31	3	25.00	32.00	Ω	100
11.32	3	39.00	0	50	100
11.33	3	50	0	Ω	100
11.34	3	25.00	31.00	Ω	100
11.35	3	10	22.00	22.00	100

Ω - Data not available for these samples.

* Evaluation on performed on selected seedlings to determine woolly apple aphid activity before onset of last infestation.