Identification of Differentially Expressed mRNA Species in *Vitis vinifera* in Reaction to Infection by *Uncinula necator*

Andreas Stephanus van der Merwe

Thesis presented in partial fulfillment of the requirements for the degree of Master of Natural Sciences at the University of Stellenbosch.

Study leader: Dr. J.T. Burger

Date of presentation: December 1999

Stellenbosch University https://scholar.sun.ac.za

Declaration

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

Date 24/11/99

Stellenbosch University https://scholar.sun.ac.za

OPSOMMING

Witroes is een van die mees belangrike wingerdsiektes en is 'n probleem in al die wingerdverbouingsgebiede. Verliesse wat veroorsaak word deur onbeheerde witroesinfeksies verplig produsente om 'n omvattende fungisied spuitprogram te volg om die siekte te beheer. In die verlede het druif-telers gepoog om natuurlike siekteweerstand in kommersiële kultivars te inkorporeer, maar was gekonfronteer met probleme soos inteling depressie, lang generasie siklus, die poligeniese aard van witroesweerstand en 'n komplekse genetiese sisteem. Gevolglik is weinig vordering gemaak om bestaande vatbare kultivars om te skakel na witroesbestande kultivars.

Vorige studies het bewys dat *V. vinifera* weerstand teen witroes veroorsaak word deur verskeie faktore soos verskille in die kutikula, 'n verhoogde aktiwitiet van ensieme betrokke in lignien biosintese, produksie van papilla, neerlegging van silika invoegsels, gelokaliseerde nekrose, en die aktivering van ensieme soos kutinase en glukanse (Pratt *et al.,* 1984, Heintz & Blaich, 1989, 1990, Eibach, 1994, Clingeleffer & Scott, 1994). In hierdie studie is bogenoemde probleem benader deur te kyk na siekteweerstand van druifkultivars op geen-aktiveringsvlak. 'n Tegniek genaamd "Differrensiële Vertonings Polimerase Kettingreaksie" (DVP) is vir hierdie doel aangewend. Bogenoemde tegniek is in 1992 deur Liang & Pardee ontwikkel en word gebruik om differensiële geen-uitdrukking van geneties identiese selle in verskillende omgewingstoestande (geïnfekteer en nie-geïnfekteer) met mekaar te vergelyk.

Die identifikasie van differensieël uitgedrukte fragmente begin by die suksesvolle verbouing van toetsplante onder identiese omgewingstoestande. Die volgende stap was om toetsplante kunsmatig te infekteer met witroes en dan die geïnfekteerde- en ongeïnfekteerde blare na 'n kort inkubasie periode te oes. Die isolasie van RNS vanaf druifblare was problematies en moes ge-optimiseer word gedurende die studie. Aangesien DVP nie voorheen in ons laboratoruim gebruik is nie, was die volgende doelwit die optimisering van dié tegniek in ons laboratoruim toestande. DVP was toe aangewend om differensieël uitgedrukte gene wat in geïnfekteerde wingerblare uitgedruk is, te identifiseer. Die nukleotied volgorde van bogenoemde fragmente is toe bepaal en vergelyk met bekende nukleotied volgordes in databassise. Die bevestiging van die differensiële uitdrukking van hierdie fragmente is gedoen deur Omgekeerde Noordelike Blot (ONB) analises.

Stellenbosch University https://scholar.sun.ac.za

Alhoewel 'n redelike hoë persentasie vals positiewe verkry is met ONB, is 25 differensieël uitgedrukte fragmente geïsoleer en vergelyk met bekende geen-volgordes in databassise. Hierdie DNS fragmente het homologie getoon met verskeie bekende geen-volgordes soos *V.vinifera, Z. mays* ferredoksin III, *C. sativus* and *B. napus* katalase, *A. thaliana* peroxidase (nie- betekenisvol) en *L. esculentum* poli-galakturonase (nie-betekenisvol).

Ons studie bevestig en versterk vorige resultate rakende die siekteweerstandsreaksie van wingerdplante in reaksie op infeksie deur witroes. Tydens die studie is reaksies waargeneem wat betrokke is by die versterking van selwande (peroksidases, katalase en prolien-ryke proteine), gelokaliseerde sel dood (ferredoksien reduktases en katalase) en die produksie van anti-fungiese bestandele (thaumatin-agtige proteine). Dit was teleurstellend dat geen differensiëel-uitgedrukte fragmente, betekenisvolle homologie getoon het met patogeen-verwante proteine soos glukanase en kutinase nie. Dit is egter moontlik dat laasgenoemde gene wel uitgedruk was, maar nie teen DVP waarnemingsvlakke nie.

Alhoewel verskeie algemene siekteweerstandsverwante gene gedurende die studie geïsoleer is, was dit teleurstellend dat geen gene wat spesifiek deur witroes infeksie geaktiveer word, geïsoleer is nie. Verskeie onbekende geen-fragmente is egter tydens die studie geïsoleer en kan moontlik nuwe en onbekende skakels wees in die siekteweerstandsrespons van wingerdplante.

Stellenbosch University https://scholar.sun.ac.za

ABSTRACT

Powdery mildew is one of the most important grapevine diseases and is a problem in all the grapevine producing areas. Losses caused by uncontrolled powdery mildew force producers to follow extensive fungicide spray programs to control the disease. Grapevine breeders has tried to incorporate natural disease resistance into commercial cultivars, but has been confronted with problems like inbreeding depression, long generation cycle, the polygenic nature of powdery mildew inheritance and a complex genetic system. As a result, not much progress has been made to convert existing susceptible cultivars to resistant cultivars.

Previous studies attributed *V. vinifrera* resistance to powdery mildew to several factors such as differences in cuticle thickness, increased activity of enzymes involved in lignin biosynthesis, production of papillae, deposition of silica incrusts, localised necrosis and the activation of enzymes like chitinases and glucanases (Pratt *et al.*, 1984, Heintz & Blaich, 1989, 1990, Eibach, 1994, Clingefeffer & Scott, 1994). We approached this problem by addressing disease resistance of grapevine cultivars to powdery mildew at the gene-activation level. Liang & Pardee (1992) develop a technique, called differential display PCR (DD-PCR), that enabled us to compare differential gene expression of identical cells under altered (infected and not-infected) conditions.

The identification of differentially expressed fragments started at the successful cultivation of test plants under identical environmental conditions. The next step was to artificially inoculate test plants with powdery mildew and to harvest the infected and control leaves after a short incubation period. The isolation of RNA from grapevine leaves was problematic and had to be optimised during this study. Because DD-PCR was not previously used in our lab, the next step was to optimise the technique to suit our lab conditions. DD-PCR was then applied to identify differentially expressed genes from infected grapevine leaves. Differentially expressed fragments were then sequenced and compared with known gene sequences in sequence databases. Verification of differential expression was done using reverse northern blots.

Although a relatively high percentage of false positives were obtained with reverse northern blots, 25 differentially expressed mRNA species were isolated and compared with known gene sequences in sequence databases. These DNA fragments aligned to several known gene sequences like, *V. vinifera* proline rich proteins 1 & 2, thaumatin-like proteins, *Z.*

Stellenbosch University https://scholar.sun.ac.za

mays ferredoxin III, *C. sativus* and *B. napus* catalase, *A. thaliana* peroxidase (not significant) and *L. esculentum* polygalacturonase (not significant).

Our study strengthens previous results concerning the grapevine defence reaction in response to powdery mildew infection. We observed reactions that are involved in the reinforcement of cell walls (peroxidase, catalase and proline rich proteins), localised cell death (ferredoxin reductases and catalase) and the production of antifungal compounds (thaumatin-like proteins). It was disappointing that no differentially expressed fragment showed significant homology with PR proteins like glucanase and chitinase. It is possible that these genes were expressed, but not at DD-PCR detectable levels.

Although several general defence-related genes were isolated during this study, it was disappointing that no genes, specifically activated by powdery mildew infection, were isolated. However, several novel differentially expressed fragments were isolated and might represent novel and important links in the grapevine defence response.

Stellenbosch University https://scholar.sun.ac.za

CONTENTS

	Acknowledgements	VI
	Abbreviations	VII
	List of Figures	IX
	List of Tables	X
1	**GENERAL INTRODUCTION AND LITERATURE REVIEW**	
1.1	**Cultivation of grapes**	1
1.1.1	Origin and early development	1
1.1.2	Botanical classification of grapes	2
1.1.3	Economic importance of grapes	2
1.2	**Powdery mildew of grapevine**	3
1.2.1	Introduction	3
1.2.2	Causal organism	3
1.2.3	Symptoms	4
1.2.4	Economic importance of powdery mildew	5
1.3	**Infection of the grapevine by powdery mildew**	6
1.3.1	Germination and development of germtube	6
1.3.2	Penetration and colonisation of plant tissue	7
1.3.3	Inoculum production	7
1.4	**Host response mechanisms of *V. vinifera* against *U. necator***	8
1.4.1	Cuticle thickness	8
1.4.2	Production of physical barriers	8
1.4.3	Activation of defence-related enzymes	9
1.5	**Introducing powdery mildew resistance genes into commercial cultivars**	9
1.5.1	Inheritance of powdery mildew resistant genes	9
1.5.2	Conventional breeding	10
1.5.3	Molecular markers	12
1.5.4	The identification and cloning of genes from grapes	13
1.6	**Molecular biology of disease resistance**	13

Stellenbosch University https://scholar.sun.ac.za

1.6.1 Generation of a signal indicative of attack by the pathogen 14
1.6.2 Recognition of the signal by the host 15
1.6.3 Signal transduction and the activation of plant defence responses 16
1.6.4 Identification of defence-related genes 18

1.7 Tools to identify differentially expressed genes 19
1.7.1 Approaches to identify differentially expressed genes 19
1.7.2 Principles of differential display 20
1.7.3 Successes obtained with differential display 22

1.8 Aims of present study 22
1.8.1 Optimise RNA isolation technique from grapevine leaves 23
1.8.2 Optimise differential display technique 23
1.8.3 Molecular analysis of identified fragments 24
1.8.4 Investigation of defence gene activation of grapevine in response to pathogen infection 25

2 MATERIALS AND METHODS 26

2.1 Introduction 26

2.2 Cultivation and infection of greenhouse plants 26

2.2.1 Cultivation of greenhouse plants 26
2.2.1.1 Collection of shoots 26
2.2.1.2 Planting and maintenance of greenhouse plants 27
2.2.2 Generation and cultivation of *in vitro* plants 27
2.2.3 Inoculation of test plants 27
2.2.3.1 Inoculation of greenhouse plants 27
2.2.3.2 Inoculation of *in vitro* plants 28
2.2.4 Harvesting of plant material 28

2.3 RNA isolation 28

2.3.1 Isolation of RNA using a standard RNA isolation protocol 29
2.3.2 Modification of RNA isolation protocol 29
2.3.3 Removal of contaminating DNA 30
2.3.3.1 Isolation of mRNA using magnetic beads 30
2.3.3.2 Differential precipitation of RNA 30
2.3.3.3 Enzymatic digestion of contaminating DNA 31

Stellenbosch University https://scholar.sun.ac.za

2.4 Production of differentially expressed fragments 31

2.4.1 Synthesis of first strand cDNA 31
2.4.2 Amplification of cDNA fragments 32
2.4.2.1 PCR conditions 32
2.4.2.2 End-labelling of anchored primers 32
2.4.3 Separation of amplified cDNA fragments by PAGE 33
2.4.3.1 Preparation of glass plates 33
2.4.3.2 Preparation of gel solution 33
2.4.3.3 Casting of gel 33
2.4.3.4 Preparation and loading of samples 34
2.4.3.5 Autoradiography 34

2.5 Identification and re-amplification of differentially expressed fragments 35

2.5.1 Identification of differentially expressed fragments 35
2.5.2 Isolation and precipitation of differentially expressed fragments 35
2.5.3 Re-amplification of isolated fragments 35
2.5.4 Isolation of re-amplified fragments 36
2.5.5 Sequencing of re-amplified fragments 36
2.5.6 Molecular analysis of differentially expressed fragments 37

2.6 Confirmation of gene expression 37

2.6.1 Synthesis of cDNA probes for Reverse Northern Blot analysis 37
2.6.2 Blotting of PCR products 38
2.6.3 Hybridisation of blotted PCR products with labelled probes 38
2.6.4 Analysis of Blots 39

3 RESULTS AND DISCUSSION 40

3.1 Introduction 40

3.2 Cultivation and infection of greenhouse plants 41
3.2.1 Selection of greenhouse plants 41
3.2.2 Selection of *in vitro* plants 42
3.2.3 Inoculation of test plants 43

Stellenbosch University https://scholar.sun.ac.za

3.3	**RNA isolation**	43
3.3.1	Optimisation of RNA isolation protocol	44
3.3.2.1	Isolation of RNA using a standard RNA extraction protocol	44
3.3.2.2	Isolation of RNA using a modified method	46
3.3.3	Removal of contaminating DNA	47
3.3.3.1	Capturing of mRNA	48
3.3.3.2	Differential precipitation of RNA	48
3.3.3.3	Enzymatic digestion of contaminating DNA	49
3.4	**Production of differentially expressed fragments**	50
3.4.1.1	cDNA template concentration	51
3.4.1.2	One-based versus two-based anchored primers	51
3.4.1.3	Optimal arbitrary primer length for differential display PCR	52
3.4.1.4	Influence of annealing temperature	55
3.4.1.5	Influence of primer ratio	56
3.4.1.6	Influence of dNTP concentration	56
3.4.1.7	End labelling of anchored primers	56
3.4.1.8	Visualisation of amplified products	57
3.4.2	Identification of differentially expressed fragments	58
3.4.2.1	Introduction	58
3.4.2.2	Control reactions	59
3.4.2.3	Differential display PCR results	60
3.4.2.4	Identification of differentially expressed fragments	61
3.4.2.5	Re-amplification of differentially expressed fragments	64
3.4.2.6	Determination of nucleotide sequences of identified differentially expressed fragments	65
3.4.2.7	Molecular analysis of sequenced fragments	65
3.5	**Confirmation of gene expression**	67
3.5.1	Reverse northern blot analysis	67
4	**POSSIBLE FUNCTION OF DIFFERENTIALLY EXPRESSED GENES IN THE *V. VINIVERA-U. NECATOR* INTERACTION**	71
4.1	Introduction	71
4.2	Possible function of differentially expressed genes in the *V. vinifera-U. necator* interaction	71

Stellenbosch University https://scholar.sun.ac.za

5	**CONCLUSIONS**	74
6	**REFERENCES**	77

Stellenbosch University https://scholar.sun.ac.za

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the following people:

My supervisors, Dr. Reon Brand and especially Dr. Johan Burger for their advice and input into the project.

The Agricultural Research Council for partly funding this project and the opportunity to study further.

Anita Burger for excellent technical and general advice.

My colleagues at Nietvoorbij for helping me with some of my duties concerning the Table grape breeding program.

Dr Jan Loubser, for allowing me to do my labwork at the Genetics Department, Stellenbosch.

Derrick and Anke for technical and general advice and for the use of their lab equipment.

My friends in the lab for all their jokes and stories.

My friends Schalk and Leatitia for putting up with me while I was finishing my thesis.

Last but by no means least, my wife, Marina for her ongoing support and encouragement.

Stellenbosch University https://scholar.sun.ac.za

ABBREVIATIONS

°C	degrees Celsius
ARC	Agricultural Research Council
BA	4 x10^{-6} M^6 N- Benyladenine
BLAST	Basic Local Alignment Search Tool
Cf-9	*Cladosporiuim fulvum R* gene
DD-PCR	Differential display PCR
DEPC	Diethylpyrocarbonate
EtOH	Ethanol
Fig.	Figure
g	gram
GRP's	glycine-rich proteins
ha	hectares
Hm1	*Cochliobolus carbonum R* gene
HR	Hypersensitive response
HRGP's	Hydroxyproline-rich glycoproteins
IBA	Indole-3-butyric acid
kb	kilo bases
L6	*Melampsora lini R* gene
LiCl	Lithium Chloride
LRR	Leucine-rich repeats
ml	milliliters
mm	millimeters
MMLV	Moloney murine leukemia virus
mRNA	Messenger RNA
N	Tobacco mosaic virus R gene
ng	nanogram
PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PG	Polygalacturonase

Stellenbosch University https://scholar.sun.ac.za

PR proteins	pathogenesis related proteins
Prf	*P. syringe* pv *tomato R* gene
PRP's	proline rich proteins
R	disease resistance
RACE	Rapid amplification of cDNA ends
RAPD	Random Amplified Polymorphic DNA
RH	relative humidity
ROS	activated oxygen species
rpm	revolutions per minute
RPM1	*P. syringae* pv *maculicola R* gene
RPS2	*P. syringae* pv *tomato R* gene
SA	salicylic acid
SAR	systemic acquired resistance
U	Unit
v/v	volume per volume
W	Watts
w/v	weight per volume
Xa21	*X. campestris* pv *oryzae R* gene
µg	microgram
µl	microlitre
µm	micrometre

Stellenbosch University https://scholar.sun.ac.za

LIST OF FIGURES

Fig. 1.1	Principles of Differential Display PCR.	21
Fig. 3.1	Isolation of RNA using a standard RNA isolation protocol.	45
Fig. 3.2	Improvement of RNA quality by reducing RNA isolation-working temperature.	46
Fig. 3.3	Isolation of RNA using the modified protocol.	47
Fig. 3.4	RAPD reaction shows presence of contaminating DNA in RNA samples.	49
Fig. 3.5	Complete digestion of unwanted DNA in DNase I treated samples.	50
Fig. 3.6	Control reactions shows absence of DNA in RNA samples.	59
Fig. 3.7	Changes in expression patterns as a result of changes in the anchored primer used for PCR.	60
Fig. 3.8	Example of differential gene expression.	62
Fig. 3.9	Differences in banding patterns of the susceptible-and resistant cultivar.	63
Fig. 3.10	Re-amplification of isolated differentially expressed fragments.	64
Fig. 3.11	Reverse northern blot with probes originating from infected RNA.	68
Fig. 3.12	Reverse northern blot with probes originating from uninfected RNA.	68

Stellenbosch University https://scholar.sun.ac.za

LIST OF TABLES

Table 3.1	Bast search results.	66
Table 3.2	Reverse northern blots results *vs.* Blast search results.	69

Stellenbosch University https://scholar.sun.ac.za

CHAPTER ONE

1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Cultivation of grapes

1.1.1 Origin and early development

Grapes have been known and cultivated by men since the earliest times. Documents concerning viticulture and winemaking in Egypt take us back some 5 000 to 6 000 years (Einset & Pratt, 1975, Krul & Mowbray, 1978). Before the beginning of the Christian era, viticulture for the production of wine and fresh fruit had considerable importance to the people form the Middle East and Mediterranean basin (Einset & Pratt, 1975). According to literature, the Old World grape (*Vitis vinifera* L.) originates from a single wild species somewhere in the region between the Black and Caspian seas (Olmo, 1976, 1986, Krul & Mowbray, 1978). From this single species, the more than 10 000 domestic cultivars that are planted today, were developed and are today still growing wild in this area (Einset & Pratt, 1975, Olmo, 1976, 1986).

From its early beginning in the Near and Middle East, the grapevine and viticulture started to spread around the Mediterranean region. As time passed, viticulture started to extend inland to the Rhone valley of France and as far north as the Rhine and Moselle valleys during the time of the Romans (Einset & Pratt, 1975, Olmo, 1976, 1986, Krul & Mowbray, 1978). From the late fourteenth century to the eighteenth century, the Old World grape rapidly spread from Europe to North America, South America, South Africa and Australia (Olmo, 1976, Krul & Mowbray, 1978). During this era, the culture and art of winemaking expanded throughout the world as grapevine cuttings were planted in several countries for the first time (Olmo, 1976, Krul & Mowbray, 1978). The first grapevine cuttings reached South Africa in the mid-seventeenth century (Huges, Hands & Kench, 1992). Jan van Riebeeck planted these cuttings for the production of wine and fresh fruit (Huges *et al.*, 1992). In South Africa, grapes are mainly grown in the Western Cape, Elephants River, Small Karoo and Southern Gariep River regions, while smaller plantings exist in the Jan Kempdorp region, Jacobsdal, Barkly West and Mpumalanga. Grapes are also planted in home gardens throughout South Africa.

Stellenbosch University https://scholar.sun.ac.za

1.1.2 Botanical classification of grapes

Grapes belong to the botanical family Vitaceae, which is made up of 11 genera and about 600 species that are widely distributed in the tropics, sub-tropics and temperate regions (Einset & Pratt, 1975). In Vitaceae, Vitis is the only genus of economic importance and the only one containing food plants.

Vitis are divided into two sub-genera. *Muscadinia* (Planch.), whose members have a somatic chromosome number of 40 and *Euvitis* (Planch.), all of whose members have 38 somatic chromosomes (Einset & Pratt, 1975, Olmo, 1976, 1986). From these two sub-genera, *Euvitis* contains nearly all the commercial cultivars and is economically more important than *Muscadinia* (Archer, 1981). However, most of the rootstocks originated from *Muscadinia* species, or crosses where *Muscadinia* species were used as one of the breeding parents (Olmo, 1986).

The *Euvitis* sub-genus can further be divided into geographical groups of species (e.g. *V. euro-asiatica, V. americaneae* and *V. asiatica orientalis*), species (e.g. *V. Vinifera*), sub-species (*V. vinifera* subsp. *Sativa*), cultivars (Pinotage) and clones (Sultana clones H4, H5 and Super H5) (Archer, 1981). However, for the purpose of this thesis it is important to note that *Vitis vinifera* is the species of economic importance and contains all the economically important scion cultivars.

1.1.3 Economic importance of grapes

Today, grapes are planted throughout the world and grapevine plantings occupy about 11 000 000 ha world-wide. On average, 21 000 000 litres of wine are produced annually (Anonymous 4, 1998). Einset & Pratt (1975) noted that the production of grapevine-related products exceeds that of any other horticultural crop. Grapes are mainly used for the making of wine and other alcoholic or non-alcoholic beverages, are consumed as fresh fruit and smaller amounts are dried and sold as raisins to various industries or consumers (Archer, 1981).

In South Africa, approximately 108 500 ha are planted with grapevine consisting of 98 203 ha wine grapes, 9 622 ha table grapes and 237 ha raisin grapes. Most of the wine and fresh grapes produced are sold on foreign markets generating valuable income and economic stability in grape producing areas (Anonymous 2, Anonymous 3, Anonymous 4, 1998). The importance of viticulture is emphasised by the fact that grapevine is the third most important horticultural crop in South Africa (Anonymous 3). In 1997, South African viticulture contributed 14.5% (R 1 362 870 000) of the gross value of South African

Stellenbosch University https://scholar.sun.ac.za

horticultural products, 6% of the gross value of all planted crops and 3.5% of the total gross value of agricultural crops in South Africa (Anonymous 3, 1998).

Although South Africa is known throughout the world for its good quality wines and fresh table grapes, infection of bunches force producers to follow certain measures to prevent losses caused by pre-and post harvest pathogenic infections. One such measure is the use of pesticides and fungicides to reduce the loss in quality and production caused by these unwanted agents. However, the economic impact of pathogens on grapevine production and product quality will be discussed later in this chapter.

1.2 Powdery mildew of grapevine

1.2.1 Introduction

Powdery mildew is caused by *Uncinula necator* (Schw.) Burr., (Heintz & Blaich, 1989, Pearson & Gadoury, 1992). Schweintitz first described this fungus in North America in 1834 and named it *Erysiphe necator*. However, endemic American species (Muscadinia sp.) contained high levels of natural resistance to this pathogen and the disease was therefore not considered as important (Pearson & Gadoury, 1992). From America it spread to Europe and the rest of the world via infected plant material and caused severe losses because *Vitis vinifera* cultivars contained low levels of natural resistance to the pathogen (Pearson & Gadoury, 1992, Délye, Laigret & Corio-Costet, 1997). In France for example, production declined in the mid-nineteenth century by 44 million hectolitres in four years, causing many ruined vineyardists to emigrate to North Africa or South America (Pearson & Gadoury, 1992). Fortunately, the discovery that powdery mildew can be controlled with sulphur in 1850 reduced the impact of powdery mildew infections and restored the annual production of wine to more or less the same level before the onset of the epidemic (Pearson & Gadoury, 1992).

Today, powdery mildew can be found in most grape-growing areas of the world, including the tropics (Pearson & Gadoury, 1992). In South Africa, powdery mildew is a problem in all grape-growing areas and producers have to follow extensive preventative control measures to reduce losses caused by powdery mildew.

1.2.2 Causal organism

This obligate biotrophic fungus is pathogenic to all the members of the Vitaceae family with the genera Ampelopsis, Cissus, Parthenocissus and Vitis appearing to be the most susceptible (Pearson & Gadoury, 1992, Evans, Whisson & Scott, 1996). *U. necator* is a member of the Erysiphales (powdery mildews) and display similar host biology

Stellenbosch University https://scholar.sun.ac.za

characteristics than most of the other mildews (Pearson & Gadoury, 1992, Evans *et al.*, 1996).

Powdery mildew is heterothallic and most populations consist of two mating types (Pearson & Gadoury, 1992, Evans *et al.*, 1996). Both mating types are not essential for survival because powdery mildew can reproduce sexually as well as asexually (Evans *et* al., 1996). The anamorph, *Oidium tuckeri* is the most common stage during the growing season and is responsible for most of the damage caused by powdery mildew infections (Gadoury & Pearson, 1990 (b), Eibach, 1994).

U. necator may overwinter as hyphae inside dormant buds of grapevine and/or as cleistothecia on the surface of the vine (Gadoury & Pearson, 1990 (a), Pearson & Gadoury, 1992). In greenhouses and in tropical climates, mycelia and conidia may survive from one season to the next on the green tissue (Chellemi & Marois, 1991, Gadoury & Pearson, 1988, Pearson & Gadoury, 1992).

Shortly after bud-break, the fungus is reactivated and covers the emerging shoots with mycelium. Conidia are abundantly produced on these infected shoots (called "flag shoots") before being dispersed to neighbouring vines by the wind (Gadoury & Pearson, 1990 (a), Cotesi *et* al., 1995). These conidia then act as secondary infection agents and are responsible for the increase in infection levels during the season (Cotesi *et* al., 1995). Cleistothecia are produced on infected leaves, shoots, and berries in late summer and are co-responsible for the production of inoculum, which is used for primary infections at the onset of the next season (Pearson & Gadoury, 1992).

1.2.3 Symptoms

The powdery mildew fungus can infect all green tissues of the grapevine (Gadoury & Pearson, 1990 (a)). Infected plant material obtain a whitish-grey, dusty appearance while young expanding infected leaves and shoots become distorted, stunted and brittle (Gadoury & Pearson, 1990(a), Pearson & Gärtel, 1985, Ferreira & Venter, 1996). Both leaf-surfaces of any developmental stage may be infected, but susceptibility declines with leaf age (Ferreira & Venter, 1996). Occasionally, the upper surfaces of infected leaves display chlorotic or colourless areas that resemble the oil-spot symptoms of downy mildew infections (Gadoury & Pearson, 1990 (a), Pearson & Gadoury, 1992). When green shoots are infected, the infected tissues appear dark brown to black in feathery patches, which later appear reddish-brown on the surface of dormant canes (Bulit & Lafon, 1978).

Stellenbosch University https://scholar.sun.ac.za

Cluster infection before or shortly after bloom may result in poor fruit set and considerable crop loss (Pool *et al.*, 1984). Berries remain susceptible to infection until the sugar content reaches about 8%. However, established infections continue to produce conidia until the berries reach a sugar level of 15% (Pool *et al.*, 1984). Infected epidermal cells die, while sub-epidermal cells continue to expand causing the berry to split providing infection sites for secondary pathogens like *Botrytis cinerea* and various other agents causing sour rot (Pearson & Gadoury, 1992, Ferreira & Venter, 1996). Moreover, infection of the epidermal cells of non-expanding berries leads to the formation of a netlike cork layer on the berry-surface (Pearson & Gadoury, 1992, Ferreira & Venter, 1996). Berries of non-white cultivars fail to colour as they start to ripen (Pearson & Gadoury, 1992). Thus, infected clusters are unmarketable as fresh fruit, and wines made of these grapes contain off-flavours (Dye & Hammett, 1976, Pool *et al.*, 1984, Amati *et al.*, 1996).

1.2.4 Economic importance of powdery mildew

Today, as we learnt from history, uncontrolled powdery mildew infections can be very destructive and have serious financial implications for producers that do not control the disease. Studies conducted in Germany revealed that the control of powdery mildew by fungicides caused a 40% to 61% increase in yields over unsprayed control vines (Pearson & Gadoury, 1992). In 1981, Sall & Tevioitdale stated that losses caused by powdery mildew infections in Californian vineyards, frequently equals the value of 10% of that state's entire crop (Pearson & Gadoury, 1992). Furthermore, Pool *et al.*, (1984) found a 40% reduction in vine size and a 65% reduction in crop yield when powdery mildew were not controlled. Infected plants also display a reduction in winter hardiness, bud-survival and fertility (Pool *et al.*, 1984). Pearson & Gadoury, (1992) determined that mechanical harvest of infected grapevines resulted in the loss of 42% leaves in comparison with 8% leaves of uninfected plants. As a result, the photosynthetic potential of infected plants is substantially reduced, which in turn reduces the capacity of infected vines to assemble storage products needed for root development and initial growth of the next season (Pool *et al.*, 1984).

Infection of table grape bunches cause a significant reduction in quality, which disqualify them for export purposes. Amati *et al.*, (1996) demonstrated that powdery mildew infections cause a decrease in colour and anthocyanin levels, but increase the level of phenolic substances in wine made from infected bunches. They concluded that powdery mildew infections are unwanted and have a negative effect on the quality of wine. Pool *et al.*, (1984) noted that the production of high quality wines is only possible when the

Stellenbosch University https://scholar.sun.ac.za

infection level of fruit is very low. Berries infected by *U. necator* tend to contain more acid than healthy berries (Ough & Berg, 1979). The fungus itself is responsible for the production of unwanted substances responsible for off-flavours in wine (Ough & Berg, 1979, Olmo, 1986, Amati *et al.*, 1996). Pool *et al.* (1984) detected the presence of hydrogen sulphide and mildew-like off-aromas in wine made from fruit with 3% or more infected berries. In South Africa, cellars do not accept grapes containing powdery mildew infections of 3% or more for winemaking purposes.

As a result, producers are forced to follow extensive preventative fungicide spray programs. These programs have negative environmental and financial implications and are only effective for a few years due to the ability of fungi to develop resistance to fungicides, (London & Myers, 1995 (a, b), Pearson & Taschenberg, 1980, MacGregor, 1995). Biological control agents such as the fungus, *Ampelomyces quisqualis*, that infects mildew colonies at the end of the season, are also killed when chemical control measures are applied to prevent or reduce powdery mildew infections. The negative impact of fungicides on the environment is a well-known fact and will not be discussed.

In 1996, South African, producers spent R27 000 000 to control powdery mildew in vineyards, which is higher than for any other grapevine disease (Anonymous 1, 1997). Chemical residue tolerances allowed on bunches, are also decreasing on markets throughout the world. Farmers are therefore forced to produce the same quality grapes with the use of less fungicides (Chellemi & Marois, 1991). This complicates their disease control programs and is not always achievable when conditions are very favourable for disease development.

The most likely answer to these problems is to increase the levels of genetic resistance in commercial cultivars through the incorporation of disease resistance genes using conventional and biotechnological breeding programs. However, detailed knowledge about host-parasite interactions is required when addressing this problem (Heintz & Blaich, 1990). Floris & Alvarez (1996) noted that a good knowledge about these aspects is essential in the development of successful breeding programs at a practical level.

1.3 Infection of the grapevine by powdery mildew.

1.3.1 Germination and development of germtube

Normal germination and initial growth of conidia depend on the presence of a dry plant surface, because free water damages the conidia or inhibits the germination process (Pratt *et al.*, 1984, Reifschneider & Boiteux, 1988 and Gadoury & Pearson, 1990 (a)).

Stellenbosch University https://scholar.sun.ac.za

From the germinated conidia, a germtube develops which grow over the leaf surface before a specialised multi-lobed infection structure (appressorium) is formed at the hypal apex (Pratt *et al.*, 1984, Heintz, 1986 and Pearson & Gadoury, 1992). Heintz (1986) demonstrated that the length of the germination tube before the formation of a specialised infection structure varies from 30μm to 120μm, depending on the nature of the substrate.

Germination, germtube growth and the formation of appressoria in some host-pathogen systems depends on tropic responses of the pathogen on the plant surface to be infected (e.g. Infection of wheat by the biotroph, *Puccinia graminis*) (Wynn, 1981). However, Heintz (1986) and Blaich, Heintz & Wind (1989), demonstrated that *U. necator* is able to produce appressoria on several artificial substrates and is therefore not dependant on the structure or chemical composition of the plant surface.

1.3.2 Penetration and colonisation of plant tissue

After firm attachment of the appressoria to the leaf surface, several penetration pegs are formed that penetrates the cuticle and cell wall mechanically (Heintz, 1986, Blaich *et al.*, 1989 and Pearson & Gadoury, 1992). Although penetration is mainly mechanical, the large diameter of the peg at cell wall level during the penetration process could involve a localised enzymatic digestion (Heintz, 1986, Heintz & Blaich, 1990). After penetration of the cuticle and cell wall, the penetration peg enlarges in the epidermal cell and forms a globular or elongated haustorium (Gadoury & Pearson, 1990 (a)). The haustorium is then used to absorb plant nutrients from the epidermal cells. The biotrophic nature of *U. necator* is confirmed by the fact that the fungus invades only the epidermal cells (Pratt *et al.,* 1984).

1.3.3 Inoculum production

Depending on the presence of both mating types and the stage of the growing season, the fungus produces asexual conidia or cleistothecia on the surface of the infected plant tissue (Pearson & Gadoury, 1992). Conidia are mainly produced in the early-and mid stages of the season but can also be produced during the end of the season, while cleistothecia are only produced during the end of the season (Gadoury & Pearson, 1990 (a, b), 1992). Depending on the environmental conditions, spores are produced from 5 to 32 days after the germination and infection of host plants by conidia or ascospores (Pearson & Gadoury, 1992).

Stellenbosch University https://scholar.sun.ac.za

1.4 Host response mechanisms of *V. vinifera* against *U. necator*.

1.4.1 Cuticle thickness

Heintz & Blaich (1989) noted that a significant difference in cuticle thickness existed in young leaves of susceptible (< 0.85μm) and resistant cultivars (> 1.1μm). However, no significant correlation exists between cuticle thickness of mature leaves and resistance. Mature leaves appeared to be generally more resistant for all the cultivars tested (Heintz & Blaich, 1989). Furthermore, the difference in resistance levels of berries and leaves can mainly be attributed to an increase in the thickness of the peripheral cells and cuticle of berries (Heintz & Blaich, 1989, Eibach, 1994). However, such differences are recognised by *U. necator* because the density of appressoria is much higher on berries (than on leaves) in an attempt to find weak zones (which exists) on the surface of the berries (Heintz & Blaich, 1989).

1.4.2 Production of physical barriers

Several host response mechanisms have been observed in grape plants during an attempted invasion by *U. necator.* Heintz & Blaich (1990) noted that the activity of certain enzymes such as peroxidases and other key enzymes in lignin biosynthesis dramatically increases during the infection process. As a result, cell walls are reinforced and thereby prevent the pathogen to advance to neighbouring cells (Heintz & Blaich, 1990). Pratt *et al.,* (1984) observed the formation of papillae and cell wall appositions in epidermal cells at, or near the infection peg, as well as in adjacent sub-epidermal cells. These papillae consist mainly of callose (polysaccharides) without lignin. Surprisingly the formation of callose is not only restricted to resistant cultivars, but are also formed in susceptible cultivars and is therefore not solely responsible for resistance. However, papillae production may play an important role in the overall plant defence reaction (Pratt *et al.,* 1984). Heintz & Blaich (1990) observed that callose deposits are not part of primary defence reactions, but are produced rather late during the infection process.

The deposition of silica incrusts in cell walls of *Vitis vinifera* after an attempted infection by *U. necator* were found to be more abundant in resistant cultivars when compared with susceptible ones (Blaich & Wind, 1989). These silica depositions also contained polyphenolic substances like resveratrol and viniferin, which are characteristic phenols of the Vitaceae (Heintz & Blaich, 1990). Blaich & Wind (1989) noted that similar silica deposits occurred when other fungi (e.g. *Plasmopara viticola*), insects, or mites attacked test plants.

Stellenbosch University https://scholar.sun.ac.za

Infected single cells or groups of cells sometimes become necrotic and shows brown accumulations of polyphenols (Heintz & Blaich, 1990). However, the frequency of such hypersensitive like reactions depends on the grape cultivar and environmental conditions during infection (Heintz & Blaich, 1990). It is important to note that cell death is localised and included only a few cells and is not as extensive as in some cases (e.g. infection of tobacco by Tobacco Mosaic Virus).

1.4.3 Activation of defence-related enzymes

In addition to the formation of these physical barriers, enzymes with digestive properties (e.g. chitinases or glucanases) can also play an important role in plant defence by digesting or weakening the fungal hyphae. Clingeleffer & Scott (1994) suggested that the levels of endogenous chitinases and glucanases could be correlated with the degree of resistance against infection by fungi like *U. necator*. Consequently, in the presence of increased levels of these enzymes, fungal growth is being inhibited by enzymatic digestion of fungal cell wall material. Moreover, a significant correlation has been found between the endogenous activities of chitinase and glucanase and the levels of resistance of artificially inoculated test plants (Clingeleffer & Scott, 1994).

It is important to note that not one of the defence reactions mentioned above would solely prevent an attempted invasion, but the cumulative effect of these reactions in conjunction with other unknown defence reactions determines the eventual level of resistance of a specific grape cultivar. The general processes involved in the overall plant defence reaction against an attempted fungal invasion will be discussed elsewhere.

1.5 Introducing powdery mildew resistance genes into commercial cultivars.

1.5.1 Inheritance of powdery mildew resistance genes

Investigations and observations regarding the inheritance of powdery mildew resistance concluded that resistance is of a polygenic nature (Eibach, 1994, Li, 1993, Staudt, 1997). This means that there is generally a very wide splitting of this characteristic in the offspring of crosses made between a susceptible and a resistant parent. However, the inheritance of polygenic characteristics can be based on additive and/or dominant gene effects (Eibach, 1994). Dominant gene effects of a polygenic character can be seen as a strong difference in the characteristic mean in the offspring from that of the parents. In contrast, additive gene effects lead to a continuous variation in the level of resistance of the offspring with the mean mid-way between the parents (Eibach, 1994, Li, 1993). Results from both studies seem to favour the important role of additive genetic

UNIVERSITEITSBIBLIOTEEK U.S.

Stellenbosch University https://scholar.sun.ac.za

inheritance, but the presence of major or dominant genes cannot be excluded for several reasons.

The screening of germplasm for powdery mildew resistance has been carried out by various groups, but comparison of these results are difficult because contradicting results were obtained (Pearson & Gadoury, 1992, Gadoury & Pearson, 1991). These differences could mainly be attributed to differences in climatic conditions, inoculation methods, infection pressures and inoculum sources. However, some heterotic groups of germplasm proved to be generally more resistant than others.

To complicate matters, Evans *et al.* (1996), detected genetic variation among different *U. necator* isolates. Gadoury & Pearson (1991) proved pathogenic specialisation of *U. necator* when different isolates were inoculated onto seedlings of resistant cultivars. Infection of test plants with inoculum consisting of a mixture of different isolates would therefore result in the more additive phenotypic nature of powdery mildew resistance. The use of heterozygous hybrids and cultivars as breeding parents could also attribute to this problem.

In contrast, studies regarding the inheritance of powdery mildew (*Sphaerotheca fuliginea*) resistance genes in melon (*Cucumis melo*) revealed the dominant or co-dominant nature of these genes against different races of this pathogen (Floris & Alvarez, 1996). Similar studies regarding the inheritance of powdery mildew (*Erysiphe cichoracearum*) resistance genes of *Arabidopsis thaliana* revealed the presence of the dominant RPW1 resistance gene in some *A. thaliana* accessions (Somerville, 1998). Results obtained with similar host-pathogen systems confirm the fact that major genes might play an important role in the determination of resistance in the *V. vinifera-U. necator* host-pathogen system. Therefore, the presence of possible dominant or co-dominant resistance genes should be taken into consideration when planning a breeding strategy to increase the levels of natural resistance of susceptible cultivars.

1.5.2 Conventional breeding

As previously mentioned, the resistance to powdery mildew in different species and cultivars of *Vitis* accessions has been investigated by various groups (Eibach, 1994, Li, 1993, Wang *et al.*, 1995 and Staudt, 1997). The low levels of natural resistance to powdery mildew of pure *V. vinifera* cultivars arose as one common conclusion in all these studies. Furthermore, due to the restricted genetic variability available, breeders were forced to use related species as sources of resistance in their breeding programs (Pratt & Einset, 1961, Olmo, 1976, 1986, Eibach, 1994, Stuadt, 1997). Species such as *V.*

Stellenbosch University https://scholar.sun.ac.za

rotundifolia, *V. solonis, V. rupestris, V. riparia* and *V. rufotomentosa* were used as donor parents in crosses made with *V. vinifera* in resistance breeding programs (Olmo, 1976, 1986, Staudt, 1997). Although viable seeds were obtained in some crossing combinations, most of the interspecific hybrids obtained is highly or completely sterile and limit the exploitation of resistance genes in related species (Olmo, 1976, 1986). Plants originating from these interspecific crosses, were back-crossed with *V. vinifera* cultivars and led to the development of complex hybrids (e.g. INRA BX 7489, Seibel 1020, and Villard Blanc) that contained high levels of powdery mildew resistance (Li, 1993, Eibach, 1994). Unfortunately, these hybrids produce low quality wines, table grapes, and cannot be used as commercial cultivars. Today, interspecific hybrids are mainly used as sources of disease resistance in conventional breeding programs (Olmo, 1986).

Progress towards the development of more resistant cultivars with acceptable quality traits are therefore slow and cumbersome (Olmo, 1976). The polygenic nature of powdery mildew inheritance, the complex genetic system, low seed germination percentage and long generation cycle of grapes strain the genetic progress made with conventional breeding programs (Pratt & Einset, 1961). The heterozygous nature of *V. vinifera* is responsible for pronounced inbreeding depression (Pratt & Einset, 1961, Gray & Meredith, 1992). Therefore, seed cannot reproduce an individual genotype and all propagation of outstanding selections must be done by vegetative propagation. The life cycle is relatively long, with a juvenile period ranging from two to six years, depending on environmental conditions and management practices (Pratt & Einsett, 1961). Thus, fruit characteristics, quality and resistance to certain diseases (e.g. Pierce's disease) cannot be confirmed until several years of fruit bearing have passed (Mortensen, 1968, Pratt & Einset, 1961). These factors combine to make the development of new resistant cultivars by breeding and selection a long, tedious and very expensive task.

In comparison with many annual crops, the introduction of specific resistance genes into an otherwise desirable cultivar by conventional breeding is not easily achieved because the long generation cycle combined with heterozygosity and inbreeding depression make back-crossing and recurrent selection difficult or impossible (Alleweldt & Possingham, 1988). Therefore, in most instances, only the F1 generation can be selected. For this reason, conventional breeding currently seeks only to combine varieties, which showed from previous test-crosses made, to be good combiners in the hope that some of the off-spring would possess an overall improvement over both parents (Gray & Meredith, 1992). In practice the off-spring possesses traits intermediate to each parent so that the high fruit quality of one parent usually cannot be directly combined with high levels of disease resistance of the other parent (Gray & Meredith, 1992). Improvement to commercially

Stellenbosch University https://scholar.sun.ac.za

acceptable levels of both traits is obtained by the production of complex hybrids originating from selection and crosses with a new parent at each generation (Gray & Meredith, 1992).

The incorporation of disease resistance is further complicated in breeding programs where disease resistant seedless cultivars are developed due to the inability to use seedless vines as female parents (Stout, 1936, Pratt & Einset, 1961, Clingeleffer, 1985). In such crosses, only the pollen parent can be seedless and therefore restricts the number and type of desirable germplasm that can be used (Stout, 1936). Thus, the development of disease resistant cultivars with good quality traits, are very difficult and a long process which enquire the availability of large areas and an extensive germplasm collection.

1.5.3 Molecular markers

The recent availability of various molecular techniques has boosted research in grapevine genetics (Reisch, 1998). Different molecular techniques can be used today to solve problems in grapevine identification and parental analysis. The application of molecular techniques for DNA fingerprinting studies, genotype identification and diversity assessment are being used extensively in grapevine breeding (Reisch, 1998). As a result, there has been progress in the assessment and understanding of genetic diversity between and within grapevine germplasm collections (Botta *et al.,* 1998, Meredith *et al.,* 1998, Malossini *et al.,* 1998). Additionally, the parentage of certain cultivars has been discovered or confirmed (e.g. Carbernet Sauvignon descends from a cross between Carbernet franc and Sauvignon blanc) (Meredith *et al.,* 1998).

Extensive linkage maps, consisting primarily of molecular markers, are being developed for various wood fruit crops (Weeden *et al.,* 1994). These maps are used to indicate the order and relative genetic distances between markers and can be used to locate genes for morphological traits (Weeden *et al.*, 1994). In the process of constructing these maps, the heterozygous nature of these crops are being utilised to produce segregating populations originating from crosses made between two heterozygous parents (Weeden *et al.,* 1994, Lodhi *et al.,* 1995 , Reisch, 1998). In long generation cycle plants like grapes, the application of marker assisted selection has great potential. Once important genes are tagged, the identification and selection of individual seedlings for desired, mapped traits such as fruit colour, flower sex and resistance to *U. necator* can take place (Reisch, 1998). Fortunately, the application of marker-assisted selection is not only restricted to the selection of monogenic traits. The application of statistical procedures to analyse markers linked to traits affected by polygenic or quantitatively inherited traits

Stellenbosch University https://scholar.sun.ac.za

makes it possible to develop markers linked to these traits (Reisch, 1998). As a result markers for quantitative traits like seedlessness, disease resistance and nematode resistance have been developed (Reisch, 1998, This *et al.,* 1998, Dalbó *et al.*, 1998, Walker pers. comm., 1998).

1.5.4 The identification and cloning of genes from grapes

According to Reisch (1998) a number of important genes have already been cloned from grapes e.g. the resveratrol-producing gene, stilbene synthase. By inserting this gene into susceptible cultivars, scientists hope to increase the natural disease resistance of the genetically modified cultivar. Positional cloning of genes based on genomic linkage maps followed by "chromosome walking" is at the moment the most popular technique used to identify and locate grapevine genes, although several other approaches are also being used (Dixon & Lamb, 1990, Reisch, 1998).

Unfortunately, when planning this study, no established segregating population, originating from crosses made between a susceptible and resistant parent existed in South Africa. The small number of offspring obtained by various groups and the long generation cycle discouraged us from crossing *Muscadinia* and *V. vinifera* types to obtain a large enough segregating population for mapping purposes. We decided not to use "conventional" biotechnological approaches and thereby excluded the necessity of a segregating population and the application of DNA marker technology. To understand why we decided to follow a different approach, it is necessary to investigate the events associated with the plant defence response in reaction to an attempted pathogen invasion.

1.6 Molecular biology of disease resistance

In plant-pathogen interactions, the development of an incompatible relationship can be summarised in three steps: (1) generation of a signal indicative of attack by the pathogen, (2) recognition of the signal by the host and (3) transduction of the signal to the cell interior, redirecting cellular metabolism towards defence responses (Somerville, 1998). In compatible reactions, one or more of these steps fail, resulting in the infection and colonisation of host tissue (Somerville, 1998). Resistance genes play an important role in steps two and three, the recognition and activation of plant defence responses (Daly, 1984, Hammond-Kosack & Jones, 1995, 1996, Hammond-Kosack, Jones & Jones, 1996, Knogge, 1996 (a), (b), Bent, 1996).

Stellenbosch University https://scholar.sun.ac.za

1.6.1 Generation of a signal indicative of attack by the pathogen

Pathogens in general utilise one or a combination of different mechanisms to penetrate the cuticle and cell wall of plant cells (Knogge, 1996 (b)). The secretion of a cocktail containing different hydrolytic enzymes such as cutinases, cellulases, pectinases and proteases is meant to enzymatically digest different components of the cuticle and outer cell walls (Agrios, 1998, Dixon & Lamb, 1990, Staskawicz, *et al.,* 1995, Knogge, 1996 (a), (b)). However, Knogge (1996 (a)) emphasises the fact that saprophytic fungi are also able to produce these enzymes. Thus, the secretion of these enzymes is therefore not the tools specifically developed by fungi to ensure successful penetration of host cells.

Alternatively, or in combination with the secretion of hydrolytic enzymes, some fungi developed the ability to mechanically pierce the cuticle and outer plant cell walls through the formation of specialised infection structures such as appressoria and penetration pegs (Heintz, 1986, Blaich & Wind, 1989, Pearson & Gadoury, 1992, Knogge, 1996 (b)). Other fungal species (e.g. some rust fungi) do not directly penetrate the plant cell, but bypass the cuticle and plant cell walls by entering the plant through natural openings or wounds (Knogge, 1996 (b)). As mentioned previously, penetration of the grapevine cuticle and epidermal cell wall is thought to be mainly mechanical, although the role of secreted hydrolytic enzymes cannot be excluded (Heintz, 1986, Blaich *et al.*, 1989, Heintz & Blaich, 1990, Pearson & Gadoury, 1992).

The production of warning signals (called elicitors) is recognised by plant surveillance systems (called receptors) and can be produced in several ways (Dixon & Lamb, 1990, Knogge, 1996 (a), Hammond-Kosack & Jones, 1995, Keen, 1997). The activity of hydrolytic enzymes by the invading pathogen result in the production of plant cell wall fragments (e.g. oligogalacturonates) that can be recognised by plant receptors (Dixon, Harrison & Lamb, 1994, Knogge, 1996 (b), Bent, 1996). Furthermore, plants may recognise an attempted fungal invasion through the presence of non-self factors present on the fungal surface (e.g. chitin and glucan fragments). The digestion of fungal cell walls by plant hydrolytic enzymes (chitinasess and glucanasess) is responsible for the production of chitin and glucan fragments that are recognised by plant receptors. These glucanase and chitinase enzymes are constitutively synthesised at low levels prior to an attempted fungal invasion (Dixon & Lamb, 1990, Knogge, 1996 (b)).

According to Keen (1997) two types of elicitors are known: (1) general elicitors which do not display differences in cultivar sensitivity within a plant species and (2) specific elicitors which function only in cultivars containing a specific disease resistance gene and confer

Stellenbosch University https://scholar.sun.ac.za

resistance against a specific race of the pathogen. General elicitors include substances associated with basic plant and pathogen metabolism (e.g. cell wall glucans, chitin oligomers and glycopeptides), while specific elicitors normally have more specific or unique substrates (e.g. proteins and peptides) and their production is a result of pathogen avirulence gene function (Keen, 1997).

1.6.2 Recognition of the signal by the host

A functional receptor is a product (direct or indirect) of a host resistance gene (*R* gene). The main function of a receptor is to initiate a cascade of defence-related signals which activates the plant defence system and thereby prevent further fungal invasion and colonisation (Lamb *et al*., 1989, Ellingboe, 1995).

Genes for resistance to the major classes of plant pathogens including viruses, bacteria, fungi and nematodes have been isolated and sequenced from different plant species. Furthermore, sequence analysis of the predicted proteins revealed that common structural domains occur in resistance genes of diverse origin and pathogen specificity (Hammond-Kosack & Jones, 1996, Hammond-Kosack *et al*., 1996, Bent, 1996, Gebhardt, 1997). Thus, the recognition of an attempted invasion by nematodes seems to be fundamentally the same as an attempted invasion by bacteria, fungi or viral pathogens (Gebhardt, 1997).

The presence of similar structural motifs in resistance gene products, suggests that these proteins may function as membrane receptors and/or components of the signal transduction pathway (Bent, 1996). It also suggests a high degree of mechanistic conservation among the pathways plants use to trigger defence responses (Hammond-Kosack & Jones, 1996, Bent, 1996, Hammond-Kosack *et al.,* 1996, Gebhardt, 1997). These structural domains found in receptors are important because they are involved in elicitor binding and the activation of downstream defence responses (Bent, 1996).

Analysis of various resistance genes (e.g. the nematode resistance gene $Hs1^{pro\text{-}1}$, RPM1 and RPS2 of *Arabidopsis*, N of tobacco, L6 from flax, Cf-9 and Prf of tomato and Xa21 of rice) revealed that the encoded proteins can be classified according to common structural motifs, irrespective of the plants species from which they originate or the pathogen to which they react (Hammond-Kosack & Jones, 1996, Hammond-Kosack *et al.,* 1996, Bent, 1996, Gebhardt, 1997). These proteins (except Xa21) originate from R genes that are in all cases part of gene-for-gene recognition systems and can be divided into three general classes (Bent, 1996, Keen 1997). The first class contains protein-kinase domains and possible membrane-anchoring myristoylation domains such as the tomato *Pto* gene

Stellenbosch University https://scholar.sun.ac.za

product (Bent, 1996, Keen, 1997). Proteins with leucine-rich repeats (LRR) domains, P-loops (amino acid consensus GXXXXGK[T/S]) and possible trans-membrane-spanning domains, such as the tomato Cf-9 gene product, fall into the second group of proteins. The last group consists of a hybrid with LRR, leucine-zipper and protein-kinase domains all in the same protein as in the *Xa-21* gene product (Bent, 1996, Keen, 1997). Although their biochemical functions have not yet been established, it has been suggested that LRR domains are involved in elicitor recognition and may activate the kinase-or P-domains to initiate signal cascades of the plant defence response (Keen 1997).

In spite of these similarities, the overall sequences of resistance genes from different plants responding to different pathogens are not highly conserved (Bent, 1996, Gebhardt, 1997). However, Leister *et al.*, (1996) utilised these conserved areas in a polymerase chain reaction (PCR) based approach as binding sites for specially designed primers and managed to isolate several gene fragments belonging to the same structural classes as *N* and *RPS2*.

It is important to note that not all receptors contain these conserved structural domains. Examples of such genes are the *HM1* gene of maize and the recessive *mlo* gene of barley (Büschges, *et al.*, 1997, Gebhardt, 1997). As additional pathogen resistance genes are isolated, new conserved structural domains will most certainly be discovered. Therefore, the existing types of resistance genes known to mankind should not be seen as representative of all types of resistance genes (Gebhardt, 1997).

1.6.3 Signal transduction and the activation of plant defence responses.

The signal transduction events that occur after recognition or elicitor-binding is complex and have not been fully clarified. However, conformational changes of receptors after elicitor binding are thought to be responsible for protein phosphorylation, the activation of protein kinases, or the combination of both (Hammond-Kosack *et al.*, 1996). This leads to the activation of intermediate signal transduction events responsible for the production of activated oxygen species (ROS), changes in ion fluxes and cellular phosphorylation (Keen, 1992, Dixon *et al.*, 1994, Bent, 1996, Mourgues, Brisset & Chevreau, 1998).

Although these intermediate signal transduction events play a vital role in signal transduction, they also represent the first defence mechanisms activated in response to elicitor recognition (Bent, 1996, Hammond-Kosack & Jones, 1996, Hammond -Kosack *et al.*, 1996, Mourgues, *et al.*, 1998). Locally, these reactions are responsible for the reinforcement of infected cell walls through the incorporation of lignin, callose or glycoproteins or by oxidative cross-linking of cell wall proteins (Mourgues, *et al.*, 1998).

Stellenbosch University https://scholar.sun.ac.za

The rapid dying of infected-and adjacent cells (called the hypersensitive response (HR)), blocks the pathogen at infection sites and is also a product of intermediate signal transduction events (Mourgues, *et al.,* 1998).

The second-line of defence include adjacent cells in which metabolic changes can be observed (Hammond-Kosack & Jones, 1996, Mourgues, *et al.,* 1998). These metabolic changes include the reinforcement of the adjacent cell walls and the activation of secondary metabolite pathways responsible for the synthesis of phytoalexins (anti-microbial compounds) (Dixon *et al.,* 1994, van de Rhee, Linthorst & Bol, 1994, Staskawicz, *et al.*, 1995, Bent, 1996, Hammond-Kosack *et al.*, 1996, Hammond-Kosack & Jones, 1996). Moreover, defence gene transcription is activated and leads to the synthesis of several proteins that are involved in the plant defence response (van de Rhee *et al.*, 1994, Bent, 1996, Hammond-Kosack *et al.*, 1996, Hammond-Kosack & Jones, 1996).

These newly synthesised proteins are mainly responsible for the hydrolysis of fungal cells or reduction of pathogen virulence through various mechanisms (Dixon & Lamb, 1990, Keen, 1992, Dixon *et al.,* 1994, van de Rhee *et al.*, 1994, Staskawicz, *et al.*, 1995, Bent, 1996, Hammond-Kosack *et al.*, 1996, Hammond-Kosack & Jones, 1996, Jones, 1996, Mourgues *et al.,* 1998). These proteins include the proteins of the phenylpropanoid pathway, enzymes involved in the production of phytoalexin, structural cell wall proteins, peroxidases, superoxide dismutases, proteinase inhibitors, thionins and pathogenesis related (PR) proteins (Collinge & Slusarenko, 1987, van de Rhee *et al.,* 1994, Matern, Grimming & Kneusel 1995). Enzymes of the phenylpropanoid pathway are involved in the synthesis of various secondary metabolites including phytoalexins (Hammond-Kosack & Jones, 1996, Bent, 1996, van de Rhee *et al,* 1994). Peroxidases play an important role in cell wall modification during an attempted fungal invasion (van de Rhee *et al.*, 1994, Hammond-Kosack & Jones, 1996). This occurs mainly though the polymerisation of lignin precursors and the cross-linking of structural cell wall proteins to polysaccharides and polyphenols (Lamb *et al.,* 1989, van de Rhee *et al.*, 1994, Hammond-Kosack & Jones, 1996). As a result, in the vicinity of the infection site, an impenetrable network is formed that prevents the advance of the invading pathogen (van de Rhee *et al.*, 1994, Hammond-Kosack & Jones, 1996). Superoxide dismutases are able to scavenge harmful superoxide radicals that are produced during stress-conditions (van de Rhee *et al.*, 1994). Proteinase inhibitors protect plant cells against pathogens and herbivorous insects (van de Rhee *et al.*, 1994, Hammond-Kosack & Jones, 1996). Thionins are small cysteine-rich proteins that are toxic to various micro-organisms (Bent, 1996, Mourgues, *et al.,* 1998). PR proteins consist of various proteins, but mainly function as hydrolytic

Stellenbosch University https://scholar.sun.ac.za

enzymes (e.g. β-glucanases, α-amylases, and chitinases) responsible for the degradation of fungal hyphae (Broglie & Broglie, 1993, Bent, 1996, Hammond-Kosack & Jones, 1996) However, a very important function of PR proteins is also the inhibition of pathogenicity or virulence factors of pathogens (Hammond-Kosack & Jones, 1996). This inhibition of pathogenicity or virulence is achieved by inhibiting pathogenic enzymes (e.g. proteases and polygalacturonases) secreted by the pathogen that are responsible for the degradation of the plant cell wall and other components (van de Rhee *et al.*, 1994, Bent, 1996, Hammond-Kosack & Jones, 1996, Mourgues *et al.,* 1998).

Several compounds produced during this second line of defence also function as long distance signals. These include PR proteins, salicylic acid (SA), jasmonates, systemin and ethylene (Sticher, Mauch-Mani & Métraux, 1997). As a result, certain resistance mechanisms, like the production of PR proteins, are induced in cells and tissues far from the infection site where they protect the plant from apprehended infection attempts by the pathogen (Sticher *et al.*, 1997, Mourgues, *et al.,* 1998). This long distance or systemic defence response, called systemic acquired resistance (SAR) not only protects the plant from the current invading pathogen, but also for a broad range of pathogens including bacteria, fungi and viruses and represents the third line of defence (Mourgues, *et al.,* 1998).

According to Staskawicz *et al.*, (1995) and Hammond-Kosack *et al.*, (1996), similar signal transduction mechanisms are involved, after the release and recognition of elicitors by different pathogens, in the activation of the plant defence reaction. Alternatively, different classes of R gene products could each be responsible for distinct signalling pathways that co-ordinate a specific defence response specially designed for individual pathogens (Hammond-Kosack *et al.*, 1996). Our understanding of defence gene activation is further complicated by the fact that biotrophic pathogens, like *U. necator,* is able to prevent the activation of a full scale defence response (Hammond-Kosack & Jones 1996). Thus, not every weapon in the defence arsenal of plants are being used during the prevention of an attempted invasion by a biotrophic pathogen. Moreover, PR proteins targeted to intercellular areas are less likely to be components of frontline defence action, but probably have their major effect, particularly against biotrophic pathogens, after significant cellular decompartmentalisation has occurred (Hammond-Kosack *et al.*, 1996).

1.6.4 Identification of defence-related genes

The over-expression of various plant defence-related genes responsible for the production of phytoalexins, PR proteins or anti-microbial peptides has been used by various groups to increase the natural disease-resistance levels of transgenic plants

Stellenbosch University https://scholar.sun.ac.za

(Mourgues, *et al.,* 1998). However, the transfer of individual defence genes generally confers only partial resistance in the transgenic plants (Mourgues, *et al.,* 1998). This is probably because a battery of defence reactions works together in a co-ordinated synergistic manner against the invading pathogen (Mourgues, *et al.,* 1998). Dilip *et al.,* (1995) noted that the constitutive co-expression of chitinase and glucanase genes in tobacco results in higher levels of resistance to a fungal pathogen than the expression of any one of the two genes alone. Moreover, the expression of both genes in tomato resulted in effective control to a *Fusarium* wilt disease, whereas the expression of either gene alone resulted in successful infection by the pathogen (Dilip *et al.,* 1995). Sufficient levels of resistance were achieved by the incorporation of only one disease resistance gene into transgenic plants, but it has been suggested that the durability of such resistance would be shorter lived when compared with transgenic plants containing two or more artificially incorporated defence genes (Dilip *et al.,* 1995).

1.7 Tools to identify differentially expressed genes

1.7.1 Approaches to identify differentially expressed genes

The major approaches to identify specific genes expressed during the plant defence reaction include differential screening, subtractive hybridisation and differential display PCR (DD-PCR) (Sunday, 1995). Up to the early nineties, differential screening and subtractive hybridisation were mainly used for this purpose, but these techniques were cumbersome and yielded limited information (Appel, Bellstedt & Gresshoff, 1999). In 1992, Liang & Pardee (1992) introduced DD-PCR that quickly replaced differential screening and subtractive hybridisation as the preferred method for the identification of differentially expressed genes.

Although DD-PCR is a multi-step process, it is based on the application of simple widely-accessible and established techniques like arbitrary PCR, polyacrylamide gel electrophoresis (PAGE) recombinant DNA technology and Northern hybridisation (Liang & Pardee, 1992, Appel *et al.,* 1999). Furthermore, if offered speed, high levels of sensitivity, reproducibility and the possibility to compare multiple conditions simultaneously (Liang & Pardee, 1992). Although other techniques were introduced after DD, it emerged as the preferred method for the identification of differentially expressed genes as reflected by a 1996 Medline database search (Appel *et al.,* 1999).

Stellenbosch University https://scholar.sun.ac.za

1.7.2 Principles of differential display

The first step in the expression of a gene is the transcription of a specific region of the gene (coding region) to messenger RNA (mRNA) (Hunt, 1994). The mRNA then serves as a template upon which ribosomes synthesise polypeptides (Hunt, 1994). Some of these polypeptides are then post-translationally modified and interact with other modified or non- modified polypeptides to form proteins. These newly formed proteins then function as enzymes in numerous metabolic pathways or as structural proteins (e.g. cell wall synthesis, structural proteins in the cytoplasm, plasmalemma or as histones in the nucleus (Hunt, 1994). Although eukaryotic organisms contain a large number of genes, only a limited number of genes are active at the same time (Liang & Pardee, 1992). Signals produced by different environmental conditions or developmental stages are responsible for the activation or silencing of different genes (Hunt, 1994). When cells with an identical DNA content are exposed to different environmental conditions, different genes would be activated resulting in differences in mRNA populations. By identifying differences in mRNA populations, genes influenced by the change in environmental conditions could be identified when other factors, influencing gene expression, are identical (Liang & Pardee, 1992).

With DD-PCR the general strategy is to amplify partial cDNA sequences from subsets of mRNA by reverse transcription and PCR (Liang & Pardee, 1992). These short sequences are then displayed on a sequencing gel. Pairs of primers are selected so that each will amplify cDNA fragments originating from about 50 to 100 mRNAs because this number is optimal for display by PAGE (see Figure 1) (Liang & Pardee, 1992).

Basically, DD-PCR is based on the comparison of mRNA species originating from genetically identical or related cells exposed to different environmental conditions (Liang & Pardee, 1992). To compare similarities between these mRNA populations, each mRNA population is divided into sub-populations and reverse-transcribed to cDNA using a specially designed primer that anneals to the 5' boundary of the poly-A tail present on most eukaryotic mRNAs. The division of each mRNA population is achieved by adding one or two additional bases (consisting of G, A or C) at the 3' end of the oligo d(T) primer used for reverse transcription. Thus, depending on the number of additional bases used, each RNA population is divided into 12 (using two additional bases) to 3 (using one additional base) sub-populations (Liang & Pardee, 1992, Liang *et al.*, 1994). The sub-division of each RNA population results in easier identification of differentially expressed fragments and also provides a system through which the total mRNA pool could be

Stellenbosch University https://scholar.sun.ac.za

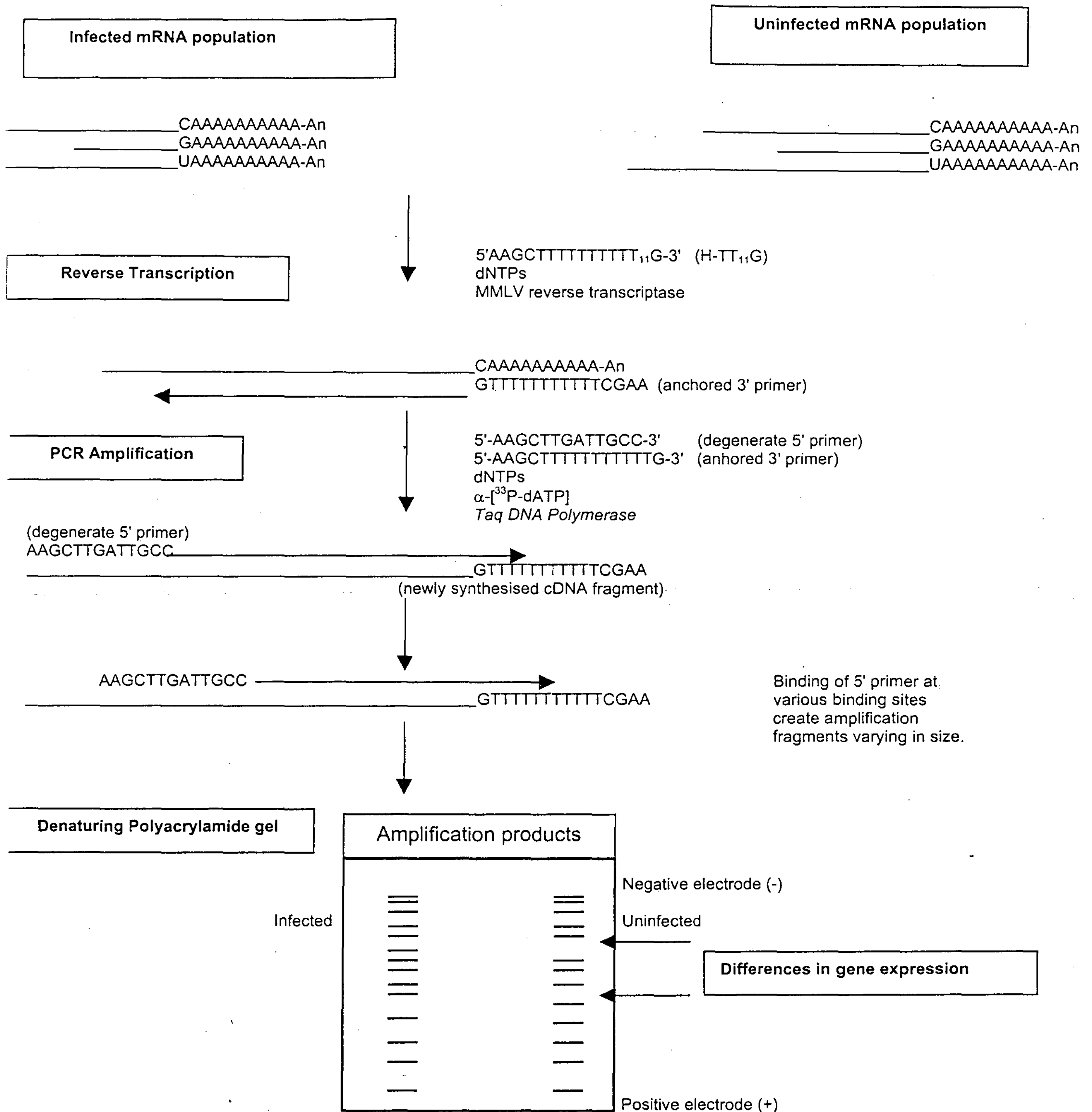

Fig 1. Differential Display PCR. Differential Display is a highly sensitive method for the identification of differentially expressed genes as detailed in the text. After the isolation of mRNA from infected and uninfected leaves, the mRNA is reverse transcribed using a anchored 3' oligo dT primer with an additional nucleotide at the 3' end of the primer (e.g. 3'C-A_{11}CGAA). By using this 3'C-A_{11}CGAA primer, only one third of the total mRNA population will be reverse transcribed. The restriction site at the 5' of the primer ensures easier manipulation of amplified fragments. The newly synthesised cDNA fragments are then amplified in a PCR reaction using the same 3' anchored primer in combination with a degenerate 13- mer 5' primer. In the example shown here, differences in gene expression can be detected as the presence of additional fragments originating from the infected mRNA population (arrows) (Liang & Pardee, 1992, Appel *et al.*, 1999).

Stellenbosch University https://scholar.sun.ac.za

screened in an orderly and efficient way for high and low abundance differentially expressed mRNAs (Liang & Pardee, 1992). It also allows side-by-side comparison of most mRNAs between or among related cells.

PCR amplification, PAGE and visualisation of gels follow the reverse transcription of these mRNAs. Amplification of cDNAs are achieved by using two different primers, a 3' primer which is the same primer used for reverse transcription and a short 5' primer which is arbitrary in sequence (Liang & Pardee, 1992, GenHunter technical manual, 1998). By changing primer combinations, the approximately 15 000 individual mRNA species, simultaneously expressed from eukaryotic cells, may be visualised by PAGE (Liang & Pardee, 1992, Sunday, 1995). After their identification and isolation, differentially expressed fragments can be used as probes, cloned or can be sequenced and compared with known sequences in databases (GenHunter technical manual, 1998). Sequences of identified fragments can also be used to design primers to screen populations segregating for disease resistance to identify molecular markers.

1.7.3 Successes obtained with differential display

The application of DD-PCR led to the identification of several differentially expressed genes. For example, four differentially expressed fragments in response to infection by *B. cinerea* have been identified in tomato (Benito, Prins & van Kan, 1996). Tawe *et al.*, (1998) detected a 40-fold increase in the mRNA level of the L-1/M-47 gene of *Caenorhabditis elegans* which encodes for the detoxification enzyme, glutathione S-transferase, after treatment with the herbicide Paraquat. A primary growth response gene, ST2/T1 is a gene whose expression is differentially regulated by different protein kinase C isozymes. This was identified using differential display PCR (Kieser *et al.*, 1995). Wilkinson *et al.*, (1995) identified three different mRNAs with enhanced expression in ripening *Fragaria ananassa* (strawberry) fruit using differential display.

1.8 Aims of present study

By using differential display, we expect to identify genes coding for enzymes like peroxidase, chitinase, glucanase, and structural cell wall proteins. Moreover, genes coding for superoxide dismutases, proteinase inhibitors, thionins and PR proteins were shown to be activated during the plant defence reaction (van de Rhee *et al.*, 1994). Although the function and importance of these genes in the grapevine defence response have not yet been established, their importance as secondary resistance mechanisms cannot be excluded. Unfortunately, several general stress-related genes are also activated during an attempted pathogenic infection and would therefore complicate the

Stellenbosch University https://scholar.sun.ac.za

identification of specific defence-related genes (Sharma & Davis, 1995, Hagen *et al.*, 1995, Benito *et al.*, 1996, Truesdell & Dickman, 1997). We do not expect to identify any receptors responsible for the activation of the plant defence response. Bent (1996) noted that biotrophic pathogens are able to prevent recognition by this first line of defence and regarded the first line of defence not as important as the second line of defence against these pathogens. Therefore, as powdery mildew is a biotrophic pathogen, we suspect that secondary defence mechanisms and gene transcription to be a very important component of the overall defence response.

No studies regarding defence gene activation in response to powdery mildew infection of grapevine were previously published. Furthermore, no publication could be found where DD-PCR was used to identify differentially expressed genes from grapevine tissue. However, it is our hypothesis that simmilar reactions will be activated during the grapevine defence reaction. Therefore, results obtained in this study would be novel and could be used in various future studies.

The aim of this study was multiple. In our hands, the isolation of RNA from grapevine leaves was problematic and needed to be optimised. The second objective was to develop and optimise the DD-PCR technique to suit our working conditions. DD-PCR was then used to identify and analyse differentially expressed fragments and to use the data obtained to investigate the grapevine gene expression in reaction to infection by powdery mildew.

1.8.1 Optimise RNA isolation technique from grapevine leaves.

The search for differentially expressed genes consists of a cascade of steps where the successful completion of each step is essential before advancing to the next step. The first and one of the most critical steps of this process, is the successful isolation of good quality mRNA, suitable for reverse transcription to cDNA. The extraction of RNA from grapevine leaves, suitable for reverse transcription, is difficult and requires special extraction conditions (N. Scott, A. Burger, pers. comm.). Attempts to isolate RNA from grapevine leaves, using several published protocols, yielded RNA of poor quality. Similar problems with the isolation of RNA from other grapevine tissues in other labs, confirm the importance and necessity of a reliable protocol (A. Burger, pers. comm.).

1.8.2 Optimise differential display technique

The next step in the cascade is the successful application of DD-PCR protocols to identify differentially expressed fragments. Although the principles and application of DD-PCR

Stellenbosch University https://scholar.sun.ac.za

seem relatively simple, several groups have experienced frustration and failure with this technique (Galindo, *et al.*, 1998). The most frequent complaints include the contamination of RNA samples with DNA, small amounts of amplification products, high false positive rate, questionable ability of DD to identify both abundant and rare mRNAs and the time-consuming nature and large amounts of RNA needed for the verification process. Galindo *et al.* (1998) noted that most of these complaints can be attributed to the design of the technique, but that the advantages still exceeded the disadvantages. In contrast, Liang & Pardee (1994) and GenHunter Technical Manual (1998) attributed failures experienced with DD-PCR, mainly to lack of optimisation under local conditions and demonstrated the ability of the technique to produce repeatable, differentially expressed fragments. Therefore, to achieve our goal, it was absolutely essential to optimise the technique for our lab conditions. Using the DD-PCR protocol under optimised conditions would ensure the production of reliable, differentially expressed fragments, suitable to use in the next step of this project and in future research. The optimisation of differential display would also ensure that the technique could be used as a standard protocol in future research.

1.8.3 Molecular analysis of identified fragments

The identification and isolation of disease-related grapevine genes can be used for various purposes in future research. Differentially expressed fragments can be used as probes to obtain full-length genes of potential economic importance. Cloning of these fragments can be used to screen an appropriate cDNA library (Sunday, 1995). Additionally, isolated differentially expressed fragments can be sequenced and compared with sequence data of known genes to determine what similarities exists (Liang & Pardee, 1992, Sunday, 1995, Mourgues *et al.*, 1998). It can also be used to establish the presence of open reading frames in the sequenced fragments (Liang & Pardee, 1992, Sunday, 1995, Mourgues *et al.*, 1998). Application of differential display would also enable us to search for promoters that are activated by an attempted fungal invasion. The availability of such promoters would enable us to activate various grapevine and non-grapevine defence genes only when transgenic grapevine plants are attacked by a pathogen.

Isolated fragments can also be used to design primers to screen populations segregating for disease resistance. This would lead to the identification of molecular markers that can be used in conventional breeding programs to select for disease resistance at a seedling stage.

Stellenbosch University https://scholar.sun.ac.za

In this study, we only focused on the identification, isolation, re-amplification, sequencing and comparison of differentially expressed fragments with known genes in sequence databases. Sequenced fragments were analysed for the presence of open reading frames, start and stop codons and promoters. No studies regarding the use of primers, designed from isolated fragments to search for molecular markers, were done.

1.8.4 Investigation of defence gene activation of grapevine in response to pathogenic infection.

Although several visual observations concerning the defence response of grapevine to powdery mildew have been made, no information is available about gene activation during this process. Information obtained during this study can hopefully be used to develop a clearer picture about genes involved in grapevine defence reactions. However, information obtained during this study has to be confirmed by a thorough molecular characterisation of the differentially expressed fragments before any conclusions about their potential role in disease resistance can be made.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER TWO

2 MATERIALS AND METHODS

2.1 Introduction

The process, through which differentially expressed genes are identified, requires the successful application of several techniques. This process starts with cultivation and inoculation of test plants. The isolation of good quality RNA, free of any contaminating DNA is the next, and one of the most crucial steps in this process. The synthesis of cDNA, from isolated RNA, is essential to provide *Taq polymerase* with a template for subsequent amplification using the polymerase chain reaction (PCR). PCR conditions need to be optimised to obtain maximum yield of reproducible fragments that represent putative differentially expressed genes. These fragments are then separated according to size by polyacrylamide gel electrophoresis (PAGE). Separated fragments are visualised by silver staining or autoradiography. Differentially expressed fragments, identified in this manner, are then isolated, re-amplified, sequenced and compared with known gene sequences in DNA sequence databases. The last step of this multi-step process is the confirmation of gene expression. This is can be done in several ways, but in this study we used only one technique, called Reverse Northern Blot analysis.

2.2 Cultivation and infection of test plants

2.2.1 Cultivation of greenhouse plants

2.2.1.1 Collection of shoots

Shoots were collected during July 1996 from the germplasm collection at the ARC-Nietvoorbij centre for Vine and Wine near Stellenbosch, South Africa. During the collection process, care was taken to ensure that only healthy shoots, free of any visible pests and diseases, were collected. Harvested shoots were then reduced to two bud cuttings and were surface sterilised by immersing the cuttings in a 2% Captan® (Zeneca™) solution for 30 minutes. These were then allowed to dry and were stored at 4°C until needed.

Stellenbosch University https://scholar.sun.ac.za

2.2.1.2 Planting and maintenance of greenhouse plants

The basal bud of each cutting was removed before planting. Submerging the top 10 mm in graftwax, sealed the tip of each cutting. Root formation was stimulated by dipping the basal end of each cutting in Seradix® B No 3 (Rhône-Poulenc, Agrichem SA) root inducing powder containing 8 g/kg 4-(indol-3-yl)-butyric acid. The cuttings were then planted in 500 mm black plastic bags containing a heat sterilised soil mix prepared from 50% hydromix, (Agrispec®) 25% bark and 25% filter sand. The cuttings were kept at 25°C ± 2°C with a relative humidity (RH) ranging from 60% to 90%. The photo-period varied between 12 to 14 hours. The light intensity was reduced to approximately 30% normal sunlight to simulate favourable conditions for powdery mildew infection.

Each cultivar was divided into two groups that were placed in separate greenhouse compartments. To prevent environmental variations between the corresponding sub-populations of each cultivar, care was taken to ensure identical environmental conditions in the two greenhouse compartments. A fungicide application was applied two weeks after bud burst and was repeated three weeks later to prevent possible fungal infections.

2.2.2 Generation and cultivation of *in vitro* plants

In vitro plants were obtained form the ARC-Nietvoorbij centre for Vine and Wine and was grown according to Burger & Gousard (1996). Briefly, buds were collected from a vineyard at Nietvoorbij, surface sterilised and transferred to petri dishes containing 4 x 10^{-6} M N^6-Benyladenine (BA) medium to encourage shoot proliferation (Burger & Gousard, 1996). The resulting shoots were rooted in jars on medium supplemented with indole-3-butyric acid (IBA) (Burger & Gousard, 1996). *In vitro* plants were grown at 25°C ± 1°C with 12 hour cool white (G.E.C. 65W) illumination.

2.2.3 Inoculation of test plants.

2.2.3.1 Inoculation of greenhouse plants

After planting, greenhouse plants were grown for 3 months before artificial inoculation with powdery mildew. Prior to inoculation, care was taken that no visible disease symptoms were present on plants in the two sub-populations of each cultivar. Fresh powdery mildew inoculum was collected from unsprayed, infected leaves of greenhouse plants cultivated in a separate greenhouse.

Stellenbosch University https://scholar.sun.ac.za

The greenhouse plants were artificially inoculated using a modified method of Evans, *et al.*, (1996). Briefly, a sterile artist's paintbrush was used to brush conidia from diseased leaves onto leaves of one population of each cultivar. Each inoculated leaf was then marked with a waterproof permanent marker for later identification. Inoculated and uninoculated plants were then incubated at 25°C ± 2°C (RH ranging between 80-90%), for three days before infected and control leaves were harvested.

2.2.3.2 Inoculation of *in vitro* plants

In vitro plants were inoculated 3 months after buds were placed on BA medium to encourage shoot proliferation. Powdery mildew inoculum was obtained from *in vitro* powdery mildew cultures. The same inoculation method as previously described, (Evans *et al.,* 1996) was used to infect *in vitro* plants. The inoculated and uninoculated *in vitro* plants were incubated at 25°C ± 1°C for three days before the collection of plant material from which RNA will be extracted.

2.2.4 Harvesting of plant material

Labelled infected and uninfected leaves were harvested and placed in separate 100 mm x 100 mm plastic bags, frozen immediately in liquid nitrogen and stored at -80°C for subsequent isolation of RNA.

Intact, infected and uninfected *in vitro* plants were removed from jars with sterile tweezers, placed in 100 mm x 100 mm marked plastic bags, frozen immediately in liquid nitrogen and stored at -80°C.

2.3 RNA isolation

Special care was taken to prevent contamination with external RNases during the RNA isolation process. Sterile gloves were always worn, glass- and plasticware were soaked in a 2% AbSolve™ (Du Pont) solution before use. Diethylpyrocarbonate (DEPC) treated double distilled, autoclaved water was used for all buffer formulations. All reagents, except Tris-based buffers, were treated with DEPC and autoclaved before use.

Stellenbosch University https://scholar.sun.ac.za

2.3.1 Isolation of RNA using a standard RNA isolation protocol

Initial attempts to extract RNA from grapevine leaves focused on the protocol described by Davies & Robinson (1996). Briefly, four grams of frozen tissue were ground to a fine powder using a mortar and pestle. The frozen powder was then added to four volumes (16 ml) of extraction buffer (5 M $NaClO_4$; 0.3 M Tris-HCl, pH 8.3; 8.5% (w/v) insoluble polyvinylpolypyrrolidone; 2% (w/v) PEG 4000; 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol) and was stirred at room temperature for 30 minutes. The resulting slurry was then placed in a 60-ml syringe plugged with a 15 mm layer of sterile glass wool. Pressure was applied slowly, the eluate was mixed with 2.5 volumes (v/v) 100% ethanol (EtOH) and left at -20°C for at least 2 hours. Nucleic acids were precipitated by centrifugation at 8300 rpm for 15 min. The pellet (containing the nucleic acids) was washed with 70% [v/v] EtOH and resuspended in 2 ml of TE buffer (10 mM Tris-HCl, pH 7.6 and 0.1 mM EDTA containing 0.2% β-mercaptoethanol). This suspension was then extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with an equal volume of chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated from the aqueous phase by adding 2.5 volumes (v/v) 100% EtOH and 0.1 volume of 3 M sodium acetate (NaOAc) and incubation at -20°for two hours. RNA was then sedimented by centrifugation at 14 000 rpm for 20 minutes. The resulting pellet was washed with 70 % EtOH and resuspended in 300 µl TE buffer. RNA quality was checked on a 1.5% agarose gel and RNA yields were measured by A_{260} absorption in a spectrophotometer (RNA concentration = OD X dilution factor X 40 $\mu g.ml^{-1}$).

2.3.2 Modification of RNA isolation protocol

The RNA isolation protocol of Davies & Robinson (1996) was modified in several ways to improve the quality of the RNA obtained. Centrifugation was done at 4°C and when possible, solutions were pre-cooled and added to RNA samples that were kept on ice. The composition of the extraction buffer was changed to 3 M $NaClO_4$; 0.2 M Tris-HCl, pH 8.3; 5% (v/v) SDS; 8,5% (w/v) polyvinylpolypyrrolidone; 2% (w/v) PEG 6000 and 1% β-mercaptoethanol. The total volume of the extraction buffer was increased to 25 ml, while less plant material (2.5-3 g) was grounded to a fine powder using liquid nitrogen. The use of a 60-ml syringe plugged with glasswool was replaced with a 15 minute centrifugation step at 14 000 rpm. The intermediate phase was then carefully removed and precipitated as described previously. The extraction of the RNA/DNA pellet with 25:24:1 phenol:chloroform:isoamyl alcohol was increased from three to at least six extractions. This ensured the complete removal of contaminating substances. The

Stellenbosch University https://scholar.sun.ac.za

precipitated, sedimented and washed RNA pellets were dried on ice and then dissolved in 100 µl DEPC-treated, ddH_20. The quality of the RNA was checked on a 1,5% agarose gel and the yield was measured at A_{260} in a spectrophotometer.

2.3.3 Removal of contaminating DNA

2.3.3.1 Isolation of mRNA using magnetic beads

Initial attempts to remove contaminating DNA from isolated RNA focused on the use of magnetic beads (Oligotex™ mRNA isolation kit) to capture mRNA from the DNA/RNA mix. Magnetic beads were used according to the manufacturer's instructions.

Briefly, total RNA (250-500 µg) was mixed with 500 µl binding buffer (20 mM Tris-HCl, pH 7.5; 1M NaCl; 2 mM EDTA and 0.2% SDS) and 30 µl Oligotex™ bead-suspension (10% (w/v) Oligotex™ suspension; 10 mM Tris-HCl, pH 7.5; 500 mM NaCl; 1 mM EDTA, 0.1% SDS and 0.1% NaN_3) that were pre-heated to 37°C. The mixture was incubated at 65°C for 3 minutes and then for 10 minutes at 25°C to allow hybridisation between the oligo-dT_{30} primer, linked to the Oligotex™ particles, and the 3' poly-A tail of the mRNA. Samples were centrifuged at 14 000 rpm for 2 minutes to sediment Oligotex™ particles containing the captured mRNA. The DNA containing supernatant was then carefully removed by pipetting and the RNA containing Oligotex™ pellet resuspended in 1000 µl wash buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl and 1 mM EDTA). The particles were sedimented by centrifugation at 14 000 rpm for two minutes. Re-suspension and sedimentation was repeated once more before the Oligotex™ pellet was mixed with 20 µl elution buffer, which was preheated to 70°C. This ensured the release of captured mRNA fragments from the Oligotex™ particles. Finally, the Oligotex™ particles were separated from mRNA by centrifugation at 14 000 rpm for two minutes. The RNA containing supernatant was then carefully removed and transferred to an RNase free microfuge tube. mRNA yield and quality was determined by A_{260} absorption and A_{260}/A_{280} absorption ratio. Isolated mRNA was stored at –80°C for future use.

2.3.3.2 Differential precipitation of RNA

RNA obtained from the modified Davies & Robinson (1996) protocol was differentially precipitated by adding 0.3 volume of 8 M LiCl (30 µl) to the isolated RNA/DNA mix and incubation at 4°C for at least 16 hours. RNA was sedimented by centrifugation at 14 000 rpm at 4°C for 25 minutes, washed and resuspended in 50 µl DEPC-ddH_2O. The RNA

Stellenbosch University https://scholar.sun.ac.za

quality was checked on a 1,5% agarose gel and yields were measured in a spectrophotometer at A_{260}.

2.3.3.3 Enzymatic digestion of contaminating DNA

Remaining traces of DNA were removed by digesting the LiCl-precipitated RNA with RNase free DNase I (Message Clean® kit, GenHunter Corp., Nashville). Total RNA (10-50 µg) was mixed with 5.7 µl, 10X Reaction buffer (100 mM Tris-HCl pH 8.4; 500 mM KCl; 15 mM $MgCl_2$ and 0.01% gelatin) and 10 units of DNase I (GeneHunter ® DNase I) in a final volume of 56.7 µl. The solution was mixed by finger tapping before incubation at 37°C for 30 minutes. The DNase I enzyme was removed by adding 40 µl phenol/chloroform (3:1), placed on ice for 10 minutes and centrifugation at 4°C for 5 minutes at maximum speed. The upper phase was carefully removed, mixed with 5 µl 3 M NaOAc pH 5.5 and 200 µl 100% EtOH, and kept at –20°C for at least 2 hours. RNA was precipitated by centrifugation at 14 000 rpm for 15 minutes at 4°C, washed with 70% EtOH and re-dissolved in 20 µl DEPC-ddH_2O. RNA quality was checked on a 1,5% agarose gel and yields were measured at A_{260} in a spectrophotometer. RNA samples were stored as 1-2 µg aliquots at –70°C.

2.4 Production of differentially expressed fragments

2.4.1 Synthesis of first strand cDNA

First strand cDNA synthesis was performed in a final volume of 20 µl, containing 250 ng mRNA or 1µg total RNA, 2 µl of one of the three one-base anchored $^{5'}$H-T_{11}V$^{3'}$ primers (H= $^{5'}$AAGCTT$^{3'}$ and V = G, C or A) (2 µM), 10 µl sterile nuclease free water, 4 µl 5x reverse transcription buffer (125 mM Tris-Cl, pH 8.3, 188 mM KCl, 7.5 mM $MgCl_2$ and 25 mM DDT) and 1,6µl dNTPs (250 µM). The mixture was heated to 65°C for 5 minutes, chilled on ice, and then incubated at 37°C for 10 minutes. Moloney murine leukemia virus (MMLV) reverse transcriptase (5 units) (RNAimage® kit, GenHunter Corp.) was then added and was mixed by finger tapping. Samples were then incubated at 37°C for 50 minutes. Heating the samples to 75°C for 5 minutes stopped the reverse transcription reaction. The synthesised cDNA fragments were stored at –20°C for later use.

Duplicate reverse transcription reactions were set up for each anchored primer to help with the identification of false positives generated during the amplification of the newly synthesised cDNA templates. Control reactions containing RNA samples with no MMLV

Stellenbosch University https://scholar.sun.ac.za

reverse transcriptase were set up to ensure that no DNA was present in the RNA, dNTPs or primers. Oligo $d(T_{11})$ primers were anchored to the 5' side of the poly A tail of mRNA by using oligo $d(T_{11})$ primers containing an extra nucleotide (excluding thymine [T]) at the 3' side of the primer. A *Hind* III restriction site ($^{5'}$AAGCTT^{3}) was included at the 5' side of the oligo $d(T_{11})$ primer for easier manipulation of differentially expressed fragments after amplification, separation and visualisation.

2.4.2 Amplification of cDNA fragments

2.4.2.1 PCR conditions

PCR was performed in a final volume of 20 µl, containing 1 µl cDNA template, 10.5 µl sterile nuclease free water, 2 µl 10X PCR buffer (160 mM $(NH_4)_2SO_4$; 670 mM Tris-HCl, pH 8.8 and 0.1% Tween-20), 0.6 µl $MgCl_2$ (50 mM), 1.6 µl dNTP (25 µM), 2µl arbitrary primer (2 µM), 2 µl $^{5'}$H-T_{11}V$^{3'}$ (H=$^{5'}$AAGCTT$^{3'}$ and V= G, C or A)(2 µM), 0.1µl α-[^{33}P]dATP (2000 Ci/mmole, NEN) and 0.2 µl *Taq* polymerase (Bio*Taq*™ DNA polymerase). The mixture was mixed by finger tapping and briefly centrifuged to collect the contents at the bottom of the tube. Amplification was done at 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 40°C for 2 minutes and 72°C for 30 seconds. A final extension period of 10 minutes at 72°C was added to ensure complete transcription of amplified fragments.

Initial reports suggested that fragments created with DD-PCR contain a large number of false positives that cannot be verified by confirmation techniques like Northern Blots, Reverse Northern Blots or Nuclear run-on analysis (Warthoe, *et al.,* 1994, Linskens *et al.,* 1995, Appel *et al.,* 1999). To minimise the generation of false positives, each reaction was done in duplicate and only fragments present in both reactions of the same sample type, was considered as being differentially expressed.

2.4.2.2 End-labelling of anchored primers

End-labelling of the anchored H-T_{11}V primer was done as described by Tokuyama & Takeda, (1995), with a few modifications. Labelling reactions (30 µl) contained 3 µl 10X One-phor-all buffer (100 mM Tris-acetate; 100 mM MgOAc and 500 mM KOAc), 15 µl of the H-T_{11}V primer (50 mM), 3 µl γ ^{33}P[ATP] (3000 Ci/mmole, NEN), 7.5 µl double distilled deionised water and 1.5 µl FPLCure™ T4 Polynucleotide Kinase (Pharmacia) and were

Stellenbosch University https://scholar.sun.ac.za

carried out at 37°C for 45 minutes in a heating block. Reactions were stopped by incubation at 65°C for 10 minutes.

Differential display PCR was carried out as described previously, except that the anchored:arbitrary primer ratio was changed to 2:1, to ensure amplification of the cDNA ends. No radioactive nucleotides were added to the reaction and the dNTP concentration was increased to 100 µM.

2.4.3 Separation of amplified fragments by PAGE

2.4.3.1 Preparation of glass plates

The amplified cDNAs were separated on a 6% polyacrylamide gel containing 7M urea. Preparation of the glass plates was done according to the Promega® technical manual for silver staining with a few modifications (Promega® technical manual, 1997). Briefly, both glass plates were placed in 0.1 N NaOH for at least two hours, rinsed with distilled water and placed vertically to dry. Dried glass plates was wiped with 100% EtOH to ensure complete removal of any unwanted residues. One glass plate (big plate) was treated with 1 ml Repelkote™, let to dry and was then briefly wiped with 100% EtOH. The two glass plates were assembled by placing two 4 mm side spacers between the plates taking special care to prevent the treated surfaces from touching each other. Three clamps were evenly spaced at each side of the assembled plates to prevent leaking of the unpolimerised gel mix.

2.4.3.2 Preparation of gel solution

Acrylamide solution (6%) was freshly prepared before casting each gel. Briefly, 31.5 g urea, 36.25 ml de-ionised water, 3.75 ml 10X TBE and 11.25 ml pre-mixed acrylamide:bis-acrylamide (19:1) were stirred until all the urea was dissolved. The acrylamide solution was then filtered through a 0.45 µM filter. Temed™ (50 µl) and 10% ammonium persulfate (250 µl) were added immediately before casting the gel.

2.4.3.3 Casting of gel

To prevent formation of bubbles and to ensure an even flow of the acrylamide solution, a 60-ml syringe was used to pour the gel. The smooth side of a 49 well sharkstooth comb was inserted for approximately 6 mm at the top of the small glass plate and secured with

Stellenbosch University https://scholar.sun.ac.za

three clamps. The gel was allowed to polymerise for at least two hours. After the polymerisation process was completed, the clamps and comb were carefully removed and the gel placed vertically in an electrophoresis apparatus. 1X TBE was added to the top and bottom wells of the apparatus. Using a 60-ml syringe with a 19-gauge needle, air bubbles and unpolymerised acrylamide was removed before pre-running the gel at 60 watts for at least 45 minutes.

2.4.3.4 Preparation and loading of samples

Samples were prepared by adding 2 µl of loading dye (95% formamide; 10 mM EDTA, pH 8; 0.09% xylene cyanole FF and 0.09% bromophenol blue) to 4 µl of each amplified sample. Samples were denatured at 94°C for 4 minutes and immediately placed on ice before loading.

To ensure complete removal of unpolymerised acrylamide and bubbles, the wells were rinsed once more before loading of the samples. To prevent cooling of the gel, care was taken to ensure that loading of samples did not exceed 20 minutes. Samples from the uninfected susceptible and resistant cultivars were loaded in adjacent lanes, while samples from infected, susceptible and resistant cultivars were loaded in adjacent lanes.

Gels were run at 60 W for approximately 3.5 hours (until the slower moving dye reached the bottom of the gel). After careful separation of the two glass plates and transfer of the gel to 3 M (Whatman™) paper, the gel was covered with plastic wrap and dried under a vacuum at 80°C for at least 1 hour.

2.4.3.5 Autoradiography

The dried gel was placed in an X-ray cassette and exposed for 12-72 hours before the film (Kodak®, Biomax MR) was developed. To ensure correct orientation of the gel with the developed film, fluorescent markers (Whitehead Scientific®) were placed on the gel, exposed to light for 2 minutes and were then developed with the film.

Stellenbosch University https://scholar.sun.ac.za

2.5 Identification and re-amplification of differentially expressed fragments

2.5.1 Identification of differentially expressed fragments

The developed X-ray film was placed on a light box to study the expression patterns of amplified cDNA samples. Special care was taken to identify differentially expressed fragments that occurred only in infected, amplified cDNA products of the resistant cultivar. However, differences in gene expression between susceptible and resistant lanes were also studied and marked accordingly. Bands of interest were identified and marked for later excision.

2.5.2 Isolation and precipitation of differentially expressed fragments

Bands of interest were excised from the X-ray autoradiograph by cutting through the film using a new ` blade. The autoradiograph was then carefully orientated with the dried gel according to the positions of the fluorescent markers. Fragments of interest were then removed by cutting the dried gel where differentially expressed bands were previously removed on the X-ray film. Excised fragments were then placed in a 1.5 ml microfuge tube and re-hydrated in 100 µl ddH_2O at 4°C for 12 hours.

Re-hydrated gel fragments were lightly vortexed for 4 hours at room temperature, boiled for 15 minutes and sedimented by centrifugation, before the DNA containing supernatant was transferred to a new 1.5 ml microfuge tube. The DNA was precipitated by adding 10 µl 3M sodium acetate, 5 µl glycogen (10 mg/ml) and 450 µl 100% EtOH and incubated at -20°C for at least 2 hours. The precipitated DNA was sedimented by centrifugation at 12 000 rpm for 15 minutes at 4°C and pellets washed with 85% EtOH and resuspended in 10 µl double distilled water.

2.5.3 Re-amplification of isolated fragments

Re-amplification was done using the same primer set and PCR conditions as described previously, except that dNTP concentrations were increased from 4 µM to 20 µM and no isotopes were added. The reaction volume was increased to 40 µl. The same primer combination, that was used to create each corresponding differentially expressed fragment, was used for the respective re-amplification reaction. Each PCR contained 4 µl of the resuspended DNA fragments.

Stellenbosch University https://scholar.sun.ac.za

To ensure that re-amplified fragments did not contain more than one fragment and were consistent with their size on the sequencing gel, thirty microliters of the PCR samples were run on a 1.5% agarose gel and stained with ethidium bromide. The yield of each re-amplified fragment was determined by comparison to known DNA quantity standards that were run on the same agarose gel (DNA standards, Life Technologies).

Although most of the re-amplification reactions yielded fragments, some reactions failed to produce any visible bands. Therefore, first round PCR products that failed to amplify were used as templates for another 40-cycle PCR. Before amplification, a 1:100 dilution was made of the first round PCR products that were then used as templates for the second round PCR. As with the first round PCR products, samples were separated with agarose, their concentrations were determined and were then excised as described above.

2.5.4 Isolation of re-amplified fragments

The excised DNA fragments were extracted from the agarose gel using a commercial kit (Qiaquick Gel Extraction kit, Qiagen), according to the manufactures prescriptions. Isolated DNA fragments were then resuspended in 30 µl ddH_2O. However, to increase the DNA concentration, the final volume of the resuspended DNA was decreased to 7 µl using a Speedy Vac for 15 minutes.

The successful retrieval of each re-amplified and gel purified fragment was confirmed by running 3 µl of each sample on a 1,5% agarose gel. The concentration of the samples was once again checked by comparison with known DNA standards as described previously.

2.5.5 Sequencing of differentially expressed fragments

The nucleotide sequence of each isolated fragment was determined using a commercial kit (Big dye terminator cycle sequencing ready reaction kit, PE Bio-systems) and an ABI Prism 377 DNA Sequencer. Approximately 200 ng (100 ng/µl) of each fragment and 5 µl (1.1 pmoles/µl) of the corresponding anchored primer were used for this purpose.

Stellenbosch University https://scholar.sun.ac.za

2.5.6 Molecular analysis of differentially expressed fragments

Nucleotides of sequenced fragments that the ABI Prism 377 DNA Sequencer failed to identify correctly were manually modified using the Chromas sequencing program (supplier unknown). Nucleotide sequences were then submitted to the Genbank, EMBL and DDBJ databases using the BLAST search program (1999 version) (Altschul *et al.*, 1997). Blast search results were presented as sequences producing significant alignments with known gene sequences, expressed sequence tags or proteins. The level of homology with known gene sequences, expressed sequence tags or proteins were indicated by an E value (the smaller the value, the more significant is the level of homology).

2.6 Confirmation of gene expression

2.6.1 Synthesis of cDNA probes for Reverse Northern Blot analysis

cDNA probes were produced according to Galindo *et al.*,(1998) with a few modifications. Infected and uninfected total RNA samples of Villard Blanc (resistant cultivar) were used for this purpose. Briefly, 6 µg RNA was mixed with 4 µl oligo d(T) primer (500 µg/ml), made up to 24 µl with DEPC treated water before incubation at 70°C for 10 minutes. After the 10-minute incubation, samples were immediately placed on ice for at least two minutes. A cocktail containing, 8 µl 5X Reverse Transcription buffer (125 mM Tris-Cl, pH 8.3; 188 mM KCl; 7.5 mM $MgCl_2$ and 25 mM DTT), 4 µl DTT, 2 µl d(G,A,T)TP mix (20 mM), 2 µl dCTP (120 µM) and 7.5 µl α-^{32}dCTP (3000Ci/mmol) (Amersham) was prepared and added to each of the RNA samples to be used as probes. After a short incubation at 42°C for 2 minutes, 4 µl Reverse Transcriptase (Superscript II™, Gibco, Life Technologies) was added, gently mixed by pipetting and incubated for 1 hour at 42°C.

The reaction was stopped, and the RNA removed from the RNA/cDNA hybrid, by adding 3 µl 3N NaOH followed by a 30-minute incubation at 68°C. A mixture containing 10 µl Tris-HCl (pH 7.4), 3 µl 2N HCl and 9 µl H_2O was then added to neutralise the reaction. Unincorporated nucleotides were removed by filtering the labelled probe through a Sephadex G50 Pasteur column.

Stellenbosch University https://scholar.sun.ac.za

2.5.6 Molecular analysis of differentially expressed fragments

Nucleotides of sequenced fragments that the ABI Prism 377 DNA Sequencer failed to identify correctly were manually modified using the Chromas sequencing program (supplier unknown). Nucleotide sequences were then submitted to the Genbank, EMBL and DDBJ databases using the BLAST search program (1999 version) (Altschul *et al.,* 1997). Blast search results were presented as sequences producing significant alignments with known gene sequences, expressed sequence tags or proteins. The level of homology with known gene sequences, expressed sequence tags or proteins were indicated by an E value (the smaller the value, the more significant is the level of homology).

2.6 Confirmation of gene expression

2.6.1 Synthesis of cDNA probes for Reverse Northern Blot analysis

cDNA probes were produced according to Galindo *et al.,*(1998) with a few modifications. Infected and uninfected total RNA samples of Villard Blanc (resistant cultivar) were used for this purpose. Briefly, 6 µg RNA was mixed with 4 µl oligo d(T) primer (500 µg/ml), made up to 24 µl with DEPC treated water before incubation at 70°C for 10 minutes. After the 10-minute incubation, samples were immediately placed on ice for at least two minutes. A cocktail containing, 8 µl 5X Reverse Transcription buffer (125 mM Tris-Cl, pH 8.3; 188 mM KCl; 7.5 mM $MgCl_2$ and 25 mM DTT), 4 µl DTT, 2 µl d(G,A,T)TP mix (20 mM), 2 µl dCTP (120 µM) and 7.5 µl α-^{32}dCTP (3000Ci/mmol) (Amersham) was prepared and added to each of the RNA samples to be used as probes. After a short incubation at 42°C for 2 minutes, 4 µl Reverse Transcriptase (Superscript II™, Gibco, Life Technologies) was added, gently mixed by pipetting and incubated for 1 hour at 42°C.

The reaction was stopped, and the RNA removed from the RNA/cDNA hybrid, by adding 3 µl 3N NaOH followed by a 30-minute incubation at 68°C. A mixture containing 10 µl Tris-HCl (pH 7.4), 3 µl 2N HCl and 9 µl H_2O was then added to neutralise the reaction. Unincorporated nucleotides were removed by filtering the labelled probe through a Sephadex G50 Pasteur column.

Stellenbosch University https://scholar.sun.ac.za

2.6.2 Blotting of PCR products

Prior to the blotting of sequenced products, a 40 µl PCR was performed from each isolated re-amplified fragment, using the same conditions as previously described for re-amplification of isolated fragments. The successful amplification of isolated fragments was checked on a 1,5% agarose gel. The amplified fragments were then dried in a SpeedyVac and resuspended in 8 µl ddH_20.

Duplicate dot blot membranes were prepared by spotting 3 µl (2 µg) of each PCR product in an identical, grid-like pattern onto two separate membranes (Hybond®-N^+, Amersham). One membrane was used for probes originating from the infected susceptible cultivar, and one for probes originating from the infected resistant cultivar. Each membrane was then air-dried for 30 minutes. Spotted DNA samples were denatured by placing the air-dried membranes (DNA side up) onto a Whatman paper pre-soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes. Membranes were then washed twice with neutralising solution (1.5 M NaCl, 1 M Tris-HCl, pH 7.4) for 5 minutes, and then rinsed in 2X SSC (3 M NaCl, 0.3 M Na-citrate). DNA was cross-linked on an UV-transilluminator for 2 minutes. Membranes were then placed in separate hybridisation tubes and 10 ml pre-hybridisation solution (10X SSC, pH 7.0; 10X Denhardts solution, 0.1 M sodium phosphate, pH 6.8; 0.2% SDS and 0.1 M EDTA, 50% formamide and boiled (0.1 mg/ml) salmon sperm DNA) was added to each hybridisation tube before incubation with rotation at 42°C for at least 18 hours.

2.6.3 Hybridisation of blotted PCR products with labelled probes

The pre-hybridisation solution was replaced by 10 ml hybridisation buffer (10X SSC, pH 7.0; 2X Denhardts solution; 0.04 M sodium phosphate, pH 6.8; 0.4% SDS and 0.1 M EDTA), 50% formamide, boiled salmon sperm DNA and boiled probe (3µg) and was incubated with rotation for 18 hours. Each membrane was then washed twice in Wash solution I (2X SSC, 0.1% SDS) for 5 minutes at room temperature and then once with Wash solution II (0.2X SSC, 0.1% SDS) for 15 minutes at 65°C. Background noise was then tested with a Geiger counter to confirm the removal of non-hybridised nucleotides from the membrane. A third wash step in Wash solution III (0.1X SSC, 0.1% SDS) at 65°C for 15 minutes was added to reduce excessive background noise. To prevent the membrane from drying, each membrane was sealed in a 100 mm X 150 mm plastic bag. The membranes were then placed in an X-ray cassette and were exposed for 96 hours.

Stellenbosch University https://scholar.sun.ac.za

2.6.4 Analysis of Blots

The X-ray film was developed as described previously. The positive signals of each membrane were compared and positive signals on each reverse northern blot originating from differentially expressed fragments were identified.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER THREE:

3 RESULTS AND DISCUSSION

3.1 Introduction

The identification of differentially expressed fragments starts at the successful cultivation of test plants in the greenhouse or laboratory. The successful inoculation of test plants is essential to ensure the activation and transcription of defence-related genes. Furthermore, the cultivation of the two populations of each cultivar under identical environmental conditions are of critical importance to ensure the accurate identification of activated defence-related genes and not genes that are influenced by differences in environmental conditions.

The isolation of good quality RNA, free of any contaminating DNA is of critical importance for the successful isolation of the above-mentioned induced, defence-related genes. Poor quality RNA reduces or inhibits the efficiency of the reverse transcription reaction, while contaminating DNA leads to the production of fragments that does not originate from expressed genes. The isolation of good quality RNA from grapevine leaves was problematic. Several published protocols served as a starting point from which a successful RNA isolation protocol was developed that met the criteria mentioned above.

Since the introduction of differential display PCR in 1992, several modifications had been proposed to extend the usefulness of the technique and to address the problems inherent with the design of the technique (Liang & Pardee, 1992, Liang *et al.,* 1994, Mou *et al.,* 1994, Appel *et al.,* 1999). With these modifications in mind, this technique had to be applied under our conditions to obtain reproducible, differentially expressed fragments. These fragments can then be used in studies to gain insight into the activation of defence-related genes of grapevine after artificial inoculation of test plants with powdery mildew. After several optimisation experiments, DD-PCR was successfully applied to identify several differentially expressed fragments.

The re-amplification and determination of the nucleotide sequences of isolated fragments were the next step in this multi-faceted procedure. Several differentially expressed fragments were re-amplified and sequenced using standard protocols as described by various groups in the literature. The nucleotide sequences of the identified fragments

Stellenbosch University https://scholar.sun.ac.za

were compared with known gene sequences that are available in databases. The last, but very important step in this process, was to confirm the expression of identified fragments. This could be done using several techniques, but in our case, the amount of RNA that was available forced us to use a technique called Reverse Northern Blot analysis. In cases where several differentially expressed fragments are identified, this technique has several advantages over conventional Northern Blot analysis, which is normally used for this purpose. The advantages of Reverse Northern Blot analysis will be discussed later.

The information obtained by this study enabled us to gain insight into defence-related gene activation of *Vitis vinifera* and to compare this process with model systems as described in the literature. Furthermore, our ability to optimise this technique and the usefulness of the technique to seek for differences in gene expression could be evaluated. The results obtained in this study could also be used in future studies where promoters, responsible for gene activation, could be identified. The characterisation of identified fragments could be used to determine the function of each gene as part of the overall defence response. Future applications of results obtained in this study will be discussed later.

3.2 Cultivation and infection of greenhouse plants

3.2.1 Selection of greenhouse plants

A comparison of induced, gene expression patterns between control and infected plants of a resistant and a susceptible cultivar formed the basis of this study. Limited availability of plant material forced us to compare gene expression patterns of only one resistant and one susceptible cultivar. The choice of a powdery mildew resistant cultivar and a powdery mildew susceptible cultivar were based on results obtained in literature and from visual observations in South African vineyards (Doster & Schnathorst, 1985, Li, 1993, and Eibach, 1994). Although the level of natural disease resistance to powdery mildew was the main factor that influenced the choice of the resistant cultivar, secondary economically important traits were also considered.

The French hybrid, Villard Blanc was chosen as the resistant cultivar and Riesling as the susceptible cultivar. Although some American researchers reported high levels of powdery mildew infections of Villard Blanc in North America, it proved to be highly resistant to powdery mildew under South African conditions (Pearson & Gladoury, 1992,

Stellenbosch University https://scholar.sun.ac.za

Reisch, personal communication). Riesling was highly susceptible to powdery mildew under South African conditions.

Several groups noted that the plant defence response is accompanied by the expression of various stress-related genes that can be activated in response to a variety of environmental stress conditions (Agrios, 1988, Dixon & Lamb, 1990, Keen, 1990, 1992, van de Rhee *et al.*, 1994, Linthorst & Bol, 1994, Staskawicz *et al.*, 1995, Bent, 1996, Hammond-Kosack & Jones, 1996, Jones, 1996). Therefore, other fungi and/or environmental stress conditions can also activate some of the genes activated during an attempted infection by *U. necator*. This implies that some defence-related genes are also activated in susceptible cultivars. In contrast to major disease resistance genes that single-handedly inhibits disease development, these stress related genes mainly function as supporting resistance genes (Staskawicz *et al.,* 1995 ,Bent, 1996 and Hammond-Kosack & Jones, 1996). The identification of differentially expressed fragments with DD-PCR could therefore include these genes, which would increase the number of differentially expressed fragments that have to be isolated, re-amplified, sequenced and confirmed by Northern Blot analysis.

To reduce the number of differentially expressed fragments, a susceptible cultivar was included in this study. We argued that by comparing the differentially expressed banding patterns of the infected populations of both cultivars, we would be able to distinguish between general stress-related genes and genes specifically activated by powdery mildew infections. This would enable us to isolate fragments only present in the infected population of the resistant cultivar and would therefore increase our chances to identify powdery mildew defence-related genes.

3.2.2 Selection of *in vitro* plants

Villard Blanc was chosen as the resistant cultivar and Muscat Seedless as the susceptible cultivar because no Riesling *in vitro* plants were available. Muscat Seedless proved to be highly susceptible to powdery mildew under South African field conditions (Personal observation).

Initially, we planned to use only greenhouse plants, but due to a broken compressor our freezer defrosted and all the cDNA synthesised from these plants were heated to room temperature for more than 48 hours. This had an influence on the quality and integrity of the cDNA fragments and we decided to discard all the synthesised cDNA samples. As no

Stellenbosch University https://scholar.sun.ac.za

greenhouse plants and inoculum were immediately available, we were forced to use *in vitro* plants. With *in vitro* plants we were able to create identical environmental conditions in which infected and control test plants were grown. All the results obtained in this study, originated from *in vitro* plants, because expression of fragments obtained from greenhouse plants could not be verified by Reverse Northern Blot analysis as no RNA or cDNA was available.

3.2.3 Inoculation of test plants

The three-day waiting period after artificial inoculation and harvesting of inoculated leaves was chosen to ensure that most of the fungal spores germinated and infected test plants. According to Gadoury & Pearson (1988, 1990 (a)), not all conidia or ascospores germinate immediately after landing on the leaf surface but have a lag period (varying in length) before they start to germinate. After landing on leaf surfaces, fungal spores absorb water that activates certain hydrolytic enzymes responsible for spore-germination (Agrios, 1988). Depending on the thickness of the spore wall, this process can take from 1 hour up to 48 hours (Agrios, 1988).

Successful germination of powdery mildew inoculum can be determined 48 hours after artificial inoculation (Gadoury & Pearson, 1988). Thus, a waiting period of 72 hours would ensure maximum germination and attempted invasion of leaf material by the fungus and provide sufficient time for test plants to activate their defence systems. The successful infection of test plants was confirmed by allowing symptoms to develop on inoculated leaves of two plants of each cultivar that were inoculated but not harvested.

3.3 RNA isolation

As mentioned previously, the isolation of RNA from grapevine is a difficult process. During the isolation process substances like polyphenols, polysaccharides, tannins and RNases must be removed or inhibited because they interfere with the isolation of RNA or cause the degradation of RNA during the isolation process (Logemann, Schell & Willmitzer, 1987, Schneibderbauer, Sandermann & Ernst, 1991, Levi, Galau, Wetzstein, 1992). Therefore, the isolation of RNA from grapevine leaves requires special conditions, to inhibit these substances, most standard nucleic acid extraction procedures do not take into account (Levi *et al.*, 1992).

Stellenbosch University https://scholar.sun.ac.za

Guanidinium isothiocyanate-based homogination buffers are a very popular starting point of most RNA isolation protocols and are used in most commercial RNA isolation kits. Unfortunately, no grapevine RNA was obtained with various guanidinium isothiocyanate-based kits when used according to the manufacturer's prescriptions. Isolation of RNA with cesium chloride centrifugation is very expensive, cumbersome and time consuming. Since several RNA isolations had to be done, we needed a quick, inexpensive and accurate method. The isolation of RNA with a conventional phenol/chloroform method met these criteria. However, in our hands, the isolation of RNA from grapevine leaves was problematic and needed to be optimised. Several published protocols served as a starting point from which a successful RNA isolation protocol was developed that suited our conditions.

3.3.1 Optimisation of RNA isolation protocol

The labour intensity and time required to perform an RNA extraction, as well as the quality and quantity of RNA, are key factors that determine the choice of an RNA extraction protocol. Although various extraction protocols were reported in the literature, the perchlorate method of Rezaian & Krake (1978) as modified by Rathejen & Robinson (1992) and Davies & Robinson (1996) worked the best under our conditions.

3.3.2.1 Isolation of RNA using a standard RNA extraction protocol

RNA quality obtained with the standard extraction protocol was of intermediate quality and contained large amounts of DNA. Yields varied from 0.5-2 μg RNA. After several attempts to synthesise cDNA from the isolated RNA, we concluded that the RNA quality was not good enough to continue with downstream applications of differential display (Fig. 3.1).

Stellenbosch University https://scholar.sun.ac.za

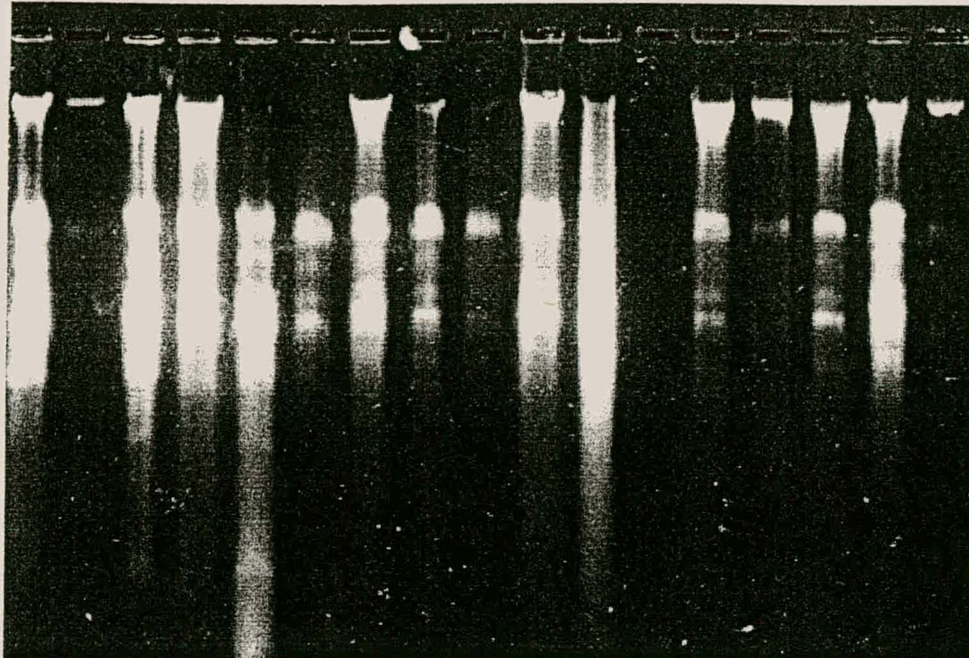

Fig. 3.1 Separation of isolated RNA in a 1,5% agarose gel. Standard RNA extraction protocols were unable to isolate good quality RNA from grapevine leaves due to the presence of substances such as polysaccharides, polyphenols, tannins and RNases. RNA obtained could not be used for cDNA synthesis.

The poor quality of RNA obtained with the standard protocol can be attributed to several factors. One of these could be the presence of contaminating substances that persisted even after their attempted removal by phenol/chloroform extraction. The brown discoloration of the extraction buffer after the 30-minute homogenisation step, suggested probable oxidation of polyphenols during this process. Furthermore, the colour of the RNA pellet prior to resuspension in TE buffer indicated that some of these contaminating substances have not been completely removed. An important factor that had a dramatic effect on RNA quality was temperature. The use of pre-cooled reagents, centrifugation at 4°C and to work on ice when possible dramatically increased the quality of the isolated RNA and was implemented routinely (Fig. 3.2)

The quality of RNA obtained with the standard extraction protocol was not good enough, even when the working temperatures was reduced to 4°C. We suspected that the ratio of extraction buffer to grounded plant material was not optimal and caused the oxidation of polyphenols during the cell disruption process. The temperature, at which the RNA

Stellenbosch University https://scholar.sun.ac.za

extraction was done, had no affect on the colour of the RNA pellet after the attempted removal of impurities.

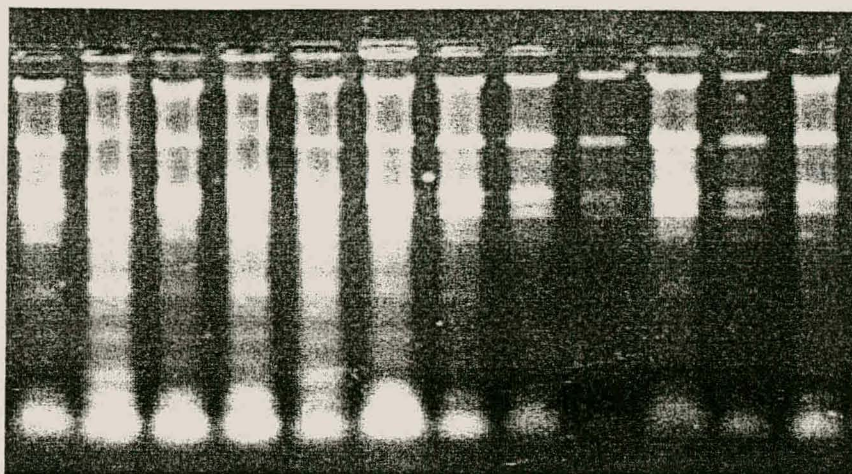

Fig. 3.2 An improvement in RNA quality of all samples was achieved by reducing RNA isolation temperatures to 4°C. Although the RNA quality improved, further improvements were needed before isolated RNA could be used for cDNA synthesis.

3.3.2.2 Isolation of RNA using the modified method

Although no dramatic changes were made to the standard RNA isolation protocol, several refinements were made to increase the quality of the isolated RNA. First, the ratio of grounded leaf material to extraction buffer was changed from 1:4 (w/v) to 1:8 (w/v) to prevent the oxidation of polyphenols during the cell disruption process. We noted no difference in RNA yield when 3 M $NaClO_4$ instead of 5 M $NaClO_4$ was used in the extraction buffer. Separation of the liquid phase from cell debris was done by replacing a filtration step through glass wool at room temperature with a 4°C centrifugation step at 14 000 rpm for 15 minutes. The complete removal of proteins and polysaccharides was achieved by increasing the number of phenol: chloroform extractions from three to at least six. This resulted in a colour change of the RNA pellet from brown to off-white and improved the long-term storage of RNA samples.

Although high quality RNA was obtained, samples were still contaminated with large amounts of genomic DNA. The fact that no RNA degradation occurred during the

Stellenbosch University https://scholar.sun.ac.za

isolation process ensured that isolated RNA could be used in downstream applications of DD-PCR to search for differentially expressed genes. However, the presence of contaminating DNA in RNA samples was unacceptable and had to be removed before RNA could be used for downstream applications (Liang & Pardee, 1992, 1995, Liang, Averboukh & Pardee, 1993, Liang *et al*., 1994) (Fig. 3.3).

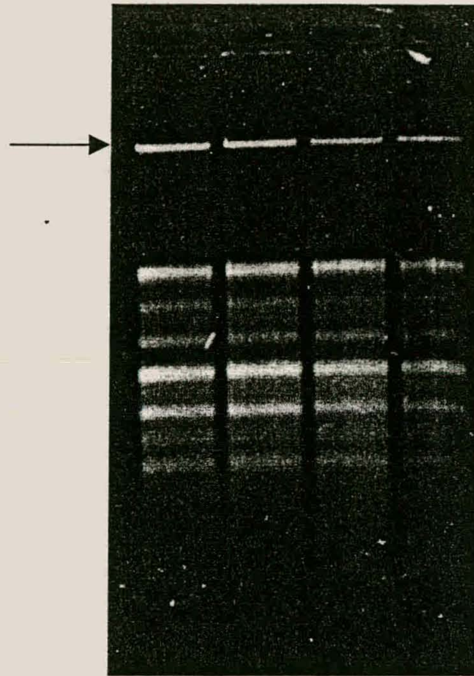

Fig. 3.3 Good quality RNA was isolated with the modified protocol, but it still contained large amounts of unwanted DNA (indicated by arrow).

3.3.3 Removal of contaminating DNA

The complete absence of DNA in RNA samples is very important because DNA contamination will lead to the amplification of fragments that does not necessarily originate from an expressed gene. Contaminating DNA would also out-compete cDNAs originating form rarely expressed mRNA species for substrate during the amplification process and would therefore increase the production of false positives (Liang & Pardee, 1992, Liang *et al*., 1993,1994).

Stellenbosch University https://scholar.sun.ac.za

3.3.3.1 Capturing of mRNA

The mRNA yields from the RNA/DNA mixture was very low and ranged from 300 ng to 500 ng for every 4 grams of leaf material used. Furthermore, A_{260}/A_{280} absorption ratios indicated a slight decrease in the quality of the isolated mRNA. The inability of the Oligotex™ suspension to capture most mRNA fragments was believed to be the main reason for low mRNA yields. As a result, differential display fragments created from cDNA originating from captured mRNA, was not reproducible and could therefore not be used to identify differentially expressed genes. The reasons for the production of non-reproducible fragments from these isolated mRNA fragments will be discussed elsewhere.

3.3.3.2 Differential precipitation of RNA

RNA is known to differentially precipitate from DNA at low salt concentrations (Wallace, 1987, Ambion Technical Manual, 1998). RNA can be precipitated by substances like ethanol, isopropanol (Wallace, 1987, Ambion Technical Manual, 1998), 2-butoxy-ethanol (Staub, Polivka & Gross, 1995) and salts like sodium chloride (NaCl) and lithium chloride (LiCl) (Ambion technical manual, 1998). The precipitation of RNA with LiCl has the advantage of not precipitating carbohydrates, proteins or DNA and is frequently use to remove inhibitors of translation, which co-purify with RNA prepared by other methods (Ambion technical manual, 1998).

The results showed that precipitation of RNA samples with LiCl resulted in the removal of large quantities of DNA. Unfortunately some DNA managed to precipitate with RNA and had to be removed before cDNA could be synthesised from the precipitated RNA (data not shown).

The lack of PCR products from LiCl-precipitated RNA can be accepted as proof for the complete removal of DNA from RNA samples (Bauer *et al.*, 1994). The presence of minute amounts of DNA in the above samples was confirmed when 20 ng of the precipitated RNA (DNA quantity unknown) was used as a template in a standard random amplified polymorphic DNA (RAPD) reaction (Fig. 3.4)

Stellenbosch University https://scholar.sun.ac.za

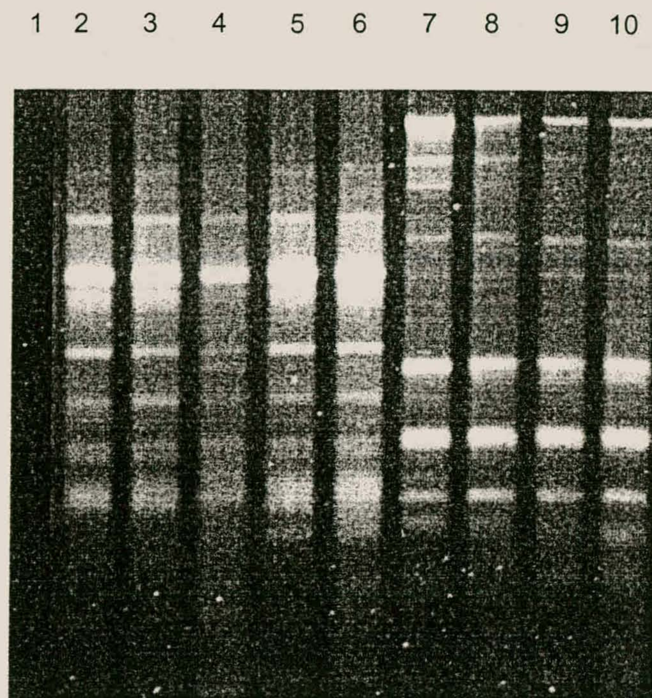

Fig. 3.4 The presence of DNA in RNA samples was confirmed in lanes 1-10 when 20 ng, LiCl precipitated RNA was used as a template in a standard RAPD reaction.

Although no degradation of RNA occurred during precipitation, a reduction in yield was observed. This loss can be ascribed to co-precipitation with DNA from the supernatant (data not shown)

3.3.3.3 Enzymatic digestion of contaminating DNA

The enzymatic digestion of unwanted DNA seemed the only solution to the problems experienced with contaminating DNA. Several researchers reported the degradation of RNA or the incomplete digestion of unwanted DNA in samples when digesting RNA samples with DNase I (Bauer *et al*, 1994). The careful selection of a DNase I source, that is absolutely RNase-free, is very important and could minimise these problems (GenHunter technical manual, 1998).

Stellenbosch University https://scholar.sun.ac.za

Results showed that no contaminating DNA was present in the DNase I treated RNA samples, while undigested RNA samples showed visible DNA bands (Fig. 3.5). Furthermore, no degradation of RNA occurred during the digestion of DNA. Amplification of RNA samples in a RAPD reaction failed to produce fragments in the digested samples while undigested samples yielded several fragments (data not shown).

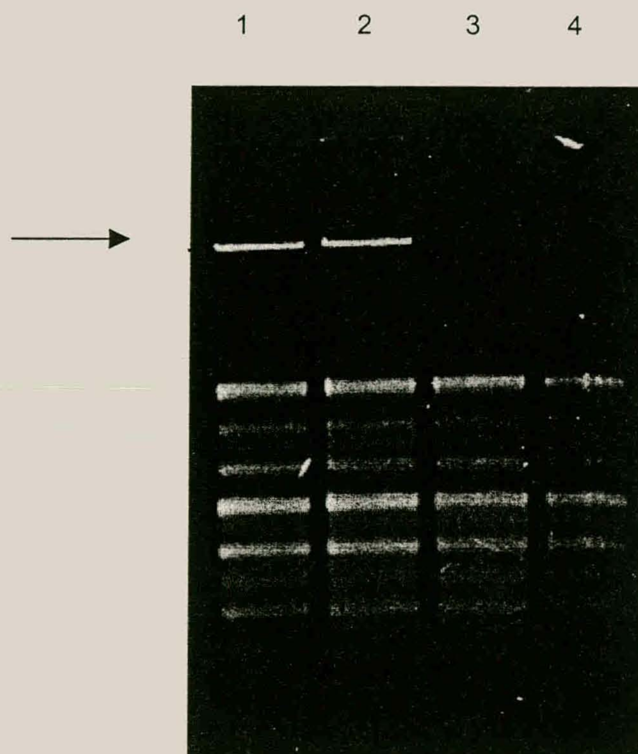

Fig. 3.5 DNase I treated RNA samples (lanes 3 & 4) shows the complete digestion of unwanted DNA. Lanes 1 & 2 shows presence of DNA (indicated by arrow) where no DNase digestions were performed.

3.4 Production of differentially expressed fragments

Although general molecular biology techniques are employed in DD-PCR, many researchers have experienced frustration and failure with this technique. As a result, several modifications have been made to address problems inherent with the design of the technique (Appel *et al.*, 1999).

Stellenbosch University https://scholar.sun.ac.za

Several parameters such as cDNA concentration, the combination, ratio and concentration of primers, dNTP concentration, annealing temperature and visualisation of the amplified fragments after PAGE had to be optimised to ensure the generation of reproducible, differentially expressed fragments (Liang & Pardee, 1992, Liang *et al.*, 1993, Bauer *et al.*, 1993, Hadman *et al.*, 1995, Sunday, 1995).

3.4.1.1 cDNA template concentration

The quality of cDNA in PCR can play an important role in the reproducibility of fragments generated by PCR. Bertioli *et al.* (1995) noted that DD-PCR shows a strong bias towards the amplification of high copy number mRNAs. Using computer model simulations it was also shown that many more bands were generated in DD-PCR than were visualised on a gel (Bauer *et al.*, 1993). Appel *et al.* (1999) noted that the competition for substrates (dNTP's in this case) during PCR will cause templates, that are present at higher levels at the start of the reaction, to reach detectable levels earlier. Excessive dilution of cDNA can thus cause a reduction in the initial template concentration and thereby reduce the number of reproducible visible bands on an acrylamide gel.

Our results with undiluted (10pmol), 10-fold (1pmol), 100-fold (0.1pmol) and 1000-fold (0.01pmol) cDNA dilutions confirmed these findings. The undiluted reactions generated the largest number of visible fragments, while a reduction in the number of reproducible fragments was observed for the diluted samples (Data not shown).

3.4.1.2 One-based versus two-based anchored primers

Anchored (3' or downstream) primers takes advantage of the poly-(A) tail present on most eukaryotic mRNAs. These primers are anchored to the 3' side of the mRNA by adding one or two additional bases that would anneal to nucleotides upstream of the poly-A tail, during the reverse transcription reaction and during PCR.

In our study, we started by using two-based anchored primers and experienced the same problems as described in literature. We found that two based anchored primers such as $^{5'}T_{11}VT^{3'}$ (V= A, G or C) with a T in the terminal 3' base position caused primer drift on the cDNA template and leads to the production of high background smears when separated by PAGE. Callard, Lescure & Mazzolini, (1994), Hadman *et al.* (1994) and Rothschild, Brewer & Bowden (1997) addressed this problem by using anchored primers consisting of $^{5'}T_{11}NV^{3'}$ (N = A, G, C or T and V= A, G or C). Similarly, Khushbeer *et al.* (1998) noted

Stellenbosch University https://scholar.sun.ac.za

that the use of anchored primers consisting of $^{5'}T_{11}VT^{3'}$ leads to an increase in background smears when resolved by PAGE due to drifting of the 5'-T anchor. This, and other factors such as the redundancy of priming, the production of false positives and the number of reverse transcriptions needed to separate the total RNA population into sub-populations forced us to look at the use on one-based anchored primers.

The use of one-based anchored primers such as $^{5'}T_{11}V^{3'}$ dramatically reduced the number or reverse transcription reactions needed to separate the total mRNA population into sub-populations. We incorporated a *Hind III* restriction site at the 5' terminus of each primer for easier manipulation after amplification and isolation of differentially expressed fragments. The problems mentioned with the use of two-based anchored primers were dramatically reduced when one-based anchored primers were used in our lab. However, some false positives and non-repeatable fragments were still produced and correspond to similar results obtained in literature (Liang *et al.,* 1994, Ross, Kumpf & Reske-Kunz, 1997, GenHunter technical manual, 1998).

Wang, Li & Feuerstein (1997) reported the use of a single, specially designed downstream primer as an alternative for both reverse transcription and DD-PCR. This allowed them to target specific genes of interest with a significant reduction in labour while decreasing the number of false positives produced. However, since we tried to identify every possible differentially expressed gene, the use of specific primers based on unique sequence motifs to be tested would lead to the possible exclusion of some fragments. We therefore did not consider this approach as an alternative to solve the above mentioned problems.

3.4.1.3 Optimal arbitrary primer length for differential display PCR

As with the 3' anchored primers, the design of the 5' arbitrary primers has been the subject of much controversy and discussion. Several modifications have been made to solve the problems experienced with the originally designed arbitrary primers. These changes included changes in arbitrary primer length and A/T content.

In our initial experiments we started by using 10-mer arbitrary primers and experienced several problems. Although we were able to visualise several fragments with these arbitrary primers, a high percentage of non-repeatable fragments were obtained. We also suspected that a large number of false positives would be obtained with northern

Stellenbosch University https://scholar.sun.ac.za

blot analysis and decided to change the arbitrary primer length to reduce the production of false positives and non-repeatable fragments.

Originally Liang & Pardee (1992) argued that the arbitrary primers should be short to ensure random and frequent annealing at various distances from the 5' ends of cDNA fragments and thereby create representative fragments of the reverse transcribed mRNA. Ideally, this annealing position should be within 500 base-pairs from the cDNA end since amplified fragments up to 500 base-pairs can be resolved with PAGE (Liang & Pardee, 1992, Appel *et al.,* 1999). Theoretically, this could be achieved by using 6-mer or 7-mer random primers. Nevertheless, primers should be long enough to allow specific cDNA amplification by PCR. The use of random 10-mer primers achieved this objective when dramatically decreased dNTP concentrations (reduction form 200 μM in standard PCR reactions to 2 μM in DD-PCR reactions) were used during amplification (Liang & Pardee, 1992).

In practice 10-mer primers display many more amplification products than predicted form their theoretical probabilities (e.g., one recognition site per 4^{10} or 1.05×10^{3} kb for any arbitrary 10 mer primer) (Liang & Pardee, 1992, Appel *et al.,* 1999). This is mainly due to 5' mismatches during the annealing of the arbitrary primers under low stringency PCR conditions. Bauer *et al.* (1993) demonstrated that annealing specificity and initiation of amplification by *Taq* polymerase is dependent on the three to six most 3' nucleotides of the arbitrary primers. Sequencing of amplified fragments confirmed the occurrence of one to four mismatches in the remaining 5' bases of the arbitrary primers without aborting amplification (Bauer *et al.,* 1993). This leads to the visualisation of fragments characteristic of 6-mer to 8-mer arbitrary primers (Liang, *et al.*, 1993). However, similar banding patterns have not been observed consistently with 10-mer arbitrary primers that contain identical 3' terminals (Bauer *et al.,* 1993, Appel *et al.,* 1999). Therefore, the 5' portion of the primer plays an important role in stabilising (or destabilising) primer annealing to the cDNA template (Appel *et al.,* 1999).

The inefficiency of 10-mers as PCR primers has been identified as a limiting factor in the sensitivity of DD-PCR. Several modifications to the arbitrary primers, in order to reduce the generation of false positives, have been proposed (Appel *et al.,* 1999). According to Graf, Fisher & Merkenschlager, (1997), the development of rationally designed primers, with an A/T content of 60-80% corresponds to the A/T content of the untranslated cDNA 5' region of near the poly-A tail. Amplification with these rationally designed primers

Stellenbosch University https://scholar.sun.ac.za

would decrease the production of 10-mer-10-mer artefacts and increase oligo (dT)/10-mer products.

We then decided to use longer arbitrary primers but several factors influenced the optimal arbitrary primer length and will briefly be discussed. In general, the use of longer arbitrary primers results in an increase in specificity. Applying this principle with DD-PCR, arbitrary primers containing 10-15 base-pairs would lead to an increase in specificity but would still manage to display the capabilities characteristic of shorter primers due to 5' mismatches (Liang & Pardee, 1992). In an attempt to increase the reproducibility of the displayed cDNA pattern, various groups increased the arbitrary primer length to 20 bases or more (Ohan & Heikkila, 1993, Linskens *et al.,* 1995 and Wang, Xiang & Feuerstein, 1995). However, when longer primers, which are designed to amplify specific genes, are used under degenerate conditions, hybridisation will take place in a non-predictable way, and thereby make the rational design of a representative set of primers impossible. Furthermore, although longer primers (>20 basepairs) can also create complex cDNA patterns, many of the amplified fragments may originate from the same cDNA template due to the "stickiness" of long primers when used under low stringency conditions (Liang *et al.,* 1994, Liang & Pardee, 1995, Appel *et al.,* 1999). The more reproducible nature of longer primers may be misleading as a result of the redundancy of amplified PCR products.

Liang & Pardee (GenHunter Technical manual, 1998) noted that several essential factors have to be kept in mind when a designing a set of arbitrary primers:

1. The theoretical consideration suggests that in order to display 50-100 mRNAs, the optimal selectivity of the arbitrary primers should be provided by no more than seven bases of each pair of primers.
2. The minimum primer length for PCR is 9 bases.
3. The arbitrary primers have to hybridise in a predictable way to rationally design a representative set of primers to display most mRNA in a cell.
4. In practice the arbitrary primers require eight to nine base-pair matches for optimal priming.
5. The redundancy (one mRNA species is represented as more than one band in each lane) should be kept to minimum.

We decided to use rationally designed 13-mer primers as described by Liang & Pardee (GenHunter technical manual, 1998). The results we obtained were similar to those obtained in literature. In our studies, the use of 13-mer arbitrary primers dramatically

Stellenbosch University https://scholar.sun.ac.za

decreased the production of non-reproducible fragments, but still managed to produce the required number of amplification products per reaction. Therefore, our results indicated that 13-mer primers are the optimal arbitrary primer length and should be used in DD-PCR. Liang *et al.* (1993) mentioned several reasons why 13-mers are much more effective as DD-PCR arbitrary primers than 10-mers. Firstly, 13-mers are able to produce highly reproducible and uniform banding patterns. Secondly, 13-mers provides excellent specificity of sequence recognition because they can be rationally designed in such a way that all primers are maximally different in their first seven 3' base sequences. The extra six, 5' bases are fixed and statistically provide an additional 1.5 base pairing for each primer (Appel *et al.,* 1999). Thus, the arbitrary 13-mers will anneal as 8-to 9-mers as required in the initial PCR amplification cycle. Thus, rational primer design allows a limited number of arbitrary 13-mers needed to ensure that most of the mRNA is represented (GenHunter technical manual, 1998).

3.4.1.4 Influence of annealing temperature

Initially, the DD-PCR technique was designed in such a way that 42°C would be optimal for product yield and specificity (Liang & Pardee, 1992). Optimisation experiments from our research tested annealing temperatures ranging from 36°C to 46°C. We tested various two-based anchored primer/10-mer arbitrary primer combinations and found that optimal primer annealing temperature depends on the primer combination. However, an annealing temperature of 40°C produced the most reproducible fragments with most of the primer combinations (data not shown). At lower temperatures the production of non-reproducible bands increased, while the number of amplified fragments at annealing temperatures of 42°C and more dramatically decreased. In our studies the primer combination T_{11}AA produced more fragments at annealing temperatures of 38°C and failed to produce any fragments at annealing temperatures of 42°C or more (Data not shown).

Bauer *et al.* (1993) noted that annealing temperatures had a significant effect on the number of bands created by PCR. Annealing temperatures lower than 40°C resulted in the generation of background smears due to the large amount of fragments created under low stringency conditions. In contrast, annealing temperatures above 42°C caused a dramatic decrease in the production of bands. Khushbeer *et al.* (1998) and Bauer *et al.* (1993) concluded that 40°C was the optimal annealing temperature for their primer combinations tested.

Stellenbosch University https://scholar.sun.ac.za

3.4.1.5 Influence of primer ratio

In our studies we tested anchored/arbitrary primer ratios of 5:1, 2:1 and 1:1 and found that a primer ratio of 1:1 produced the most reproducible bands. The results we obtained are similar to results obtained in literature. Although the initial DD-PCR protocol used a primer ratio of 5:1 (anchored to random primer), it has been modified by various groups mainly due to the production of many oligo-(dT) artefacts. The production of these artefacts could be suppressed by decreasing the anchored primer concentration (Liang & Pardee, 1992, Graf *et al.,* 1997). Rothschild *et al.* (1997) and Khushbeer *et al.* (1998) used a 2:1 (anchored to random) ratio, while Graf *et al.* (1997) suggested a primer ratio of 1:1 or 1:2 (anchored to random) for rationally designed primers.

3.4.1.6 Influence of dNTP concentration

Liang & Pardee (1992) concluded that the production of false positives was dramatically decreased, and the specificity of amplification increased, by decreasing the dNTP concentration from 200 μM (which are normally used in arbitrary PCR) to 2 μM. Lowering the dNTP concentration improves the specificity of DNA amplification, and is necessary for labelling PCR products with radioactive nucleotides to provide high resolution and visualisation after PAGE. Khushbeer *et al.* (1998) showed that specific amplification and visualisation of amplified fragments are possible when low (6 μM) dNTP concentrations are used in combination with an end-labelled anchored primer. In contrast, Rothschild *et al.* (1997) increased the dNTP concentration to 100 μM when the anchored primer was end-labelled and still managed to get reproducible fragments. Our experience confirmed that low dNTP concentrations (2–to 6 μM) are essential to obtain reproducible fragments, even when labelled primers are used.

3.4.1.7 End labelling of anchored primers

As mentioned previously, the absence of all contaminating DNA is essential to prevent the production of false positives. To overcome the presence of contaminating DNA in RNA samples, researchers sought different ways to remove DNA from RNA samples. While differential precipitation is unable to remove all the contaminating DNA from RNA samples, some researchers noted the inability of some RNase-free DNA digesting enzymes to digest all the DNA without a reduction in RNA yield and quality.

Stellenbosch University https://scholar.sun.ac.za

The use of an end-labelled oligo-(dT) primer instead of random labelling is one way to ensure that only products originating from mRNA, is visualised (Hadman *et al.,* 1995, Tokuyama & Takeda, 1995, Ambion technical manual, 1998, Rothschild *et al.,* 1997 , Khushbeer, *et al.,* 1998, Appel *et al.,* 1999). However, since contaminating DNA may still be present, undesired amplification products may be present (although not visible) and care must be taken with this approach. Firstly, the presence of DNA may out-compete rare cDNA species for substrate. The presence of invisible bands (surrounding candidate bands) may complicate re-amplification and cloning (Appel *et al.*, 1999). Hadman *et al.*, (1995) noted that the use of labelled oligo-(dT) primers only allow bands from the very 3' end of the mRNA, which contain less informative, untranslated sequences, to be detected. Furthermore, some mRNA species do not contain a poly-A tail and would therefore not be amplified when a labelled oligo-(dT) primer is used, while the same mRNA species may produce fragments originating from arbitrary primer/arbitrary primer amplification (P. Gresshoff, personal communication).

The production of non-reproducible fragments increased significantly when labelled oligo-(dT) primers were used during our optimisation process and was therefore not used to search for differentially expressed genes in this study.

3.4.1.8 Visualisation of amplified products

Several detection methods have been proposed to visualise DD-PCR amplification products. These include the incorporation of radioactive nucleotides, silver staining of amplified products, chemiluminescence and separation on agarose gels (Liang & Pardee, 1992, Lohmann, Schickle & Bosch, 1995, An *et al.,* 1996, Luehrsen, *et al.,* 1997, Rompf & Günter, 1997). As for radioactive detection methods, ^{33}P emerged as the label of choice, although ^{32}P and ^{35}S are still routinely being used (Liang & Pardee, 1995, Appel *et al.,* 1999).

Lohmann *et al.* (1995) and Appel *et al.* (1999) showed that the detection of multiple amplified fragments with silver staining is possible although not the same level of sensitivity, as with autoradiography, is achieved. Rompf & Günter (1997) demonstrated the separation and visualisation of 5-25 bands with agarose gel electrophoresis (in comparison with 50-100 bands obtained with PAGE).

The use of fluorescent primers or nucleotides and the detection of amplified fragments on automated sequencers was developed to increase the rate at which certain mRNA pools

Stellenbosch University https://scholar.sun.ac.za

could be screened for differences in gene expression (Luehresen *et al.*, 1997). However, since differentially expressed fragments cannot be isolated and re-amplified from the gels of an automated sequencer, the DD-PCR reaction have to be repeated with radio-active nucleotides and separated before bands of interest can be isolated. When fluorescent oligo-(dT) primers are used for amplification, the same disadvantages apply as with labelled primers.

During our optimisation experiments, silver staining, agarose gel electrophoresis and autoradiography was tested as detection methods. ^{33}P emerged as the detection of choice. Although amplified fragments were detected with silver staining, there was a notable reduction in the number of amplified fragments. Agarose gel electrophoresis yielded significantly less fragments than detected by autoradiography (data not shown).

3.4.2 Identification of differentially expressed fragments

3.4.2.1 Introduction

Differential expression of amplified cDNA fragments is determined by visual inspection of autoradiograms to identify fragments present in only one cDNA population. Although the method is believed to be non-quantitative, differences in band intensities can be regarded as differences in the level of gene expression (Sunday, 1995). In contrast, Guimarães *et al.* (1995) noted that significant differences in the level of gene expression might be undetectable with 40 cycles of PCR (as in our case) due to template saturation. In our study we focused on the identification of fragments present only in infected cDNA amplification products of the resistant cultivar but completely absent in the control populations. We also compared the expression patterns of the susceptible and resistant cultivars to reduce the number of fragments to be isolated. We argued that by comparing the banding patterns of the infected populations of both cultivars, we would be able to distinguish between general stress-related genes and genes specifically activated by powdery mildew infections. This would enable us to isolate fragments only present in the infected population of the resistant cultivar and would therefore increase our chances to identify powdery mildew defence-related genes.

Stellenbosch University https://scholar.sun.ac.za

3.4.2.2 Control reactions

Several control reactions were performed to ensure that amplification products originated from expressed genes. The amplification of reverse transcription reactions where no MMLV reverse transcriptase was added failed to produce any amplification products and confirmed results obtained with previous control reactions (amplification of RNA samples in a RAPD reaction) (Fig. 3.6). Contamination of RNA samples (or reagents) with DNA would result in the presence of amplification products in samples where no MMLV reverse transcriptase were used in reverse transcription reactions. These unwanted fragments originate from the amplification of contaminating DNA. Furthermore, expression patterns of amplification products were dependent on both primers and changed when the same arbitrary primer was used with different anchored primers and *vice versa* (Fig. 3.7).

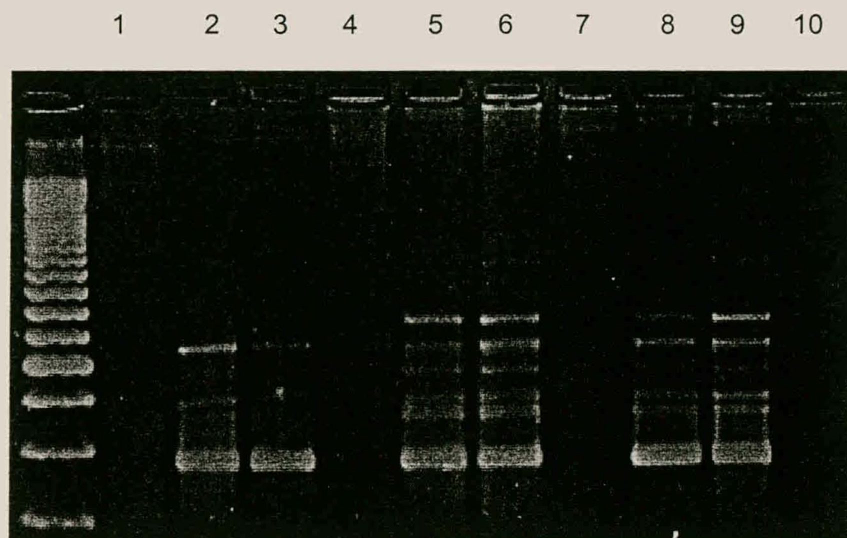

Fig. 3.6 Control RAPD reactions to ensure that no contaminating DNA is present in RNA samples. Lanes 1, 4 and 7 represents RNA samples where no MMLV reverse transcriptase were used and lanes 2, 3, 5, 6, 8 and 9 represents cDNA synthesised from the same RNA samples used in lanes 1, 4 and 7. The absence of bands in lanes 1, 4 and 7 show that no contaminating DNA is present in RNA samples. The presence of bands in lanes 3, 3, 5, 6, 8 and 9 shows successful synthesis of cDNA from RNA. A control reaction (lane 10) was included to ensure the absence of DNA in reagents used for PCR.

Stellenbosch University https://scholar.sun.ac.za

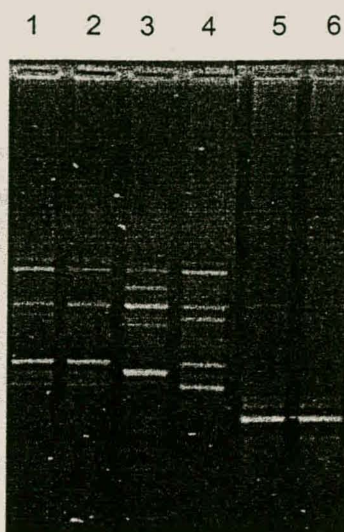

Fig. 3.7 Changes in expression patterns as a result of changes in the anchored primer used for PCR. In lanes 1 and 2 the anchored primer $^{5'}T_{11}C^{3}$ was used, $^{5'}T_{11}G^{3}$ in lanes 3 and 4 and $^{5'}T_{11}A^{3}$ in lanes 5 and 6.

3.4.2.3 Differential display PCR results.

The total number of amplified fragments visualised by autoradiography, varied from 30-200 fragments (depending on the primer combination) and correlated well with results obtained elsewhere (Liang & Pardee, 1992, Bauer *et al.,* 1993). Moreover, depending on the primer combination used, the overall reproducibility of the amplified fragments varied from 80% to 95% (using the same or duplicate reverse transcription reactions). In contrast, Liang & Pardee (1992) noted that amplified fragments always displayed a reproducibility of more than 95%, regardless of the primer combination used. Since several modifications have been made by various research groups to increase DD-PCR reproducibility (which we applied) but never reported levels of reproducibility better to what we found, no extra efforts were made to increase the reproducibility of our experiments.

Amplification products varied from less than 100 base pairs to 1000 base pairs in size with the majority of amplification products lying between 200 base pairs and 600 base pairs, corresponding well with results found in the literature (Appel *et al.,* 1999).

Approximately 8000 amplified fragments were visualised by PAGE and autoradiography using 24 arbitrary- and three one-based anchored primers (72 reactions with an average

Stellenbosch University https://scholar.sun.ac.za

of 110 fragments per reaction). According to Liang & Pardee (1992, Liang *et al.,* 1994), Sunday (1995), and Guimarães *et al.* (1995) this corresponds to approximately half (52%) of the total number of expressed mRNA species in eukaryotic cells. A comparison of our total number of amplified fragments with theoretical estimations of the number of arbitrary primers necessary to cover most of the genes expressed in a cell confirmed these results. GenHunter Technical Manual (1998) indicated that 20 arbitrary primers in combination with three one-based anchored primers (60 reactions) would amplify approximately 56% of all the potentially expressed genes and that 80 arbitrary primers would be needed to amplify most (96%) of the potentially expressed mRNA species.

The total number of primer combinations needed to identify the genes of interest cannot be predicted, but depends on the results obtained with initial primer combinations used. Wilkinson *et al.* (1995) used only 3 primer combinations to identify five mRNA's, showing ripening-enhanced expression in strawberry, while Ikeda *et al.* (1997) needed 240 different primer combinations to identify one aquaporin-like gene from the *Brassica* self-incompatibility response. In our study, 30 differentially expressed fragments were identified with 72 different primer combinations and were sufficient for the purpose of this study.

3.4.2.4 Identification of differentially expressed fragments

Direct comparisons between lanes were made to identify differentially expressed fragments. Since duplicate reactions of each primer combination were performed, only fragments present in both lanes were regarded as a truly expressed fragment (Fig 3.8)

As expected, a very low percentage (0.38%) of the 8000 amplification products was differentially expressed (Fig. 3.10). This corresponded well with similar studies in the literature (Appel *et al.,* 1999). Initially, we planned to compare expression patterns of the infected resistant and infected susceptible cultivars, as well as the expression patterns of the infected resistant cultivar with the expression patterns of the unifected resistant cultivar. By comparing the banding patterns of the infected populations of both cultivars, we hoped to distinguish between general stress-related genes and genes specifically activated by powdery mildew infections. This would enable us to isolate fragments only present in the infected population of the resistant cultivar and would therefore increase our chances to identify powdery mildew defence-related genes. Since no overall similarities between the populations existed, comparison of expression patterns of the susceptible and resistant cultivars was not possible. Expression patterns of the two

Stellenbosch University https://scholar.sun.ac.za

cultivars, with the first four primer combinations tested, were very different and provided no guarantee that fragments of similar size originated from the same expressed gene. Further analysis of samples from the susceptible cultivar was therefore discontinued (Fig. 3.9).

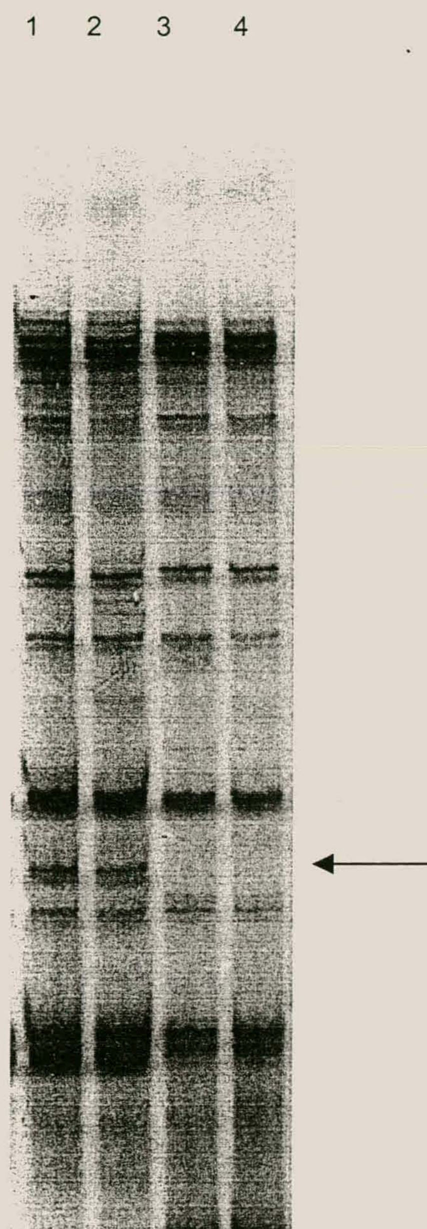

Fig. 3.8 Example of differential gene expression indicated by arrow. Lanes 1 and 2 represents the infected resistant cultivar and lanes 3 and 4 represent the uninfected resistant cultivar.

Stellenbosch University https://scholar.sun.ac.za

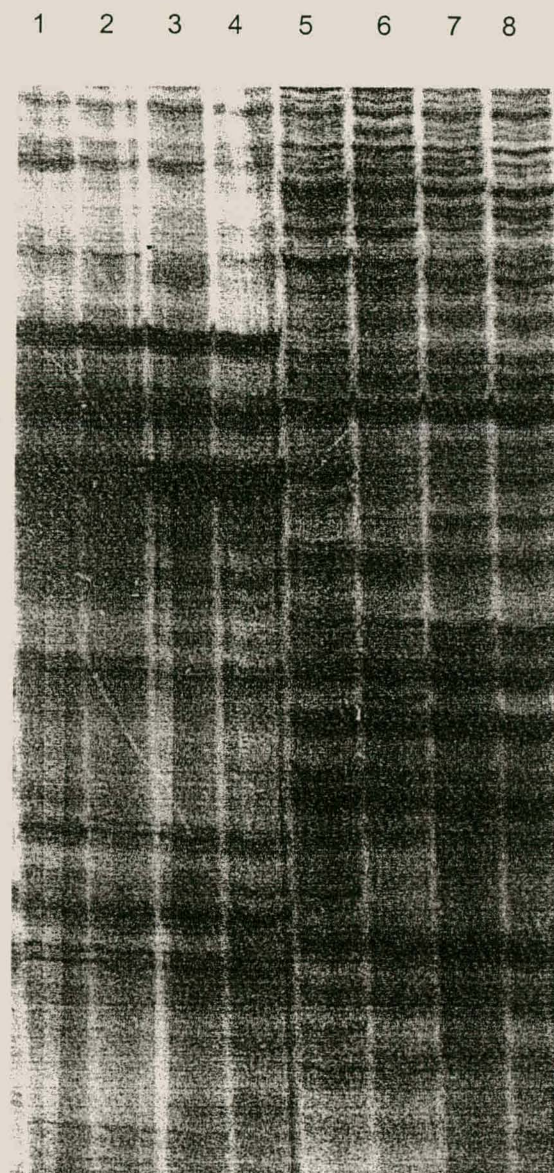

Fig. 3.9 Banding patterns from the susceptible (lanes 1-4) -and resistant cultivar (lanes 5-8) were very different and could not be used to distinguish between general stress-related genes and genes specifically activated by powdery mildew infections.

Stellenbosch University https://scholar.sun.ac.za

3.4.2.5 Re-amplification of differentially expressed fragments

About 83% (25 out of 30) of the fragments managed to amplify during the first round PCR (Fig. 3.10). Unfortunately, fragments failing to yield any products during the first round PCR, also failed during the second round PCR and were omitted from further studies. Comparison of re-amplified products with known DNA quantities revealed that re-amplification reactions yielded products ranging from 30 ng DNA to 250 ng DNA.

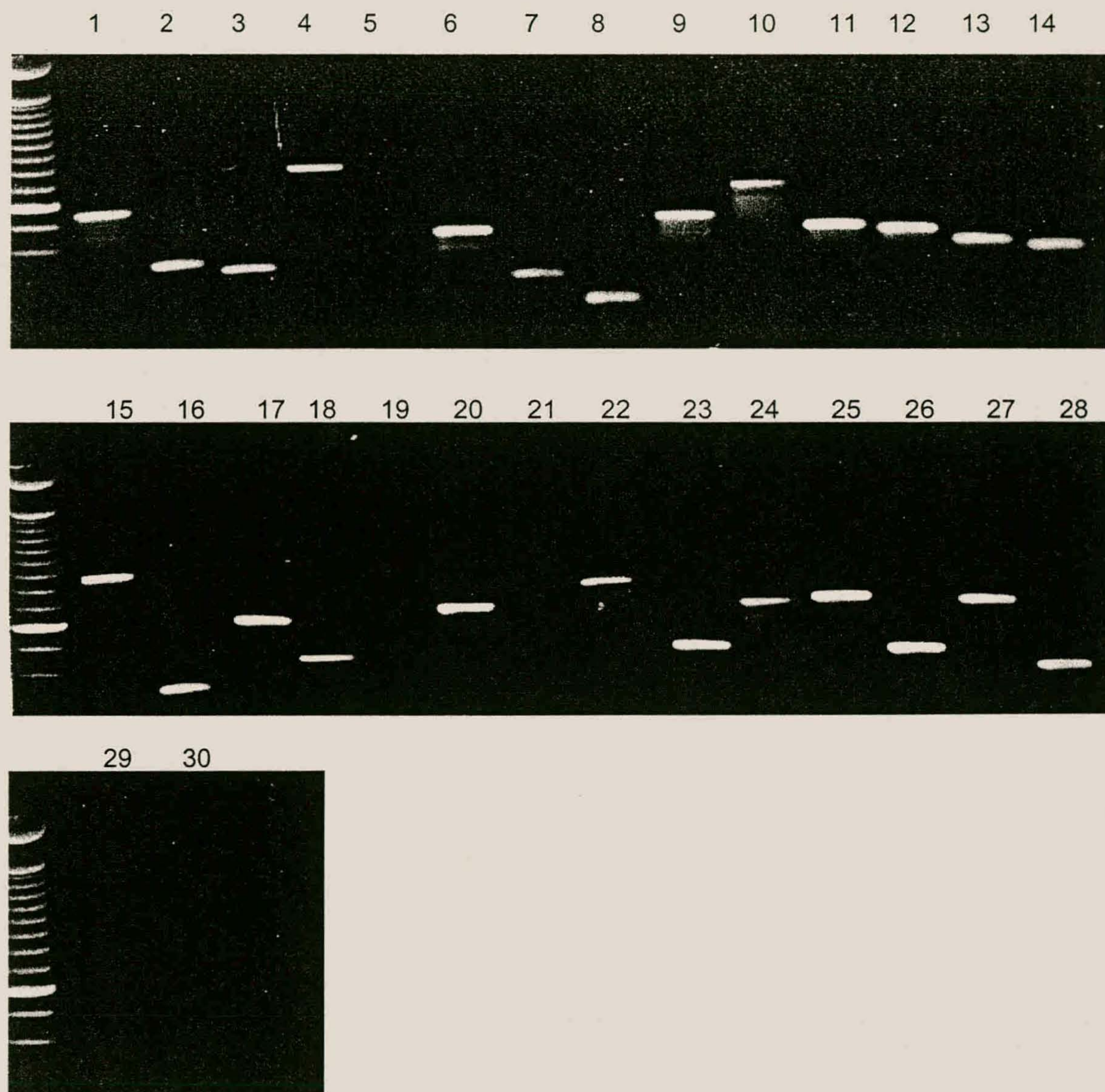

Fig. 3.10 Re-amplification of isolated differentially expressed fragments. Failed re-amplifications can be seen in lanes 5, 19, 21, 29 and 30.

Stellenbosch University https://scholar.sun.ac.za

Integrity of excised fragments was confirmed by comparing fragment sizes of re-amplification products in agarose gels with those of original fragments in acrylamide gels. Since re-amplification yielded single fragments of correct size, the use of PAGE for removal of unwanted bands was unnecessary.

3.4.2.6 Determination of nucleotide sequences of identified differentially expressed fragments.

Sequencing results confirmed previous results and showed that most (20/25) of the re-amplified fragments had very little background (contaminating nucleotides or DNA fragments). Three fragments had some background and it was necessary to do manual base-calling of their nucleotide sequences obtained from the ABI Prism 377 DNA Sequencer. Base-calling of these nucleotide sequences was done using the Chromas sequencing program (Technelysium). Sequencing results showed that two differentially expressed fragments each contained two similar sized fragments after PAGE and strengthen similar complaints from literature (Bauer *et al.*, 1993, Liang *et* al., 1993, Callard *et al.,* 1994). No conclusive nucleotide sequence could be determined from these fragments and they were therefore discarded.

3.4.2.7 Molecular analysis of sequenced fragments

Nucleotide sequences were submitted to the Genbank, EMBL and DDBJ databases using the BLAST search program (Altschul *et al.,* 1997). In literature, one of the complaints frequently raised is that amplification products identified as candidate differentially expressed mRNA species do not usually contain the complete coding regions of genes and are therefore less informative (Sunday, 1995, Galindo *et al.*, 1998, Appel *et al.*, 1999). Our results confirmed these complaints and are summarised in Table 1. Blast search results showed that four sequenced fragments showed significant (E<0.01) (E-value shows significance of aligment, the smaller the E-value, the more significant the aligment) the alignments with genes that can play an important role in the plant defence reactions. Two fragments showed insignificant alignments with possible defence-related genes. One fragment showed significant homology with a fragment that is not defence-related (See Table 1). Results also indicated that various fragments contained short sequences with low levels (E> 0.01) of alignment with human, *Drosophila*, mouse and other unrelated organisms. It was decided that sequenced fragments with E values >0.01 would be regarded as novel sequences.

Stellenbosch University https://scholar.sun.ac.za

Table 3.1 Blast search results.

Fragment No	Fragment Size	No of significant hits	E-value	Homology Percentage	Bases Aligned	Blast alignment Hits
1	260	None	-	-	-	-
2	1756	None	-	-	-	-
3	1659	None	1.1	100	17	*A. thaliana* mRNA for peroxidase.
4	470	None	-	-	-	-
5	336	2	e-135 e-138 0.053	97 97 100	232 226 21	*V. vinifera* proline rich protein 1, *V. vinifera* proline rich protein 2, *O.sativa* mRNA for thaumatin-like protein.
6	327	2	e-144 e-138 0.031	96 96 100	289 283 21	*V. vinifera* proline rich protein 1, *V. vinifera* proline rich protein 2, *O.sativa* mRNA for thaumatin-like protein
7	264	None	-	-	-	-
8	220	None	-	-	-	-
9	546	3	2e-27 2e-15 2e-15	80 79 79	284 204 204	*C. sinensis* mRNA for non-photosynthetic ferredoxin, *Z. mays* ferredoxin III isoprotein mRNA, *Z. mays* DNA for ferredoxin III.
10	33	None	-	-	-	-
11	390	2	4e-09 0.001	81 83	129 77	*G. hirstum* mRNA for ribosomal protein, *A. thalinana* DNA from chromosome 5
12	347	None	-	-	-	-
13	166	None	-	-	-	-
14	338	2	2e-05 0.008	100 100	25 21	*C. sativus* mRNA for catalase, *B. napus* catalase.
15	230	None	-	-	-	-
16	250	None	-	-		-
17	341	None	-	-	-	-
18	347	None	-	-	-	-
19	1110	None	-	-	-	-
20	190	None	-	-	-	-
21	180	None	-	-	-	-
22	1150	None	-	-	-	-
23	299	None	3.6	100	17	*L. esculentum* polygalacturonase mRNA.
24	1106	None	-	-	-	-
25	1323	None	-	-	-	-

e-135, e-138, e- 144, etc. < e= 0.01 and therefore significant.

Stellenbosch University https://scholar.sun.ac.za

A general complaint with DD-PCR is that when sequences generated by this technique are compared with known gene sequences, limited information is obtained. Techniques to circumvent this problem include 5'RACE (rapid amplification of cDNA ends). The 5' RACE offers the possibility to obtain full-length genes from differentially displayed amplification products (Frohman, Dush & Martin, 1994). This approach was successfully used in one of the first reports using DD-PCR for the identification of differentially expressed genes in plant tissues (Goormachtig *et al.*, 1995). Haag & Raman (1994) successfully replaced the oligo-dT primer with a secondary primer during the DD-PCR to generate useful information of sea urchin mRNA (which contains long untranslated 3' regions).

No studies regarding the presence of possible promoters were done because Blast search results indicated that isolated fragments contained mainly untranslated 3' ends of differentially expressed genes. However, the same approach as mentioned above can be used to search for promoters.

The possible function of identified fragments that showed sequence homology with known defence-related genes will be discussed in the next chapter.

3.5 Confirmation of gene expression

3.5.1 Reverse northern blot analysis

Expression of induced mRNA fragments identified by DD-PCR was verified by Reverse Northern Blots. In contrast to conventional northern blot protocols, Reverse Northern protocols uses labelled probes generated from RNA to hybridise with DNA (PCR products or clones) in conditions very similar to Southern Blot hybridisation (Mou *et al.*, 1994, Zhang, Zhang & Liang, 1996, Galindo *et al.*, 1998). Reverse Northern blots has the advantage that much less mRNA is needed to confirm the expression of differentially expressed genes. Furthermore, Reverse Northerns are able to verify differential expression of numerous fragments with the use of only two probes, one originating from the treated (in our case, infected) RNA population and one from the control (uninfected) population (Fig. 3.11 and Fig. 3.12). It also prevents the hybridisation of PCR products with contaminating DNA present in RNA samples (as with conventional Northern blot analysis). Additionally, time consuming preparations needed to create an RNase free environment, is excluded. The transfer of total RNA to membranes from formamide gels

Stellenbosch University https://scholar.sun.ac.za

is no longer necessary. The overall time needed to verify differentially expressed fragments in comparison with conventional northern blot protocols are also reduced.

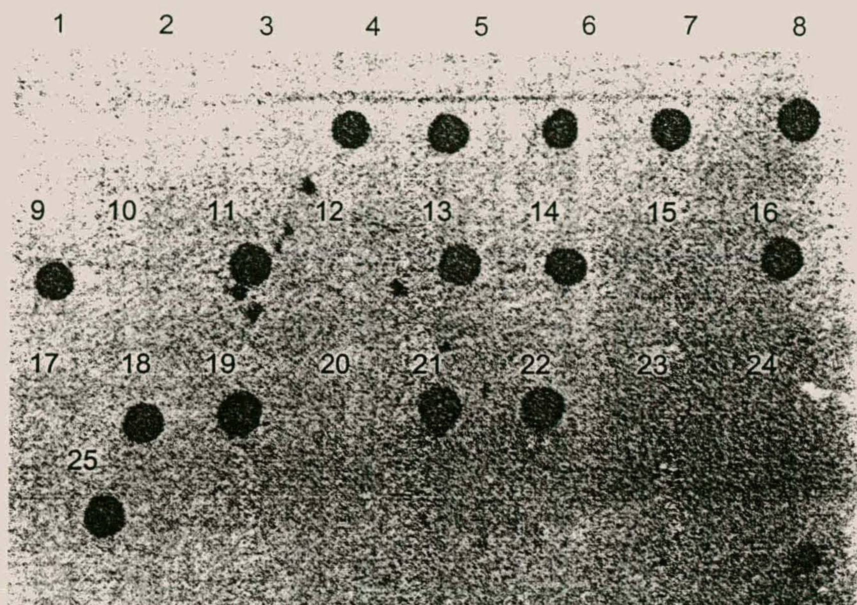

Fig. 3.11 Reverse northern blot with probes originating from infected RNA.

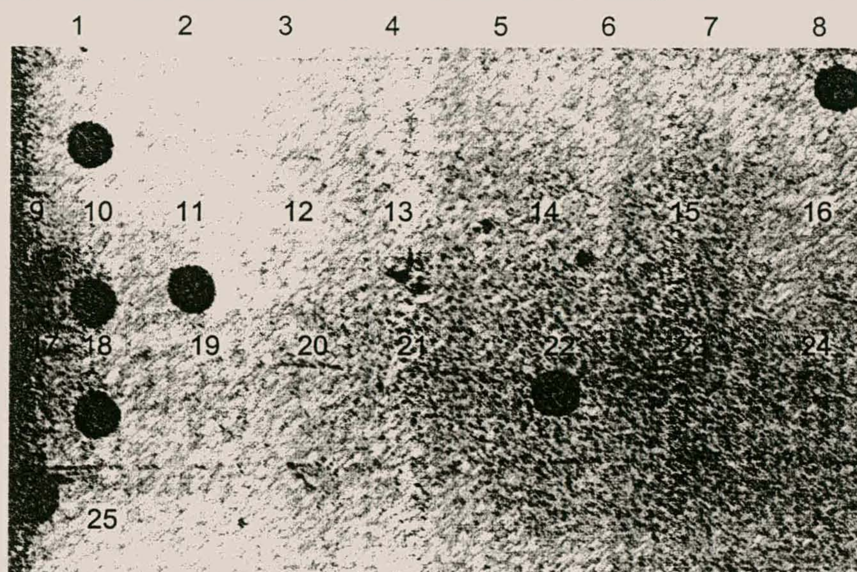

Fig. 3.12 Reverse northern blot with probes originating form uninfected RNA.

The reverse northern blot data is summarised in Table 3.2. Our results showed fifteen positive signals from probes originating from the infected RNA and seven probes originating from the uninfected RNA. Five false positive signals (positive signals obtained from infected and uninfected RNA) were obtained. Two positive signals were only present in the uninfected mRNA and were also regarded as false positives.

Stellenbosch University https://scholar.sun.ac.za

Table 3.2 Reverse northern blot results *vs.* Blast search results.

Fragment	Infected RNA Probe	Uninfected RNA Probe	Sequence results
1	-	-	Novel
2	-	+	Novel
3	-	-	*A. thaliana* mRNA for peroxidase
4	+	-	Novel
5	+	-	*V. vinifera* proline rich protein 1, *V. vinifera* proline rich protein 2, *O.sativa* mRNA for thaumatin-like protein.
6	+	-	*V. vinifera* proline rich protein 1, *V. vinifera* proline rich protein 2, *O.sativa* mRNA for thaumatin-like protein
7	+	-	Novel
8	+	+	Novel
9	+	-	*C. sinensis* mRNA for non-photosynthetic ferredoxin, *Z. mays* ferredoxin III isoprotein mRNA, *Z. mays* DNA for ferredoxin III.
10	-	+	Novel
11	+	+	*G. hirstum* mRNA for ribosomal protein, *A. thalinana* DNA from chromosome 5
12	-	-	Novel
13	+	-	Novel
14	+	-	*C. sativus* mRNA for catalase, *B. napus* catalase
15	-	-	Novel
16	+	-	Novel
17	-	-	Novel
18	+	+	Novel
19	+	-	Novel
20	-	-	Novel
21	+	-	Novel
22	+	+	Novel
23	-	-	*L. esculentum* polygalacturonase mRNA
24	-	-	Novel
25	+	+	Novel

Stellenbosch University https://scholar.sun.ac.za

Although a high rate of false positives were obtained, most of the genes that can play an important role in the plant defence-reaction (5, 6, 9 & 14) only gave a positive signal from probes originating from infected RNA. Homology search results indicated that fragments 3 and 23 also aligned with defence-related genes but with e-values that is not significant. No signal was detected from these two fragments with reverse northern blot analysis. Fragments 2, 8, 10, 11, 18, 22 and 25 gave positive signals with probes originating from uninfected RNA and would therefore represent gene expression that has to do with normal cellular functions and not with defence-related, differentially expressed genes.

The high rate of false positives (46%) obtained from our reverse northern blot analysis confirm complaints raised by various researchers in literature (Callard *et al.*, 1994, Zhang *et al.*, 1996). A possible reason for such a high level of false positives can be attributed to probes used with reverse northern blot analysis. Probes are generated from a mixture of mRNA species that may lead to non-specific annealing to short fragments of target DNA and thereby increase the number of false positives.

Of the 25 fragments analysed by reverse northern blot analysis, eight (32%) failed to produce any detectable signal. This failure of reverse northern blot probes to produce signals could be technical. For instance, it could be due to shorter labelled probes (e.g. fragment 20) and to high stringency conditions during hybridisation and washing. It is also possible that the products matched low level mRNA's beneath the detection limit of the reverse northern blot analysis (Sunday, 1995).

Stellenbosch University https://scholar.sun.ac.za

CHAPTER FOUR

4 POSSIBLE FUNCTION OF DIFFERENTIALLY EXPRESSED GENES IN THE *V. VINIFERA- U. NECATOR* INTERACTION.

4.1 Introduction

Resistance in many plant-pathogen interactions is accompanied by the rapid deployment of a multi-component defence response (Dixon *et al.,* 1994). The individual components of this include the HR, the production of chemical weapons like anti-microbial phytoalexins and hydrolytic enzymes and structural barriers like lignin and hydroxyproline-rich cell wall proteins. Moreover, it would appear that the *de novo* transcription of the plant genes encoding the various components of the defence response is an early determinative event (Dixon *et al.,* 1994). Although biotrophic pathogens like *U. necator* are able to prevent the activation of a full scale defence response, some components of the overall defence response are activated and are able to prevent further invasion by the pathogen (Bent, 1996). Previous studies attributed *V. vinifera* resistance to powdery mildew to several factors such as differences in cuticle thickness, increased activity of enzymes involved in lignin biosynthesis, production of papillae, deposition of silica incrusts, localised necrosis and the activation of enzymes like chitinases and glucanases (Pratt *et al.*, 1984, Heintz & Blaich, 1989, 1990, Eibach, 1994, Clingeleffer & Scott, 1994).

Our study concentrated on induced gene expression of grapevine in reaction to infection by powdery mildew. The fact that *R* genes from diverse plant species with specificity for a wide variety of viral, bacterial and fungal pathogens often encode structurally similar proteins, suggested a high degree of mechanistic conservation among the pathways that plants use to trigger defence responses (Jones, 1996). Although different races of *U. necator* were found that differs in pathogenicity, inoculations were done using a mixture of different races. This and the fact that tolerance conferred by Villard Blanc appears to be multigenic horisontal resistance are the main reasons why no R gene homologues were found during this study.

4.2 Possible function of differentially expressed genes in the *V. vinifera-U. necator* interaction.

The DNA fragments isolated form *V. vinifera* using DD-PCR showed homology with several sequences. These sequences include *V. vinifera* proline rich proteins 1 & 2, thaumatin-like proteins, *Z. mays* ferredoxin III, *C. sativus* and *B. napus* catalase, *A. thaliana* peroxidase (not significant) and *L. esculentum* polygalacturonase (not significant).

Stellenbosch University https://scholar.sun.ac.za

Sequence alignment results of differentially expressed fragments indicated that two fragments showed significant homology with *V. vinifera* proline rich proteins 1 & 2. In both dicots and monocots, three types of structural cell wall proteins have so far been identified. These are the extensins and related hydroxyproline-rich glycoproteins (HRGP's), glycine-rich proteins (GRP's) and the proline rich proteins (PRP's) (van de Rhee *et al.*, 1994, Hammond-Kosack & Jones, 1996). These proteins play a role in protection against stress conditions and their expression has been found to be induced by pathogen attack and wounding in dicots (van de Rhee *et al.*, 1994, Hammond-Kosack & Jones, 1996). Our result therefore strengthens previous observations that the reinforcement of cell walls is part of the *V. vinifera* plant defence reaction. However, the reinforcement of cell walls would not be regarded as specific to the powdery mildew defence reaction, but rather as a general defence-related reaction.

Tattersall, van Heeswijck & Hoj, (1997) noted that although the exact role of a *V. vinifera* thaumatin-like protein (VVTL1) is unknown, the timing of its accumulation correlates with the inability of *U. necator* to initiate new infections in the berry. Van de Rhee *et al.* (1994) noted that thaumatin-like proteins cause lysis of fungal sporangia and inhibition of hyphal growth through the disruption of the fungal plasma membrane. Our results showed sequence homology with thaumatin-like proteins and the possible role of these proteins in the *V. vinifera* plant defence reaction could therefore not be ignored.

Catalase enzymes are responsible or the conversion of H_2O_2 to H_2O and O_2 (Hammond-Kosack & Jones, 1996). The production of reactive oxygen species (ROS) like H_2O_2 is often the first response activated in many incompatible reactions and are responsible for membrane damage, is directly toxic to pathogens, may contribute to structural reinforcement op plant cell walls, is essential in the production of lignin polymer precursors and increases the activity of various hydrolytic enzymes (Hammond-Kosack & Jones, 1996). In grapevine only localised cell death have been observed, therefore these ROS have to be quickly degraded to prevent damage to neighbouring cells. Catalase could be responsible for the inactivation of H_2O_2 and be involved in the reinforcement of cell walls. Catalase-related defence-reactions would also be regarded as a general defence-related reaction, and not as a reaction specific to infection by powdery mildew.

Ferredoxin reductases are electron carriers involved in photosynthesis and other metabolic pathways. Loulakakis & Roubelakis-Angelakis (1997) isolated a ferredoxin-dependant glutamate synthase, an enzyme with a central role in the assimilation of ammonia in higher

Stellenbosch University https://scholar.sun.ac.za

plants, from *V. vinifera*. Apart for the accumulation of ammonia levels that may be toxic to the pathogen, accumulation of ammonia at toxic levels can also cause plants cells to die and thereby stops fungal colonisation and infection of adjacent cells or tissues. In general, ferredoxin-dependant enzymes could have the same function as the NADPH oxidase complex in the plant defence response (Bent, 1996, Hammond-Kosack & Jones, 1996). The NADPH oxidase complex is responsible for the production of O_2^- that is used for the production of ROS (Hammond-Kosack & Jones, 1996). Once again, this could be regarded as a general defence-related response and not specific to powdery mildew infection.

Peroxidase enzymes are responsible for the cross-linking of cell walls, lignification, wound healing, phenol oxidation and pathogen defence (Lagrimini *et al.,* 1993). Although homology search data did not detect significant alignment with known peroxidases, it is known that various peroxidase isozymes exist in both monocots and dicots and are derived from a large peroxidase gene family. Since the reinforcement of cell walls has been detected as part of the *V. vinifera* defence response, it is very likely that the fragment isolated, represents a novel peroxidase isozyme with only short conserved domains related to the larger peroxidase isozyme family.

L. esculentum polygalacturonase sequence alignment was too insignificant to propose a function for this enzyme in *V. vinifera*. Giovannoni *et al.,* (1990) suggested that PG may play an important role in the developmentally induced susceptibility to pathogens that accompanies tomato fruit ripening.

Although several general defence-related genes were isolated during this study, it was disappointing that no genes, specifically activated by powdery mildew infection, were isolated. However, several novel differentially expressed fragments were isolated and might represent novel and important links in the grapevine defence response.

Stellenbosch University https://scholar.sun.ac.za

CHAPTER FIVE

5 CONCLUSIONS

The objective of this study was to identify and isolate defence-related genes that play an important role in the grapevine defence reaction, and in particular against powdery mildew. To achieve this goal, a starting point was to identify differentially expressed mRNA species in response to powdery mildew infection. DD-PCR was selected as the most suitable technique to achieve this objective. The aim of this study was multiple. In our hands, the isolation of RNA from grapevine leaves was problematic and needed to be optimised. The second objective was to develop and optimise the DD-PCR to suit our working conditions. The next objective was then to use DD-PCR to identify and analyse differentially expressed fragments and to use the data obtained to investigate the grapevine defence-reaction when infected by powdery mildew.

The optimisation of an RNA isolation protocol was successful and yielded good quality RNA suitable to use in downstream applications. Optimisation of DD-PCR was achieved under our lab conditions, but we experienced similar frustrations as described in literature. These frustrations could mainly be attributed to the design of the technique and included a high percentage of false positives obtained with reverse northern blot analysis, production of more than one, similarly-sized fragments during re-amplification and the limited usefulness of information derived from differentially expressed fragments. PAGE and autoradiography visualised approximately 8000 amplified fragments using 24 arbitrary and three one-based anchored primers. This corresponds to approximately half (52%) of the total number of expressed mRNA species in eukaryotic cells. Thirty fragments were identified as differentially expressed, but only twenty-five yielded visible fragments during re-amplification reactions.

Sequence results showed that twenty fragments contained very little background and could be directly used for sequence alignment. Three fragments contained some background and it was necessary to manually interpret sequence data. Sequence results showed that two fragments were actually mixes of similarly-sized fragments and was omitted from the study.

After submission to Genbank, EMBL and DDBJ databases using the Blast search program, four sequenced fragments showed significant alignment with genes that might play an

Stellenbosch University https://scholar.sun.ac.za

important role in the plant defence reaction (*V. vinifera* proline rich proteins 1 & 2, thaumatin-like proteins, *Z. mays* ferredoxin III, *C. sativus* and *B. napus* catalase). Two fragments showed non significant alignments with possible defence-related genes (*A. thaliana* peroxidase and *L. esculentum* polygalacturonase) and one fragment showed significant homology with a fragment that is not defence-related. Various sequences showed no significant alignment with any known gene sequence.

Reverse northern blot analysis yielded fourteen positive signals from probes originating from the infected RNA and seven signals from probes originating from the uninfected RNA. Five false positive signals (positive signals obtained from infected and uninfected RNA) were obtained. Two positive signals were only present in the uninfected mRNA and were also regarded as false positives. The high rate of false positives (50%) obtained from our reverse northern blot analysis confirm complaints raised by various researchers in literature (Callard *et al.*, 1994, Zhang, *et al.*, 1996). Seven differentially expressed fragments (28%) failed to produce any detectable signal with reverse northern blot analysis. This failure of reverse northern blot probes to produce signals could be technical. For instance, it could be due to shorter labelled probes (e.g. fragment 20) and to high stringency conditions during hybridisation and washing. It is also possible that the products matched low level mRNA's beneath the detection limit of the reverse northern blot analysis (Sunday, 1995).

Our study strengthens previous results concerning the grapevine defence reaction in response to powdery mildew infection. We observed reactions that are involved in the reinforcement of cell walls (peroxidase, catalase and proline rich proteins), localised cell death (ferredoxin reductases and catalase) and the production of antifungal compounds (thaumatin-like proteins). It was disappointing that no differentially expressed fragment showed significant homology with PR proteins like glucanase and chitinase. It is possible that these genes were expressed, but not at DD-PCR detectable levels.

Although several general defence-related genes were isolated during this study, it was disappointing that no genes, specifically activated by powdery mildew infection, were isolated. However, several novel differentially expressed fragments were isolated and might represent novel and important links in the grapevine defence response. Our hypothesis, that simmilar defence related reactions will be activated during the grapevine defence reaction was therefore, partially proven. Thorough characterisation and determination of the molecular function of novel sequences would help prove our hypothesis.

Stellenbosch University https://scholar.sun.ac.za

The isolation of full-length fragments and gene characterisation of these unknown sequences would be the next step in this process. Several molecular techniques are available for this purpose. As mentioned previously, the 5' RACE technique offers the possibility to obtain full-length genes from differentially displayed amplification products. Specific primers can be synthesised from sequence data and be used to amplify full-length fragments of interest. Isolated fragments can be used as probes in Southern blot analysis to isolate full-length fragments. When full-length fragments have been obtained, sequence data of these fragments can be used to search for significant alignment with known genes in sequence databases. Novel genes should then be characterised by transformation and expression in tobacco to determine their metabolic function. This may even lead to the incorporation of these genes into important grapevine cultivars and thereby increase their level of natural resistance to powdery mildew and other fungal diseases.

Stellenbosch University https://scholar.sun.ac.za

REFERENCES

Agrios, G.N. 1988. How plants defend themselves against pathogens. pp 97-113 in: Agrios, G.N (ed.) *Plant Pathology.* Academic Press, California.

Alleweldt, G. and Possingham, J.V. 1988. Progress in grapevine breeding. *Theoretical Applied Genetics* 75: 669-673.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J. S., Zhang, Z., Miller, W. and Lipman, D.J. 1997. "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402.

Amati, A., Piva, A., Castellari, M. and Arfelli, G. 1996. Preliminary studies on the effect of *Oidium tuckeri* on the phenolic composition of grapes and wines. *Vitis* 35: 149-150.

Ambion Technical Manual. 1998. *Http://www. ambion.com/RNA/html/working.html*

An, G., Luo, G., Veltri, R.W. and O'Hara, S.M. 1996. Sensitive, nonradioactive differential display method using chemiluminescent detection. *BioTechniques* 20: 342-346.

Anonymous 1, 1998. Product usage tables-Fungisides. *Annual report-* DOS marketing, Pretoria.

Anonymous 2, 1998. Macro-economic impact of the wine industry on the western cape. *Study done by Conningarth consultants.*

Anonymous 3, 1998. Gross value of agricultural production. *Abstracts of Agricultural Statistics.* National Department of Agriculture.

Anonymous 4, 1998. *Suid-Afrikaanse wynbedryf statistiek* 22: 1-26.

Appel, M., Bellstedt, D.U. and Gresshoff, P.M., 1999. Differential display of eukaryotic mRNA: meeting the demands of the new millennium? *Journal of Plant Physiology* (in review).

Stellenbosch University https://scholar.sun.ac.za

Archer, E., 1981. Classification, anatomy and morphology. Burger, J. and Deist, J., (eds.) pp 1-115 in: *Wingerdbou in Suider-Afrika.* Trio Rand S.A., Litho N'dabeni.

Bauer, D., Warthoe, P., Rhode, M. and Strauss, M. 1994. Detection and identification of expressed genes by differential display. *PCR-Methods Applied* 1994 edition: 421-437.

Bauer, D., Müller, H., Reich, J., Riedel, H., Ahrenkiel, V., Warthoe, P. and Strauss, M. 1993. Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). *Nucleic Acids Research* 21: 4272-4280.

Benito, E.P., Prins, T. and van Kan, J.A.L. 1996. Application of differential display RT-PCR to the analysis of gene expression in a plant-fungus interaction. *Plant Molecular Biology* 32: 947-957.

Bent, A.F. 1996. Plant disease resistance genes: Function meets structure. *The Plant Cell* 8: 1757-1771.

Bertioli, D., Schlichter, U.H.A., Adams, M.J., Burrows, P.R., Steinbiss, H.-H. and Antoniw, J.F. 1995. An analysis of differential display shows a strong bias towards high copy number mRNAs. *Nucleic Acids Research* 23: 4520-4523.

Blaich, R., Heintz, C. and Wind, R. 1989. Studies on conidial germination and initial growth of the grapevine powdery mildew *Uncinula necator* on artificial substrates. *Applied Microbiology* and *Biotechnology* 30: 415-421.

Blaich, R. and Wind, R. 1989. Inducible silica incrusts in cell walls of *Vitis* leaves. *Vitis* 28: 73-80.

Botta, R., Schneider, A., Akkak, A., Scott, N.S. and Thomas, M.R. 1998. Within cultivar variability studied by morphometrical and molecular marker based techniques. *Abstract-7th International Symposium on Grapevine Genetics and Breeding.*

Broglie, R. and Broglie, K. 1993. Chitinases and plant protection. pp 411-421 in: Fritig, B. and Legrand, M. (eds.) *Mechanisms of Plant Defence Responses.* Kluwer Academic Publishers, Netherland.

Stellenbosch University https://scholar.sun.ac.za

Bulit, J. and Lafon, R. 1978. Powdery Mildew of the vine. pp 525-548 in: D.M. Spencer (ed.) *The Powdery Mildews.* Academic Press, New York.

Burger, P. and Goussard, P.G. 1996. *In vitro* culture of ovules and embryo's from seedless grapes (*Vitis vinifera L.*). *S. Afr. J. Enol. Vitic* 17: 31-37.

Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F. and Schulze-Lefert, P. 1997. The Barley *Mlo* Gene: A novel control element of plant pathogen resistance. *Cell* 88: 695-705.

Callard, D., Lescure, B. and Mazzolini, L. 1994. A method for the elimination of false positives generated by the mRNA differential display technique. *BioTechniques* 16: 1096-1103.

Chellemi, D.O. and Marois, J.J. 1991. Effect of fungicides and water on sporulation of *Uncinula necator. Plant Disease* 74: 455-457.

Clingeleffer, P.R. 1985. Breeding table grape varieties. *The Australian Grapegrower & Winemaker* (April edition): 117-119.

Clingeleffer, P.R. and Scott, N.S. 1994. Integration of molecular techniques and conventional breeding. *Abstract*: *6th International symposium on Grape Breeding.*

Collinge, D.B. and Slusarenko, A.J. 1987. Plant gene expression in response to pathogens. *Plant Molecular Biology* 9: 389-410.

Cotesi, P., Gadoury, D.M., Seem, R.C. and Pearson, R.C. 1995. Distribution and retention of cleistothecia of *Uncinula necator* on the Bark of Grapevines. *Plant Disease* 79: 15-19.

Dalbó, M.A., Ye, G.-N., Weeden, N.F. and Reisch, B.I. 1998. QTL analysis of disease resistance in interspecific hybrid grapes. *Abstract-7th International symposium on Grapevine Genetics and Breeding.*

Daly, J.M. 1984. The role of recognition in plant disease. *Annual Review of Phytopathology* 22: 273-307.

Stellenbosch University https://scholar.sun.ac.za

Davies, C. and Robinson, S.P. 1996. Sugar accumulation in grape berries. Cloning of two putative vacuolar invertase cDNAs and their expression in grapevine tissues. *Plant Physiology* 111: 275-283.

Délye, D., Laigret, F. and Corio-Costet, M-F. 1997. RAPD analysis proves insight into the biology and epidemiology of *Uncinula necator. Phytopathology* 87: 670-677.

Dilip, J.E., Marque, J., Whassam, P.-E. and Porter, C. 1995. Transgenic plants and disease resistance: Important considerations. *Canadian Plant Journal* 3: 214-227.

Dixon, R.A., Harrison, M.J., Lamb, C.J. 1994. Early events in the activation of plant defence responses. *Annual Review of Phytopathology* 32: 479-501.

Dixon, R.A. and Lamb, C.J. 1990. Molecular communication in interactions between plants and microbial pathogens. *Annual Review of Plant Physiology and Molecular Biology* 41: 339-367.

Doster, M.A. and Schnathorst, W.C. 1985. Comparative susceptibility of various grapevine cultivars to the powdery mildew fungus *Uncinula necator. American Journal of Enology and Viticulture* 36: 101-104.

Dye, M.H. and Hammett, K.R.W. 1976. Effects on fruit yield and on powdery mildew infection of fungicide and bactericide sprays applied to grapes during flowering. *N.Z. Journal of Experimental Agriculture* 5: 63-65.

Eibach, R. 1994. Investigation about the genetic resources of grapes with regard to resistance characteristics to powdery mildew (*Oidium tuckeri*). *Vitis* 33: 143-150.

Einset, J. and Pratt, C. 1975. Grapes. pp 130-148 in: Janick, J. and Moore, J.N. (eds.) *Advances in Fruit Breeding*. Purdue University Press, West Lafayette, Indiana.

Ellingboe, A.H. 1995. Genic Interactions in Hosts and Pathogens. pp 34-82 in: (Mills, D., Kunoh, H., Keen, H.T. and Mayama, S. (eds.) *Molecular aspects of pathogenicity and resistance: Requirement for signal transduction.* APS Press, St. Paul.

Evans, J.K., Whisson, D.L. and Scott, E.S. 1996. An experimental system of characterising isolates of *Uncinula necator. Mycological Research* 100: 675-680.

Stellenbosch University https://scholar.sun.ac.za

Ferreira, J.H.S. and Venter, E. 1996. Grapevine Diseases and Pests. LNR Infruitec/Nietvoorbij Institutue.

Floris, E. and Alvarez, J.M. 1996. Nature of resistance of seven melon lines to *Spaerotheca fuliginea*. *Plant Pathology* 45: 155-160.

Frohman, M.A., Dush, M.K. and Martin, G.R. 1994. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* 85: 8998-9002.

Gadoury, D. M. and Pearson, R.C. 1988. Initiation, development, dispersal and survival of cleistothecia of *Uncinula necator* in New York Vineyards. *Phytopathology* 78: 1413-1421.

Gadoury, D.M. and Pearson, R.C. 1990(a). Germination of Ascospores and Infection of *Vits* by *Uncinula necator. Phytopathology* 80: 1298-1203.

Gadoury, D.M. and Pearson, R.C. 1990(b). Ascocarp dehiscence and ascospore discharge in *Uncinula necator. Phytopathology* 80: 393-401.

Gadoury, D. M. and Pearson, R.C. 1991. Heterothallism and pathogenic specialisation in *Uncinula necator. Phytopathology* 81: 1287-1293.

Galindo, J.E., Poirier, G.M.-C., Guo, P., Huvar, A., Wagaman, P.C., Zhu, J., Tench, J., Wan, J.S. and Erlander, M.G. 1998. Differential display of RNA. pp 225-236 in: Caetano-Anollés and G., Gresshoff, P.M. (eds.) *DNA markers: protocols, applications and overviews.* Wiley-Liss, New York.

Gebhardt, C. 1997. Plant genes for pathogen resistance-variation on a theme. *Trends in Plant Science* 2: 243-244.

Stellenbosch University https://scholar.sun.ac.za

GenHunter Corporation. 1998. Your complete source for differential display: *1998/1999 catalouge*. GenHunter Corporation, Nashville.

Giovannoni, J.J., DellaPenna, D., Lashbrook, C.C., Bennet, A.B. and Fischer, R.L. 1990. Expression of a chimeric polygalacturonase gene in transgene rin (ripening inhibitor) tomato fruit. pp 217-227 in: Bennet A.B. and O'Niel, S.D. (eds.) *Horticultural Biotechnology*. Wiley-Liss, Inc., New York.

Goormachtig, S., Valerio-Lepiniec, M., Szczyglowski, K., van Montagu, M., Holsters, M. and de Bruijn, F.J. 1995. Use of differential display to identify novel *Sesbania rostratra* genes enhanced by *Azorhizobium caulinodans* infection. *Mol. Plant-Microbe Interact.* 8: 816-824.

Graf, D., Fisher, A.G. and Merkenschlager, M. 1997. Rational primer design greatly improves differential display-PCR (DD-PCR). *Nucleic Acids Research* 25: 2239-2240.

Gray, J.D. and Meredith, C.P. 1992. Grape. pp 229-261 in: Hammerschlag, F.A. and Litz, R.E. (eds.) *Biotechnology in Agriculture* (Series #8): *Biotechnology of Perennial Fruit Crops*. CAB International Press, L.A.

Guimarães, M.J., Lee, F., Zlotnik, A. and McClanahan, T. 1995. Differential display by PCR: Novel findings and applications. *Nucleic Acids Research* 23: 1832-1833.

Haag, E. and Raman, V. 1994. Effects of primer choice and source of *Taq* DNA polymerase on the banding patterns of differential display RT-PCR. *BioTechniques* 17: 226-228.

Hadman, M., Adam, B.-L., Wright, G.L. and Bos., T.J. 1995. Modifications to the differential display technique reduce background and increase sensitivity. *Analytical Biochemistry* 22: 383-386.

Hagen, H.-E., Kläger, S.L., Chan, V., Sakanari, J.A., McKerrow, J.H. and Ham, P.J. 1995. *Simulium damnosum* s.l.: Identification of inducible serine proteases following an *Onchocera* infection by differential display reverse transcription PCR. *Experimental Parasitology* 81: 249-254.

Stellenbosch University https://scholar.sun.ac.za

Hammond-Kosack, K.E., Jones, D.A. and Jones, D.G. 1996. Ensnaring microbes: the components of plant disease resistance. *New Phytopathology* 133: 11-24.

Hammond-Kosack, K.E. and Jones, D.G. 1995. Plant disease resistance genes: unravelling how they work. *Canadian Journal of Botany* 73: 495-505.

Hammond-Kosack, K.E. and Jones, D.G. 1996. Resistance gene-dependent plant defence responses. *The Plant Cell* 8: 1773-1791.

Heintz, C. 1986. Infection mechanisms of grapevine powdery mildew (*Oidium tuckeri*): Comparative studies of the penetration process on artificial membranes and leaf epidermis. *Vitis* 25: 215-225.

Heintz, C. and Blaich, R. 1989. Structural characters of epidermal cell walls and resistance to powdery mildew of different grapevine cultivars. *Vitis* 28: 153-160.

Heintz, C. and Blaich, R. 1990. Ultrastructural and histochemical studies on interactions between *Vitis vinifera* L. and *Unicinula necator* (Schw.) Burr. *New Phythology* 155: 107-117.

Hunt, A.G. 1994. Messenger RNA3' end formation in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 45: 47-60.

Huges, D., Hands, P. and Kench, J. 1992. A Historical prespective. pp 11-22 in: Jones, J.P. and Hunt, G. *South African Wine*. Struik Publishing Group (Pty) Ltd.

Ikeda, S., Nasrallah, J.B., Dixit, R., Preiss, S. and Nasrallah, M.E., 1997. An aquaporin-like gene required for the *Brassica* self-incompatibility response. *Science* 276: 1564-1566.

Jones, J.D.G. 1996. Plant disease resistance genes: Structure, function and evolution. *Current Opinion in Biotechnology* 7: 155-160.

Keen, N.T. 1990. Gene-for-Gene complementarity in plant-pathogen interactions. *Annu. Rev. Genet.* 24: 447-463.

Keen, N.T. 1992. The molecular biology of disease resistance. *Plant Molecular Biology* 19: 109-122.

Stellenbosch University https://scholar.sun.ac.za

Keen, N.T. 1997. Elicitor generation and receipt-the mail gets through, but how? pp 379-389 in: Crute, I.R., Holub, E.B. and Burdon, J.J. (eds.) *The Gene-for-Gene Relationships in Plant-Parasite Interactions.* CAB International, New York.

Khushbeer, M., Foltz, L., Mahoney, W.C. and Schueler, P.A. 1998. Interaction and effect of annealing temperature on primers used in differential display RT-PCR. *Nucleic Acids Research* 26: 854-856.

Kieser, A., Goodnight, J., Kölch, W., Mischak, H. and Mushinski, J.F. 1995. Identification of the primary growth response gene ST2/T1, as a gene whose expression is differentially regulated by different protein kinase C isozymes. *FEBS Letters* 372: 189-193.

Knogge, W. 1996(a). Molecular basis of specificity in host/fungus interactions. *European Journal of Plant Pathology* 102: 807-816.

Knogge, W. 1996(b). Fungal infection of plants. *The Plant Cell* 8: 1711-1722.

Krul, W.R. and Mowbray, G.H. 1978. Grapes. pp 396-433 in: Sharp, Evans, Ammirato and Yamad (eds.) *Handbook of plant cell culture: Crop species.* McGraw-Hill, New York.

Lagrimini, M.L., Vaughn, J., Erb, W.A. and Miller, S.A. 1993. Peroxidase overproduction in tomato: Wound-induced polyphenol deposition and disease resistance. *HortScience* 28: 218-221.

Lamb, C.J. Lawton, M.A., Dron, M. and Dixon, R.A. 1989. Signals and transduction mechanisms for activation of plant defences against microbial attack. *Cell* 56: 215-224.

Leister, D., Ballvora, A., Salamini, F. and Gebhardt, C. 1996. A PCR-based approach for isolating pathogen resistance genes form potato with potential for wide application in plants. *Nature Genetics* 14: 421-429.

Levi, A., Galau, G. and Wetzstein, H. 1992. A rapid procedure for the isolation of RNA from high-phenolic-containing tissues of peach. *HortScience* 27: 1316-1318.

Stellenbosch University https://scholar.sun.ac.za

Li, H. 1993. Studies on the resistance of grapevine to powdery mildew. *Plant Pathology* 42: 792-766.

Liang, P, Averboukh, L. and Pardee, A.B. 1993. Distribution and cloning of eukaryotic mRNAs by means of differential display, refinements and optimisation. *Nucleic Acids Research* 21: 3269-3275.

Liang, P. and Pardee, A.B. 1995. Recent advances in differential display. *Curr. Opin. Immunol.* 7: 247-280.

Liang, P. and Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257: 957-971.

Liang, P., Zhu, W., Zhang, X., Guo, Z., O'Connel, P., Averboukh, L., Wang, F. and Pardee, A.B. 1994. Differential display using one-base anchored oligo-dT primers. *Nucleic Acids Research* 25: 5763-5764.

Linskens, M.H.K., Feng, J., Andrews, W.H., Enlow, B.E., Saati, S.M., Tonkin, A.L., Funk, W.D. and Villeponteau, B. 1995. Cataloging altered gene expression in young and senescent cells using enhanced differential display. *Nucleic Acids Research* 23: 3244-3251.

Lodhi, M.A., Daly, M.J., Ye, G.-N., Weeden, N.F. and Reisch, B.I. 1995. A molecular marker based linkage map of *Vitis*. *Genome* 38: 786-794.

Logemann, J., Schell J. and Willmitzer, L. 1987. Improved method for the isolation of RNA from plant tissues. *Analytical Biochemistry* 163: 16-20.

Lohmann, J., Schickle, H. and Bosch, T.C.G. 1995. REN display, a rapid and efficient method for nonradioactive differential display amplification in thermal cycling. *BioTechniques* 20: 342-346.

London, L. and Myers, J. 1995(a). Agrichemical usage in the southern region of South Africa. *South African Journal of Science* 91: 509-514.

Stellenbosch University https://scholar.sun.ac.za

London, L. and Myers, J. 1995(b). Agrichemical usage patterns and workplace exposure in the major farming sectors in the southern region of South Africa. *South African Journal of Science* 91: 515-522.

Loulakakis, K.A. and Roubelakis-Angelakis, K.A., 1997. Molecular cloning and characterization of cDNAs coding for ferredoxin-dependent glutamate synthase from *Vitis vinifera*. *Physiologia Plantarum* 101: 220-228.

Luehrsen, K.R., Marr, L.L., van der Knaap, E. and Cumberledge, S. 1997. Analysis of differential display RT-PCR products using fluorescent primers and genescan™ Software. *BioTechniques* 22: 168-174.

MacGregor, A. 1995. Maintaining the competitive edge-avoiding unacceptable pesticide residues in table grapes. *4th Australian Table Grape Industry Technical Conference.*

Malossini, U., Grando, M.S., Roncador, I. and Mattivi, F. 1998. Parentage analysis and characterisation of some Italian *Vitis vinifera* L. crosses. *Abstract-7th International symposium on Grapevine Genetics and Breeding.*

Matern, U., Grimming, B. and Kneusel, R.E. 1995. Plant cell wall reinforcement in the disease-resistance response: molecular composition and regulation. *Canadian Journal of Botany* 73: 511-517.

Meredith, C.P., Bowers, J.E., Siret, R., This, P. and Boursiquot, J.M. 1998. A single pair of parents proposed for a group of varieties in North-eastern France. *Abstract-7th International symposium on Grapevine Genetics and Breeding.*

Mortensen, J.A., 1968. The inheritance of resistance to Pierce's diseases in *Vitis. Journal of the American Society for Horticultural Science* 95: 331-337.

Mou, L., Miller, H., Li, J., Wang, E. and Chalifour, L. 1994. Improvements to the differential display method for gene analysis. *Biochemical and Biophysical Research Communications* 199: 564-569.

Mourgues, F. Bisset, M.-N. and Chevreau, E. 1998. Strategies to improve plant resistance to bacterial diseases through genetic engineering. *TibTech* 16: 203-210.

Stellenbosch University https://scholar.sun.ac.za

Ohan, N.W. and Heikkila, J.J. 1993. Reverse transcription-polymerase chain reaction: An overview of the technique and its applications. *Biotech. Adv.* 11: 13-29.

Olmo, H.P. 1976. Grapes. pp 294-298 in: Simmons, N.W. (ed.) *Evolution of Crop Plants.* Longman, Londen.

Olmo, H.P. 1986. The potential role of (*vinifera* x *rutundifolia*) hybrids in grape variety improvement. *Experimenta* 42: 921-926.

Ough, C.S. and Berg, H.W. 1979. Powdery Mildew sensory effect on wine. *American Journal of Enology and Viticulture* 30: 321-326.

Pearson, R.C., and Gadoury, D.M. 1992. Powdery Mildew of Grape. Chaube, Siroh and Mukhopadhayay (eds.) in: *Plant Diseases of International importance.* Vol II *Diseases of fruit crops.* New Jersey, New York.

Pearson, R.C., Gärtel, W. 1985. Occurrence of Hyphae of *Uncinula necator* in Buds of Grapevine. *Plant Disease* 69: 149-151.

Pearson, R.C. and Taschenberg, E.F. 1980. Benomyl-resistant strains of *Uncinula necator* on grapes. *Plant Disease*: 64: 677-680.

Pool, R.M., Pearson, R.C., Weiser, M.J., Lakso, A.N. and Seem, R.C. 1984. Influence of powdery mildew on yield and growth of Rosette grapevines. *Plant Disease* 68: 590-593.

Pratt, C., Goffinet, M.C., Welser, M.J., Pearson, C.R. 1984. Powdery mildew of *Vitis*: Papillae (wall appositions) as a host response to infection. *Vitis* 23: 225-229.

Pratt, C., Einset, J. 1961. Grapes. pp 130-164 in: Janick, J. and Moore, J.N. (eds.) *Advances in Fruit Breeding.* Purdue University Press, West Lafayette, Indiana.

Promega Technical Manual, 1997. DNA Silver Staining System, pp 1-12.

Rathejen, A.H. and Robinson, S.P. 1992. Aberrant processing of polyphenol oxidase in a variegated grapevine mutant. *Plant Physiology* 99: 1619-1625.

Stellenbosch University https://scholar.sun.ac.za

Reifschneider, F.J.B., Boiteux, L.S. 1988. A vacuum-operated settling tower for inoculation of powdery mildew fungi. *Phytopathology* 78: 1463-1265.

Reisch, B.I. 1998. Molecular markers-the foundation for grapevine genetic mapping, DNA fingerprinting and genomics. *Abstract-7th International Symposium on Grapevine Genetics and Breeding.*

Rezaian, M.A. and Krake, L.R. 1987. Nucleic acid extraction and virus detection in grapevine. *Journal of Virological Methods* 17: 277-285.

Rompf, R. and Günter, K. 1997. mRNA Differential display in agarose gels. *BioTechniques* 23: 28-32.

Ross, R., Kumpf, K. and Reske-Kunz, A.B. 1997. PCR-Amplified cDNA probes for verification of differentially expressed genes. *BioTechniques* 22: 894-897.

Rothschild, C.B., Brewer, C.S. and Bowden, D.W. 1997. DD/AP-PCR: Combination of differential display and arbitrarily primed PCR of Oligo(dT) cDNA. *Analytical Biochemistry* 245: 48-54.

Schneibderbauer, A., Sandermann, H. and Ernst, D. 1991. Isolation of functional RNA from plant tissues rich in phenolic compounds. *Analytical Biochemistry* 197: 91-95.

Sharma, Y.K. and Davis, K.R. 1995. Isolation of a novel *Arabidopsis* ozone-induced cDNA by differential display. *Plant Molecular Biology* 29: 91-98.

Somerville, S. 1998. Molecular mechanisms of Host-Pathogen interactions. *Http://plantbiology.stanford.edu/plantbiology.html/shauna.html.*

Staskawicz, J.B., Ausubel, M.F., Baker, B.J., Ellis, J.G. and Jones, J.D. 1995. Molecular Genetics of Plant Disease Resistance. *Science* 268: 661-667.

Staub, U., Polivka, H. and Gross, H.J. 1995. Two rapid microscale procedures for isolation of total RNA from leaves rich in polyphenols and polysaccharides: Application for sensitive detection of grapevine viroids. *Journal of Virological Methods* 52: 209-218.

Stellenbosch University https://scholar.sun.ac.za

Staudt, G. 1997. Evaluation of resistance to grapevine powdery mildew (*Uncinula necator* [Schw.] Burr., anamorph *Oidium tuckeri* Berk.) in accessions of *Vitis* species. *Vitis* 36: 151-154.

Sticher, L., Mauch-Mani, B. and Métraux, J.P. 1997. Systemic acquired resistance. *Annual Review of Phytopathology* 35: 235-270.

Stout, A.B. 1936. Breeding for hardly seedless grapes. *Proceedings of the American Society for Horticultural Science* 34: 416-420.

Sunday, M.E. 1995. Differential display RT-PCR for identifying novel gene expression in the lung. *Am. J. Physiol.* 269: 273-284.

Tawe, W.N., Eschbach, M.-L., Walter, R.D. and Henkle-Dührsen, K. 1998. Identification of stress-responsive genes in *Caenorhabditis elegans* using RT-PCR differential display. *Nucleic Acids Research* 26: 1621-1627.

Tattersall, D.B., van Heeswijck, R. and Hoj, P.B. 1997. Identification and characterisation of a fruit-specific Thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. *Plant Physiology* 114: 759-769.

This, P., Lahogue, F., Adam-Blondon, A.F., Doligez, A. and Bouquet, A. 1998. Towards marker assisted selection of seedlessness in grapevine. *Abstract-7th International symposium on Grapevine Genetics and Breeding.*

Tokuyama, Y. and Takeda, J. 1995. Use of ^{33}P-Labeled primer increases the sensitivity and specificity of mRNA differential display. *BioTechniques* 18: 424-425.

Truesdell, G.M. and Dickman, M.B. 1997. Isolation of pathogen/stress-inducible cDNAs from alfalfa by mRNA differential display. *Plant Molecular Biology* 33: 737-743.

van de Rhee, M.D., Linthorst, H.J.M. and Bol, J.F. 1994. Pathogen-induced gene expression. pp 249-283 in: Amarjit, S.B. (ed.) *Stress-induced gene expression in plants.* Harwood Academic Publishers, Switzerland.

Wallace, D.M. 1987. Precipitation of Nucleic Acids. *Methods of Enzymology* 152: 41-46.

Stellenbosch University https://scholar.sun.ac.za

Wang, X., Li, X. and Feuerstein, G.Z. 1997. Use of novel downstream primers for differential display RT-PCR. *BioTechniques* 24: 382-386.

Wang, X., Li, X., Feuerstein, G.Z. 1995. Direct Sequencing of DNA isolated from mRNA differential display. *BioTechniques* 18: 448-452.

Wang, Y., Liu, Y., He, P., Chen, J., Lamikanra, O. and Lu, J. 1995. Evaluation of foliar resistance to *Uncinula necator* in Chinese wild *Vitis* species. *Vitis* 34: 159-164.

Warthoe, P., Bauer, D., Rohde, M. and Strauss, M. 1994. Detection and identification of expressed genes by differential display. *Current Protocols,* 1994 edition.

Weeden, N.F., Hemmat, M., Lawson, D.M., Lodhi, M., Bell, R.L. and Manganaris, A.G. 1994. Development and application of molecular marker linkage maps in woody fruit crops. pp 269-273 in: Schmidt, H. and Kellerhals, M. (eds.) *Progress in Temperate Fruit Breeding.* Kluwer Academic Publishers, Netherland.

Wilkinson, J.Q., Lanahan, M.B., Conner, T.W. and Klee, H.J. 1995. Identification of mRNAs with enhanced expression in ripening strawberry fruit using polymerase chain reaction differential display. *Plant Molecular Biology* 27: 1097-1108.

Wynn, W.K. 1981. Tropic and taxic responses of pathogens to plants. *Annual Review of Phytopathology* 19: 237-251.

Zhang, H., Zhang, R. and Liang, P. 1996. Differential screening of gene expression difference enriched by differential display. *Nucleic Acids Research* 24: 2454-2455.