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ANALYSIS OF GENES DIFFERENTIALLY EXPRESSED IN FUERTE AVOCADO FRUIT IN RESPONSE TO *COLLETOTRICHUM GLOEOSPORIOIDES* INFECTION

ARNAUD THIERRY DJAMI TCHATCHOU

A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the Degree of **DOCTOR OF PHILOSOPHY**

May, 2012

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university



30 May 2012

Arnaud Thierry Djami Tchatchou Date

ABSTRACT

The anthracnose pathogen, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., is a major cause of disease in the avocado industry, causing significant economic losses, and infects all cultivars. In South Africa, Fuerte and Hass varieties are the most widely grown. Identification of genes differentially expressed in avocado during infection with the fungus represents an important step towards understanding the plant's defence responses and would assist in designing appropriate intervention strategies. In this study, 454 sequencing and analysis of the transcriptome of infected Fuerte avocado fruits were performed using the Roche 454 GS FLX Titanium platform. cDNA libraries enriched for differentially expressed genes were constructed from unharvested and harvested avocado fruit tissues collected after 1, 4 and 24 h post-infection and after 3, 4, 5 and 7 day post-infection, then sequenced.The expression profiles of the genes expressed were measured by a hierarchical clustering algorithm.Subsequently, quantitative real-time PCR was employed to measure the expression of some candidate resistance genes to anthracnose disease and to validate the sequencing results. The single sequencing run produced 215 781 reads from the transcriptome. A total of 70.6 MB of sequence data was generated and subjected to BLAST searches of which about 1500 genes encoding proteins predicted to function in signal transduction, transcriptional control, metabolism, defence, stress response, transportation processes and some genes with unknown functions were identified. The expression profiles studies showed that many expressed genes were either up or down regulated after infection in avocado fruits when compared to the uninfected sample. Salicylic acid and ethylene were identified to be involved in the signalling networks activated in avocado fruit during *C. gloeosporioides* infection. This study showed that avocado is able to respond to *C. gloeosporioides* infection by exhibiting a sophisticated molecular system for pathogen recognition and by activating structural and biochemical defence mechanisms.

DEDICATION

I am glad to dedicate this thesis to the Father Lord GOD who gave me life, strength, knowledge, encouragement, his grace, his favour, his blessings and who allowed this work to be done. May all the glory be given to him for this achievement in the name of my Loving, Lord and Saviour JESUS-CHRIST.

ACKNOWLEDGEMENTS

I would like to take this opportunity to express my deepest gratitude to some precious people who have been immensely supportive during the course of completing this research project.

- First and foremost to my supervisor, Professor Colin J. Straker who gave me the opportunity to leave my country Cameroon to join his research team in order to pursue my PhD studies under his invaluable guidance and for his unstinting financial support, his always welcoming attitude for discussion, his advice, enthusiasm and expertise throughout the course of this study. I am very grateful for the opportunity he granted me to travel abroad and present my work during the VII World Avocado Congress.

- To Prof Chrissie Rey for all her technical assistance and advice.

- To Dr Neolani van den Berg and Mr Mahomed Waheed from FABI , University of Pretoria for their collaboration and technical advice

- To Mrs. Farhahna Allie (Fluffy) my friend and colleague for her technical assistance and contribution to my learning in molecular biology.

- To Mr. Mthuthuzeli Zamxaka, Dr Joseph Mafofo, Mr Marimani Musa, Dr Hamilton for their assistance and guidance.

- To Mr. and Mrs. Taylor the owner of Roodewal farm (Nelspruit) for providing fresh avocado fruits used in this study.

- Special thanks to the National Research Foundation, South Africa (ICD2007052500003: 2008-2011), the University of the Witwatersrand Postgraduate Merit Award, the Bradlow Award and the Oppenheimer Memorial Trust for their financial support**.**

- To Dr. Neossi Serge for his love, support and who was the first person to encourage me to pursue my studies in South Africa and who did all the follow up during my application process.

- To my lovely mother Mrs. Nfomen Tchatchou Henriette for her love, her sacrifices and who was always there for me. May this achievement be a source a great joy to you and a thanks giving to our Lord Jesus-Christ.

- To my precious grand mother Mama Magni Lucienne who took care of me from the primary school until I obtained my matric which opened door to me for university. I don't have enough word to express all my gratitude for all that you have done to me and to God who kept you alive.

- To Dr. and Mrs. Kwawou for being my parents in South Africa, who treated me just as their son and for all their love, support, encouragement and for being a shoulder I could always rely on.

- To my lovely sister Christelle Djacheu, my brothers Cooper, Idriss, Dalhil for being there as a source of encouragement.

- To my family members: Mr. and Mrs. Tchantchou, Mr. and Mrs. Nzogue, Mr. and Mrs. Bayeck, Mr. and Mrs. Matip, Mr. Djapa, Mr. and Mrs. Ntime and Dr. Tiedeu Barbara for all their support.

- To all my Christian family for all their support and love throughout the duration of my studies.

- Above all, I thank God for everything and for everyone who brought a contribution for the achievement of this work. To him be all glory and honour.

PUBLICATIONS ARISING FROM THIS WORK

Peer-reviewed Journals

- A.T. Djami-Tchatchou and C. J. Straker**.** 2012. **The isolation of high quality RNA from avocado (*Persea americana* Mill.) fruit.** South African Journal of Botany, 78:44-46.

- A.T. Djami-Tchatchou, C. J. Straker and Farhana van Schalk. 2012. **454 Sequencing for the identification of genes differentially expressed in avocado fruit (cv. Fuerte) infected by C*olletotrichum gloeosporioides***. Journal of Phytopathology. doi: 10.1111/j.1439-0434.2012.01925.x

- A.T. Djami-Tchatchou and C. J. Straker**. The molecular basis of resistance in avocado to C*olletotrichum gloeosporioides*.** (Manuscript in preparation).

Conference Proceedings

A.T. Djami-Tchatchou and C. J. Straker. 2011. **Identification of genes differentially expressed in avocado fruits (cv. fuerte) infected by C*olletotrichum gloeosporioides* using Roche 454 GS FLX Titanium Platform.** Proceedings VII World Avocado Congress 2011. Cairns, Australia. 5 -9 September 2011.

Conference presentations

- A.T. Djami-Tchatchou and C. J. Straker. Identification of genes differentially expressed in avocado fruits (cv. Fuerte) infected by C*olletotrichum gloeosporioides* using Roche 454 GS FLX Titanium Platform.

Oral presentation at the VII World Avocado Congress 2011. 4-9th September 2011, Cairns, Australia.

- A.T. Djami-Tchatchou and C. J. Straker **.** Analysis of genes differentially expressed in Fuerte avocado fruit in response to *Colletotrichum gloeosporioides* infection**.**

Oral presentation at the University of the Witwatersrand, School of Molecular and Cell Biology. 25th October 2011, Johannesburg, South Africa.

- A.T. Djami-Tchatchou and C. J. Straker. 454 sequencing for the identification of genes differentially expressed in avocado fruit (cv. Fuerte) infected by C*olletotrichum gloeosporioides.*

Oral presentation at the Molecular Biosciences Research Thrust Research Day, University of the Witwatersrand.7th Dec 2011, Johannesburg, South Africa.

AWARDS

-First Prestige award received in August 2011: Awarded for the 1st publication accepted in a peer reviewed journal on the 21 April 2011 emanated from my PhD thesis by the School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa

- **Second Prestige award received in May 2012:** Awarded for the 2nd publication accepted in a peer reviewed journal on the 18 April 2012 emanated from my PhD thesis by the School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg, South Africa

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BLASTN	basic local alignment search tool (nucleotide)
BLASTX	basic local alignment search tool (protein)
bp	base pair
cDNA	complentary DNA
Ct values	crossing points
CTAB	cetyltrimethyl ammonium bromide
DEPC	diethyl pyrocarbonate;
dNTP	deoxyribonucleotide triphosphate;
DTT	dithiothreitol;
EtOH	ethanol
EST	expressed sequence tag
E value	expectation value
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
ET	ethylene
EREBP	ethylene responsive element binding protein
FW	fresh weight
HR	hypersensitive response
ISR	induced systemic resistance
JA	jasmonate
LiCl	lithium chloride
LRR	leucine rich repeat
MAPK	mitogen-activated protein kinases

NO	nitric oxide
PCR	polymerase chain reaction
PR	pathogenesis-related proteins
PVP	polyvinylpyrrolidone
qPCR	real time PCR
R	resistance
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
RT	room temperature
SA	salicylic acid
SAAGA	South African Avocado Growers Association
SABP	salicylic acid binding protein
SAR	systemic acquired resistance
SIPK	salicylic acid induce protein
ssDNA	single-stranded DNA
Tris	hydroxymethyl methylamine
v/v	volume/volume

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

Avocados (*Persea americana* Mill.) which originated in Central America and Southern Mexico (Chen *et al*., 2008) and are recognized today to be one of the most sought after food sources worldwide. They are produced on a large scale in more than 30 countries around the world and because of their appeal it is an industry that continues to grow. Avocado fruits are known throughout the world to be good for health and nutritious. In nutrition, they are pressed for oil, blended into dessert drinks, used to make guacamole and in sushi, given as wedding gifts, eaten with ice cream, added to salads, or eaten alone (IFAS, 2006). This fruit contains one to two times more protein than any other fruit, is rich in minerals such as manganese, phosphorous, iron and potassium, but is low in sodium, and also contains the vitamins niacin, vitamin E, vitamin C, ß-carotene, thiamin, riboflavin, nicotinic acid and folate (Rainey *et al*., 1994). Besides their nutritional value, avocados contribute to maintaining good cholesterol (HDL) and are effective in lowering bad blood cholesterol (LDL) (Naveh *et al.*, 2002). In cosmetics, avocado oil has great powers of penetration compared to other vegetable oils due to the fact that it conveys the vitamins and the nourishment to the glands that lie just beneath the skin. In addition, it is particularly rich, nourishing and invaluable in creams and lotions for sunburn and sensitive skin. Avocado oil has therapeutic benefit because of its healing, regenerative and moisturizing properties and is also reputed to be beneficial in reducing age spots and the healing of scars (Eyres *et al*., 2001).

Avocados are a commercially valuable crop whose trees and fruit have been the object of much research throughout the world, particularly in South Africa. For example, Sanders and Korsten (2003) undertook research to determine the virulence of *C. gloeosporioides* isolates collected during a market survey of post-harvest disease incidence on avocados and mangoes and their cross-inoculation potential with each other as well as on papayas, strawberries, peppers, guavas and citrus. Research by Prusky and co-workers have demonstrated an excellent correlation between resistance of unripe avocado fruit to *C. gloeoesporiodes* and the presence of antifungal fatty acid derivatives in the fruit peel (Prusky and Keen, 1993).

Avocado is grown in the subtropical environment where like others crops it is susceptible to many pathogen attacks such as fungi which commonly lead to important reductions in yield and quality of avocado fruit. For example, postharvest fungal diseases like anthracnose and black spot limit the shelf-life of most fruit crops due to their high susceptibility (Pernezny *et al*., 2000). In South Africa, avocado black spot (Cercospora spot) caused by *Pseudocercospora purpurea* (Cooke) is still the most serious pre-harvest disease (Boyum and Bard, 2002). The phytopathogenic fungus, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., responsible for anthracnose is the most important post-harvest pathogen which attacks a wide variety of tropical and subtropical fruits (Coate *et al.,* 1993) and causes significant economic losses in tropical, sub-tropical and temperate regions and it also reduces the shelf life of fruits during storage, transport and hampers market access (Freeman *et al*., 1998). In addition, these pathogens have the ability to infect the fruits and remain quiescent until post-harvest, when the typical symptoms develop, extending losses to merchants and

consumers (Freeman, 2000). In South Africa, these losses represent a substantial portion of avocado export revenue (Donkin and Oosthuyse, 1996).

Plant disease problems are becoming more extensive because of the development of pathogen resistance to fungicides and the withdrawal of pesticides due to environmental reasons related to pollution (Agrios, 2005). Plants have evolved their own powerful defence mechanisms to prevent and limit disease on developing fruit. These include biochemical and physical barriers to pathogen invasion, which could be constitutive or inducible in nature (Agrios, 2005). Consequently, there is a great interest in determining the natural resistance mechanisms of unripe fruits to fungal pathogens and the extension of its effectiveness to fruits after harvest (Ziogas and Girgis, 1993). Plant disease resistance is a prerequisite for the successful use of crop species in modern agriculture. Conventional breeding approaches have been very successful in introducing resistance genes from wild populations into commercial crop cultivars harvest (Ziogas and Girgis, 1993). In general, the use of resistant cultivars is the most suitable method for disease control although the extensive use of pesticides and fungicides remains the main strategy of disease control (Quirino and Bent, 2003).

The focus on molecular biology over recent years has improved our understanding of plant-pathogen interactions through the identification of a number of endogenous resistance genes and analysis of signalling pathways leading to the hypersensitive response and systemic acquired resistance. The breakthrough has enabled more sophisticated breeding strategies in commercial cultivars to be employed using marker-assisted breeding (Ayliffe and Lagudah,

2004).Transgenic classical breeding approaches were used to introduce single dominant genes such as *Vf* and others into apple plants for resistance against apple scab (Gygax *et al*., 2004). The understanding of disease resistance today due to the completion of rice and *Arabidopsis* genome sequences (Goff *et al*., 2002) and current sequencing of crop plant genomes associated with improved knowledge of plant defence response mechanisms through functional analyses will result in the development of novel pesticides capable of activating plant defence responses.

The present review introduces avocado and the disease anthracnose. In addition, an overview of pathogen attack and plant defence response mechanisms is presented. Finally, the literature review is put into context through discussion of the aims of the project and experimental approaches adopted in this study to elucidate defence response mechanisms in avocado fruits.

1.2 THE HOST: AVOCADO

1.2.1 Classification

Avocado is classified as follows (Naveh *et al.,* 2002):

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Order : Laurales

Family : Lauraceae

Genus : *Persea*

Species : *americana*

1.2.2 Origin and distribution

The cultivated avocado originated in Southern Mexico, Central and South America where its fruit has been used as an integral part of the local diet for thousands of years (Snowdon, 1990). The Aztecs considered avocados an aphrodisiac and called it huacatl, meaning testicles, referring to the way they hang from the trees and the fruit's shape. In Peru, Chile, and Ecuador avocado is called palta, an Incan name. Spanish-speaking people also call it aguacate, cura or cupandra. They are known as avocat or avocatier in France and as abogado in Spain. The avocado is botanically classified into three races: West Indian (*Persea Americana* Miller var. *americana*), Mexican (*Persea Americana* Miller var. *drymifolia* Blake), and Guatemalan (*P. nubigena*var. *guatemalensis*L. Williams) (Bergh and Lahav, 1996).

The avocado is grown commercially not only in America and throughout tropical America and the larger islands of the Caribbean but in Polynesia, the Philippines, Australia, New Zealand, Madagascar, Cameroon, Mauritius, Madeira, the Canary Islands, Algeria, tropical Africa, South Africa, southern Spain and southern France, Sicily, Crete, Israel, Egypt and others countries. Avocados were introduced to South Africa between 1652 and 1700 by Dutch settlers, who brought seedlings from the West Indies and other Dutch colonies (Durand, 1990). In South Africa, the Mexican and West Indian varieties are of little

economic importance. The natural hybrid of the Mexican and Guatemalan races is Fuerte, the most popularly grown cultivar (Durand, 1990).

In South Africa, avocado production is concentrated mainly in the warm subtropical areas of the Limpopo and Mpumalanga provinces in the North East of the country between latitudes 22 ^{o}S and 25 ^{o}S. They are also grown also grown commercially in certain areas of KwaZulu-Natal. The harvest season for South African avocados is between March and October. Most of the major cultivars are available over an extended period during the season because of the climatic variability between growing regions. For instance in the northern regions, Fuerte is harvested from mid-March to May, and is harvested in July and August in KwaZulu-Natal (Donkin, 2007).

1.2.3 Description and varieties

According to the description of Morton (1987), the avocado tree is usually 9 m, but sometimes is 18 m or more, with a trunk 30 to 60 cm in diameter, (greater in very old trees) or it may be short and spreading with branches close to the ground. Almost evergreen, leaves being shed briefly in dry seasons at blooming time, the leaves are alternate, dark-green and glossy on the upper surface, whitish on the underside, variable in shape (lanceolate, elliptic, oval, ovate or obovate), 7.5 to 40 cm long. The Mexican race is strongly anise-scented. Small, pale-green or yellow-green flowers are borne profusely in racemes near the branch tips. They lack petals but have 2 whorls of 3 perianth lobes, more or less pubescent, and 9 stamens with 2 basal orange nectar glands. The fruit, pear-shaped, often more or less necked,

oval, or nearly round, may be 7.5 to 33 cm long and up to 15 cm wide. The skin may be yellow-green, deep-green or very dark-green, reddish-purple, or so dark a purple as to appear black, and is sometimes speckled with tiny yellow dots: it may be smooth or pebbled, glossy or dull, thin or leathery and up to 1/4 in (6 mm) thick, pliable or granular and brittle. In some fruits there is a thin layer of soft, bright-green flesh immediately beneath the skin, but generally the flesh is entirely pale to rich-yellow, buttery and bland or nutlike in flavour. The single seed is oblate, round, conical or ovoid, 5 to 6.4 cm long, hard and heavy, ivory in colour but enclosed in two brown, thin, papery seed coats often adhering to the flesh cavity, while the seed slips out readily. Due to the lack of pollination or other factors some avocado fruits are seedless (Nakasone and Paull, 1998).

The avocado's flesh is deep green near the skin, becoming yellowish nearer the single large, inedible ovoid seed. At the moment of harvesting, the flesh is hard but softens to a buttery texture later.The fruits of Mexican varieties are higher in oil content and small with paper-thin skins that turn glossy green or black when ripe. The fruit of Guatemalan types produce medium ovoid or pear-shaped, pebbled green fruits that turn blackish-green when ripe and have intermediate oil content. West Indian types produce smooth, round, enormous, glossy green fruits that are low in oil (Bergh and Lahav, 1996).

There are many varieties of avocado fruit, each characterized with a particular flavour and texture. The most popular varieties are: Hass, Fuerte, Gwen, Pinkerton, Reed, Bacon and Zutano. A particular preference is given to Fuerte and Hass in South Africa (Donkin, 2007).

The Fuerte variety is mostly oval shaped and is green when ripe. Hass is medium sized and rounded and during ripening turns to a purple colour.

1.2.4 Marketing and international trade

Avocado is cultivated in nearly 50 countries and is the sixth-most important subtropical crop in the world (Demirkol, 1995). According to the FAO the five world's leading producers of avocados in 2005 were: Mexico, Indonesia, the United States of America, Colombia and Brazil. South Africa was at the 15th position among the highest producing countries of avocado in the world (Figure 1.1) (FAO, 2005). Although high producers, Brazil and Mexico do not export a great deal of fruit because 97 % of all fruit produced in Mexico is for domestic use, and cultivars produced in Brazil are not acceptable for export because of the inferior fruit quality (van Zyl and Ferreira, 1995)

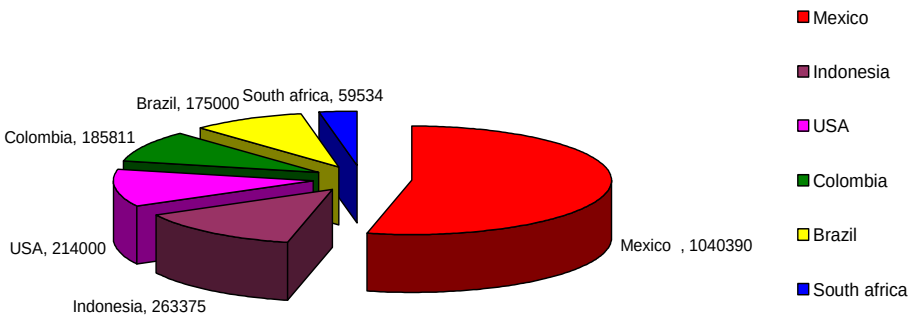


Figure 1.1. Major Producers of Avocado **fruit (cv. 'Fuerte') [(adapted from FAO (2005)].** The quantity of avocado fruit produced by each country is expressed in metric tonnes.

In South Africa, the expansion of the avocado industry started in 1970s, with plantings of approximately 2000 ha in 1970 increasing to approximately 12 000 ha in 2003 (Donkin, 2007). In 2001, South Africa was classified as the leading exporter of avocados worldwide (Witney**,** 2002), but in 2011 South Africa was classified 4thexporter of avocados worldwide with Spain, after Mexico, Chile and Peru (Naamani, 2011). Every year about 60% of the total avocado crop is exported with 97 % destined for the European Union. Chile, Israel, Kenya, Mexico, Spain and Peru are the major competitors of South Africa in the European markets (Witney**,** 2002). The varieties exported are Hass and Fuerte with a small amount of Pinkerton. In the European Union, South Africa's largest markets for avocados are France, Germany and the United Kingdom. In South Africa, the avocado industry is led by the South

African Avocado Growers Association (SAAGA) funded by voluntary levies from all members and whose goal is to improve the economic viability of the production, packaging and marketing of avocados. It is also a responsibility of SAAGA, in association with the National Department of Agriculture, to determine the quality standards for export, ensuring that the exported fruits are of good quality as the maturity, size and blemish levels are concerned and also ensuring that those fruits meet the standards of the country of destination (SAAGA, 2005). One of the challenges of the South African industry is to access new markets such as China, Japan and USA, within the official protocols of the target government. The industry plans an increase in annual exports of roughly 12,000 metric tonnes by 2012 and expects to continue promotion in Europe (Witney, 2002).

1.3 ANTHRACNOSE OF AVOCADO

1.3.1 **The pathogen**: *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc.

The filamentous form of the fungal plant pathogen *C. gloeosporioides*, which is the anamorph, or asexual stage of the fungus causes anthracnose disease on various temperate, subtropical, and tropical fruits such as apple, avocado, citrus, mango, guava, papaya, passion fruit and grapes (Alahakoon *et al*., 1994; Swart, 1999).

1.3.2 Taxonomy and biology of *Colletotrichum gloeosporioides*

Kingdom: Fungi

Phylum: Ascomycota

Class: Sordariomycetes

Subclass: Incertae sedis

Order: Phyllachorales

Family: Phyllachoraceae

Genus: *Colletotrichum*

Species: *gloeosporioides*

C. gloeosporioides produces abundant conidia on infected leaves, inflorescences, and on mummified aborted fruit. Conidia are spread by splashing rain and cause new infections on leaves, blossoms and fruit. In the infected fruit in the field, the fungus remains quiescent until the fruit is harvested and then it becomes activated and the lesions begin to develop and to enlarge. In storage, however, the fungus does not move from one fruit to the next.

The fungus produces hyaline, one- celled, ovoid to oblong, slightly curved or dumbbell shaped conidia, 10-15 µm in length and 5-7 µm in width. Masses of conidia appear pink or salmon coloured. The waxy acervuli that are produced in infected tissue are subepidermal, typically with setae, and simple, short, erect conidiophores. Environmental conditions suitable to the pathogen are high temperatures 28 ^{o}C being optimal, and high humidity. Spores must have free water to germinate; germination is negligible below 97% relative humidity. Spores are only released from acervuli when there is an abundance of moisture.

Splashing from rain is a common means of spread. Severity of disease is related to weather and the fungus is relatively inactive in dry weather. Sunlight, low humidity and temperature extremes (below 18°C or greater than 25 ^{o}C) rapidly inactivate spores (Scot, 2008).

Swart (1999) showed that there are distinct differences between avocado and mango isolates of *C. gloeosporioides* in South Africa. Morphologically, isolates of *C. gloeosporioides* from avocado and mango are different phenotypically from isolates of others countries with colony colours ranging from pale salmon pink to dark gray green. However there are only a few differences between appressoria of different isolates, which are produced terminally from germ tubes by most isolates with a mean length of 14.6 µm. The *C. gloeosporioides* isolates of both avocado and mango produce mainly cylindrical conidia with a tapered base and obtuse apex out of the four different types of conidial shapes identified. The length of conidia varies considerably but their width remains relatively constant. There is no correlation between the length/width ratio of conidia and the pathogenicity of isolates when inoculated into avocado and mango fruits (Sanders and Korsten, 2003). The shape and size of conidia, production of perithecia, morphology of setae (if present), and colony growth characteristics were the criteria traditionally used to identify isolates in the *Colletotrichum* genus (Gunnel and Gubler, 1992).

1.3.3 Host-specificity and life cycle of *Colletotrichum gloeosporioides*

Host-specificity is evident in *Colletotrichum;* some species have a wide host range while others are restricted to a specific host or host families, genera, species, or even cultivars within those families (Freeman, 2000). Specificity has been reported within *C. gloeosporioides* species on *Stylosantes* in Australia where two distinct biotypes different in doubled stranded RNA, morphology, molecular karyotypes, restriction fragment length polymophisms are both host-specific (Manners *et al*., 1992). The differentiation between *Colletotrichum* species on the basis of their host specificity may not be reliable, because several species such as *C. gloeosporioides, C. dematium, C. acutatum,* and *C. graminicola* infect a broad range of host plants. For instance, studies showed that isolates of *C. gloeosporioides* and *C. acutatum* both infect peach and mango fruits (Adaskaveg and Hartin, 1997; Freeman *et al*., 1998; Swart, 1999). Potential cross-infection worked successfully under artificial inoculation conditions between different species of *Colletotrichum* and a variety of tropical, subtropical, and temperate fruits. In addition, *Colletotrichum gloeosporioides* isolates from some tropical fruit crops can also cross-infect detached leaves and fruits with the infection depending on inoculum density (Anderson *et al*., 1996).

During the asexual stage of the life cycle of most ascomycetes, including *Glomerella*, they are typically haploid and become diploid during the sexual phase (Skipp *et al*., 1995). The life cycle of *Colletotrichum* species (Figure 1.2) comprises a sexual and an asexual stage. In general, the sexual stage is responsible for the genetic variability and the asexual stage

accounts for the propagation of the fungus. *Glomerella cingulata* is one of the rare species of *Colletotrichum* which produces the sexual stage (Bryson *et al*., 1992). *Glomerella* teleomorphs of *Colletotrichum* can exhibit many sexual systems but they are present in only 11 out of 20 *Colletotrichum* species due to the fact that sexual recombination in most *Colletotrichum* species is rare in nature. Besides this, *Glomerella* sexual reproduction is more complex than is usual for most ascomycete fungi. *C. gloeosporioides* exhibits a wide range of sexual expression in which self-fertile (homothallic) forms readily form perithecia in culture and ascospores give rise to cultures the same as the parental form. In the plus strains which resemble the original parent, perithecia, and ascospore progeny are irregularly produced and the minus strains produce fertile perithecia (Skipp *et al*., 1995). A ridge of perithecia is produced along the interaction zone by the plus and minus strains, which leads to the suggestion that *C. gloeosporioides* is fundamentally self-sterile (heterothallic) It has also been shown that *C. gloeosporioides* can operate a complete parasexual cycle (Skipp *et al*., 1995)

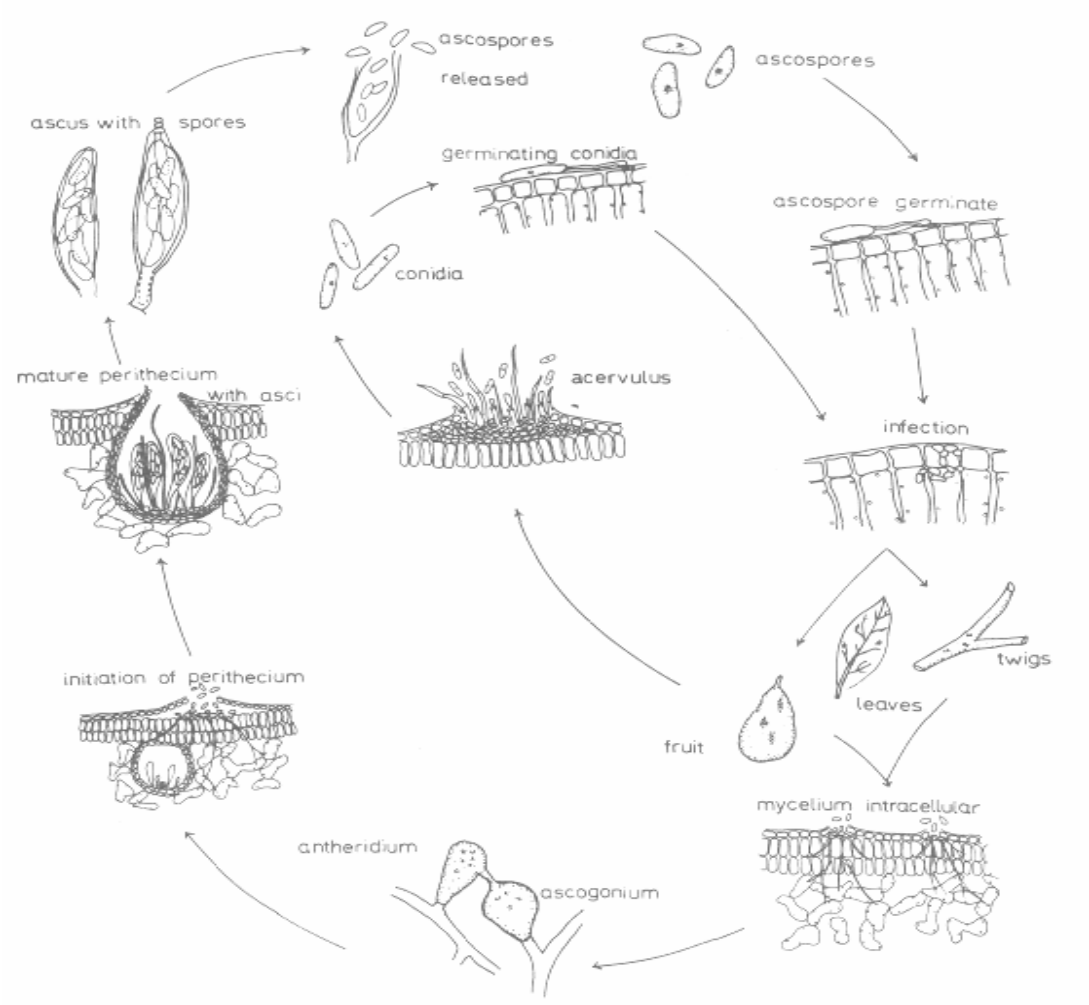


Figure 1.2 Schematic presentation of the life cycle of C*olletotrichum gloeosporioides* on avocados (Kotzé, 1978).

1.3.4 *Colletotrichum gloeosporioides* infection, symptomology and epidemiology

Anthracnose is a serious disease and causes post harvest losses of the avocado fruit all over the world. The interaction of the fungus and avocado is a complex one. The infection process starts when a conidium lands on the surface of the fruit; it adheres and germinates to produce a germ tube, which develops a terminal appressorium. Then an infection peg emerges and penetrates into the outer wax layer and the cuticle of the fruit skin. When it reaches this hemibiotrophic stage, it ceases growth and remains quiescent until fruit ripening (Jeffries *et al*., 1990; Giblin and Coates, 2007). During this stage of dormancy or quiescence, due to the presence of preformed antifungal compounds in the exocarp of unripe fruits known as dienes, the fungus is unable to colonise further (Prusky *et al*. 1993). After harvest, diene levels decrease which correlate with the resumption of fungal growth, and subsequent disease development. Infection pegs during ripening penetrate through the epidermal cell walls to the lumen of the cells, which results in maceration and cell death. The production of plant cell wall degrading enzymes such as pectate lyase is coupled to the transition from the hemibiotrophic stage to necrotrophic attack (Coates *et al*., 1993). Rainfall is associated with the infections of fruit which occur from fruit-set until harvesting. The main sources of inoculum are dead leaves entangled in the tree canopy, defoliated branch terminals, mummified inflorescences and flower bracts (Dodd *et al*., 1992). In the orchard the conidia are spread by means of irrigation and light rain, heavy dew, with rainy weather being conducive to conidium production, dispersal and infection (Prusky, 1994).

Lesions of various sizes, dark in colour, can occur anywhere on avocado fruits and expand rapidly in size, affecting the skin and pulp. Symptoms may appear rapidly, within 1 or 2 days, on fruits that sometimes appeared to have no blemishes at the time of harvest. After fruit harvest and during ripening, darkly coloured, rounded lesions usually appear in latent infections on fruit skins. Symptoms may form on the unripe fruits while they are still on the trees (Menge and Ploetz, 2003). Fruits developing symptoms before ripening may drop prematurely. In some cases, skin symptoms are more difficult to detect on avocado cultivars with dark coloured skins. Some symptoms may be related to fruit injury or openings created during harvesting (Whiley *et al*., 2002). In the beginning the lesions on the fruit skin are small light brown, and circular, later enlarging and becoming slightly sunken in their centres, while their colour turns to dark brown or black (Figure 1.3) (Scot, 2008). Under very humid conditions leaf and stem symptoms (spots or blight) can develop. *C. gloeosporioides* is considered to be a weak pathogen of avocado fruit, due to the fact that it requires some types of wound created by some other means, in order to penetrate the fruit and subsequently cause disease (Pernezny *et al*., 2000)

In South Africa most of the orchards are planted in Fuerte and Hass varieties (74%) (Donkin, 2007) and anthracnose affects all varieties, (Witney, 2002), but Fuerte is the most susceptible cultivar (Darvas and Kotze, 1981).

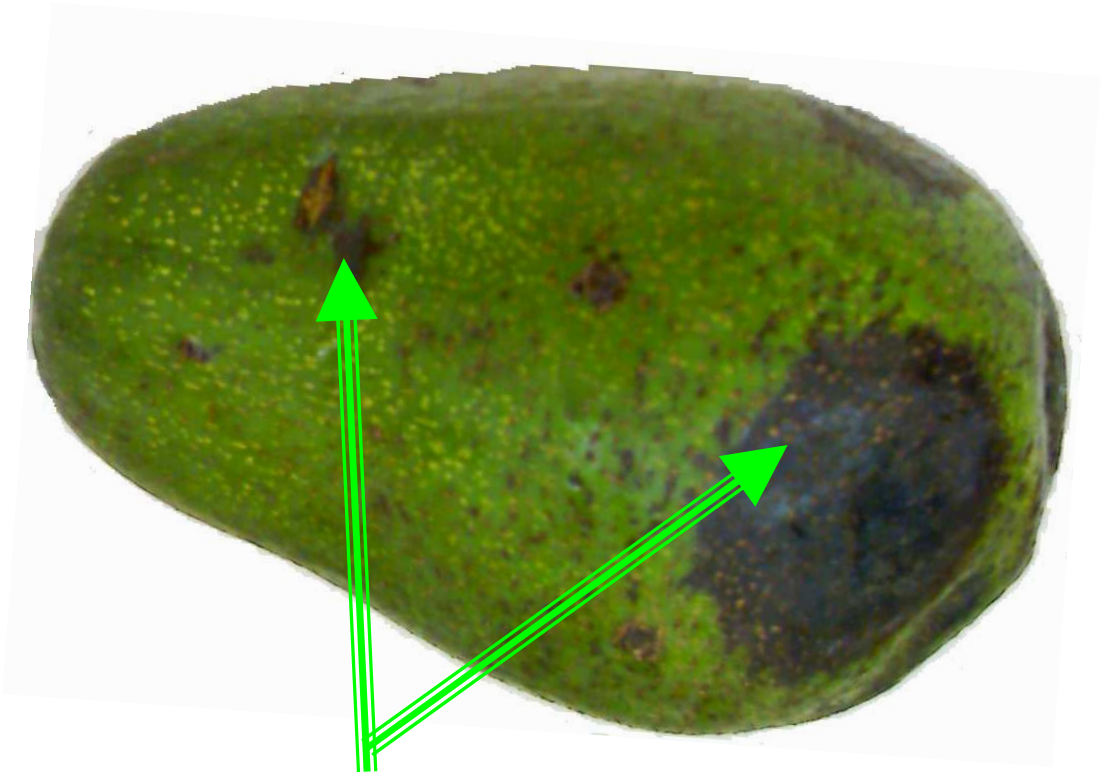


Figure 1.3. Fuerte avocado fruits exhibiting symptoms of anthracnose

1.3.5 Control of Anthracnose disease

Anthracnose disease needs to be controlled in order to maintain the quality and abundance of food, feed, and fibre produced by growers around the world. Since *C. gloeosporiodes* remains quiescent in the cuticle of unripe fruit, different approaches may be used to prevent, mitigate or control plant diseases: these include cultural control, chemical control, biological control and use of resistant cultivars.

1.3.5.1 Cultural control

Good anthracnose control depends on good control of other diseases (especially Cercospora spot) and avoidance of cuts and bruises to the fruit in handling. Fruits showing any sign of anthracnose should not be packed in cartons with healthy fruits. Harvesting fruits in an immature condition may substantially contribute to anthracnose appearance in the market place, because the fungus may be carried on the immature fruit and will subsequently invade the flesh through small cracks made during post-harvest handling procedures (Pernezny*et al*., 2000, Agrios, 2005). Tactics for managing anthracnose disease of avocado include selection of avocado cultivar and planting location, cultural practices within orchards, care in fruit handling, and control of fruit storage conditions and marketing (Scot, 2008) .

1.3.5.2 Chemical control

Control of *C. gloeosporioides* and other postharvest avocado and mango pathogens needs an adequate preharvest spray programme in place with a main focus to reduce the inoculum and prevent latent infection (Darvas *et al*., 1987). In South Africa, sprays with benomyl and copper fungicides (cupric hydroxide or copper oxychloride) are the registered pre-harvest chemical treatments used with mist blowers on a monthly basis to control anthracnose on avocado (Willis and Mavuso, 2009). For the registered post-harvest chemical treatments, spray and dip applications of prochloraz and thiabendazole with a combination of benomyl were used to control anthracnose on avocado (Sanders *et al*., 2000). In the early 1960`s, disease control was revolutionized by the introduction of benzimidazole fungicides such as benomyl, carbendazim and thiophanate. However, the prolonged use of these fungicides resulted in selection for resistant pathogen genotypes, which remained predominant for

several years after discontinued use. *Botrytis cinerea* in vineyards was one of the fungi with short life cycles identified among the first cases of resistance. Later other resistant fungi were identified in some plants such as: *Penicillium digitatum* and *Penicillium italicum* from citrus, *Venturia inaequalis* from apple, and *C. gloeosporioides* from rambutan and mango (Sanders *et al*., 2000). Prochloraz and thiabendazole are the current registered post-harvest chemical treatments used to control anthracnose on avocados (Sanders *et al*., 2000). Recently, ultra-low volume (ULV) application of fungicides developed as thermal fogging was used in the control of diseases on Fuerte avocado fruits. The results showed that the ULV machine (TracFog 100F) has the ability to effectively control the disease by using 50% less copper oxychloride needed per hectare, by also reducing application time due to one tank mixture doing more hectares compared to the mist blowers (Van Niekerk and Mavuso, 2011). This technology was also used successfully to control disease in some tropical crops such as bananas, cocoa and rubber (Mabbet, 2007).

The use of chemicals to control plant disease has contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, their use is increasingly restricted due to public concern over the environmental pollution caused by excessive use and misuse of those agrochemicals.

1.3.5.3 Biological control

Effective biocontrol agents offer great potential for alternative methods that are economical and suited for adoption by the small-scale avocado industry. Some potential biocontrol agents have been identified to have roles in the control of plant diseases. Kefialew and Ayalew (2008) isolated the following potential biocontrol agents from mango fruits: *Brevundimonas diminuta*, *Stenotrophomonas maltophilia*, *Enterobacteriaceae*, *Candida membranifaciens* and possible new yeast species. The cell suspensions and culture filtrates of these isolates inhibited spore germination and hyphal growth of *C. gloeosporioidesin vitro*. In addition, the isolates significantly reduced severity of anthracnose on artificially inoculated mango fruit (Kefialew and Ayalew,2008). Various *Bacillus* spp., originally isolated from leaf and fruit surfaces, effectively controlled Cercospora spot of avocado, anthracnose of avocado, stem end rot of avocado and post-harvest decay. Integrated treatments involving antagonists combined with quarter-strength or recommended dosage of fungicides, disinfectants or natural plant extracts also effectively suppress post-harvest diseases of avocado (Korsten, 2004). AvoGreen, a pest monitoring programme, was also used previously to manage pests by combining methods that include ecological and toxicological factors with emphasis on biological controls and economic thresholds (Stevens, 1997).

A variety of biological controls are available for use, but further development and effective adoption will require a greater understanding of the complex interactions among plants, people and the environment.

1.3.5.4 Resistant varieties

In general, plant disease incidence can be reduced by appropriate cultivation practices, the use of pesticides, the use of antagonistic organisms and the use of disease-resistant cultivars; the latter remains the most inexpensive and safest method for disease control (Quirino and Bent, 2003). Resistance is defined as an incompatible interaction between host and pathogen. Incompatibility, involving processes in the plants that retard or inhibit pathogen growth, can be dependent on a host resistance gene and a pathogen avirulence gene (Agrios, 2005). Pathogen infection is established due to an inadequate defence response of the host to the invading pathogen in terms of timing and intensity. Plant defence response has generally been described as involving activation of signalling molecules such as salicylic acid and defence pathways, ultimately leading to changes in gene expression and defined cellular responses (Dangl and Jones, 2001). The plant resistance is considered durable when it remains effective in a cultivar despite widespread cultivation in an environment favouring the disease. Single genes and multiple genes with cumulative effects variously control durable resistance in different pathosystems. The breakthrough in the understanding of plant-pathogen interactions through the identification of resistance genes has enabled more sophisticated breeding strategies in commercial cultivars to be employed using marker-assisted breeding (Ayliffe and Lagudah, 2004). Through this, many disease resistant crop varieties have been generated by traditional breeding. For instance transgenic and classical breeding approaches were used to introduce a single dominant gene *Vf* against apple scab into apple plants (Gygax *et al*., 2004).

1.4 PLANT DEFENCE MECHANISMS AGAINST PARASITIC ATTACK

1.4.1 Pathogen recognition by plant cells

The capacity of plants to invoke defence reactions is mediated by the initial recognition of pathogens (Dixon *et al*., 1994; Schenk *et al*., 2000). Plants exhibit a sophisticated molecular system for recognition of and response to a potential pathogen. Firstly the presence of a pathogen is recognised by the plant when pathogen derived molecules (elicitors) bind to receptors. The recognition is done by the detection of elicitors that originate from the pathogen or are plant cell wall degradation products (Agrios, 2005).

Plants are capable of activating a large array of defence mechanisms in response to pathogen attack. The speed of their activation is a crucial factor determining the success of these mechanisms. Consequently, there is considerable interest in understanding recognition and control of pathogen attack and expression of defence mechanisms. In host specific resistance, perception involves receptors with high degrees of specificity for pathogen strains, which are encoded by constitutively expressed defence resistance (R) genes, located either in the cytosol or on the plasma membrane (Martin, 1999; Dangl and McDowell, 2006). In a single plant species, large repertoires of distantly related individual R genes with diverse recognitional specificities are found (Ellis *et al*., 2000). Individual R genes have narrow recognition capabilities and they trigger resistance when the invading pathogen expresses a corresponding avirulence (Avr) gene. In the pathogens, Avr genes from different pathogen

classes are structurally very diverse and have different primary functions. Specific recognition of the pathogen by the plant requires the presence of matching Avr and R genes in the two species and is thought to be mediated by ligand receptor binding (Glazebrook, 1999).

Plant disease R genes encode proteins that serve in the recognition of specific pathogen-derived Avr proteins and initiate signal transduction pathways leading to defence responses. Some studies suggest that recognition specificity of R proteins is determined by either a protein kinase domain or by a region consisting of leucine-rich repeats. R genes conferring resistance to pathogens seem to use multiple signalling pathways, some of which involve distinct proteins and others which converge upon common downstream effectors. A promising strategy to improve disease resistance in plants is the manipulation of R genes and their signalling pathways by transgenic expression (Martin, 1999).

In addition to the Avr-R gene interactions, plants possess a broader, more basal, surveillance involving sensitive perception systems for numerous microbe-derived molecules, which mediate activation of plant defence responses in a non-cultivar-specific manner and have been described as general elicitors. These molecules are named as PAMPs for pathogen-associated molecular patterns, and they are shared by large groups of pathogens (Gómez-Gómez, 2004). General elicitors involved in the activation of the first line of plant defences have been isolated from viral, bacterial, fungal and oomycete pathogens. They act as signalling compounds at low concentrations, have diverse structures and include polygalacturonides, β-glucans, chitosan, lipids and proteins (Ebel and Cosio, 1994). A few of

them can be viewed as molecular signatures of microbial invaders and therefore as potential PAMPs. Examples include, the elicitor PaNie from the pathogenic oomycete *Pythium aphanidermatum* and other fungi (Veit *et al*., 2001); the elicitor Pep-13, an internal peptide fragment conserved among different oomycetes; transglutaminases (Brunner *et al*., 2002), which are surface exposed and consequently accessible to association with an extracellular plant receptor (Sacks *et al*., 1995); fungal chitin (Day *et al*., 2001); lipopolysaccharides and complex glycolipids that are integral components of the cell surface of Gram-negative bacteria, which activate immune responses in mammals and plants (Akira *et al*., 2001; Erbs and Newman, 2003). Two other bacterial PAMP-like elicitors are a bacterial cold shock protein (CSP) present in several bacteria (Felix and Boller, 2003) and bacterial flagellin (Felix *et al*., 1999). Structural homologs present in oomycetes, fungi and bacteria, like the necrosis-inducing *Phytophthora* protein 1 have been characterized as PAMPs (Fellbrich *et al*., 2002). All these molecules are made by fungi or bacteria, but not by plant cells, and their recognition by the plant receptors can signal the presence of potential phytopathogens.

1.4.2 Plant defence signalling networks

In most eukaryotic organisms there is a link between pathogen recognition responses and signal transduction cascades (Nürmberger and Scheel, 2001). Defence signalling pathways that lead to defence responses are activated after elicitor binding to receptors. Intracellular signalling is instigated when the occupied receptor, directly or indirectly, activates a downsteam effector enzyme to produce a specific second messenger. In its turn, the second messenger binds to and activates, for example, a protein kinase that is at the start of a kinase

cascade. This leads to different responses, such as transcriptional activation of defence related genes (Laxalt and Munnik, 2002).

Receptor-mediated recognition at the site of infection initiates cellular and systemic signalling processes that activate multicomponent defence responses at local and systemic levels, resulting in rapid establishment of local resistance and delayed development of systemic acquired resistance (Scheel, 1998). The earliest reactions of plant cells include changes in plasma membrane permeability leading to calcium and proton influx and potassium and chloride efflux (McDowell and Dangl, 2000).

Ion fluxes subsequently induce extracellular production of reactive oxygen intermediates, such as hydrogen peroxide, superoxide and hydroxyl free radical, catalyzed by a plasma membrane-located NADPH oxidase and/or apoplastic-localized peroxidases (Somssich and Hahlbrock, 1998). The initial transient reactions are, at least in part, prerequisites for further signal transduction events resulting in a complex, highly integrated signalling network that triggers the overall defensive response. The role of calcium is shown in experiments with calcium channel inhibitors, which, preventing increases of cytosolic calcium concentrations, delay the development of the hypersensitive response. Heterotrimeric GTP-binding proteins and protein phosphorylation/ dephosphorylation are probably involved in transferring signals from the receptor to calcium channels that activate downstream reactions (Legendre *et al*., 1992). The changes in ion fluxes trigger localized production of reactive oxygen intermediates and nitric oxide, which act as second messengers for hypersensitive response induction and defence gene expression (Piffanelli *et al*., 1999). Synergistic interactions

between reactive oxygen intermediates, nitric oxide (NO) and salicylic acid (SA) have been postulated (McDowell and Dangl, 2000). Other components of the signal network are specifically induced phospholipases, which act on lipid-bound unsaturated fatty acids within the membrane, resulting in the release of linolenic acid, which serves as a substrate for the production of jasmonate, methyl jasmonate and related molecules via a series of enzymatic steps. Most of the inducible, defence-related genes are regulated by signal pathways involving one or more of the three regulators jasmonate, ethylene (ET) and SA (Hammond-Kosack and Parker, 2003).

Thus, experimental evidence suggests that defence signalling is complex and evolves an interplay between protein kinases, phospholipids, and defence signalling molecules such as NO, reactive oxygen species , SA, jasmonic acid (JA) and ET (Thomma *et al*., 2001; Hammond-Kosack and Parker, 2003). Hydrogen peroxide (H_2O_2) and NO play a role in cross-talk and convergence points between pathways (Kumar and Klessig, 2000).

1.4.2.1 Mitogen-activated protein kinases and defence signalling

Mitogen-activated protein kinases (MAPK) are serine/threonine-specific protein kinases that transduce extracellular stimuli such as mitogens, osmotic stress, heat shock and proinflammatory cytokines into intracellular responses in cells. In the cells, protein kinases covalently attach phosphate to the side chain of tyrosine, serine or threonine of specific proteins. Such phosphorylation of proteins can control their interaction with

other proteins and molecules, their location in the cell, their enzymatic activity, and their propensity for degradation by proteases. MAPK phosphorylate specific serines and threonines of target protein substrates that regulate various cellular activities ranging from gene expression, mitosis, cell movement, cell differentiation, proliferation, metabolism, and programmed death (Pearson *et al*., 2001).

Signal transduction networks allow cells to perceive changes in the extracellular environment and to mount an appropriate response. MAPK cascades are major components downstream of receptors or sensors that form a crucial link that transduces extracellular stimuli into intracellular responses, from extracellular receptors (Inne, 2001; Zhang and Klessig, 2001). MAPK cascades regulation and function have been evolutionarily conserved from unicellular organisms to complex organisms. They are organized in a three-kinase architecture consisting of a MAPK, the last kinase in the cascade, a MAPK activator (MEK, MKK, or MAPK kinase), and a MEK activator (MEK kinase, MEKK or MAPK kinase kinase). MAPK kinase that is activated by extracellular stimuli phosphorylates a MAPK kinase on its serine and threonine residues, and this MAPK kinase activates a MAPK through phosphorylation on its serine and tyrosine residues (Tyr-185 and Thr-183 of ERK2). Signal transduction is achieved by sequential activation and phosphorylation of the components specific to a respective cascade (Zhang and Klessig, 2001).

MAPKs are differentially activated by cytokines, hormones and growth factors. Studies showed that MAPK modules may be activated by a variety of stress stimuli including UV irradiation, wounding, ozone, heat shock, salinity, osmolarity, DNA damage and reactive

oxygen species and bacterial products such as lipopolysaccharides. Some cellular activities such as gene expression, metabolism, cytoskeletal functions and other cellular regulatory events are controlled by the activation of MAPK in response to these stimuli. In addition MAPK from several plant species were also shown to be activated during plant responses to elicitors or pathogens (Madhani and Fink, 1998; Zhang and Klessig, 2001).

A breakthrough in our understanding of plant MAPKs was made with the map-based cloning of *FL*agellin *S*ensing (*FLS2*) (Gómez-Gómez and Boller, 2000). In the *Arabidopsis FLS2* pathway, the flagellin peptide present in the extracellular media interacts with the extracellular LRR FLS2 domain. It has been shown that the FLS2 kinase activity is directly or indirectly in charge of the phosphorylation and activation of a MAP kinase cascade, which might further induce the activation of WRKY type transcription factors (Figure 1.4) (Asai *et al.*, 2002; Gómez-Gómez, 2004).

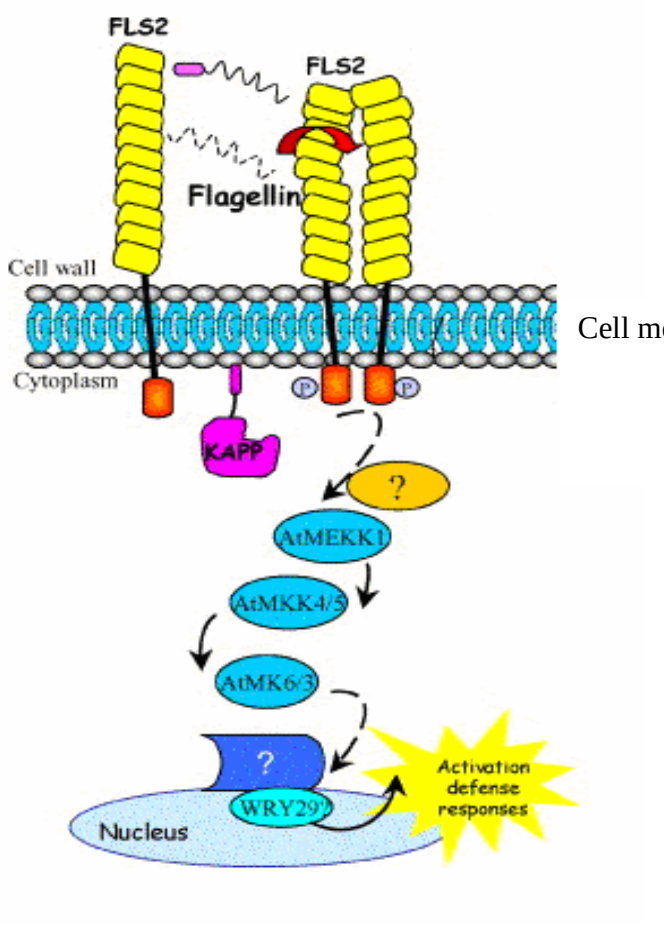


Figure 1.4. Main elements of the signal transduction pathway involved in flagellin signalling in *Arabidopsis* (modified from Gómez-Gómez, 2004).

In the *Arabidopsis* FLS2 pathway, the flagellin peptide present in the extracellular media interacts with the extracellular LRR FLS2 domain. This interaction, which may also involve additional components, leads to heterodimerisation or dimerisation of the receptor complex and activation of the FLS2 kinase domain. A kinase-associated protein phosphatases (KAPP) is a negative regulator in this pathway. The FLS2 kinase activity is responsible for the phosphorylation and activation of the *Arabidopsis thaliana* mitogen kinase kinase 1

(AtMEKK1) which then phosphorylates *Arabidopsis thaliana* mitogen kinase kinase 4 and 5 (AtMAKK4/5). These kinases in turn phosphorylate and activate *Arabidopsis thaliana* mitogen kinase 6 and 3 (AtMK6/3) and lead to the activation of the transcription factor WRY29, which activates the transcription of defence genes.

1.4.2.2 Nitric oxide

Nitric oxide (NO) is an important diffusible molecular messenger in animals and plants involved in many physiological and pathological processes within the organism, both beneficial and detrimental. NO is an important mediator in plant defence signalling and it is also involved in number of diverse signal functions in plants (Wendehenne *et al*., 2004; Delledonne, 2005). NO has been identified as an essential molecule that mediates hypersensitive cell death and defence gene activation in plants. In addition to this role, studies showed that it can also play an important role as an intercellular signal contributing to spread of hypersensitive responses (HR) by inducing cell death (Romero-Puertas *et al*., 2004; Zeidler *et al*., 2004).

In one study (Crampton, 2006), cDNA microarray analysis was applied to examine the effects of NO on a non-model cereal plant, pearl millet, and to identify genes that are up and down regulated following exogenous application of a NO donor. The results revealed that pearl millet responds to treatment with a NO donor, and alters the expression profiles of a number of transcripts. Comparison of pearl millet NO responsive genes with *Arabidopsis thaliana* NO responsive genes showed very little overlap. Most of the genes exhibiting

significant differential expression in pearl millet have not been previously implicated in NO signalling in plants (Crampton, 2006).

1.4.2.3 Reactive oxygen species

Reactive oxygen species (ROS) are chemically-reactive molecules containing oxygen such as: hydrogen peroxide ($H_2O_{2)}$, superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($O_2^{\bullet}$). ROS can be either inorganic or organic and are highly reactive and toxic due to the presence of unpaired valence shell electrons, and can lead to the oxidative destruction of cell structures. They are synthesized in plants during normal unstressed photosynthetic and respiratory metabolism taking place in chloroplasts and mitochondria. They are also generated by cytoplasmic, membrane-bound, or exocellular enzymes involved in redox reactions (Foyer *et al*., 1994; Wojtaszek 1997; Grene, 2002). However, when conditions become unfavourable, for instance during times of environmental stress such as ultra violet or heat exposure, excess light energy, dehydration, low temperature, ROS levels can increase dramatically. Such stresses can lead to significant damage to cell structures manifested in inactivation of enzymes or cell death, if the amount of ROS generated exceeds the capacity of the scavenging systems. ROS are also generated by exogenous sources such as ionizing radiation (Dat *et al*., 2000; Grene, 2002)

Plant cells contain several enzymatic and non enzymatic antioxidant scavenging systems, which are in charge of ROS detoxification to avoid cellular damage. These include enzymes such as superoxide dismutases (SODs), glutathione peroxidases, ascorbate

peroxidases (APXs), and catalases (CATs) as well as small molecule antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E) and glutathione (Mittler, 2002; Muller et al., 2007).

Although potentially damaging, ROS have been shown to serve as second messengers or catalysts in plants to promote plant resistance to pathogens in several ways. Some of the better studied downstream responses promoted by ROS are the oxidative cross linking of cell wall hydroxyproline rich proteins to reinforce the cell wall against pathogen degrading enzymes, the stimulation of defence-related genes, the induction of phytoalexin production and promotion of the programmed cell death leading to the formation of the hypersensitive response (HR) of infected cells. (Low and Merida, 1996; 1997; Mullineaux *et al*., 2000; Neill *et al*., 2001). During defence responses, ROS are produced by amine oxidases in the apoplast and plasma membrane-bound NADPH oxidases and cell wall-bound peroxidases (Mahalingam and Fedoroff, 2003; Laloi *et al*., 2004). At the early stage of plant's defence responses, the oxidative burst occurs which is a rapid and transient production of large amounts of ROS at the site of infection (Wojtaszek, 1997).

1.4.2.4 Salicylic acid

SA is synthesized by the plants either via isochorismate synthase (ICS), or via phenylalanine ammonia-lyase (PAL) pathway (Wildermuth *et al*., 2001). Previous work has suggested that oxidative stress caused by ultraviolet light or ozone also triggers SA biosynthesis (Yalpani *et al*., 1994).

SA is involved in the activation of various plant defence responses following pathogen attack by playing an important signalling role. These responses include the potentiation of host cell death, the containment of pathogen spread, the induction of local acquired resistance (LAR) and systemic acquired resistance (SAR) (Chamnongpol *et al*., 1998; Reymond and Farmer, 1998; Nawrath and Métraux, 2002).

SA is the key defence response component in *Arabidopsis* (McDowell and Dangl, 2000). It has been demonstrated that transgenic plants having the bacterial *Nah*G gene, which encodes for the salicylate hydroxylase enzyme, responsible for the inactivation of SA by converting it to catechol, were compromised in their ability to activate SAR (Ryals, 1996). SA was shown to play a key role in the stimulation of the translocation of NPR1 to the nucleus where it is assumed to act as a transcriptional co-factor for the expression of PR-1 (Ham *et al.*, 2007). The accumulation of SA induces the PR gene systems, notably PR-1 and PR-5 (Krzymowska *et al.*, 2007).

SA mediates these effects through varied mechanisms which can involve alterations in the synthesis or activity of certain enzymes, increase in defence gene expression, potentiation of several defence responses and the generation of free radicals. Many genes encoding products involved in the SA-mediated defence pathway(s) have been isolated through the analysis of mutant plants exhibiting aberrant responses to pathogen infection. Additionally, it has been suggested that certain defence responses can be activated via a SA-independent pathway(s) (Demsey *et al*., 1999).

1.4.2.5 Jasmonic acid

Jasmonic acid (JA) is derived from the fatty acid linolenic acid. It is a member of the jasmonate class of plant hormones. It is biosynthesized from linolenic acid by the octadecanoid pathway (Turner *et al*., 2002). Jasmonates, especially phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA) are involved in the regulation of plant growth, including embryogenesis, pollen and seed development, and root growth (Farmer *et al*., 2003; Liechti *et al*., 2006). It has been demonstrated that JAs also mediate resistance to insect pathogen attack, and abiotic stress responses to wounding and ozone (Kunkel and Brooks, 2002; Voelckel and Baldwin, 2004). Another study suggests that, 12-oxo-phytodienoic acid, a cyclopentenone precursor of JA, can also stimulate the expression of defence genes (Farmer *et al*., 2003).

Arabidopsis mutants impaired in the perception or synthesis of JA exhibit enhanced susceptibility to a variety of pathogens, such as the fungi *Alternaria brassicicola, Botrytis cinerea,* and *Pythium* sp., and the bacterium *E. carotovora* (Norman-Setterblad *et al*., 2000; Thomma *et al*., 2001). JA in some cases seems to contribute to plant resistance against biotrophs, however, JA responses are generally considered effective in defence against necrotrophic pathogens (Turner *et al*., 2002; Farmer *et al*., 2003). *Arabidopsis* constitutive expression of the *vsp1* (*cev1*) mutant exhibits constitutive JA signalling and increased defence against the fungus *E. cichoracearum* and the bacterium *P. syringae* pv. *maculicola* (Ellis C. *et al*., 2000).

The subsequent signal transduction and perception of JA is still unclear. A receptor for JA has not yet been characterized (Liechti *et al*., 2006). However, a central element of the JA signalling pathway seems to be the COI1 (Coronatine Insensitive 1) protein (Feys *et al*., 1994; Xie *et al*., 1998). *Arabidopsis coi1* mutants of are male-sterile, fail to express JA-regulated genes, and are susceptible to pathogens (Thomma *et al*., 1998). COI1 is an F-box protein that forms an active SCF^{COI1} complex, which together with the 27 COP9 signalosome (CSN) contribute to JA signalling (Devoto *et al*., 2002; Xu *et al*., 2002). *In vivo,* this machinery works as an ubiquitin ligase complex that removes repression from JA-responsive defence genes. It targets regulatory proteins, including transcriptional repressors, to ubiquitin-proteasome-mediated protein-degradation (Devoto *et al*., 2002; Xu *et al*., 2002; Feng *et al*., 2003). Plants with reduced CSN function, like the *coi1* mutant, exhibit a JA-insensitive root elongation phenotype and an absence of specific JA-induced gene expression (Feng *et al*., 2003). Interestingly, the auxin receptor TIR1 which was recently characterised, is an F-box protein that, like COI1, forms an ubiquitin protein ligase SCFTIR complex (Dharmasiri *et al*., 2005). Thus, it is tempting to speculate that COI1 could act like TIR1 as a receptor for JA.

1.4.2.6 Ethylene

Ethylene (ET) is a gaseous organic compound that is a plant hormone involved in many aspects of the plant life cycle, such as seed germination, root hair development, fruit ripening, organ senescence and root nodulation (Bleecker and Kende, 2000). It is also involved in the defence response to pathogen attack and the regulation of plant responses to abiotic stresses including wounding, hypoxia, ozone, chilling, freezing and those induced by flooding or drought (Johnson and Ecker, 1998; O'Donnell *et al*., 2003). Five ethylene receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) have been identified in *Arabiodpsis* and were shown to take part in ET signalling (Hua and Meyerowitz, 1998; Sakai *et al*., 1998).

ET contributes in various plant disease resistance pathways, but depending on the plant species and pathogen, its role can be totally different. A deficiency of ET signalling in the plant may lead either to an increase in susceptibility or resistance. This evidence was demonstrated by the fact that the reduction of ET sensitivity in soybean produces less severe chlorotic symptoms when infected with the virulent strains *Pseudomonas syringae* pv *glycinea,* while virulent strains of the fungi *Septoria glycines* and *Rhizoctonia solani* lead to more severe symptoms (Hoffman *et al*., 1999).

1.4.2.7 Interaction between the JA, SA and ET signalling pathways

Studies of the changes in the expression levels of 2375 selected genes following pathogen infection or SA, ET and JA treatment showed that some genes are affected or respond to at least two defence signals (Schenk *et al*., 2000). There is a substantial network of regulatory interaction and coordination among different components of plant defence signalling pathways. The SA-dependent and JA-ET-dependent pathways share common pathways and interact with each other, either positively or negatively, besides the fact that they induce resistance to different pathogens and regulate the expression of different pathogenesis-related (PR) genes (Feys and Parker, 2000; Schenk *et al*., 2000; Lorenzo and Solano, 2005).

Some pathogens can induce plant defence responses via activation of the JA and ET signalling pathways. Both pathways co-operate to regulate the expression of many genes and at least some jasmonate-inducible genes are not inducible in plants unable to produce or sense ET (Reymond and Farmer, 1998). A significant advance in our understanding of this evidence was made by Penninckx and coworkers (Penninckx *et al*., 1998) who showed that, *Arabidopsis* plants with defects in JA signalling (*coi1*) or ET perception (*ein2*) fail to induce a subset of *PR* gene expression, the plant defensin gene *PDF1.2*, a basic chitinase (*PR-3*), and an acidic heveinlike protein (*PR-4*), resulting in enhanced susceptibility to some pathogens. Interestingly, the majority of other responses mediated by the JA and ET pathways are specific to only one of the signals, but JA and ET signalling pathways are required to induce *PDF1.2*. The interaction between JA and ET signalling pathways is likely to be downstream, possibly at the level of the specific defence gene promoters, because only

a small subset of genes is affected by both signals. Also, both signalling pathways may regulate independently some particular process involved in pathogen defence response. For instance, it has been shown that only the JA signalling pathway is involved in the induction of the defence compound 3-indolylmethylglucosinolate in *Arabidopsis* when infected by the pathogen *Erwinia carotovora* (Brader *et al*., 2001), confirming that that ET and JA pathways may also function independently in mechanisms of pathogen defence response.

The SA dependent response can be triggered by a pathogen that obtains nutrients from living plant tissue, while the ethylene-jasmonate-dependent pathway is activated by pathogens that kill plant cells to obtain nutrients. In addition, it was suggested that ethylene-jasmonate and salicylic acid pathways are mutually inhibitory. Such cross-talk probably implies a capacity for a selective defence against specific types of parasites (Odjakova and Hadjiivanova, 2001).

The interactions between SA and ET signalling appear to be complex, and there are a number of examples of synergistic action. A null mutation in the *EDR1* gene has been shown to increase resistance to *Erisyphe cichoracearum* and *Pseudomonas syringae*, and induces rapid activation of defence related genes such as *PR-1* (Frye *et al*., 2001). This disease resistance induction is independent of the JA and ET pathways and depends on the SA-induced defence response pathway. However, *PR-1* gene expression, which is SA-dependent, is highly induced by ET treatment in *edr1* mutant plants, whereas it is almost

absent in wild-type plants. This shows that there is significant interaction between the ET and SA-dependent pathways.

Some evidence also indicates that SA and JA act synergistically in plant defence responses via the activation of the signalling pathways (Van Wees *et al.*, 2000). SA dependent systemic acquired resistance (SAR) is fully compatible with the JA dependent induced systemic resistance (ISR). Simultaneous activation of both pathways led to an elevated level of protection against pathogen attack.

In *Arabidopsis thaliana*, SAR and ISR function together against many pathogens including the foliar pathogen *Pseudomonas syringae* pv. *tomato*. When SAR and IRS are activated simultaneously, they confer effective protection against *Pseudomonas syringae* pv. *tomato*. This protection is absent in *Arabidopsis* genotypes blocked in either SAR or ISR. It has been shown that in plants expressing SAR, there is no effect on the SAR biomarker gene *PR-1* when the ISR is induced. These findings suggest that the SAR and the ISR pathway which both require the key regulatory protein NPR1 are compatible and that there is no significant cross-talk between these pathways. Plants expressing both types of induced resistance did not show elevated *Npr1* transcript levels, indicating that the constitutive level of NPR1 is enough to favour simultaneous expression of SAR and ISR. These results showed that the enhanced level of protection is established through synergistic activation of complementary, NPR1-dependent defence responses that are both active against the pathogen *Pseudomonas syringae* pv. *tomato*. (Van Wees *et al*., 2000)

1.4.3 Resistance mechanisms

Plants defend themselves against pathogens by a combination of weapons from two arsenals: the passive or pre-existing defence mechanism and the active or induced defence.

1.4.3.1 Passive resistance mechanisms

Pathogens have evolved weapons to facilitate their invasion of plants. Plant defences may be structural or chemical. The first mechanical barrier to infection is the plant's surface, which the pathogen must adhere to and penetrate if it is to cause infection (Dangl and Jones, 2001; Agrios, 2005). Some of the constitutive structural defences are familiar features of plants and are present even before the pathogen comes in contact with the plant. Such structures include the amount and quality of wax and cuticle that cover the epidermal cells, lignification of woody tissues, the structure of the epidermal cell walls, the size, location, and shapes of stomata on lower leaf surfaces and lenticels, and the presence of tissues made of thick-walled cells that hinder the advance of the pathogen on the plant and may initially stop establishment of infection structures as well (Heath, 2000a; Dixon, 2001; Kamoun, 2001). Waxes on leaf and fruit surfaces form a water repellent surface, which prevent the formation of a film of water on which pathogens might be deposited and germinate or multiply. The toughness and thickness of the outer wall of epidermal cells are apparently important factors in the resistance of some plants to certain pathogens: thick, tough walls hinder the direct penetration of fungal pathogens (Agrios, 2005; Nürnberger and Lipka, 2005).

Although structural characteristics may provide a plant with various degrees of defence against attacking pathogens, it is clear that the resistance of a plant against pathogen invasion depends not so much on its structural barriers as on the substances produced in its cells before or after infection. At the biochemical level, many plants produce secondary metabolites that are not only involved in some aspects of the plant life cycle such as plant growth or development but some of these chemicals probably aid in resistance to insect pests, and some have antimicrobial activity and play a key role in defence against pathogens. Some of these constitutive chemicals involved in plant disease resistance are known as proinhibitins such as alkaloids, phenolics and phytoanticipins (Nürnberger and Lipka, 2005). Phytoanticipins are low molecular weight compounds present in plants before challenge by pathogens are produced after infection solely from pre-existing constituents (Dixon, 2001). Saponins are a class of phytoanticipins that destroy membrane integrity in saponin-sensitive parasites, and are stored in an inactive form in the vacuoles of the plant cell, becoming active when hydrolase enzymes are released following wounding or infection (Agrios, 2005). If all these preformed plant weapons are not sufficient to stop pathogen invasion the plant activates inducible defences.

1.4.3.2 Active resistance mechanisms

Induced defence responses involve both physical factors and biochemical reactions such as hypersensitive necrotic responses and tissue and cellular modifications. The biochemical reactions take place in the cells and tissues of the plant and the synthesis and accumulation of antimicrobial reactive oxygen species, phytoalexins, and translation products from pathogenesis-related genes lead to the localized reinforcement of cell walls and hypersensitive, programmed cell death. These biochemical reactions are toxic to pathogens and can also create conditions that inhibit growth of the pathogen in the plant (Dixon, 2001; Nürnberger and Lipka, 2005).

1.4.3.2.1 Hypersensitive response

The hypersensitive response (HR) is regarded as one of the mechanisms, used by plants, to restrict the spread of infection by microbial pathogens to other parts of the plant (De Wit, 1992; Kwang-Hyung *et al*., 2004). It involves a complex form of programmed cell death (Greenberg and Yao, 2004). HR synergistically works with the resistance response (RR) which involves the co-ordinated activation of many defences that limit the growth of the pathogen in the plant (Greenberg, 1997).

The HR is triggered by the plant when it recognizes a pathogen. It is characterized by the rapid death of cells in the local region surrounding an infection and the changes in their metabolic activities as well. Their respiration decreases and becomes very slow or completely stopped, followed by the accumulation of toxic compounds (Agrios, 2005).

These lead to an inhibitory effect or an unfavourable condition for the further growth and spread of the pathogen around the site of infection. In some cases, HR restricts or kills the invading pathogen, and in other cases, it seems to signal the induction of a cascade of induced defences (Jeong, 2005; McDowell, 2004). In addition, the plant system or those cells surrounding the infection can also accumulate newly produced antifungal chemicals known as phytoalexins which are small molecular weight compounds. Studies showed that phytoalexins are produced when there is microbial attack or under conditions of stress, and are completely absent in healthy tissues (Grayer and Tetsuo, 2001). In addition, the HR activates the expression of many plant genes including those encoding enzymes of the phenolic pathway, peroxidases, glucanases, chitinases and hydroxyproline-rich glycoproteins (Dixon and Lamb, 1990).

The HR can restrict the growth or kill the invading biotrophic and hemibiotrophic pathogens that require nutrition from living plant cells for at least part of their infection cycle (Richael and Gilchrist, 1999). But for non biotrophic pathogens that do not require their host cells to stay alive, cell death alone cannot restrict their growth. However for a complete defence response of the plant, HR has to be supported by many other induced defence mechanisms that typically occur within the dying cells and in their adjacent living neighbours (De Wit, 1992; Hu *et al*., 1998; Molina *et al*., 1999; Heath, 2000b).

It has been suggested that signals that condition adjacent cells to become responsive to pathogen elicitors are released by HR cell death (Graham and Graham, 1999). Additionally those signals can activate systemic resistance throughout the plant. In *Arabidopsis*, systemic acquired resistance depends on secondary oxidative bursts in distant tissues and the

formation of ‘micro-HRs’; showing that the cell death component of the HR may function more as a signalling system than as a direct defence mechanism (Alvarez *et al*., 1998).

1.4.3.2.2 Systemic acquired resistance

Systemic acquired resistance (SAR) involves broad spectrum, long-lasting, plant defence responses that primes the whole plant against subsequent infection. It is a process through which the plant is induced to produce greater than normal amounts of pathogenesis-related proteins with antimicrobial activity such as chitinases, β-1-3-glucanases, defensins and peroxidases that form a broad host defence system against phytopathogens (Uknes *et al*., 1992; Plymale *et al.*, 2007). Additionally, the defence response involved also includes some physical changes such as cell wall lignification and papilla formation (Schneider *et al*., 1996). During SAR, one or several translocated signals involved in the stimulation of resistance mechanisms are produced in the uninfected parts of the plant. As a result, a first infection predisposes the entire plant to be more resistant to further infections. Studies demonstrated that ROS are often generated as warning signals within the cell or to the neighbouring cells, triggering various reactions such as the structural development of SAR (Dixon and Lamb, 1997; Grayer and Tetsuo, 2001).

Evidence has accumulated that SA is a signal for systemic resistance (Ryals *et al*., 1996; Schneider *et al*., 1996; Sticher *et al*., 1997; Yang *et al*., 1997). Studies to discern the factors responsible for stimulating and controlling SAR reported that SA produces resistance in plants to an array of pathogens (Gaffney *et al*., 1993; Delaney *et al*., 1994; McDowell and

Dangl, 2000; Park *et al*., 2007). SA has long been recognized as one signal leading to SAR due to the fact that its concentration increases dramatically in concert with the induction of *PR* genes and resistance after pathogen infection (Malamy *et al*., 1990; Métraux *et al*., 1990; Durrant and Dong, 2004). The role of SA as a signal in SAR induction was documented by experiments using transgenic tobacco to over express the enzyme salicylate hydroxylase, encoded by the nahG gene of *Pseudomonas putida* (Gaffney *et al*., 1993). This enzyme catalyzes the conversion of SA to catechol, which is not an active SAR inducer, by removing the carboxyl group from SA and replacing it with a hydroxy group in a very specific reaction that utilizes NADH as a cofactor (Gaffney *et al*., 1993; Delaney *et al*., 1994). The plants expressing nahG gene do not accumulate SA in response to pathogen infection and are unable to induce an SAR response to pathogens. These experiments underline the direct involvement of SA in SAR signalling, but they do not address whether SA is the long-distance, phloem-mobile signal for SAR. In *Arabidopsis* plants, another study showed that ^{18}O-labeled SA is transported from pathogen-inoculated leaves of tobacco to systemic, noninoculated leaves, showing that SA itself is the signal (Shulaev *et al*., 1995). SA was also suggested to be converted to volatile methyl salicylate (MeSA), which could induce resistance not only in distal tissues of the infected plant but also in neighbouring plants (Shulaev *et al.*, 1997).

Although the above studies indicate that SA is mobile and therefore could function as the long distance SAR signal, an increasing body of evidence arguing against SA as the mobile systemic signal also exists. In *Pseudomonas syringae* infected cucumber, the signal for SAR and increased peroxidase activity moved out of the inoculated leaf before

increases in SA levels were detected in the petiole (Smith *et al*., 1998). This detachment of leaves from *P. syringae*-infected plants before SA levels rose did not block SAR development (Rasmussen *et al*., 1991). Others studies showed that potato and rice can produce high SA concentrations under non-inducing conditions (Coquoz *et al*., 1995; Silverman *et al*., 1995). In a recent study over one and a half million cDNA reads were assembled into 34,800 transcript contigs from American chestnut and 48,335 transcript contigs from Chinese chestnut. The number of genes tagged, the length of coding sequences, and the numbers of tagged members within gene families showed that the cDNA dataset provides a good resource for studying the American and Chinese chestnut transcriptomes. *In silico* analysis of transcript abundance identified hundreds of GDTA in canker versus healthy stem tissues. These DTA genes belong to various pathways involving cell wall biosynthesis, reactive oxygen species (ROS), salicylic acid (SA), ethylene, jasmonic acid (JA), abscissic acid (ABA), and hormone signalling. DTA genes were also identified in the hypersensitive response and programmed cell death (PCD) pathways. These DTA genes are candidates for host resistance to the chestnut blight fungus, Cryphonectria *parasitica* (Barakat *et al*., 2012).

1.4.3.2.3 Cell wall fortifications and vascular occlusion

Pathogens have to overcome the plant cell wall to reach the cell, or sometimes can penetrate through a natural opening or wound. The cell walls of many plants are able to modify, produce or accumulate defence-related compounds that reinforce the walls and lead to an increase in resistance. The plant cell walls in reaction to infection can produce or deposit

substances such as amino acids, hydroxyproline, callose, glycoproteins, phenolic compounds (lignins and suberin), or mineral elements (silicon and calcium). Many of these substances react and cross-link with one another in order to form more insoluble cell wall structures that confine the pathogen and further increase resistance to penetration. Besides these substances, plant cells can also respond to invading pathogens by producing vascular occlusions such as tyloses and gels which aim to cut off the transpiration stream in the xylem, consequently immobilising the pathogen (Agrios, 2005).

Lignification is a mechanism for disease resistance in plants, which lead to an ultra-structurally modified reinforced cell wall (Walter, 1992). Lignin is an amorphous, three dimensional polymer whose composition and properties are different from both carbohydrates and proteins (Agrios, 2005). It is found in the middle lamella, as well as in the secondary cell wall of xylem vessels and the fibres that strengthen plants. In addition lignin occupies the spaces in the cell wall between pectin components, cellulose and hemicellulose and confers rigidity and mechanical strength to the cell wall, waterproofs xylem elements and allows for defence strategies against pathogen attack (Chabannes *et al*., 2001). The lignification process enables the cell wall to become more resistant to mechanical pressure applied during penetration by fungal appressoria as well as more water resistant and thus less accessible to cell wall-degrading enzymes (Zeyen *et al*., 2002). In response to invasion by fungal pathogens, plants assemble cell wall apposition, also called callose papillae on the inner side of cell walls at the sites of attempted penetration of biotrophic fungi such as powdery mildew (Zeyen *et al*., 2002). Callose is made by a few cell types, however it is synthesized made by most cells in response to wounding and during attempted penetration

by invading pathogens such as fungal hyphae (Agrios, 2005). Callose is a plant polysaccharide containing high proportion of glucose residues linked together through β-1,3-linkages, and is termed a β-glucan. Its synthesis is catalyzed by callose synthase or β-1, 3-glucan synthase and its degradation by β-1,3-glucanase in the cell wall (Bell and Hemsley, 2000). Callose synthesis can be initiated in a Ca^{2+} dependent or in a Ca^{2+} independent manner. Studies showed that localised deposition of callose may begin when Ca^{2+} influx increases and activates callose synthase (Bowles, 1990).

1.4.3.2.4 Phytoalexins and phenolic compounds involved in defence response

Phytoalexins are low molecular mass, lipophilic, toxic, antimicrobial substances produced rapidly around sites of incompatible pathogen infections and in response to abiotic stimuli such as chemical or mechanical injury and to an extensive array of abiotic elicitors (Agrios, 2005). During compatible plant-pathogen interactions, phytoalexins accumulate; but they are tolerated, detoxified or suppressed by the pathogens (Mert-Türk, 2002). For example, it was previously demonstrated that a phytoalexin (camalexin) is accumulated during both incompatible and compatible interactions in *Arabidopsis thaliana* following the infection with *Peronospora parasitica* (Mert-Türk *et al*., 1998).

Phytoalexin biosynthesis occurs after primary metabolic precursors are diverted into secondary metabolic pathways. The diversion of phenylalanine into the synthesis of various flavonoid phytoalexins arises from the *de novo* synthesis of enzymes, such as PAL, an enzyme that controls a key branch points in the phenylpropanoid biosynthetic pathway (Hammond-Kosack and Jones, 2000). Phytoalexins are produced in healthy plant cells,

surrounding infected or wounded cells. Their accumulation is induced by substances diffusing from damaged cells into the neighbouring healthy tissue (Agrios, 2005). The resistance is induced when the concentration of phytoalexins is sufficient to inhibit the growth of the pathogen. When leaves of rice were inoculated with the rice blast fungus *Magnaporthe grisea*, the rice leaf phytoalexins sakuraneith and momilactone A were produced within three days (Grayer and Tetsuo, 2001). In addition, it was shown later that during interaction between resistance rice cultivars and the blast fungus *Magnaporthe oryzae*, the induction of phytoalexin biosynthetic genes and the accumulation of phytoalexins started at 2 days postinoculation (dpi) which led to a hypersensitive response and the inhibition of fungal growth, whereas in the susceptible rice cultivars the accumulation of phytoalexins was delayed and was several times lower compared to the resistant rice cultivars. Detoxification of phytoalexins is a strategy used by the fungus to invade the host plant and prompt induction of phytoalexins upon HR is a tactic used by the resistant rice cultivars to block invasion of the fungus (Hasegawa *et al*., 2010).

It may well emerge for many plant-pathogen interactions that phytoalexins are synthesized in response to pathogens infection in order to reduce the severity of secondary infections or the overall development of virulent pathogens (Hammond-Kosack and Jones, 2000).

Plant phenolics are secondary metabolites that constitute one of the most common and widespread groups of substances in plants; they are synthesized from the shikimate phenylpropanoid-flavonoid pathways (Randhir *et al*., 2004). Phenolics usually are stored in the epidermal cells as well as subepidermal cells of leaves and shoots and central vacuoles of

guard cells during normal processes of differentiation. Phenolic compounds are known to have multi physiological properties, which are needed by plants for growth, pigmentation, reproduction, resistance to pathogens and for many other functions (Puupponen-Pimia, 2001). Generally, the role of phenolic compounds defence is related to their antibiotic, unpalatable properties. In plants, during pathogen infection or injury, phenolic-storing cells burst and a chemical reaction leads to the oxidation of plant phenolics, which serve to lignify and/or suberize the site of infection. Some phenolics occur constitutively and function as preformed phenolics that are synthesized during the normal growth of plant tissues. Others are induced phenolics that are synthesized by plants in response to physical injury, pathogen invasion or abiotic stress (Nicholson and Hammerschmidt, 1992). It has been also shown that during vascular defence, phenolic compounds can also react as signalling molecules. The first study that demonstrated that phenolics can provide disease resistance was the case of onion scales which contains sufficient quantities of catechol (I) and protocatechuic acid (II) to prevent onion smudge disease caused by *Colletotrichum circinans.* It has been shown that the spore germination of *Colletotrichum circinans* is reduced to below 2% in the coloured outer onion scales of resistant onion varieties containing enough of these two phenols, whereas in the susceptible varieties lacking these two phenols, the spore germination rate was 90% (Vincenzo *et al.*, 2006).

1.4.3.2.5 Pathogenesis-related proteins

Pathogenesis-related (PR) proteins were originally discovered and classified in tobacco plants hypersensitively reacting to tobacco mosaic virus infection (van Loon and van Kammen, 1970); but a number of different PR proteins have been found since then in other plant species including many monocots and dicots and appear to be ubiquitous in higher plants (Stintzi *et al*., 1993). PR proteins play an important role in plant defence against pathogen attack, seed germination and in general help the plant to adapt to stressful environment (Odjakova and Hadjiivanova, 2001; Aglika, 2005). They can be induced both locally and around the infection site and systemically away from the initial infection site. PR proteins are monomers (with a few exceptions) of rather low molecular mass (8-50 kDa) which display very characteristic physicochemical properties which aid in their detection and isolation. The PR proteins are either extremely acidic or extremely basic and have typical physicochemical properties that make them resistant to acidic pH and proteolytic cleavage and thus survive in the unfavourable environments where they occur, which include vacuoles, cell walls or intercellular species (Stintzi *et al*., 1993). Different PR proteins families function in various biological activities though generally they all secrete their effector proteins into either the intercellular space (acidic proteins) or the vacuole (basic proteins).

Initially, five main groups of PR proteins (PR-1to PR-5) were characterized by both biochemical and molecular genetic techniques in tobacco. They are grouped into families consisting of several members with similar properties (Bol *et al*., 1990). Thereafter, in 1994

a unifying nomenclature for PR proteins was proposed on the basis of their grouping into families within which they share amino acid sequences, serological relationships, and enzymatic or biological activity. Eleven families (PR-1 to PR-11) were recognized and classified for tobacco and tomato, with the families PR-8 and PR-10 being also present in cucumber and parsley respectively (Van Loon *et al*., 1999). Afterwards, three novel families PR-12, PR-13 and PR-14 were recognized in radish, *Arabidopsis* and barley respectively (Van Loon and Van Strien, 1999). Criteria used for the introduction of new families into PR proteins are based on the fact that: firstly, the protein must be induced by a pathogen in tissues that do not normally express it, and secondly, induced expression must occur in at least two different plant-pathogen combinations, or expression in a single plant-pathogen combination must be confirmed independently in different laboratories (Van Loon and Van Strien, 1999). Currently on the basis of their primary structures, enzymatic properties and immunologic relationships, PR proteins have been classified into seventeen families (PR-1 through 17) (Okushima *et al*., 2000; Christensen *et al*., 2002) (Table 1.1).

PR-1, proteins with small size (usually 14-17 kD) and antifungal activity is the most abundant group of PR proteins induced by pathogens or SA, and is commonly used as a marker for SAR. Their limited antifungal activity suggests a function in plant defence, but its mode of action or relationship to other proteins is unknown. The PR-2 family consists of endo-β-1, 3-glucanases, which are able to hydrolyze β-1, 3-glucans, a biopolymer found in fungal cell walls. The PR-3, PR - 4, PR -8 and PR -11 families are all classified as endochitinases belonging to various chitinase classes (I – VII) (Van Loon and Van Strien, 1999). In plant defence mechanisms, these chitinases degrade chitin, the major structural

component of fungal cell walls. The PR-5 family belongs to the thaumatin-like proteins and osmotin-like proteins with homology to permatins that permeabilise fungal membranes (Vigers *et al*., 1991). PR-6 is a group of proteinase inhibitors involved in defence against insects and other herbivores, microorganisms and nematodes. PR-7 has so far been characterized only in tomato, where it is a major PR protein and acts as an endoproteinase. The PR-9 family of peroxidases is likely to function in strengthening plant cell walls by catalysing lignin deposition in reaction to microbial attack. The PR-10 family is structurally related to ribonuclease, however their capability to cleave viral mRNA remains to be demonstrated. The PR-12 defensins, PR-13 type thionins, PR-14 type lipid transfer proteins, PR-15 type oxalate oxidase and PR-16 type oxalate-oxidase-like proteins all may have antifungal and antibacterial activity, exerting their effect on the target microorganism at the level of the plasma membrane (Bohlmann, 1994; García-Olmedo *et al*., 1995; Broekaert *et al*., 1997). The PR-17 family was found in tobacco but their properties are still unknown (Okushima *et al*., 2000).

Table 1.1 The families of pathogenesis related proteins [(adapted from Van Loon and Van Strien, 1999)].

Family	Type member	Properties	Gene symbol
PR-1	Tobacco PR-1a	Unknown	*Ypr1*
PR-2	Tobacco PR-2	β-1,3-glucanase	*Ypr2*, [*Gns2* ('*Glb*')]
PR-3	Tobacco P, Q	Chitinase type I,II, IV,V,VI,VII	*Ypr3*, *Chia*
PR-4	Tobacco 'R'	Chitinase type I, II	*Ypr4*, *Chid*
PR-5	Tobacco S	Thaumatin-like	*Ypr5*
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	*Ypr6*, *Pis* ('*Pin*')
PR-7	Tomato P_{69}	Endoproteinase	*Ypr7*
PR-8	Cucumber chitinase	Chitinase type III	*Ypr8*, *Chib*
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase	*Ypr9*, *Prx*
PR-10	Parsley 'PR1'	Ribonuclease-like	*Ypr10*
PR-11	Tobacco 'class V' chitinase	Chitinase, type I	*Ypr11*, *Chic*
PR-12	Radish Rs-AFP3	Defensin	*Ypr12*
PR-13	Arabidopsis THI2.1	Thionin	*Ypr13*, *Thi*
PR-14	Barley LTP4	Lipid-transfer protein	*Ypr14*, *Ltp*
PR-15	Barley OxOa (germin)	Oxalate oxidase	*Ypr15*
PR-16	Barley OxOLP	Oxalate oxidase-like	*Yrp16*
PR-17	Tobacco PRp27	Unknown	*Yrp17*

1.4.4 Plant disease resistance genes

1.4.4.1 Transcription factors

Transcriptional activation of genes is a crucial part of the plants defence system against pathogens. This activation by the signals in the cell nucleus leads to *de novo* synthesis of a variety of proteins and antimicrobial compounds. The architecture of the promoters leads to the differences in the expression patterns of pathogen-responsive genes. Transcription factors that have an important role in defence response belong to WRKY, bZIP, ethylene responsive element binding proteins (EREBP), Whirly and Myb protein families. EREBS, WRKY and Whirly proteins appear to be unique to plants whereas other transcription factors such as bZIP and Myb proteins also have counterparts in animals (Rushton and Somssich, 1998).

WRKY proteins are a large family of transcription factors which appear to exist exclusively in plants. Studies revealed that the WRKY family consists of more than 90 members in rice and 74 members in *Arabidopsis thaliana* (Ülker and Somssich, 2004). All these proteins share in common, a DNA-binding region of approximately 60 amino acids in length (the WRKY domain) which contains the conserved amino acid sequence motif WRKYGQK, adjacent to a novel zinc-finger motif. WRKY factors show high binding affinity to a DNA sequence characterised by the signature sequence (C/T) TGAC (T/C) known as the W box (Eulgem *et al*., 2000). W-box-dependent binding activity requires both the invariable WRKY amino-acid signature and the cysteine and histidine residues of the WRKY domain, which tetrahedrally coordinate a zinc atom. The invariable WRKY amino-acid signature and the

cysteine and histidine residues of the WRKY domain, which tetrahedrally coordinate a zinc atom, are required for the W-box dependent binding activity (Ülker and Somssich, 2004).

In many plants species, the transcription of the WRKY gene is strongly and quickly up regulated in response to pathogen attack, wounding or abiotic stresses. In tobacco, multiple WRKY genes are induced after infection with bacteria or tobacco mosaic virus, or treatment with fungal elicitors SA or H_2O_2 (Yoda *et al*., 2002; Takemoto *et al*., 2003). In addition, 49 out of 72 tested WRKY genes in *Arabidopsis* responded to bacterial infection or SA treatment (Dong *et al*., 2003); it may also happen that an even higher percentage is induced throughout the whole plant (Kalde *et al*., 2003). Several lines of evidence also demonstrate a specific role of WRKY transcription factors in defence-induced MAPK kinase signalling cascades (Wan *et al*., 2004). Two *Arabidopsis* WRKY factors (AtWRKY22 and AtWRKY29) have been characterized as important downstream components of a MAPK pathway that give resistance to both fungal and bacterial pathogens (Asai, 2002).

1.4.4.2 The concept of gene-for-gene resistance

According to the gene-for-gene model for plant disease resistance, an incompatible reaction results from the interaction of the product of a plant resistance gene with the product of the corresponding avirulence gene that induces the chain of signal transduction events which leads to the activation of defence mechanisms, thus stopping the pathogen growth (Agrios, 2005). The results of this concept is that the products of resistance genes in gene-for-gene interactions work as receptors for specific ligands synthesized by the pathogen, either directly or indirectly through expression of avirulence genes. This specific receptor-ligand

recognition event, elicits through signal transduction, a complex cascade of defensive responses observed as the resistant phenotype (Staskawicz *et al*., 1995; Gachomo *et al*., 2003). Further study demonstrated that there are many exceptions to the gene-for-gene model and that most interactions between R and Avr genes are indirect, involving perception of pathogen derived proteins within a complex (Hammond-Kosack and Parker, 2003)

1.4.4.3 Resistance genes

Resistance genes based on their predicted protein structural characteristics can be classified into six classes whose products seem to activate a similar range of defence mechanisms (Table 1.2). These six classes of R genes are those coding for: TIR/NBS/LRR proteins, detoxifying enzymes, kinases, extracellular receptors, G protein couple receptor and receptor kinases (Ayliffe and Lagudah, 2004). R genes structure and loci discovery provides insight into R gene evolution and function which can lead to new strategies for plant disease control. An increasing number of R genes have been isolated from plants such as the *Pto* gene from tomato which encodes a ser/thr protein kinase that confers resistance to *Pseudomonas syringae* carrying the avirulence gene *avrPto* (Martin *et al.,* 1993); the tomato *Cf-x* genes which confer resistance to *Cladosporium fulvum* and which encode single pass membrane proteins with extracellular LRRs (Jones, 1994); the rice resistance gene *Xa21* which confers resistance against the bacterial pathogen *Xanthomonas oryzae* pv. *Oryzae* (Song *et al*., 1995) and the RRS-1 gene from *Arabidopsis* which confers resistance to *Ralstonia solanacearum* (Meyers *et al.*, 1999).

The genes that encode NBS–LRR proteins constitute the largest and most diverse family of resistance genes in plants (Wroblewski *et al.*, 2007). The NBS-LRR genes encode for a family of proteins with a centrally located nucleotide-binding site (NBS) and a C-terminal leucine-rich repeat (LRR) region (Figure 1.4). This family of plant resistance genes function in a classical gene-for gene interaction in which pathogen elicitors are recognised by the C-terminal LRR receptor region and a (HR) hypersensitivity response is activated. Amongst plant species, NBS-LRR proteins can be subdivided into two subgroups based on deduced N-terminal structural features; firstly those containing an amino terminus domain with homology to the intracellular signalling domains of the *Drosophila* Toll protein and mammalian interleukin (IL)-1 receptors (TIR-NBS-LRR) and secondly those with a leucine zipper or coiled-coil domain (CC-NBS-LRR) (McHale *et al.*, 2006). Several subfamilies of the CC-NBS-LRR class vary in the location of coiled-coil domain and in size. Studies showed that only CC-NBS-LRR is present in monocotyledonous species, whereas both subgroups are found in dicotyledonous species, with TIR-NBS-LRR genes being the more abundant class (Dangl and Jones, 2001; Ayliffe and Lagudah, 2004). A recent study of strawberry plant defence mechanisms showed the ability of a strawberry plant to respond efficiently to pathogens. It relies firstly on the physiological status of injured tissue (pre-formed mechanisms of defence) and secondly on the general ability to recognize and identify the invaders by surface plant receptors, followed by a broad range of induced mechanisms, which include cell wall reinforcement, production of reactive oxygen species, phytoalexin generation and pathogenesis-related protein accumulation (Amil-Ruiz *et al*. 2011).

Table 1.2. The six classes of plant resistance genes [(adapted from Hammond-Kosack and Jones (2000); Hammond-Kosack and Parker (2003); Ayliffe and Lagudah (2004)].

Class	R protein predicted features	Gene	Plant	Pathogen	Pathogen
1a	Detoxifying enzymes	*Hm 1*	Maize	*Helminthosporium maydis (race1)*	Necrotrophic fungus
2a	Intracellular proteins kinase	*Pto*	Tomato	*Pseudomonas syringae pv tomoato (avrPto)*	Extracellular bacteria
2b	Intracellular proteins kinase with 2 tandem kinase domains	*Rpg1*	Barley	*Puccinia graminis f.sp.tritic*	Biotrophic intracellular fungus
3a	TIR-NBS-LRR	*N*	Tobacco	Tobacco mosaic virus	Intracellular virus
		RPP1, RPP4, RPP5	Arabidopsis	*Peronospora parasitica (avrRPP1, avrRPP4, avrRPP5)*	Biotrophic intracelular Oomycete
3b	TIR-NBS-LRR-NLS-WRKY	*RRS-1*	Arabidopsis	*Ralstonia solanacearum*	Extracellular bacteria
3c	CC-NBS-LRR	*RPS2*	Arabidopsis	*Pseudomonas syringae pv maculicola (avrRpt2)*	Extracellular bacteria
		Mla1/Mla6	Barley	*Blumeria graminis f.sp. hordei (race 1, race 6)*	Biotrophic fungus
		Rp1-D	Maize	*Puccinia sorghi*	Biotrophic intracelleular fungus
		Rp3	Maize	*Puccinia sorghi*	Biotrophic intracellular fungus
		Lr10, Lr21	Wheat	*Puccinia triticina*	Biotrophic intracellular fungus
		Pm3	Wheat	*Blumeria graminis*	Biotrophic intracellular fungus
3d	NBS-LRD	*Pi-ta*	Rice	*Magnaporthe grisea (avrPita)*	Biotrophic fungus
4a	Extracellular LRR with single membrane spanning Region and short cytoplasmic carboxyl terminus (eLRR-TM)	*Cf-9,Cf-2,Cf-4,Cf-5*	Tomato	*Cladosporium fulvum (Avr 9, avr2, avr4, avr5)*	Biotrophic extracellular fungus
4b	CC- eLRR-TM-ECS	*Ve1*	Tomato	*Verticillium albo-atrum*	Extracellular fungus
	eLRR-TM-PEST-ECS	*Ve2*	Tomato	*Verticillium albo-atrum*	Extracellular fungus
5	Extracellular LRR with single membrane spanning Region and cytoplasmic kinase domain (eLRR-TM-kinase)	*Xa-21*	Rice	*Xanthomonas oryzae pv oryzae (alla races)*	Extracellular bacteria
6	G protein coupled receptor	*mlo*	Barley	*Blumeria graminis f. sp. hordei*	Biotrophic fungus

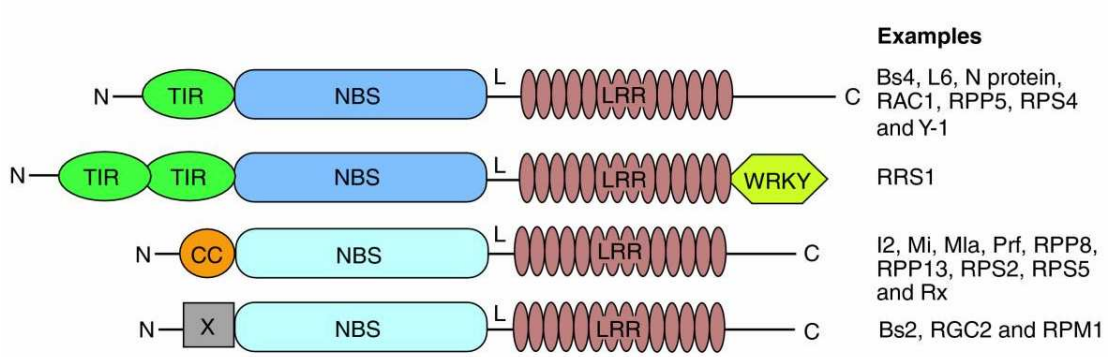


Figure 1.5. Schematic representation of the major domains of the NBS-LRR protein (adapted from McHale *et al.*, 2006). The proteins included in this representation with each configuration are shown on the right. Bs4, I2, Mi, and Prf are from tomato; L6 from flax; N from tobacco; RAC1, RPP5, RPS4, RRS1, RPP8, RPP13, RPS2, RPS5, and RPM1 from *Arabidopsis*; Y-1 and Rx from potato; Mla from barley; RGC2 from lettuce; Bs2 from pepper. N, amino terminus; TIR, Toll/interleukin-1 receptor-like domain; CC, coiled-coil domain; X, domain without obvious CC motif; NBS, nucleotide binding site; L, linker; LRR, leucine-rich repeat domain; WRKY, zinc-finger transcription factor-related domain containing the WRKY sequence; C, carboxyl terminus.

Recently (Vargas *et al*., 2012), transcriptomic, histological, and biochemical studies of the early events were performed during the infection of maize (*Zea mays*) with *Colletotrichum graminicola*, a model pathosystem for the study of hemibiotrophy. Time-course experiments revealed that mRNAs of several defence-related genes, reactive oxygen species, and antimicrobial compounds all begin to accumulate early in the infection process and continue to accumulate during the biotrophic stage. The authors also identified several novel putative fungal effectors and studied their expression during anthracnose development in maize. The results of this study demonstrated a strong induction of defence mechanisms occurring in maize cells during *C. graminicola* infection, even during the biotrophic development of the pathogen (Vargas *et al*., 2012).

1.5 COMPONENTS OF AVOCADO DEFENCE MECHANISMS

1.5.1 Preformed antifungal compounds in avocado fruits

In the past years, several investigations revealed that natural sources of resistance are present in avocado fruit; as in other plant species, avocado has the ability to respond to pathogen attack and possesses a preformed and/or inducible defence strategy. Prusky and colleagues (1982) demonstrated that unripe avocado fruits are resistant to *C. gloeosporioides* due to the presence of high concentrations of preformed antifungal chemicals. Extracts derived from the exocarp of freshly harvested fruits inhibited 78% of fungal growth as compared with 7% for extracts from ripe fruits displaying disease symptoms (Prusky *et al*., 1982). The major antifungal compound that conditions the fungal quiescence in the fruit was shown to be 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene. Subsequent research led to the discovery of a second antifungal compound, which was subsequently purified from unripe avocado fruits (peel and flesh) and identified as 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-ene. This monoene was less fungitoxic than the diene (Prusky *et al*., 1991a).

Later, three other antifungal compounds were isolated from the peel of immature avocado fruit and identified as 1,2,4-trihydroxyheptadec-16-yne; 1,2,4-trihydroxyheptadec-16-ene and 1-acetoxy-2, 4-dihydroxyheptadec-16-ene, which together with the two previously described diene and monoene compounds constitute the antifungal chemical arsenal of the unripe fruit peel (Adikaram *et al*., 1993). (E,Z,Z)-1-Acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene was also isolated from Fuerte avocado idioblast cells and shown to inhibit *C. gloeosporioide* germination and germ tube elongation (Domergue *et al*.,

2000). The main action of these antifungal compounds is to maintain the fungus in a quiescent stage, which is a phenomenon where infection takes place in the early stage of fruit development, but the disease appears later on in the near-mature or mature stage of the avocado fruit (Guyot *et al.* 2005).

In order to confirm the antifungal properties of these compounds, a recent study revealed that inoculation of harvested and unharvested Fuerte avocado fruits with *C. gloeosporioides* lead to an increase in the antifungal diene and triene levels, particularly at 2 days post infection (Marimani, 2011). The infection process of the fungus was more efficient and striking in harvested fruits, which contain low antifungal diene contents, than in unharvested fruits that are more resistant to the fungus due to their higher concentration of diene contents (Marimani, 2011). The use of systemic resistance inducers, such as potassium silicate in avocado fruits infected with *C. gloeosporioides*, increased the presence of antifungal compounds and potentially decreased anthracnose incidence (Bosse *et al*., 2011).

1.5.2 Molecular basis of resistance in avocado

Recently, Mitter *et al*. (2011) used genetic modification to deliver RNA silencing based resistance to *Phytophthora cinnamomi* in avocado through the transformation of avocado somatic embryos of selected rootstocks. This technique is well adapted to produce avocado plants with disease resistance to *Phytophthora* root rot (Mitter *et al*., 2011).

The activity of β-1,3-glucanases was highly up regulated in avocado rootstock resistant clones infected with *P. cinnamomi*, 6 hours post inoculation, with no significant response

in the more susceptible avocado rootstock clone (Christie *et al*., 2011). Subsequently, confocal microscopy showed limited spread of hyphae in the resistant clones 12 days post inoculation and abundant hyphae in the susceptible rootstock. These studies have opened up an understanding of resistance mechanisms to *P. cinnamomi* in avocado rootstocks (Christie *et al*., 2011).

The first avocado transcriptome study of differentially expressed genes in avocado root infected by *P. cinnamomi* using the Roche 454 GS FLX Titanium platform has been reported by Mahomed *et al*. (2011). 454 Pyrosequencing of uninfected and infected tolerant avocado root cDNA libraries was conducted followed by bioinformatics analysis. A total of 2.0 Mb of sequence data were generated by the single sequencing run, consisting of 9953 reads assembled into 371 contigs. Many genes were identified and predicted to be involved in: cellular processes, defence mechanisms, cellular components, stress responses, ribosome structure, cell wall related proteins, protein binding, mitochondrial proteins, ATP binding, signal transduction, translation and ribosomal structure, chaperones, carbohydrate metabolism, intracellular trafficking, transcription, cytoskeleton, inorganic ion transport and metabolism. Some of the identified genes are those coding for fructose-bisphosphate aldolase, metallothionein, pathogenesis related protein, thaumatin, universal stress proteins and many genes of unknown function. This study was the first step in elucidating *P. cinnamomi* and avocado root interaction on a molecular level (Mahomed *et al*., 2011). Their data is currently the only EST data that has been generated for avocado rootstocks, and the ESTs identified have already been useful in identifying defence-related genes as well as providing gene information for other studies looking at processes such as ROS regulation and hypoxia in avocado roots. Those EST data will aid in the elucidation of the avocado transcriptome and

identification of markers for improved rootstock breeding and screening (Mahomed and van den Berg, 2011).

Very few genetic resources are available for avocado excepting studies on genetic relationships and the molecular characterization of the flowers and fruits (Chernys and Zeevaart, 2000; Chanderbali *et al.*, 2008). Chernys and Zeevaart (2000) monitored the expression of the 9-cis-epoxycarotenoid dioxygenase gene family during the ripening process in avocado and the regulation of abscisic acid biosynthesis. The expression of two of the genes studied (PaNCED1 and PaNCED3) was induced during fruit ripening and the expression of the last gene studied (PaNCED2) was constant during the wilting of leaves and ripening fruits (Chernys and Zeevaart, 2000).

Some avocado genes have been cloned and sequenced such as AVOe3 mRNA, a ripening-related gene (McGarvey *et al*., 1990), and cytochrome P 450, (O'Keefe *et al*., 1992), Twenty three cDNA clones wth homologies to cysteine proteinase inhibitor, endochitinase, polygalacturonase and stress-related proteins (Dopico *et al*., 1993), polygalacturonase (Kutsunai *et al*., 1993) and cellulose (Tonutti *et al*., 1995) have been identified.

1.6 AIMS OF THE PROJECT

Resistance to anthracnose disease may involve both major and minor genes in avocado fruit. Identifying and understanding all these important elements would result in better resistant cultivars being bred and ultimately better quality fruit being produced. But, there is limited genetic information as the avocado genome has not yet been fully sequenced. Therefore, this project aimed to identify a broad spectrum of avocado genes differentially expressed following *C. gloeosporioides* infection. Moreover, the study will enhance our understanding of the molecular basis of defence response mechanisms and signalling networks involved in avocado fruit against *C. gloeosporioides* attack and could also contribute to the design of effective alternative disease management strategies at a time when there is movement away from continued use of agrochemicals for disease prevention.

We focused on comparing cDNA libraries generated from healthy avocado fruit and fruit infected with *C. gloeosporioides,* using the Roche 454 GS FLX Titanium Platform. The Fuerte cultivar was used to establish a model genetic system for the avocado fruit. As although all avocado varieties are susceptible to anthracnose to varying degrees (Section1.3.4) there is already a good body of knowledge on the resistance response to *C. gloeosporioides* in Fuerte involving preformed antifungal compounds (Section 1.5.1). Fuerte is one of the most commercially important varieties. It was necessary to include both pre- and post-harvest fruits because *C. gloeosporioides* causes quiescent infections in unripe fruit due to high concentrations of the preformed antifungal compounds (Section 1.3.4), and this study could elucidate the genetic and molecular control of this mechanism. The strategy undertaken here does not require prior sequence knowledge or genome reference, and relies exclusively on publicly available software and basic

scripting tools. This study is the first exploration of differentially expressed candidate genes in Fuerte avocado fruit following infection with *C. gloeosporioides.*

The specific objectives of this project were:

- The observation of the *C. gloeosporioides* infection process on infected avocado fruits.
- The development of an efficient procedure to extract high quality and quantity of total RNA from avocado fruit.
- *De novo* transcriptome sequencing and analysis of both unharvested and harvested uninfected and infected avocado fruits (cv. Fuerte) using 454 Sequencing.
- The identification and characterization of defence related genes involved in avocado fruit (cv. Fuerte) response to *C. gloeosporioides* infection.
- The construction of a model of resistance/susceptibility for Fuerte avocado fruit.

In addition to the literature review, the thesis is presented as a compilation of five other chapters as follow:

Chapter 2: The observation of *Colletotrichum gloeosporioides* infection on infected avocado fruit (cv. Fuerte)

Chapter 3: The isolation of high quality RNA from the fruit of avocado (*Persea americana* Mill.)

Chapter 4: 454 Sequencing for the identification of genes differentially expressed in avocado fruit (cv. Fuerte) infected by C*olletotrichum gloeosporioides*

Chapter 5: Resistance responses of avocado fruit to *Colletotrichum gloeosporiodes* infection

Chapter 6: Conclusions

CHAPTER 2

THE OBSERVATION OF *COLLETOTRICHUM GLOEOSPORIOIDES* INFECTION ON INFECTED AVOCADO FRUIT (cv. Fuerte)

2.1 INTRODUCTION

In South Africa, avocados (*Persea americana* Mill.), comprise a large portion of the subtropical fruit industry (Donkin, 2007). In the subtropical environment, where avocado is grown, fungal diseases cause important reductions in yield and quality of avocado fruit (Pernezny *et al*., 2000). Fuerte avocados seem to be the most susceptible type of South African avocado to post-harvest infection and fruit rot, followed by Edranol, Hass and Ryan (Darvas & Kotze 1981). When the avocado fruits are minimally ripe, the symptoms of these post-harvest diseases first appear but they can become quite severe as the fruits continue to ripen (Hopkirk *et al.* 1994).

The phytopathogenic fungus, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. is the most important post-harvest pathogen attacking a wide variety of tropical and subtropical fruits (Coates *et al*., 1993). At the post-harvest level, disease caused by *C. gloeosporioides*, is referred to as anthracnose. Anthracnose causes lesions in the avocado fruit. Bruising and lesions in the skin aids the entry of other microorganisms into the fruit that may cause post-harvest fungal diseases (Sivanathan and Adikaram 2008). When *C. gloeoesporiodes* attacks avocado fruits, its conidia germinate on the fruit surface and form appressoria and infection pegs that penetrate the fruit cuticle until the epidermal cell wall is approached (Coates *et al*., 1993; Zamora-Magdaleno *et al.* 2001). At this stage, the infection becomes quiescent, where infection occurs early in fruit development, but the disease symptoms appear in the near-mature or mature stage (Guyot *et al.* 2005). The preformed antifungal compounds in the peel and flesh of unripe avocado fruits are responsible for the quiescence phase (Prusky and Keen, 1993). During fruit ripening, a transition to a necrotrophic stage occurs, after the decrease in the levels of the preformed antifungal compounds in the fruit. Infection pegs

during ripening penetrate through the epidermal cell wall to the lumen of the cells, which results in softening and cell death. The production of plant cell wall degrading enzymes causes a transition from the hemibiotrophic stage to necrotrophic attack (Coates *et al*., 1993). *C. gloeosporioides* can produce endopolygalacturonase (Prusky *et al*. 1989; Yakoby *et al*., 2000), pectin lyase A (*pnlA*) (Bowen *et al*., 1995; Templeton *et al*., 1994), pectin methyl esterase (Ortega 1996), and pectate lyase B (*pel*B) (Wattad *et al*., 1997) during the colonization of infected tissue. These enzymes can be regarded as pathogenicity factors.

The purpose of this investigation was to observe and confirm the infection cycle of *C. gloeoesporiodes* on harvested and unharvested avocado fruit using scanning electron microscopy and confocal laser scanning microscopy in parallel with the main objective of the project which was to analyse differential gene expression in avocado induced by *C. gloeoesporiodes* infection. The identity of the fungal isolate used has already been confirmed from spore morphology and PCR with genomic sequence analysis by Marimani (2011).

2. 2 MATERIALS AND METHODS

2. 2.1 Study site

The experimental study site was at Roodewal farm in Nelspruit, Mpumulanga, South Africa (25°25′54.62″S 30°56′15.34″E) (Figure 2.1) on orchard 7, which contained Fuerte avocado trees. Orchard 7 was approximately 2.5 hectares and the trees were young and small (approximately 2-3 metres in both height and width) with approximately 2-3 metres between each tree. The size and distance between the trees allowed some ease in controlling the amount of each treatment per tree. The age of the fruits was approximately 240 days after fruit set just before the first seasonal harvest of the Fuerte crop.

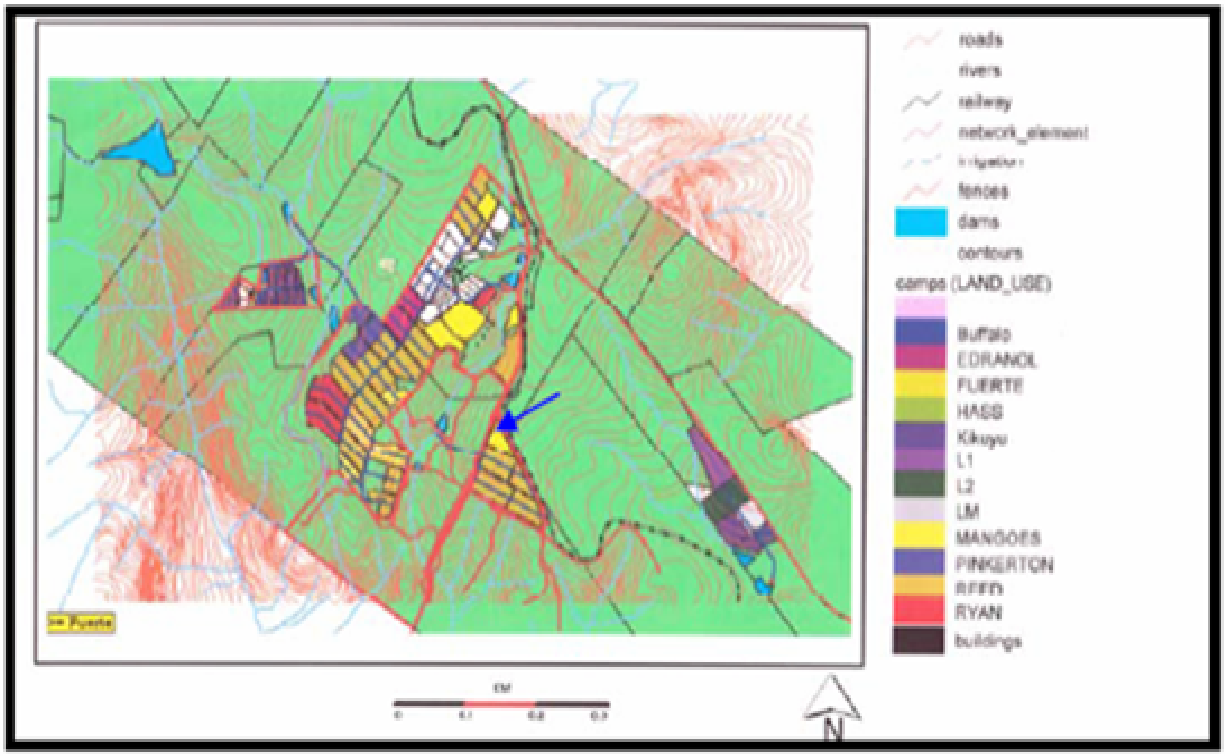


Figure 2.1. Geographic map of Roodewal farm, Nelspruit, South Africa, generated on ESRI ArcExplorer 1.1. Orchard 7 indicated by arrow.

2. 2. 2 Fungal Inoculum

The fungal isolate of *C. gloeoesporiodes* was isolated from an anthracnose lesion on a Fuerte avocado fruit (Giovanelli, 2008). The fungus (10 mm) was cultured on 5% w/v Malt Extract Agar (MEA) (Merck); a modified version of the Modified Merlin Norkrans (MMN) medium (malt extract 3.0 g, glucose 20.0 mg, ammonium hydrogen phosphate [$(NH_4)_2HPO_4$] 250.0 mg, potassium dihydrogen phosphate [KH_2PO_4] 500.0 mg, hydrated magnesium sulphate [$MgSO_4.7H_2O$] 150.0 mg hydrated calcium chloride [$CaCl.2H_2O$] 65.0 MG, ferrous chloride (1 %) [$FeCl_2$] 1.2 ml, sodium chloride [NaCl] 25 mg, thiamine HCl 0.1 g, peptone 750 mg, agar 15.0 g); and oatmeal agar (OA) (oatmeal [Jungle Oats] 60.0g, agar 12.5 g). The plates were incubated for two weeks at 25 °C and the different media monitored for optimal spore production. Spore suspensions (conidia) of the fungus were prepared by adding 10 ml of 0.05 % Tween-80 (Sigma-Aldrich) to the fungal cultures. Spores were harvested by using a glass spreader and filtered through four layers of sterile cheesecloth to remove the fungal mycelial debris, centrifuged at 10 000 *g* (Spectrafuge 24D, Labnet) for 1 min and adjusted to 1 x 10^7 spores per ml^{-1} storage buffer using a haemocytometer (Marienfeld, Germany) (Prusky *et al*., 1990) and stored at -20°C.

2.2.3 Plant Materials and Inoculation

In the field, the experiment used unharvested (inoculated and uninoculated) and harvested (inoculated and uninoculated) fruits. Before inoculating, the fruit surface was sterilized by immersion in 1 % sodium hypochlorite (JIK) for 10 min followed by rinsing in sterile distilled water three times and was left to air dry. Afterwards the fruits were wiped with 70% ethanol (Merck) and allowed to air dry (Giovanelli, 2008).

For in *planta* inoculations, the fruits were wounded and inoculated with 100 µl of *C. gloeosporioides* spore suspension (1 x 10^7 spores per ml^{-1}) using a sterilized needle of 2 mm length and 1 mm thick. The inoculated fruits were covered with a clean plastic bag in which distilled water was sprayed to maintain humidity. For the post harvest inoculation, after the sterilization steps, the fruits were wounded and inoculated with 100 µl of the spore suspension. Fruits were incubated at 25 °C inside aluminium trays covered with aluminium foil and humidity was maintained by placing a Petri dish filled with sterile distilled water at the centre of each tray (Kwang-Hyung *et al*., 2004). One uninoculated fruit was used as a control for both unharvested and harvested experiments. The control fruits for both unharvested and harvested fruits were treated similarly to infected fruits but were inoculated with 100 µl of sterile distilled water. The control fruit was placed in a separate tray to prevent the spread of infection.

Symptom development was monitored daily from day 1 up to 7 day post inoculation on both inoculated and uninoculated fruits and tissues were harvested after 1, 2, 3, 4, 5 and 7 days post-infection.

2.2.4 Confocal laser scanning microscopy (CLSM)

Avocado peels were cleared and stained using a procedure described by Brundrett *et al*. (1993). The peels were cleared in 2.5% KOH at 90°C by leaving them to stand until discolouration of solution occurred, approximately 10 min. After clearing, peels were captured on a sieve, rinsed with water and acidified by soaking in 1% HCl for 24 h.

Acidified peels were stained with a concentration of 0.05% Trypan Blue in acidic glycerol solution (50% v/v glycerol and 1% HCl) for 15 min at 90°C. Trypan Blue stained lignified or suberised cell walls in peels, especially xylem, endodermis and exodermis cells. Staining

quality was substantially improved by destaining peels in acidic glycerol for several days prior to observation, to allow excess stain to leach out. Tissue was mounted onto 76 X 26 mm slides (B&C, Germany) with 22 X 50 mm cover-slips (Deckgläser). The slides were viewed under a confocal laser scanning microscope (ZEISS LSM 410) at 488 nm (for the green colour) at 25 X magnification. Fluorescein isothiocyanate was used for excitation.

2.2.5 Scanning Electron Microscopy (SEM)

SEM was performed using a method described by Palhano *et al*. (2004), with some modifications. Pieces of fruit peel were excised using a sterile scalpel and fixed with 2 % (v/v) glutaraldehyde (Merck) in 0.1 M potassium phosphate buffer (pH 7.0) for 3 h. After fixing, tissues were briefly rinsed in 0.1 M potassium phosphate buffer (pH 7.0) and sequentially dehydrated in a graded ethanol series (10, 30, 50, 70 and 90 % for 15 min (two changes of each) and in 100 % (v/v) ethanol (Merck) for 1 h. The ethanol was removed from the tissues by critical point drying (HITACHI, HCP-2 Critical point Dryer). Tissues were mounted on stubs using graphite and allowed to air-dry, covered with carbon and coated with gold, and observations were made with a JEOL JSM-840 instrument operated at 15 kV.

2.2.6 Partial test of Koch's postulate

The fungus was re-isolated from infected fruits using the tissue transplanting technique and cultured on MEA and compared to the original culture, to confirm that no other fungus was present and that the symptoms observed, were caused by the inoculate.

2. 3. RESULTS

2.3.1 Observation of the infection process of *C. gloeosporioides* in avocado using Confocal Laser Scanning Microscopy (CLSM)

Observations from CLSM did not show any fungal structures or spores characteristic of *C. gloeosporioides* in any of the inoculated sites from 1 h to 5 dpi. However, an increased number of ellipsoid spores were observed at 7 dpi but without any fungal hyphae (Figure 2.2). In the uninoculated fruit (controls) fungal structures and spores were not observed at any time point.

2.3.2 Observation of the infection process of *C. gloeosporioides* in avocado using SEM

The SEM showed no fungal structures in any of the four inoculated sites from 1 h to 1 dpi. Few dispersed ellipsoid spores, which characterize C. *gloeosporioides*, without any fungal hyphae were observed on the inoculated fruit surfaces of harvested fruits starting from 2 dpi (Figure 2.3 B) and 3 dpi (Figure 2.3 C). Starting from 3 dpi, fungal hyphae started to appear on inoculated fruit surfaces of harvested fruits (Figure 2.3 D). Some of those fungal hyphae were attached to the fruit surface with or without spores and others were forming a mycelium. At 4 dpi in most harvested fruits, an increased number of spores were observed which resulted from a successful hyphal colonization of the fruit surfaces (Figure 2.4 A, B). At 4 dpi in harvested fruit, severe tissue destruction and damage had commenced with some hyphae protruding out of the lenticels, which resulted in cell wall and cuticle destruction (Figure 2.4 C, D).

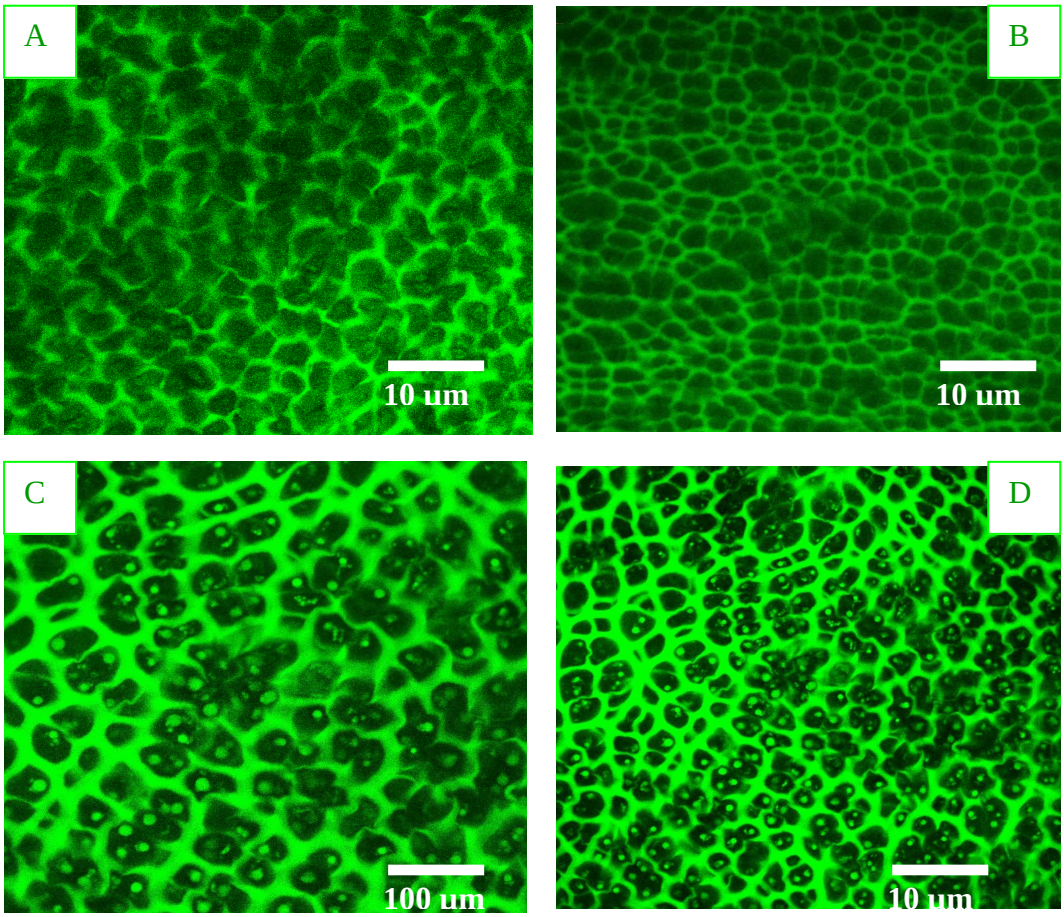


Figure 2.2. Confocal laser scanning micrographs of avocado fruit from 1 d to 7 d post inoculation with *C. gloeosporioides*. (A) Uninoculated fruit (control). (B) Harvested inoculated fruit surface at 5 dpi. (C-D) *C. gloeosporioides* spores on the surface of inoculated harvested fruit at 7 dpi.

By 7 dpi there was an increase in the density of mycelium and in the number of spores observed on the fruit surface (Figure 2.5 D). However, in some inoculated fruits, cell wall and cuticle destruction was increased, as more exocarp destruction occurred, thereby, increasing the exposure of the mesocarp layer and (Figure 2.5 B, C) and hyphae were observed emerging through many lenticels (Figure 2.5 A). In none of the control fruits treatments, were any fungal structures observed for up to 7 dpi, showing that there was no

fungal infection and or contamination (Figure 2.3 A). In general, symptom development on unharvested fruit was much less severe and delayed until 5 to 7 d compared with harvested fruit (data not shown).

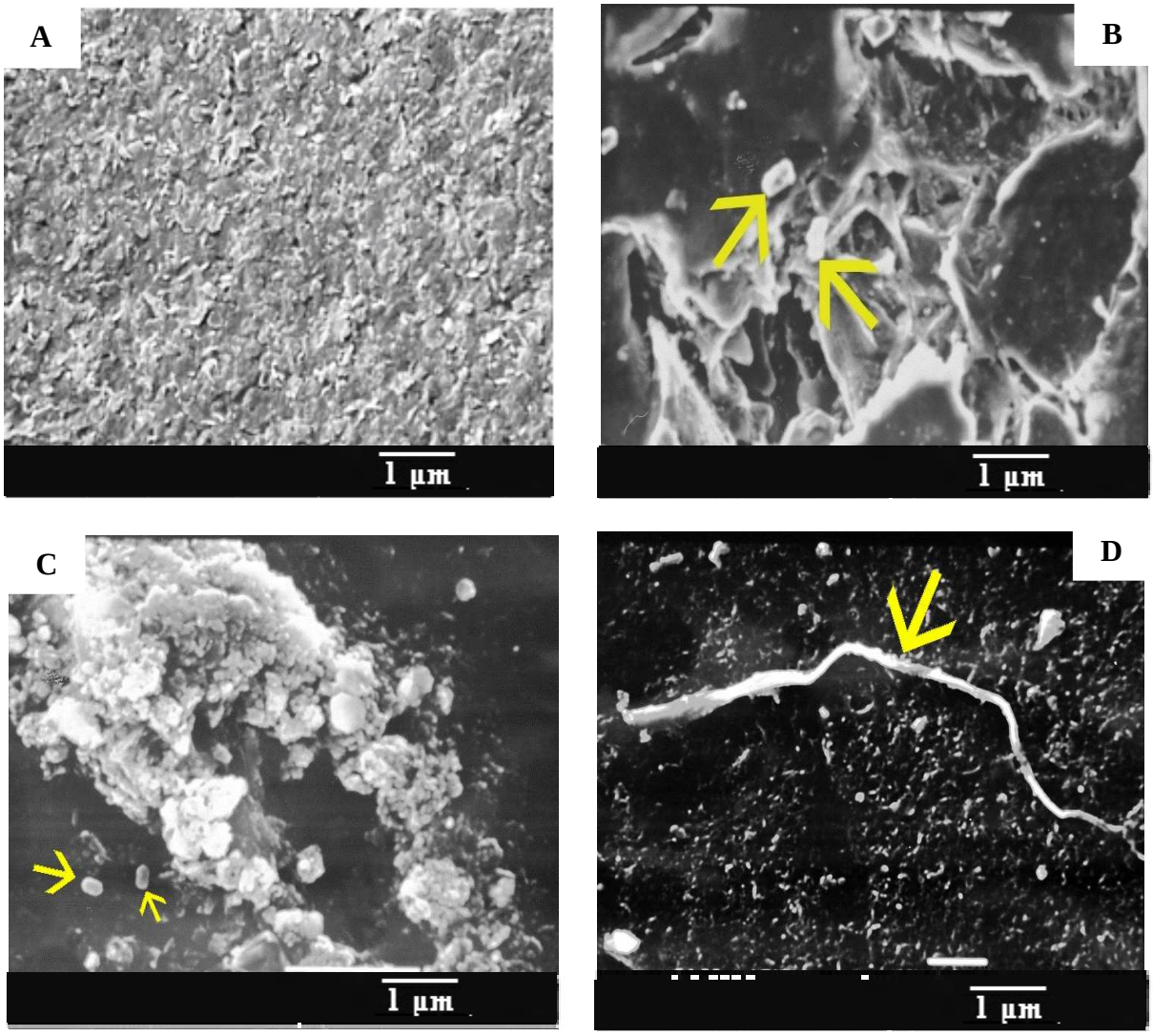


Figure 2.3. Scanning electron micrographs of harvested avocado fruit infected with *C. gloeosporioides* from 2 d post inoculation (dpi). (A) Uninoculated fruit (control) at 2 dpi. (B) Ellipsoid spore of *C. gloeosporioides* on the inoculated fruit surface at 2 dpi and at (C) 3 dpi. (D) Surface hyphae protruding out of the inoculated fruit surface at 3 dpi.

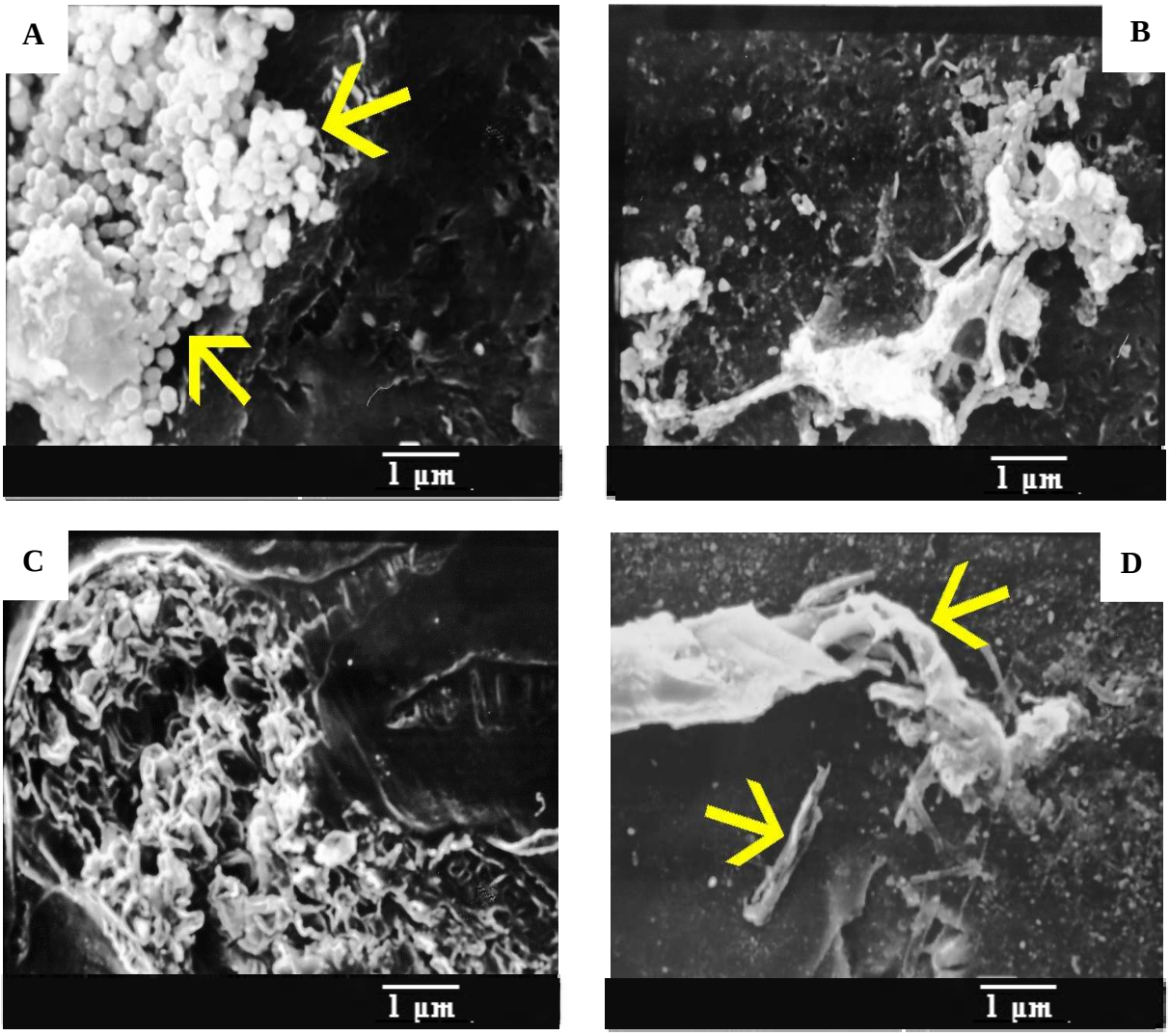


Figure 2.4. Scanning electron micrographs of harvested avocado fruit infected with *C. gloeosporioides* at 4 and 5 dpi. (A) An increased number of *C. gloeosporioides* spores on the surface at 4 dpi. (B) Surface hyphae interacting with each other to form a mycelium at 4 dpi. (C) Destruction of the exocarp, leading to subsequent exposure of the mesocarp layer at 4 dpi. (D) Hyphae protruding out of lenticel at 5 dpi.

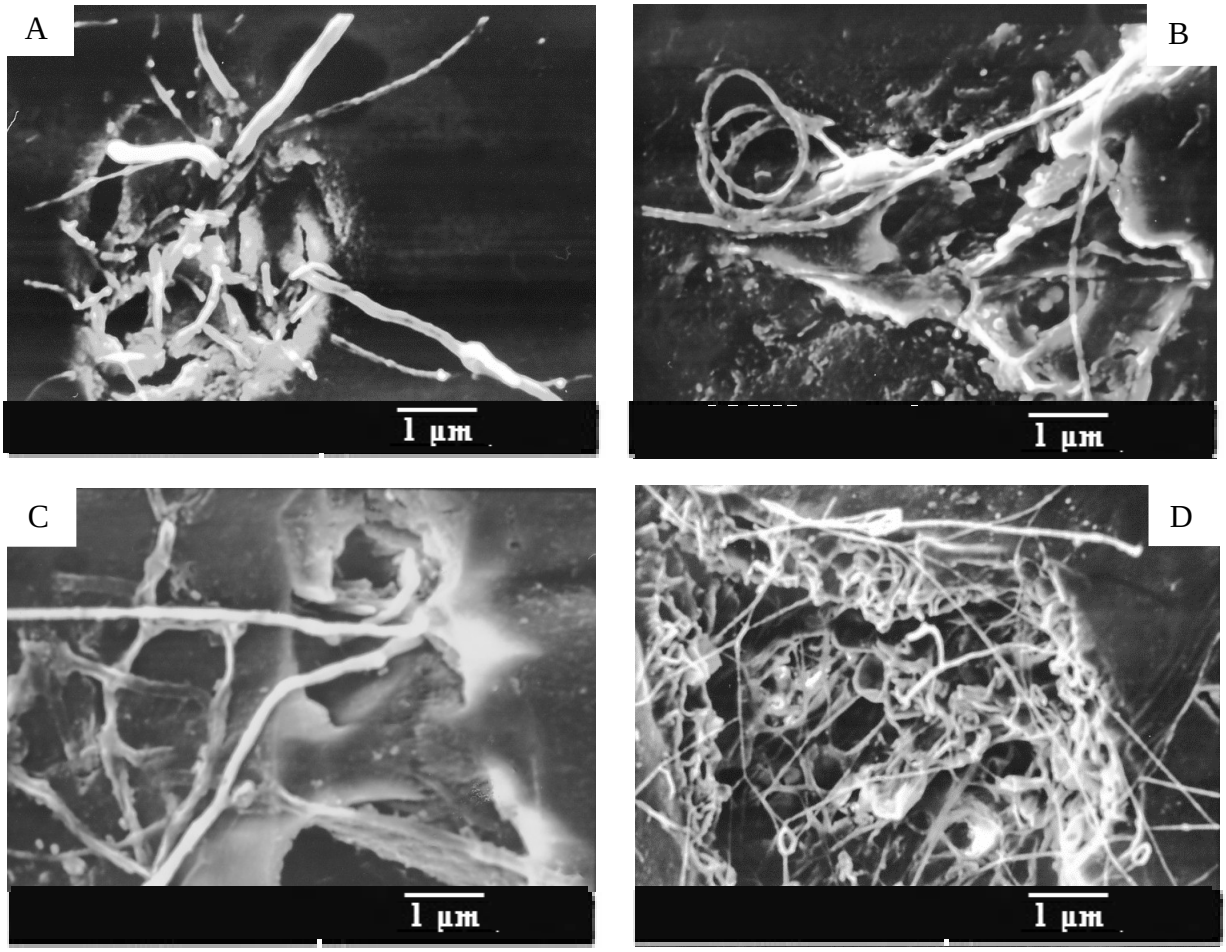


Figure 2.5. Scanning electron micrographs of harvested avocado fruit infected with *C. gloeosporioides* at 7 dpi. (A) Hyphae protruding out of lenticel. (B-C) Increased exposure of the mesocarp layer, due to an increase in the destruction of the fruit exocarp layer. (D) An increased number of hyphae interacting with each other to form a mycelium.

2.3.3 Partial test of Koch's postulate

The isolates of *C. gloeoesporiodes* re-isolated from infected avocado fruits expressing symptoms of anthracnose were grown on MEA at 25 °C as seen on Figure 2.6.

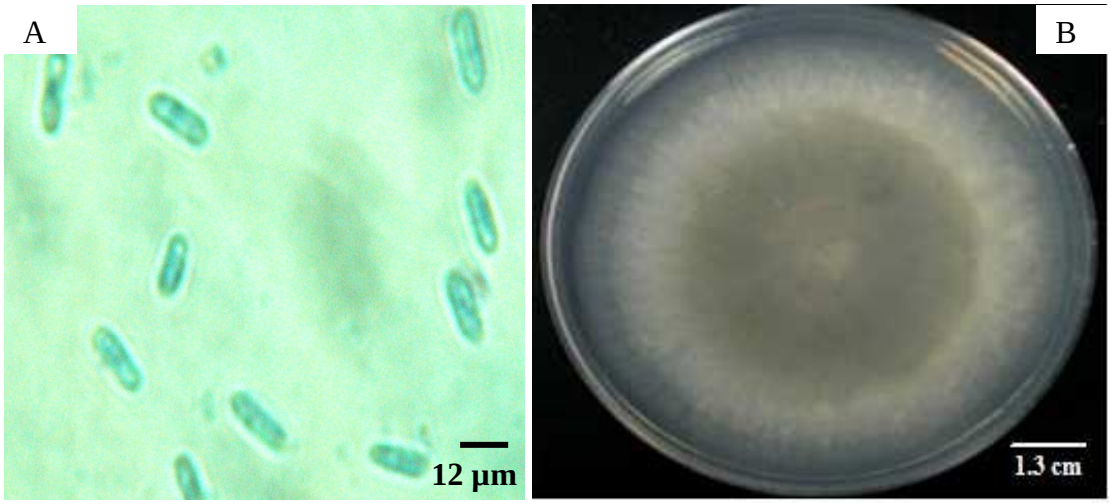


Figure 2.6 Morphological identification of *Colletotrichum* gloeosporioides from ellipsoid spores examined under the light microscope using high power (A) and Culture of *C. gloeoesporiodes* isolated from infected avocado fruits expressing symptoms of anthracnose (B). Spores were ellipsoid in morphology with an average size of 11.9 X 4.7 µm.

2.4 DISCUSSION

No anthracnose symptoms were noted for up to 7 dpi in unharvested fruits and lesions were less severe. This suggested that *C. gloeosporioides* had formed quiescent infections in unharvested fruits. Various causes have been considered as an explanation for quiescent infections by a pathogen in unripe fruits: insufficient enzyme production by the fungus, its nutritional requirements and the presence of antifungal compounds that inhibit pathogen development in unripe but not in ripening fruits (Guyot *et al.* 2005). The involvement of antifungal compounds in quiescent infections of unripe fruits was suggested to be the result of either their induction or their presence as preformed compounds (Prusky and Keen, 1993). Fungal inhibition during quiescent infections of *C. gloeosporioides* on unripe avocado fruits was suggested to result from the presence of the preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (Prusky *et al*., 1982). This is considered to be the same compound as AVII found by Sivanathan and Adikaram (1989) who also found three other less toxic compounds. All these compounds decrease to a subfungitoxic level upon harvesting of the fruit, thus allowing the pathogen to recommence growth (Guyot *et al.* 2005). The decline in diene concentration is linked to the rapid increase in lipoxygenase activity upon harvesting which catalyses the oxidation of diene (Karni *et al*., 1989). Marimani (2011) monitored the levels of antifungal diene and triene compounds and observed a rapid increase in these compounds 1-2 days after inoculation of both harvested and unharvested Fuerte fruits with *C. gloeosporioides* 240 days after fruit set, followed by a decline within 7 days to uninoculated control or below control levels; however, levels in unharvested fruit were higher. In a bioassay, Marimani (2011) demonstrated the ability of these compounds to inhibit conidial germination and germ tube growth of *C. gloeosporioides.*

Moreover, it has been also shown in other plant species that unripe fruits in contrast to ripening fruits posses extremely high concentrations of pre-formed antimicrobial compounds. This arsenal of constitutive resistance may accumulate in the immature pericarp at concentrations up to 1 mg g^{-1} fruit fresh weight, and include 5-substituted resorcinols such as 5-(12-cisheptadecenyl)-resorcinol in mango, dienes such as 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15 diene in avocado, and saponins like a-tomatine in tomato (Prusky and Plumbley, 1992; Prusky, 1996).

In harvested fruits, although no fungal structures were observed from 1 h to 1 dpi, ellipsoid spores characteristic of *C. gloeosporioides* were observed on the fruit surface from 2 dpi (although these might have originated from the inoculum). After inoculation, the ungerminated, aseptate conidium of *Colletotrichum* would attach to the cuticle and germinate, with or without septation, to produce a germ-tube. (Akinwunmi and Latunde-Dada, 2001). By 3 dpi fungal hyphae emerged, most of which successfully invaded and colonized the fruit surface to form a mycelium. During this period of fungal penetration and invasion, an increased number of spores were observed on the fruit surfaces. Extensive fruit damage and destruction were observed at 5 and 7 dpi, as an increased number of hyphae invaded the fruit surface. Consequently, some hyphae caused fruit damage by protruding out of the lenticels, resulting in cell wall and cuticle destruction. This finding is similar to previous studies which stated that many *Colletotrichum* species initially establish infection by means of a brief biotrophic phase, associated with large intracellular primary hyphae. They later switch to a destructive, necrotrophic phase, associated with narrower secondary hyphae, which ramify throughout the host tissue (Latunde-Dada *et al*., 1996; Wharton and Julian, 1996; Kim, 1998).

In conclusion, inoculation of harvested and unharvested 'Fuerte' avocado fruits with *C. gloeosporioides* at approximately 240 day after fruit set causes major infection symptoms in harvested fruit compared with unharvested fruits which presented few symptoms, probably due to their high concentrations of antifungal compounds (triene and diene). The fruits showed no invasion by other fungi, confirmed *C. gloeosporioides* as the causal agent of anthracnose symptoms and allowed the transcriptome expression analysis to proceed.

THIS CHAPTER HAS BEEN PUBLISHED AS: A. T. Djami-Tchatchou and C. J. Straker. 2012. **The isolation of high quality RNA from the fruit of avocado (*Persea americana* Mill.).** South African Journal of Botany, 78:44-46.

CHAPTER 3

THE ISOLATION OF HIGH QUALITY RNA FROM THE FRUIT OF AVOCADO (*Persea americana* Mill.)

3.1 INTRODUCTION

Extraction of RNA of high quantity and quality is a preliminary step for many investigations in plant molecular biology such as northern blot hybridization, mRNA purification, PCR amplification, cDNA synthesis and cDNA library construction (Hu *et al*., 2002). However, isolation of RNA from some plant tissues is difficult due to the presence of high amounts of secondary products, such as polysaccharides and polyphenolic compounds, which can co-precipitate or bind to RNA and result in poor yields (Gasic *et al*., 2004).

Avocado (*Persea americana* Mill.), an important sub-tropical export crop for South Africa, produces a climacteric fruit which, in addition to mainly monounsaturated and polyunsaturated oils, contains high levels of proteins and structural polysaccharides (Bergh, 1992; Naveh *et al*., 2002), and polyphenols (López-Gómez, 2002). Several methods, using either phenol or CTAB in the extraction buffer or high-molarity guanidinium salts, have been developed for the extraction of RNA from plant tissues containing high levels of polyphenolic compounds and polysaccharides (Asif *et al*., 2006; Chang *et al*., 1993; Hu *et al*., 2002; Jaakola *et al*., 2001; Liu *et al*., 1998; López-Gómez and Gómez-Lim, 1992; Manning, 1991; Pandit *et al*., 2007; Salzman *et al*., 1999; Valderrama-Cháirez *et al*., 2002). In preliminary experiments, we tested four of these protocols (Hu *et al*., 2002; López-Gómez and Gómez-Lim, 1992; Pandit *et al*., 2007; Valderrama-Cháirez *et al*., 2002) and one commercially available RNA extraction kit (RNeasy Plant Mini Kit, Qiagen) to extract RNA from the skin (exocarp) and flesh (mesocarp) of avocado fruit but the results were unsatisfactory for all the protocols because of the poor quality and quantity of the resulting total RNA, although the one described by Valderrama-Cháirez et al. (2002), gave better

results in terms of RNA quantity compared with the others. Gasic et al. (2006) had successfully modified the method of Chang et al. (1993) to extract RNA from apple tissues. In order to achieve an improvement in both yield and purity of RNA from avocado fruit we used the CTAB/NaC1 method of Chang et al. (1993) and modified it by replacing PVP K 30 with PVP K 40, removing spermidine from the extraction buffer and including a simple polysaccharide precipitation step that does not affect the RNA yield but removes contaminating polysaccharides.

This research reports on the effectiveness of this modified method to isolate total RNA from the skin and flesh of avocado fruit and the product's suitability for cDNA synthesis and other subsequent gene expression studies.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Fruits of avocado (*Persea americana* Mill. cv. Fuerte.) were collected from the orchards of Roodewal farm near Nelspruit, Mpumalanga Province, South Africa (25°25′54.62″S 30°56′15.34″E). The age of the fruits was approximately 240 days after fruit set just before the first seasonal harvest of the Fuerte crop. The skin and flesh (± 5 g) were immediately sliced off, frozen in liquid nitrogen, and stored at –80 °C until needed.

3.2.2 RNA extraction protocol

DEPC–treated water was used for all solutions. Frozen tissues were ground to powder with a mortar and a pestle in liquid nitrogen. The powder (0.5–1g) was transferred to sterile centrifuge tubes containing 10 ml of pre-warmed (65 °C) sterile extraction buffer (2%

CTAB, 2% PVP K 40, 100 mM Tris-HCl pH 8.0, 25 mM EDTA, 2 M NaCl, and 400 µl 2% β-mercaptoethanol added just before use) and incubated at 65 °C for 5–10 min to lyse the cells completely. Following the addition of 15 ml of chloroform: isoamyl alcohol (24:1 [v/v]) tubes were vortexed and centrifuged at 5 000 rpm for 20 min at room temperature. After centrifugation, the upper phase was transferred to a new tube with 15 ml of chloroform: isoamyl alcohol, then vortexed and centrifuged again for 20 min at 5 000 rpm. After the second centrifugation, the supernatants (12 ml) were transferred to sterile tubes following the addition of 3 ml of 10 M LiCl (1/4-volume). After overnight incubation at -20 °C, the tubes were centrifuged (10 000 rpm, 30 min, 4 °C) and the pellets re-suspended in 700 µl NaCl-sodium dodecylsulphate-Tris-EDTA buffer (1 M NaCl, 0.5% SDS, 10 mM Tris-HCl, pH 8.0, 1mM EDTA), pre-warmed at 60 °C, and incubated for a few minutes at 60 °C to ensure complete re-suspension of the RNA-pellet in the buffer. The suspension was transferred to a 2 ml microcentrifuge tube and extracted with 700 µl of chloroform: isoamyl alcohol followed by a spin down at 10 000 rpm for 10 min at room temperature. The upper phase (600 µl) was transferred to a 2 ml microcentrifuge tube containing 1.2 ml of 100% EtOH and the RNA was precipitated at –70 °C for 1 h. After incubation for the precipitation of nucleic acids, the tubes were centrifuged (30 min, 13 000 rpm, 4°C). Nucleic acids were recovered by washing with 1 ml of 70% EtOH (v/v) and centrifuging at 10 000 rpm for 2 min, air dried and re-suspended in 100 µl DEPC water.

The RNA yield was measured with a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA) and the 260/280 and 260/230 absorption ratios were verified as quality indexes. The RNA was examined by electrophoresis on 2% agarose /TAE gels containing EtBr to assess its integrity. One volume of the 2 X RNA loading buffer was mixed with one volume of the RNA sample at 70 ◦C for 10 min. After cooling on ice for 3

min, the samples were loaded and run on the gel in the 1 X TAE electrophoresis buffer at 80 V.

3.2.3 Protocol for the synthesis of double-stranded cDNA from total RNA

Doubled strand cDNA was synthesized starting with the total RNA, using a cDNA Synthesis System Kit (Roche, Mannheim, Germany), following the manufacturer's instructions. First strand synthesis was carried out in a reaction volume of 21 µl containing 10-20µg RNA, 2µl Oligo(dT)$_{15}$ primer (200 pmol/µl) and water, PCR grade. The samples were incubated at 70°C for 10 min in a waterbath and immediately placed on ice. Then the following components were added: 8µl RT-buffer, 4µl DTT (0.1M), 2µl AMV (25u/µl), 1µl Protector RNase Inhibitor (25u/µl), 4 µl dNTP-mix (10mM). After mixing gently, the samples were incubated at 42°C for 60 min and immediately placed on ice to terminate the reaction.

Second strand synthesis followed immediately in a reaction volume of 150 µl containing 40 µl of the cDNA mixture from the first strand reaction, 30 µl 5x second strand buffer, 1.5 µl 10 mM dNTP-mix, 6.5 µl second strand enzyme blend (mixture of DNA polymerase I, Escherichia coli ligase and RNase H) and 72 µl water, PCR Grade. The reaction was mixed gently and incubated at 16 ºC for 2 hours, followed by the addition of 20 µl (20U) T_4 DNA polymerase and incubation for 5 min. The reaction was stopped by adding 17 µl EDTA, 0.2M (pH 8.0). The residual RNA was digested from the ds cDNA reaction by adding 1.5 µl (15U) RNase I followed by incubation at 37°C for 30 min. Then 5 µl (0.25U) Proteinase K was added to the reaction and incubated at 37°C for 30 min.

Ds cDNA was purified by adding 200 µl phenol to the reaction, then vortexed for 10 s and centrifuged at 14 000 rpm for 15 s. The supernatant was transferred into a new tube. The remaining phenol phase was washed with 50 µl TE (to minimize loss of DNA) vortexed for 10 s and centrifuged at 14 000 rpm for 15 s. The supernatant obtained was combined with the previous one in to a new tube, then 200 µl phenol/chloroform/isoamylalcohol (25:24:1) was added, vortexed for 10 s and centrifuged at 14 000 rpm for 15 s. The supernatant was transferred to a new tube followed the addition of 200 µl chloroform/isoamylalcohol (24:1), vortexed for 10 s and centrifuged at 14 000 rpm for 15 s. The supernatant was saved and this step was repeated, then the cDNA contained in the supernatant was precipitated by adding 0.6 volume of 5 M NH_4OAc and 2.5 volume of cold 100% EtoH (-15 to -25^oC) and stored at -70 ^oC for 1 hour. After the DNA was pelleted by centrifugation at 14 000 rpm for 10 min, then the pellet was washed by overlaying 300 µl of cold 70 % EtoH (-15 to -25^oC) and centrifuged at 14 000 rpm for 10 min. The final pellet was air dried and dissolved in 50 µl water, PCR Grade.

The size distribution of double-stranded cDNA was monitored by running a 2% agarose gel after measuring the yield with a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA).

3.3 RESULTS

3.3.1 Total RNA isolation

Avocado fruits were inoculated with *C. gloeosporioides* as reported in Chapter 2. Total RNA was extracted from pre and post harvest fruits at 1, 4 and 24 h post inoculation for the early response and at 3, 4, 5 and 7 days post-infection for the late response. RNA extraction at each time point was performed in triplicate. RNA was also extracted from uninfected avocado fruit. The relative yields of total RNA per gram of tissue ranged from 87.76 to 174.94 μg g^{-1} of fresh weight. The A260/280 ratios for isolated RNA varied from 2.09 to 2.15 and the A260/230 ratios from 2.06 to 2.18 (Table 3.1).

Table 3.1 Yield and purity of total RNA extracted from avocado fruit (*Persea americana* Mill.)

Sample	RNA yield (μg g^{-1} FW)	Absorbance ratios	
		A260/A280	A260/A230
C	118.94 ± 11.01	2.14 ± 0.01	2.13 ± 0.05
EU	92.45 ± 6.44	2.15 ± 0.11	2.14 ± 0.02
LU	174.94 ± 8.31	2.14 ± 0.01	2.18 ± 0.02
EH	147.49 ± 5.51	2.13 ± 0.01	2.17 ± 0.03
LH	87.76 ± 11.07	2.09 ± 0.01	2.06 ± 0.05
Skin	86.83 ± 6.01	2.10 ± 0.01	2.07 ± 0.06

Results are expressed as mean ± S.E.M of 3 biological replicates. C, control; EU, early unharvested flesh; LU, late unharvested flesh; EH, early harvested flesh; LH, late harvested flesh.

3.3.2 Total RNA gel electrophoresis

Furthermore, the RNA (50 μg g^{-1} FW) integrity was assessed by the sharpness of ribosomal RNA bands visualized by non-denaturing 2% agarose/TAE gel electrophoresis. For all RNA samples tested, distinct 28S and 18S ribosomal RNA bands without degradation were observed, (Figure 3.1).

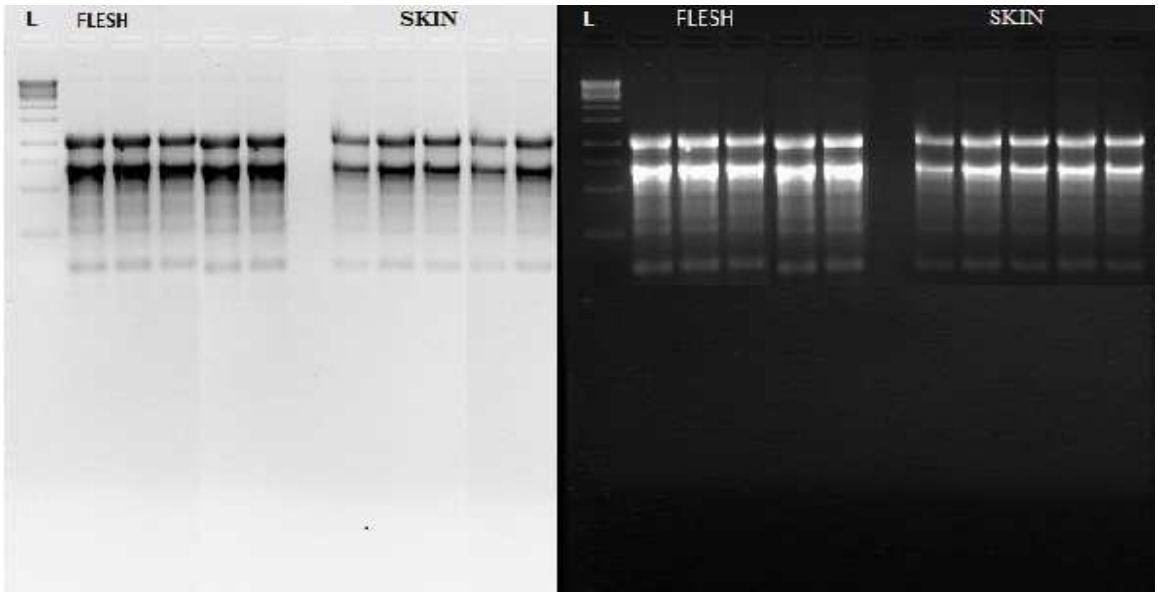


Figure 3.1. **Total RNA from avocado fruit flesh separated on 2% non-denaturing agarose gel containing EtBr and photographed under ultraviolet light**. L, RiboRuler™ High Range RNA Ladder.

3.3.3 Doubled strand cDNA synthesis and gel electrophoresis

The RNA obtained was used for cDNA synthesis using the AMV reverse transcriptase with the synthetic oligonucleotide, oligo $(dT)_{15}$ primer. The cDNA was successfully synthesised without any amplification with a good yield (1.12 ± 0.26 μg g^{-1} FW) in a total volume less than 50 μl.

The reverse transcription products resolved on 2% agarose/TAE gel electrophoresis exhibited clear bands (Figure 3.2). The resulting cDNA was used to construct a 454 library. One library was constructed from the control sample and four libraries from the infected samples (early unharvested, early harvested, late unharvested and late harvested).

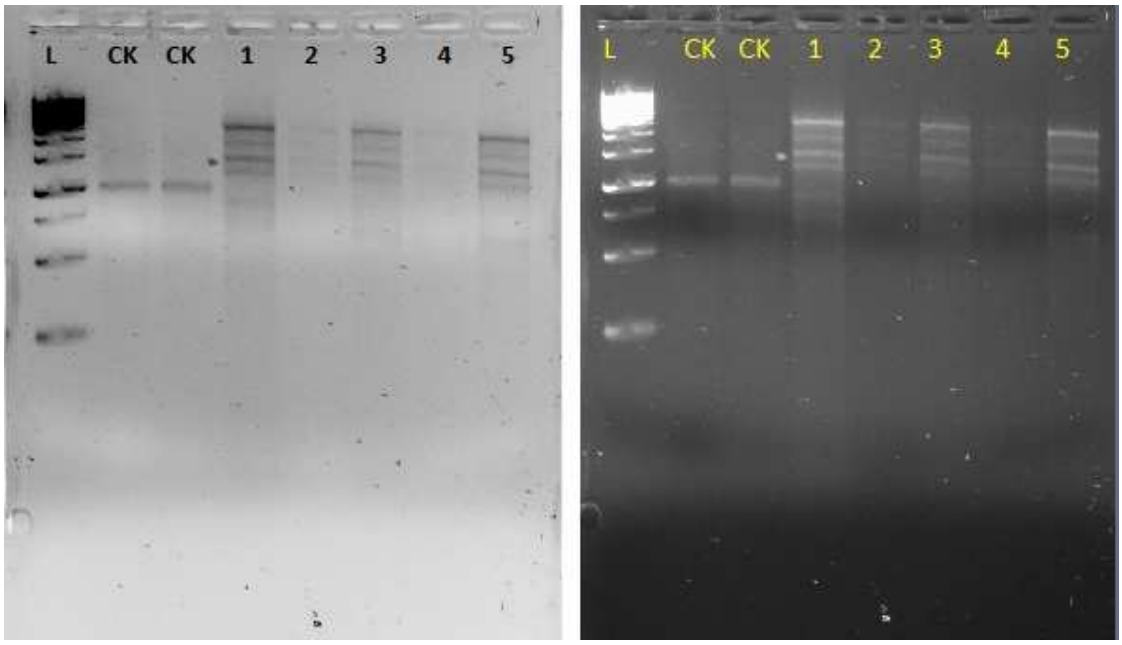


Figure 3.2. **cDNA resulting from reverse transcription of total RNA from avocado fruit with Oligo $(dT)_{15}$ primer separated on 2% non-denaturing agarose gel containing EtBr and photographed under ultraviolet light**. Lane L, 1-kb DNA size ladder; Lane CK, cDNA of a control RNA (Neo mRNA) from the cDNA Synthesis System Kit (Roche); Lane 1-5 are the cDNA products obtained from different avocado RNA extracts.

3.4 DISCUSSION

The effectiveness of an RNA extraction protocol can be monitored through the quantity, quality and integrity of the resulting RNA. The major problem in the extraction of RNA from tissue of many plants is contamination by polyphenols and polysaccharides which can bind irreversibly to nucleic acids and co-precipitate with RNA, so to successfully isolate a pure intact total RNA, the binding of these compounds to the nucleic acids needs to be prevented (Suzuki *et al*., 2003). Use of the phenol and high-molality guanidinium salts procedures, which are commonly used for RNA extraction from plant tissues, failed to recover RNA of high quality with good quantity. This indicated that the RNA might be lost by binding to polysaccharides, polyphenolics or other unknown components in the homogenate during extraction. In addition, it is known that guanidinium/guanidine salts are protein denaturants, but are not effective in dissociating RNA from non-protein complexes after binding (Mason *et al*., 2007). Even the protocol reported by Liu et al. (1998), which includes ice-cold potassium acetate to precipitate genomic DNA and secondary metabolites, produced very low amounts of RNA (data not shown). Another attempt to isolate total RNA from avocado fruit was based on the procedure of Valderrama-Cháirez et al. (2002). The RNA yield was higher than 90 $\mu g\ g^{-1}$ FW but was contaminated with proteins and polysaccharides as shown by the low values of the A260/280 (0.74-0.81) and A260/230 (0.54-1.57) ratios. The A260/280 ratio remained consistently low and did not improve with multiple LiCl precipitations, nor did the A260/230 ratio, even after a clean-up process using the RNeasy MinElute Cleanup Kit (Qiagen).

To solve this RNA isolation problem encountered in avocado fruit we slightly modified the procedure of Chang et al. (1993). They had developed an efficient method by modifying some established techniques which allowed the isolation of total RNA from pine tree tissue

without the use of toxic and expensive chemicals such as phenol, guanidium isothiocyanate and guanidium hydrochloride or the need for ultracentrifugation. To prevent the problem of the RNA co-precipitation PVP K 40 and β-mercaptoethanol were added in the extraction buffer as reducing reagents with CTAB as the detergent and extraction with chloroform-isoamylalcohol instead of phenol to remove proteins. The problem of polysaccharide contamination was solved by using 2 M NaCl instead of less than 1 M in the extraction buffer and 1.0 M NaCI in the NaCl-sodium dodecylsulphate-Tris-EDTA buffer to dissolve the RNA pellet. The increase of the NaCl concentration in the buffers helps to remove polysaccharides (Fang *et al*., 1992) and dissolves the CTAB-RNA complex, in order to allow more CTAB and polysaccharides to be removed in the chloroform extraction. In addition the precipitation step overnight at -20 °C instead of 4 °C improves the quality of RNA to be recovered.

The high-quality of the RNA obtained in this study was confirmed by the A260/A280 absorbance ratio whose values were always between 2.12 and 2.15, indicating that RNA was relatively free of protein and polyphenol contamination; and the A260/230 ratio which was higher than 2.0, indicating that RNA was of high purity and without polysaccharide contamination (Logemann *et al*., 1987; Manning, 1991) (Table 3.1). Similar results were obtained when RNA was extracted from different apple tissues also using an extraction buffer containing CTAB, PVP and β-mercaptoethanol (Gasic *et al*., 2004). Furthermore, the RNA integrity was assessed by the sharpness of ribosomal RNA bands visualized by non-denaturing 2% agarose/TAE gel electrophoresis (Figure 3.1). For all RNA samples tested, distinct 28S and 18S ribosomal RNA bands were observed, suggesting that RNA was relatively intact and was also relatively free of RNases (Figure 3.1). Similar results were observed by Valderrama-Cháirez et al. (2002) when they isolated RNA from ripe

mango mesocarp. Finally, the RNA quality was tested by cDNA synthesis using the AMV reverse transcriptase with the synthetic oligonucleotide, oligo $(dT)_{15}$ primer. The cDNA was successfully synthesised without any amplification with a good yield and the reverse transcription products resolved on 2% agarose/TAE gel electrophoresis exhibited clear bands (Figure 3.2). These results demonstrated that total RNA obtained was of sufficient quality to be used for downstream transcriptome analysis. This is a first report of the extraction of high quality total RNA from avocado fruit.

THIS CHAPTER HAS BEEN PUBLISHED AS:

A. T. Djami-Tchatchou, C. J. Straker and F. Allie. 2012. **454 Sequencing for the identification of genes differentially expressed in avocado fruit (cv. Fuerte) infected by C*olletotrichum gloeosporioides.*** Journal of Phytopathology**,** doi: 10.1111/j.1439-0434.2012.01925.x

CHAPTER 4

454 SEQUENCING FOR THE IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN AVOCADO FRUIT (cv. Fuerte) INFECTED BY *COLLETOTRICHUM GLOEOSPORIOIDES*

4.1 INTRODUCTION

4.1.1 Genetics of avocado

Avocado is a sub-tropical fruit-tree with 12 pairs of chromosomes (2n=24) and a small haploid genome size of 907 Mbp, just six times that of *Arabidopsis thaliana* (Arumuganathan and Earle, 1991). Despite its importance little information is available on the genetics of avocado. The lack of genetic information has led to difficulties in improving production and storage of the fruit, because most of the important problems of production have a genetic basis. In order to gain knowledge of the plant's genome, ESTs (Expressed Sequence Tags) projects have been generated, which are based on sequencing a great number of cDNAs obtained from cDNA libraries, generated from different structures and stages of plant development or from plant responses to biotic or abiotic stress (López-Gómez *et al.*, 2007). López-Gómez and co-workers (2007), generated cDNA libraries of fruit and seed, and also genomic libraries of the mesocarp of avocado criollo fruit (*Persea americana* var. d*rymifolia*) at 8 months of development. In that study, they sequenced the cDNA libraries, and their results revealed that 4% of the genes are involved in senescence, 6% of the genes showed no similarity to any sequence reported in the databases, 6% related to pathogen response, 8% are related to lipid synthesis, 14% are related to fruit ripening, 20% are related to unknown function and 42% of the sequenced genes are related to metabolism (López-Gómez *et al.*, 2007).

The ripening of avocado fruit, which starts at the removal of the fruit from the tree, involves differential gene expression and a series of complex biochemical and physiological changes that lead to a soft, edible fruit. The ripening behaviour of climacteric fruit such as avocado is explained by two mechanisms (Christoffersen *et al*., 1982). Firstly

the process is regulated at the level of the *de novo* synthesis of a specific set of mRNAs which leads to the biochemical events that involve ripening. The second mechanism proposes that all the essential enzymes (genes) necessary for ripening are present and need only to be activated but there is also the expression of specific genes related to ripening (Christoffersen *et al*., 1982). Later, Christoffersen *et al*. (1984**)** showed that in ripening avocado fruits there is the accumulation of cellulase mRNA during the climacteric which results in the build-up of cellulase enzyme activity. The increase of cellulase activity during ripening is a manifestation of differential gene expression in the avocado fruit.

4.1.2 Gene expression studies of parasitized plants

Over the last decade a number of studies have been done on gene expression in parasitized plants, some with model host plants such as *Arabidopsis thaliana* or tobacco (*Nicotiana tabacum*) (Joel and Portnoy, 1998; Westwood *et al*., 1998; Griffitts *et al*., 2004). The search for differentially expressed genes in plants during pathogen infections led to a breakthrough in the understanding of the molecular processes involved in infection and contributed to the development of future biotechnological strategies to improve production and storage of the fruit.

Recently, investigation of the *Mikania micrantha* and *Cuscuta campestris* interaction have been carried out to determine the differentially expressed genes involved in the host plant response to a parasitic plant attack (Li *et al*., 2009). Genes expressed upon parasitization by *C. campestris* at early post-penetration stages were investigated by construction and characterization of subtracted cDNA libraries from shoots and stems of *M. micrantha*. Three hundred and three presumably up-regulated ESTs were identified and classified in functional categories, such as metabolism, cell defence and stress, transcription factor,

signal transduction, transportation and photosynthesis. Moreover, the expression of various *M. micrantha* genes involved in metabolism and biosynthesis of carbohydrates, nitrogen and fatty acids was induced during *C. campestris* infection at early post-penetration stages (Li *et al*., 2009).

Casado-Díaz *et al*. (2006) analysed strawberry genes differentially expressed in response to *Colletotrichum* infection. To obtain a wide spectrum of differentially expressed genes, crown tissue was collected at 1, 3, 5 and 7 days after each treatment. For each time point, a pool of crowns was obtained and messenger RNA was extracted from every pooled crown sample. Then subtractive libraries representing differentially expressed transcripts were produced and reverse northern blotting was used to identify ESTs. The results indicated that a large number of strawberry genes involved in signalling, transcriptional control and defence and many genes with unknown functions have altered expression in response to *Colletotrichum acutatum* infection. The findings yielded a first insight into some of the genes responding to this plant-pathogen interaction (Casado-Díaz *et al*., 2006).

4.1.3 Avocado and anthracnose

Anthracnose, caused by *C. gloeosporioides,* is the most severe post harvest disease of avocado fruit. The pathogen infection may extend to the leaves and stems of avocado and it also colonizes dead avocado plant parts suspended in the tree canopy on the ground. Anthracnose reduces avocado fruit shelf life and negatively affects fruit quality, taste and marketability (Coate *et al.*, 1993; Bernstein *et al*., 1995; Freeman *et al*., 1998; Pernezny *et al*., 2000). Two distinct types of diseases occur: those affecting developing fruit in the field (pre harvest) and those damaging mature fruit during storage (post harvest). The ability to cause latent or quiescent infections has grouped *Colletotrichum* among the most important

post harvest pathogens (Bailey *et al*., 1992). The elucidation of gene expression profiles in the infected tissue may bring more understanding of how avocado fruits react at the molecular level to *C. gloeosporioides* and therefore may contribute to the development of strategies to improve its production and storage.

4.1.4. Next-generation sequencing technology

Over the last few years, next-generation sequencing (NGS) technologies have been used as powerful approaches for discovering new genes and analysing gene expression profiles in plant tissues. They have also led to a revolution in genomics and genetics and provided cheaper and faster delivery of sequencing information (Mardis, 2008; Morozova and Marra, 2008). Today there are four commercially available NGS technologies: 454 Life Sciences (acquired by Roche), Solexa (acquired by Illumina), ABI SOLID (acquired from Agencourt Biosciences) and Helicos Biosciences. On the basis of the lengths of the sequence reads produced and their specific features, generally, they can be grouped into two classes. Solexa, ABI SOLID and Helicos all produce very short reads in very large quantities, while the 454 platform can produce a more moderate amount of sequence, but with much longer read lengths (Rounsley *et al*., 2009; Wheeler *et al*., 2008). Furthermore, for model organisms such as *Arabidopsis* where a wealth of genomic information is available in the Genbank, Solexa and ABI SOLID technologies are most frequently used, as transcriptome reads can be mapped to the reference transcriptome or genome. However, for a non-model organism (such as avocado) the Roche 454 pyrosequencing platform is used because the longer reads generated are more suitable for *de novo* assembly and annotation (Shendure and Ji, 2008; Kumar and Blaxter, 2010).

In two thousand and five, 454 GS20 was the first commercial NGS platform released which could produce about 200,000 reads with an average read length of 100 bases per run (Chi, 2008; Schuster, 2008). Since then, a rapid improvement in accuracy, read length and throughput has been experienced by 454 sequencing technology. The newest 454 sequencing platform, the GS FLX Titanium, can be used to generate one million reads with an average length of 400 bases at 99.5% accuracy per run. Nowadays, the 454 pyrosequencing technique is the most widely used new platform in *de novo* sequencing and analysis of transcriptomes in non-model organisms (Meyer *et al*., 2009; Li *et al*., 2010; Sun *et al*., 2010). For example, to study the profile of gene expression in *Salvia miltiorrhiza* and elucidate its functional genes, the 454 GS FLX platform was used to produce a substantial EST dataset from the roots of *S. miltiorrhiza* (Li *et al.,* 2010). In that study 454 sequencing produced a total of 46 722 ESTs with an average read length of 414 bp. Then 454 ESTs were combined with the *S. miltiorrhiza* ESTs from GenBank and were assembled into 18 235 unigenes. The annotation of 73% of these unigenes using BLAST searches showed that 27 unigenes were found to be involved in tanshinone biosynthesis, and 29 unigenes involved in phenolic acid biosynthesis. 70 putative genes were found to encode cytochrome P450 and 577 putative transcription factor genes were identified (Li *et al*., 2010).

The sequencing of cDNA instead of genomic DNA focuses analysis on the transcribed portion of the genome, which reduces the size of the sequencing target space. Many applications have been elucidated through transcriptome sequencing such as: gene expression profiling, genome annotation, and rearrangement detection to non coding RNA discovery and quantification. The versatility of the transcriptome sequencing data can be analyzed simultaneously to provide insight into the level of gene expression, the structure of genomic loci, and sequence variation present at loci.

Recently a first 454 transcriptome sequencing project was done to study genes differentially expressed in avocado roots infected by *Phytophthora cinnamomi*. Since there is no genome data available for avocado, the study did not rely on the sequence coverage. But the identification and characterisation of the targets genes were done based on the percentage of sequence similarity to other sequences of Genbank and by comparing the gene expression in the uninfected and infected avocado roots (Mahomed and Van den Berg, 2011).

In the current study we also focused on comparing the transcriptomes generated from healthy avocado fruit and avocado fruits infected with *C. gloeosporioides* during pre and post harvest using the Roche 454 GS FLX titanium platform. Since *C. gloeosporioides* causes quiescent infections in unripe fruit maintained by high concentrations of preformed antifungal compounds (Prusky *et al*., 1990; Domergue *et al*., 2000), it was necessary to include both pre and post harvest fruits in the experimental design. The strategy undertaken here does not require prior sequence knowledge or genome reference, and relies exclusively on publicly available software and basic scripting tools. To the best of our knowledge, this study is the first to discover differentially expressed candidate genes in avocado fruit following infection with *C. gloeosporioides.*

4.2 MATERIALS AND METHODS

4.2.1 Previous preparation prior to sequencing

The preparation of the fungal inoculum, plant materials, inoculation, total RNA and synthesis of double stranded cDNA from total RNA were performed as outlined in Chapter 2 and 3 (Materials and methods).

4.2.2 454 library construction and sequencing

Total RNAs from infected samples collected after 1h, 4h and 24h were pooled together for the early response and the infected samples collected after 3, 4, 5 and 7 day post infection (dpi) were also pooled together for the late response. This was done for both unharvested and harvested fruits separately after *C. gloeosporioides* infection. cDNA of 2.100, 0.874, 0.907, 1.036 and 0.612 µg of the control, early unharvested (EU), late unharvested (LU), early harvested (EH) and late harvested (LH) samples respectively were submitted for 454 sequencing. The sequencing reactions were conducted by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). cDNA of the control and infected samples of each time point were used to construct a 454 library following the supplier's instructions (Roche Diagnostics, Mannheim, Germany). The cDNA samples were then fragmented into smaller pieces by nebulization using a nebulizer. The nebulized DNA samples were purified on a column from the Qiagen MinElute PCR Purification following the supplier's instructions. Then the fragment end repair was performed for each sample as follows: 9 µl of end repair mix (RL10 x PNK Buffer, RL ATP, RL dNTP, RL T4 Polymerase, RL PNK and RL Taq Polymerase) was added to each sample containing 16 µl of purified nebulized DNA, then vortexed for 5 s and spun for 2 s. Then the samples were run on a thermocycler with the lid on at 25˚C for 20 min, at 72°C for 20 min and 4°C on hold. Once the end repair was completed, 1 µl of RL adaptor and 1 µl of RL ligase were added in each sample then vortexed for 5 s and centrifuged for 2 s. Afterwards, the samples were incubated at 25 °C for 10 min. In the meantime the agencourt AMPure beads were prepared following the supplier's instructions (Roche Diagnostics). After incubation the samples were added to 125 µl of AMPure beads prepared and vortexed for 2 s then centrifuged for 2 s followed with incubation at room temperature (RT) for 5 min. Then all the samples were put on the Magnetic Particle Concentrator (MPC). When the beads had fully ‘pelleted’ on the wall of

the tube, the supernatant was carefully removed. Then 100 µl of TE buffer, 500 µl of sizing solution were added then pipetted up and down and incubated at RT for 5 min. The samples were put on the MPC and when the beads had fully pelleted on the wall of the tube, the supernatant was carefully removed. Each sample was kept on the MPC and the beads were washed twice by adding 1 ml of 70% ethanol. After discarding the ethanol, the pellet was dried at RT for 2 min. Then each sample was removed from the MPC andn 53 µl of TE Buffer was added, vortexed for 5 s and centrifuged for 2 s. Each sample was placed again in the MPC. After the beads were pelleted on the wall of the tube, 50 µl of the supernatant containing the cDNA library was transferred to a new tube. An aliquot of the DNA library was diluted for each sample to a working stock of 1×10^7 molecules/µl, in TE Buffer. The adaptors ligated on to each resulting fragment provided priming sequences for both amplification and sequencing, forming the basis of the single-stranded template library.

The cDNA libraries (DNA fixed to the beads) were then amplified by PCR using one of the GS FLX Titanium the emulsion-based clonal amplification (emPCR amplification) kits following the supplier's instructions (Roche Diagnostics). Briefly the amplification program with the lid set to track within 5°C of the block temperature ran as follows: 1× (4 min at 94°C) 50× (30 s at 94°C, 4.5 min at 58°C, 30 s at 68°C), 10°C on hold. After the amplification, bead recovery and DNA bead enrichment were performed following the manufacturer's instructions (Roche Diagnostics). Then a proper amount of sequencing primer for the sample type was added and vortexed. Each DNA-bound bead was placed into a well on a pico titer plate, a fiber optic chip. A mix of enzymes such as ATP sulfurylase, luciferase, and DNA polymerase were also packed into the well. The Pico titer plate was placed into the GS FLX System for sequencing. The 6 samples (Control, EU, LU, EH and LH) were pooled together then sequenced using Roche 454 GS FLX titanium platform

sequencing and therefore, the individual samples had to be separated based on their individual tags. The basic technique of this sequencing procedure is described by Marguiles *et al* (2005).

4.2.3 Transcript assembly and analysis

The data from the 454 read sequences of each sample were assembled into contigs using the proprietary Roche 454 Newbler Assembler software. Reads from each library were assembled separately. Each sample set was then put through a stringent assembly process and the assembled reads corresponded to contigs, which in all likehood, corresponded to transcripts. For every sample set, not all reads were associated into contigs. These unassembled reads likely corresponded to transcripts as well, but in very low copy number. This could be due to a low expression level of these transcripts.

The cDNA sequences were annotated using CLC Workbench software (CLC bio, Cambridge, MA) and BLAST [Basic Local Alignment Search Tool (Altschul *et al.*, 1990)]. Similarities at the nucleotide level were identified using BLASTN and protein similarities were identified using the non-redundant protein databases BLASTX (Altschul *et al.*, 1990). Each gene was placed in a functional category based on the putative function of the gene product.

4.2.4 Statistical analysis of gene expression data

Because many sequences obtained from the 454 cDNA mapped reads showed similarity to the same protein, the statistical analysis of genes obtained from those reads was done using

the statistical analysis software GraphPad inStat 3. Results were expressed as mean ± standard error of mean (SEM.) and the statistical significance differences between the groups was determined by One-way Analysis of Variance (ANOVA) followed by Dunnett's multiple comparison test as post test. P values less than 0.05 were considered significant.

4.2.5 Hierarchical clustering of gene expression data

The expression profiles of the genes obtained from the mapped read was measured by hierarchical clustering algorithm using Hierarchical Clustering Explorer 3.0 software (http://www.cs.umd.edu/hcil/) which is visualization software for interactive exploration of multidimensional datasets. During hierarchical clustering analysis, genes were grouped into clusters based on the similarity in the expression patterns or profiles (Eisen *et al*., 1998) in both uninfected and infected avocado fruits. The hierarchical clustering algorithm was based on the combination of the average linkage analysis (unweighted pair group method with arithmetic mean) and Pearson's correlation coefficient (Tang *et al*., 2001). All the computational details used are described in the cluster manual available at http://www.cs.umd.edu/hcil/multi-cluster/hce3-manual/hce3_manual.html.

4.3 RESULTS

4.3.1 Inoculation of avocados fruit with *Colletotrichum gloeosporioides*

Symptoms of anthracnose developed 3 days after inoculation. These symptoms were characterized by black fruit rot and spots, as well as fluffy white mycelium which

developed on the wounded inoculated and adjacent uninoculated areas. These results showed that the inoculation technique was effective in causing disease (Chapter Two).

4.3.2 454 sequencing and *de novo* assembly

cDNA samples prepared from total RNA were sequenced using the 454 GS-FLX platform. This single sequencing run produced 215 781 reads from avocado fruit transcriptome, with an average sequence length of 252-300 nucleotides. Using the proprietary Roche 454 Newbler Assembler software, *de novo* assembly of the reads produced contigs, representing avocado fruit transcripts. A total of 70.6 megabases (MB) of sequence data were generated resulting in the assembly of about 1500 contigs. More specifically, 11.4 MB of healthy transcriptome sequence, 11.5 MB of EU transcriptome sequence, 8.3 MB of LU transcriptome sequence, 23.9 MB of EH transcriptome sequence and 15.5 MB of LH of transcriptome sequence was generated.

4.3.3 Sequences analysis

The comparison between the healthy and infected transcriptomes enabled us to identify a large number of candidate pathogen response genes. We focused on comparing the transcriptomes generated from uninfected fruits and infected fruits from each time point (EU, EH, LU and LH). We first determined how many times a gene was represented in each of the libraries based on the number of reads for each unigene count. Putative functions of each of the genes were determined by comparing their sequence with other sequences present in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The selection

criteria of each gene was based on the percentage of maximum identity (range of the percentage = 51-100 %) and its identity as a gene in plants. Then we determined which genes were common in the healthy (uninfected) and infected (EU, EH, LU and LH) transcriptomes.

4.3.3.1 Mapped reads

During the assembly process, there are certain reads called mapped reads which align to other reads at each time point. A total of 709 genes obtained from these reads present in both uninfected and infected samples were found to be differentially expressed after *C. gloeosporioides* infection. During each time point, some of these genes were up or down regulated or remained without any change. Putative functions of each of the genes were determined by comparing their sequence with other sequences present in public GenBank databases using BLASTN and BLASTX programme. The top-scoring genes were used to group the transcripts by their putative function. Of the 709 sequences of the genes analysed, 639 showed significant similarity to previously known plant gene sequences and 70 had no significant similarity to plant genes in the database. Of the 639 sequences exhibiting similarity to plant genes, 358 had similarity to genes for senescence associated proteins, 114 had similarity to genes for cytochromes (cytochrome P450 like_TBP protein, cytochrome P450 monooxygenase, cytochrome c oxidase and cytochrome f), 102 had similarity to genes for a hypothetical proteins, 18 showed similarities to genes encoding proteins involved in metabolism, 14 showed similarity to plant defence and stress-related protein genes, 9 showed similarity to transcription factor and cellular communication genes, 9 had similarity to expressed protein genes, 8 had similarity to genes for proteins

involved in photosynthesis and cell structure and 7 showed similarity to genes for proteins involved in electron transport.

Table 4.1 represents a selection from the 639 cDNA sequences exhibiting differential expression in response to *C. gloeosporioides* infection, to show their expression per time point. Selection was based on high percentage identity to the reference gene. Leucine rich repeat genes were up regulated in early unharvested sample and down regulated in late unharvested and harvested samples. Cytochrome P450 monooxygenase genes were generally up regulated in all infected samples with only one of these genes being down regulated in all infected samples (Table 4.1). Transcription factor WRKY36 was up regulated during early response in harvested fruit and down regulated in during late response in both unharvested and harvested fruits. One senescence-associated protein gene was up regulated in all the infected samples whereas the pattern for the others was to be down regulated in early and late unharvested samples and up regulated in all early harvested samples and most late harvested samples (Table 4.1). Stress related protein such as heat shock protein was up regulated during early response in unharvested fruit and during late response in harvested fruit and down regulated in late unharvested fruit. Defence genes, such as those for an endochitinase and glucanase, tended to be down regulated in infected harvested fruit but unexpressed in infected unharvested fruits (Table 4.1). Genes for enzymes involved in metabolism (e.g. respiration) were down regulated in all unharvested fruits but up regulated in all harvested fruits. The sequences of some selected genes shown in Table 4.1 are presented in the Appendix 1).

Table 4.1. Summary of some selected candidate genes differentially expressed in avocado fruits in response to *C. gloeosporioides* infection with their different expression per time point (reads mapped to the individual transcripts for every time point). Genes up regulated (shaded in yellow) and genes down regulated (shaded in green).

Name	Similar sequence from database BLASTn (Accession num)	Max Identity %	Similar sequence From database BLASTx (Accession num)	E-value	Copies per time-point expressed				
					Cont	Early Unharvest	Late Unharvest	Early harvest	Late harvest
SIGNAL TRANSDUCTION AND TRANSCRIPTION FACTORS									
Gene00001	Cinnamomum camphora large subunit ribosomal gene (DQ008772.1|)	99	Leucine rich protein [Arachis hypogaea] (ABH09320.1)	4e-04	107	145	41	88	83
Gene00609	Daphnandra micrantha 26S ribosomal RNA gene |DQ008629.1)	96	Transcription factor WRKY36 [Physcomitrella patens subsp. Patens (XP_001775684.1)	8.7	7	7	4	9	3
CYTOCHROME PROTEINS									
Gene00132	Gomortega keule 26S ribosomal RNA gene (AY095460.1)	98	Cytochrome P450 monooxygenase [Pyrus communis] (AAR25996.1)	2e-06	12	40	20	49	30
Gene00144	Cryptocarya meissneriana 26S ribosomal RNA gene (DQ008627.1)	98	Cytochrome c oxidase subunit 5B, mitochondrial precursor (ACN10266.1)	7e-05	8	19	9	22	6
Gene00301	Glycine max catalase (cat4) mRNA, complete cds(AF035255.1)	96	Cytochrome P450 like_TBP [Nicoti tabacum] (BAA10929.1)	8e-49	45	63	29	58	45
Gene00475	Phoebe excelsa chloroplast ndhF gene (AB442055.1)	100	Cytochrome P450-like TBP protein [Lilium longiflorum] (ABO20848.1)	6e-04	10	24	7	14	19
Gene00614	Hernandia nymphaeifolia 26S ribosomal RNA gene (AY095462.1)	100	Cytochrome P450 like_TBP [Nicoti tabacum] (BAA10929.1)	3e-23	4	1	3	3	2
HYPOTHETICAL PROTEINS									
Gene00082	Peumus boldus 26S ribosomal RNA gene (AY095466	96	Hypothetical protein [Arabidopsis thaliana] (BAF01964.1)	2e-48	126	207	178	262	167
Gene00213	Doryphora sassafras 26S ribosomal RNA gene (DQ008630.1)	98	Hypothetical protein LOC10038298 [Zea mays] (NP_001169136.1)	2e-31	31	26	13	30	25
Gene00264	Musa acuminata subsp. burmannicoides isolate (EU418634.1)	100	Hypothetical protein SORBIDRAFT_3036s002010 [Sorghum bicolor] (XP_002488947.1)	2e-06	28	42	14	44	22
Gene00343	Liriodendron tulipifera chloroplast, complete genome (DQ899947.1)	100	Hypothetical protein SORBIDRAFT_0070s002020 [Sorghum bicolor] (XP_002489102.1)	3e-35	19	13	9	20	19

Table 4.1 (Continued)

Name	Similar sequence from database BLASTn (Accession num)	Max Identity %	Similar sequence From database BLASTx (Accession num)	E-value	Copies per time-point expressed				
					Cont	Early Unharvest	Late Unharvest	Early harvest	Late harvest
DEFENCE/STRESS									
Gene00654	Cananga odorata large subunit ribosomal RNA gene (DQ008784.1)	100	Catalase [Arabidopsis thaliana] (CAA45564.1)	4e-24	1	1	1	2	0
Gene00509	Magnolia denudata 26S ribosomal RNA gene (AF389256.1)	100	Endo-1,4-beta-glucanase [Malus x domestica] (AAQ55294.1)	1e-25	7	0	0	0	1
Gene00405	Persea americana mRNA for endochitinase (Z78202.1	100	Endochitinase [Persea americana] (CAB01591.1)	4e-65	5	0	0	1	2
Gene00308	Laurus nobilis 26S ribosomal RNA gene (DQ008626.	100	Heat shock protein [Cucumis sativus (ADF30255.1)	7e-79	2	3	1	2	3
Gene00653	Strombosia grandifolia 26S ribosomal RNA gene (DQ790225.1)	100	Metallothionein-like protein [Arabidopsis thaliana] (CAA44630.	8.5	1	1	0	3	0
SENESCENCE ASSOCIATED PROTEINS									
Gene00091	Calycanthus occidentalis 26S ribosomal RNA gene (AY095454.1)	95	Putative senescence-associated prote [Trichosanthes dioica] (ABN50032.1)	6e-30	135	187	184	349	236
Gene00225	Persea americana mRNA for fructose-bisphosphate(emb|AJ133146.2)	100	Putative senescence-associated prote [Cupressus sempervirens] (ACA30301.1)	1e-42	57	28	29	132	88
Gene00407	Zea mays clone 10282 mRNA sequence (DQ244722.1	80	Putative senescence-associated prote [Pisum sativum] (BAB33421.1)	5e-15	51	5	15	127	80
Gene00473	Avocado cellulase (endo-(1-4)-beta-n-glucanase (M17634.1)	100	Putative senescence-associated prote [Lilium longiflorum] (ABO20851.1)	0.003	5	2	1	5	2
Gene00282	Eucalyptus grandis chloroplast, complete genome (HM347959.1)	100	Senescence-associated protein [Liliu longiflorum] (BAB33421.1)	1e-30	13	2	8	39	11
Gene00496	Striga asiatica isolate St505 zinc-binding dehydrogena mRNA, (DQ445137.1)	100	Senescence-associated protein [Pisu sativum] (BAB33421.1)	3e-17	6	5	3	21	15
Gene00504	Phyllostachys edulis cDNA clone: bphyst035g20, full insert sequence (FP092404.1)	100	Senescence-associated protein [Pice abies] ACA04850.1	2e-44	32	19	23	61	35
Gene00617	Persea americana mRNA for metallothionein-like emb (AJ133145.1)	100	Senescence-associated protein [Pisu sativum] (BAB33421.1)	1.00	70	16	33	74	32

Table 4.1 (continued)

Name	Similar sequence from database BLASTn (Accession num)	Max Identity %	Similar sequence from database BLASTx (Accession num)	E-value	Copies per time-point expressed				
					Cont	Early Unharvest	Late Unharvest	Early harvest	Late harvest
METABOLISM									
Gene00237	Arabidopsis thaliana clone 34690 mRNA, (AY087376	72	Fructose-bisphosphate aldolase [Per americana] (CAB77243.2)	2e-81	7	4	1	13	9
Gene00237	Arabidopsis thaliana clone 34690 mRNA, complete sequence(AY087376.1)	82	Fructose-bisphosphate aldolase [Per americana](CAB77243.2)	2e-102	7	4	1	13	9
PHOTOSYNTHESIS AND CELL STRUCTURE									
Gene00447	Gyrocarpus americanus 26S ribosomal RNA (DQ008624.1)	87	Chloroplast hypothetical protein [Ze mays subsp. mays] (YP_588293.1)	1e-33	1	8	0	6	2
Gene00665	Liriodendron tulipifera chloroplast, complete genome (DQ899947.1)	95	Photosystem I assembly protein Ycf [Zea mays] (NP_043035.1)	1e-18	1	2	2	0	0
ELECTRON TRANSPORT AND EXPRESSED PROTEINS									
Gene000453	Gomortega keule 26S ribosomal RNA(AY095460.1)	62	Aquaporin NIP6-1 [Medicago truncatula](XP_003604211.1)	4e-06	0	1	1	9	0
Gene0037	Laurus nobilis 26S ribosomal RNA(DQ008626.1)	98	Expressed protein [Arabidopsis lyrat subsp. lyrata] (XP_002884233.1)	3e-27	50	80	30	106	63
Gene0043	Laurus nobilis 26S ribosomal RNA(DQ008626.1	98	Expressed protein [Arabidopsis lyrat subsp. lyrata] (XP_002884233.1)	2e-27	87	120	51	144	83
Gene00532	Calycanthus occidentalis 26S ribosomal RNA gene (AY095454.1)	100	NAD-dependent sorbitol dehydrogenase 3 [Malus x domestic (AAP69752.1)	3e-40	0	3	0	2	3
UNKNOWN FUNCTION									
Gene00086	Gyrocarpus americanus 26S ribosomal RNA gene , partial sequence (AY095454.1)	98	Unknown [Zea mays] (ACR36970.1)	3e-58	17	16	2	14	9

4.3.3.2 Unmapped reads

During the assembly process, there were reads that did not map with others reads at each time point in both uninfected and infected samples. Unmapped reads do not align to other reads because they are either very different from other reads or are too low in frequency. These unmapped reads also represent genes and/or parts of the genes. The unmapped reads were also quantified in order to determine the number of copies expressed per time-point. The function assignment of these genes was done based on similarity after comparing their sequences to the non-redundant protein databases BLASTX program. This analysis revealed that some unmapped reads were expressed in both infected and uninfected samples but not at each time point, others unmapped reads were only expressed in the infected samples (coding for genes predicted to be induced) and other unmapped reads were expressed only in the uninfected samples. The quantification of unmapped reads expressed in both infected and uninfected samples but not at each time point i.e how many times an unmapped gene read from EU, LU, EH and LH samples map or do not map to the unmapped reads from the control is presented in Table 5.2 (Chapter 5). However, in this chapter, Table 4.2 merely summarises whether selected candidate genes were expressed or not in infected avocado fruits and the time point of expression. Functional groups include genes that are known to be involved in defence, transcription, regulation, signal transduction, oxidative burst, stress response, transportation, metabolism, protein synthesis and photosynthesis. Some sequences were considered as being of unknown/unclassified function.

Mitogen-activated protein kinase (MAPK) and leucine rich repeat receptor-like protein kinase were expressed in all the infected samples (Table 4.2). Salicylic acid binding protein was expressed in all the infected harvested samples, whereas calcium dependent protein

kinase and calcium ion binding protein were expressed in both unharvested and harvested samples during early response but only in the harvested infected samples during late response (Table 4.2). Chaperone protein dnak (heat shock protein 70) was expressed in all the infected samples. A number of others genes likely to be involved in plant defence response were also expressed in infected samples, such as: endo-1,4-D-glucanase expressed in unharvested samples during early response and in harvested sample during late response; endochitinase expressed in unharvested samples during early response and in harvested samples during early and late response. Endopeptidase was expressed in both unharvested and harvested samples during late response and aspartic proteinase was expressed only in the harvested samples. The genes for a number of proteins involved with the oxidative burst were also expressed, mostly in infected unharvested fruits. Among proteins predicted to be involved in proteins synthesis, elongations factor 1 was expressed in all the infected samples and acetyl-CoA carboxylase carboxyltransferase, involved in metabolism, was expressed only in all infected unharvested samples, whereas stearoyl-acyl-carrier protein desaturase was expressed only in infected unharvested fruits.

4.3.3.3 Genes induced by *C. gloeosporiodes* infection

During the assembly process, a group of reads from the infected samples that did not map with the reads from the uninfected samples are considered to code for genes induced after *C. gloeosporioides* infection. These genes were classified according to their putative function after comparing their sequence with other sequences present in public GenBank databases using BLASTX program (Figure 4.1).

Based on the protein similarity in GenBank, of the 218 induced genes the largest set of these genes 52 (24%) was assigned to defence, followed by the second large group of 36 (17%) assigned to unclassified genes. Thirty-four genes were found to have significant similarity to signal transduction/cellular communication genes, 17 had similarity to genes involved in oxidative burst and energy, 14 had similarity to genes involved in metabolism, 14 had similarity to genes involved in protein synthesis, 12 showed similarity to transcription factor genes, 12 genes showed similarity with genes involved in transportation, 11 genes showed similarity to stress related protein genes. Finally 8 genes had similarity to genes coding for proteins involved in photosynthesis while genes involved in regulation (4) and cell structure (4) constituted the smallest group, each comprising 2% of the sequences (Figure 4.1). A complete list of genes (and their respective reference sequences), induced by *C. gloeosporioides* irrespective of time point, is given in Appendix 2. The quantification to see how many times they were expressed in the infected samples was not performed during the assembling process.

Table 4.2. Summary of some selected candidate genes expressed in infected avocado fruits in response to *C. gloeosporioides* infection with (1) for expressed and (0) not expressed

Name	**BLASTx** Accession num of similar sequence	**Similar sequence from database (Putative function)**	**E-value**	**Max Identity** %	Expression			
					Early Unharvest	**Late Unharvest**	**Early harvest**	**Late harvest**
SIGNAL TRANSDUCTION								
Gene 01	XP_002532559.1	Calcium-dependent protein kinase, putative [Ricinus communis]	8e-25	68	1	0	1	1
Gene 02	XP_002521983.1	Calcium ion binding protein, putative [Ricinus communis]	9e-21	45	1	0	1	1
Gene 03	XP_002880383.1	Kinase family protein [Arabidopsis lyrata subsp. lyrata]	0.094	73	1	1	1	1
Gene 04	AAF66615.1	LRR receptor-like protein kinase [Nicotiana tabacum]	3e-33	75	1	1	1	1
Gene 05	NP_201509.1	Mitogen-activated protein kinase kinase kinase 19 [Arabidopsis thaliana]	7 e-21	60	1	1	1	1
Gene 06	AAR87711.1	Salicylic acid-binding protein 2 [Nicotiana tabacum]	1 e-10	63	0	0	1	1
TRANSCRIPTION FACTOR								
Gene 08	NP_001152266.1	Basic helix-loop-helix (bHLH) transcription factor [Zea mays]	1e-23	72	1	0	0	0
Gene 09	ACF60482.1	bZIP transcription factor [Oryza sativa Japonica Group]	1e-08	57	1	0	0	0
Gene 10	AAS68190.1	Myb transcription factor [Vitis vinifera]	6e-27	89	1	0	1	1
Gene 11	AAD10237.1	TATA-box binding protein [Phaseolus vulgaris]	3e-06	100	0	0	0	1
Gene 12	CAD56217.1	Transcription factor EREBP-like protein [Cicer arietinum]	0.069	100	1	1	1	1
Gene 13	AAM63665.1	Transcription factor, putative [Arabidopsis thaliana]	2e-33	89	1	1	1	1
OXIDATIVE BURST								
Gene 14	ACO37154.1	ACC oxidase [Stenocereus stellatus]	4 e-21	88	0	0	1	0
Gene 15	XP_002533075.1	Glutathione peroxidase, putative [Ricinus communis]	6e-12	79	1	0	0	0
Gene16	AAF61392.1	Glutathione S-transferase [Persea americana]	8 e-43	73	1	1	1	0
Gene 17	ACG39782.1	NADH-cytochrome b5 reductase [Zea mays]	9 e-35	90	1	1	1	1
Gene 18	BAD83480.2	NADH dehydrogenase subunit 4 [Nicotiana tabacum]	2 e-16	100	1	1	0	0
Gene 19	YP_784442.1	NADH-plastoquinone oxidoreductase subunit 1 [Drimys granadensis]	6 e-23	100	1	1	0	1

TABLE 4.2 (continued)

Name	BLASTx Accession num of similar sequence	Similar sequence from database (Putative function)	E-value	Max Identity %	Expression			
					Early Unharvest	Late Unharvest	Early harvest	Late harvest
STRESS RESPONSE								
Gene 20	ABE79560.1	Chaperone protein dnak (heat shock protein 70) [Medicago truncatula]	7e-19	53	1	1	1	1
Gene 21	NP 192977.2	Stress-inducible protein, putative [Arabidopsis thaliana]	2e-22	77	1	0	1	1
Gene 22	XP_002514902.1	Stress associated endoplasmic reticulum protein, putative [Ricinus communis]	2e-09	100	1	0	0	1
Gene 23	NP_191404.2	Universal stress protein family protein [Arabidopsis thaliana]	8 e-29	86	1	0	1	1
DEFENCE								
Gene 24	AAK15049.1	Asparaginyl endopeptidase [Vigna radiata]	2 e-20	66	1	1	0	0
Gene 25	NP_172655.1	Aspartic proteinase A1 [Arabidopsis thaliana]	4 e-28	77	0	0	1	1
Gene 26	AAD30292.1	Catalase 3 [Raphanus sativus]	5 e-57	76	1	1	1	1
Gene 27	ADQ39593.1	Class II chitinase [Malus x domestica]	4 e-06	82	1	0	0	1
Gene 28	BAB82473.1	Chitinase 3 [Triticum aestivum]	2e-34	82	0	1	0	0
Gene 29	ABX79341.1	Cysteine protease [Vitis vinifera]	4 e-46	81	1	1	1	0
Gene 30	ABK78689.1	Cysteine proteinase inhibitor [Brassica rapa]	2 e-15	75	0	1	1	1
Gene 31	CAB01591.1	Endochitinase [Persea Americana]	2 e-58	77	1	0	1	1
Gene 32	ABY58189.1	Endo-1,4-D-glucanase [Persea americana]	6 e-48	100	1	0	0	1
Gene 33	ACG44564.1	Endopeptidase Clp [Zea mays]	4 e-28	52	0	1	0	1
Gene 34	EE84132.1	Enhanced disease resistance 2 protein [Arabidopsis thaliana]	0.014	51	1	0	0	0
Gene 35	AAF97315.1	Lipoxygenase [Arabidopsis thaliana]	4e-08	72	1	0	0	0
Gene 36	XP_002527223.1	Oligopeptidase A, putative [Ricinus communis]	5 e-09	86	0	0	1	0
Gene 37	ABA33845.1	Pathogenesis-related protein 6 [Zea diploperennis]	3e-16	59	1	0	0	1
TRANSPORTATION								
Gene 38	CAB41144.1	H+-transporting ATPase-like protein [Arabidopsis thaliana]	3 e-23	81	0	0	0	1
Gene 39	XP_002526521.1	Peptide transporter, putative [Ricinus communis]	2 e-15	62	0	1	0	1
Gene 40	XP_002526529.1	Protein transport protein sec23, putative [Ricinus communis]	7e-11	89	0	0	1	1
CELL STRUCTURE AND COMPONENT								
Gene 41	ADN34200.1	Annexin [Cucumis melo subsp. melo]	2e-16	70	1	0	0	0
Gene 42	ACS28251.1	Cell division control protein [Nicotiana glutinosa]	2e-33	90	1	1	1	1
Gene 43	NP_564367.1	Integral membrane HRF1 family protein [Arabidopsis thaliana]	1e-20	89	0	0	1	0

TABLE 4.2 (continued)

Name	BLASTx Accession num of similar sequence	Similar sequence from database (Putative function)	E-value	Max Identity %	Expression			
					Early Unharvest	Late Unharvest	Early harvest	Late harvest
METABOLISM / PHOTOSYNTHESIS								
Gene 44	ABI18045.1	Acetyl co-A carboxylase [Strombosia grandifolia]	4 e-10	81	1	1	0	0
Gene 45	ADO64899.2	Acetyl-CoA carboxylase carboxyltransferase beta subunit [Theobroma cacao]	1e-19	90	1	1	0	0
Gene 46	YP_004021302.1	ATP synthase CF1 alpha subunit [Theobroma cacao]	6 e-90	99	0	0	1	0
Gene 47	ACG42565.1	Calcium homeostasis regulator CHoR1 [Zea mays]	8e-17	68	1	0	0	0
Gene 48	ACG59771.1	Chloroplast aspartate aminotransferase [Triticum aestivum]	3e-14	94	0	0	1	0
Gene 49	AAL77589.1	Chloroplast ribose-5-phosphate isomerase [Spinacia oleracea]	1e-17	64	1	0	0	0
Gene 50	XP_002532986.1	Flavonol synthase/flavanone 3-hydroxylase, putative [Ricinus communis]	1. e-04	65	1	0	0	0
Gene 51	CAP12013.1	Photosystem II protein Z [Coffea myrtifolia]	2 e-06	94	1	0	1	1
Gene 52	AAF15308.1	Stearoyl-acyl-carrier-protein desaturase [Persea americana]	9 e-20	100	0	0	1	1
Gene 53	ACO40485.1	Terpene synthase [Actinidia deliciosa]	3e-17	58	1	0	0	0
Gene 54	ACG45528.1	Ubiquitin-protein ligase [Zea mays]	2e-17	76	1	1	1	0
PROTEIN SYNTHESIS/REGULATION								
Gene 55	BAB90396.1	ADP-ribosylation factor [Oryza sativa Japonica Group]	5e-05	100	0	0	1	0
Gene 56	ADB93067.1	Aquaporin [Jatropha curcas]	3e-31	87	1	0	0	0
Gene 57	AAM12952.1	Auxin-regulated protein [Zinnia violacea]	2e-06	53	1	0	1	1
Gene 58	XP_002528028.1	Elongation factor 1-alpha, putative [Ricinus communis]	9 e-51	100	1	1	1	1
Gene 59	CAA71882.1	Elongation factor 2 [Nicotiana tabacum]	9e-15	94	0	0	1	0
Gene 60	XP_002513404.1	Eukaryotic translation elongation factor, putative [Ricinus communis]	3e-35	89	0	0	1	0
Gene 61	AAZ75913.1	Ribosomal protein L16 [Coffea humilis]	0.014	100	1	1	0	0
Gene 62	BAD83474.2	Ribosomal protein S3 [Nicotiana tabacum]	0.001	100	1	0	1	0
FUNCTION: UNCLASSIFIED								
Gene 63	ADC35365.1	Alpha-expansin 2 [Coffea arabica]	5 e-25	90	0	1	0	1
Gene 64	NP_193002.4	Endomembrane family protein 70 [Arabidopsis thaliana]	2 e-12	94	0	1	0	1
Gene 65	XP_002888340.1	Predicted protein [Arabidopsis lyrata subsp. lyrata]	8.6	73	0	0	1	0
Gene 66	XP_002519112.1	Protein binding protein, putative [Ricinus communis]	5 e-13	51	0	0	0	1
Gene 67	NP_001077933.1	Ubiquitin fusion degradation 1 [Arabidopsis thaliana]	9.1	93	1	0	0	1
Gene 68	ACU24411.1	Unknown protein product [Glycine max]	3 e-06	92	0	1	0	1

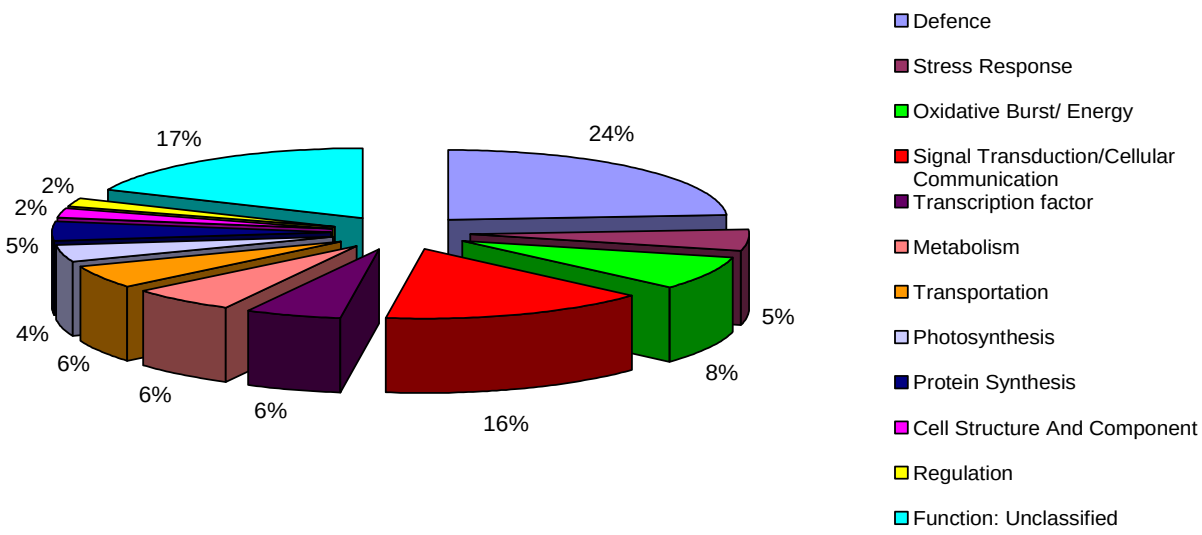


Figure 4.1 Exploded pie chart showing the distribution of induced genes with functional categories.

4.3.3.4 Unexpressed genes in infected fruit

Finally, others reads from the uninfected sample which did not map during the assembly process with the reads from the infected samples code for genes predicted to be repressed or not expressed in the infected samples. The function assignment of these genes was done based on homology after comparing their sequences to the non-redundant protein databases BLASTX program (Table 4.3). Important genes expressed only in uninfected avocado, which will be discussed further, are glycosyltransferase, (R)-limonene synthase, cytosolic NADP-malic enzyme and genes involved with jasmonic acid metabolism.

Table 4.3. Summary of unexpressed genes in avocado fruits in response to *C. gloeosporioides* infection with their putative function

Name	Accession num of similar sequence	Similar Sequence From database (BLAST X)	PUTATIVE FUNCTION	E-value	Max Identity %
Gene 1	AAM21683.1	1-Aminocyclopropane-1-carboxylate synthase 1 [Persea americana]	Lyase (metabolism	1e-27	96
Gene 2	ABI34672.1	bZIP transcription factor bZIP53 [Glycine max]	Transcription	0.001	64
Gene 3	XP_002534067.1	Calcium-activated outward-rectifying potassium channel, putative [Ricinus communis]	Ion transport	1e-23	83
Gene 4	ADN33942.1	Chloroplast outer envelope protein 34 [Cucumis melo subsp. melo]	Photosynthesis	5e-45	84
Gene 5	CAC00657.1	Common plant regulatory factor 6 [Petroselinum crispum]	Regulation	2e-06	70
Gene 6	ABB86962.1	Cytosolic NADP-malic enzyme [Malus x domestica]	Oxidation	2e-21	95
Gene 7	ADD09620.1	Dehydration responsive element binding protein [Trifolium repens]	Stress	0.059	90
Gene 8	NP_001150497.1	Early nodulin 93 [Zea mays]	Cell structure	3e-08	73
Gene 9	ACN38307.1	Eukaryotic translation initiation factor 4e [Carica papaya]	Protein synthesis	1e-39	72
Gene 10	BAF46299.1	Extensin like protein [Ipomoea nil]	Cell structure	5e-18	74
Gene 11	XP_002960850.1	Glycosyltransferase CAZy family GT14 [Selaginella moellendorffii]	Biosynthesis of cell walls	5e-41	76
Gene 12	AAF04915.1	Jasmonic acid 2 [Solanum lycopersicum]	Signal transduction	1e-38	66
Gene 13	ACG35116.1	Legumin-like protein [Zea mays]	Nutrient Reservoir Activity(metabolism)	3e-31	74
Gene 14	XP_002533356.1	(R)-Limonene synthase[Ricinus communis]	Lyase (metabolism)	3.8	66
Gene 15	XP_002525554.1	Negative cofactor 2 transcriptional co-repressor, putative [Ricinus communis]	Transcription	1e-08	93
Gene 16	XP_002521801.1	Patellin-3, putative [Ricinus communis]	Transport /cell Division	6e-27	82
Gene 17	BAD46097.1	Putative wall-associated kinase 4 [Oryza sativa Japonica Group]	Signal transduction	1e-21	69
Gene 18	CAB77245.1	Putative seed imbibition protein [Persea americana]	Protease (defence)	2e-50	90
Gene 19	NP_001147686.1	Seven-transmembrane-domain protein 1 [Zea mays]	G protein-coupled receptors (signal transduction)	7e-11	60
Gene 20	CAA78365.1	Tobacco pre-pro-cysteine proteinase [Nicotiana tabacum]	Cysteine-Type Endopeptidase Activity	1e-38	70

4.3.4 Statistical analysis

The results of the statistical analysis of gene expression in fruits infected with *C. gloeosporiodes* compared with that in uninfected control fruits are shown in Table 4.4.

Table 4.4. Statistical analysis of gene expression in avocado fruits per time point. The values are the means ± SEM of the total number of expressed gene sequences within each group.

Genes (number)	Expression per time point				
	Control	**EU**	**LU**	**EH**	**LH**
Senescence-associated protein (358)	51.00 ± 3.65	40.32 ± 4.17*****	37.31 ± 3.29******	110.62 ± 7.71******	71.09 ± 5.00******
Cytochromes (114)	36.96 ± 4.35	49.72 ± 6.09******	23.12 ± 2.59******	66.56 ± 7.98******	42.99 ± 5.35
Hypothetical proteins (102)	28.24 ± 4.50	36.42 ± 6.57	19.54 ± 3.63*****	53.27 ± 9.09******	33.27 ± 5.78
Metabolism (18)	8.61 ± 4.13	11.00 ± 4.36	9.11 ± 5.55	8.67 ± 2.73	6.55 ± 2.46
Defence/ Stress (14)	26.93 ± 9.24	32.64 ± 14.71	15.64 ± 5.76	56. 14 ± 20.99*****	38.79 ± 15.37
Transcription factor/signal transduction (9)	48.33 ± 16.67	54.33 ± 22.01	26.78 ± 10.48	77.89 ± 29.07	51.56 ± 17.98
Expressed protein (9)	25.25 ± 10.89	35.38 ± 15.43	15.63 ± 6.202	42.13 ± 19.30	24.50 ± 11.25
Photosynthesis (8)	12.63 ± 8.81	15.63 ± 8.78	17.63 ± 12. 21	15.13 ± 5.17	8.38 ± 4.96
Electron transport (7)	14.29 ± 7.37	24 ± 11.61	10.86 ± 5.46	28.86 ± 16.43	17.43 ± 9.73
Unknown protein (70)	102.54 ± 11.57	115.16 ± 14.35	78.64 ± 9.79	213.69 ± 6.77******	135.63 ± 17.32*****

Control (uninfected), EU-early unharvested, LU-late unharvested, EH-early harvested, LH-late harvested

Analysis was by One-way ANOVA and Dunnett's test. ***** represents significantly different from control at $p < 0.05$; ****** represents significantly different from control at $p < 0.0001$.

For most of these genes, gradual changes are noticed in their expression across time points. Based on the statistical analysis, genes for senescence associated proteins are down regulated in the unharvested samples and are up regulated in the harvested samples. Genes coding for cytochromes are down regulated in the LU samples and are up regulated in both early unharvested and harvested samples. The expression of hypothetical protein genes is down regulated in the LU samples and is up regulated in the EH samples and the and the expression of defence/stress related protein genes is up regulated in the EH infected

samples. The expression of genes predicted to be involved in metabolism, signal transduction, transcriptional activation, photosynthesis, electron transport and expressed proteins do not show any statistically significant changes in any of the infected samples compared to the uninfected samples.

4.3.5 Hierarchical Cluster Analysis

4.3.5.1 Mapped reads

Hierarchical clustering analysis was used to group together genes with similarity in the expression patterns into clusters, in both uninfected and infected avocado fruits (Figure 4.2). The clustering analysis resulted in the generation of hierarchical series of clusters represented graphically by a binary tree called a dendrogram which reflects the relationships among genes. In this tree, each data point corresponds to a terminal node of the binary tree with the similarity of the subtree indicated by the distance from the root to a subtree; genes or groups of genes with similar expression patterns are adjacent.

The dendogram was combined with a colour mosaic display in which each data point is represented by a colour that qualitatively reflects the original experimental gene expression. In the colour mosaic display the organization of rows and columns varies according to the clustering results. In addition, in this colour mosaic display reflecting gene expression, large contiguous patches of colour correspond to groups of gene sharing similar expression patterns under the experimental condition (uninfected or infected). By default, in hierarchical clustering explorer, a bright red colour represents higher levels of gene expression, a bright green colour represent lower levels of gene expression whereas the black colour represents the middle value or represents genes equally expressed. The colour becomes darker as a value gets closer to the middle value between the red and the green

lines. Finally, a large contiguous patch of a colour indicates that most of the values are near extremes and represent group of genes that share similar expression patterns over multiple time points. For example, there is a dramatic increase in expression of a large group of gene families in the early unharvested (EU) sample, whereas the late unharvested (LU) sample shows a down regulation of approximately the same group of gene families and the late harvested (LH) fruits show a down regulation of some of the same groups of genes and an upregulation of more distantly related genes (Figure 4.2). These changes in patterns of gene expression can also be observed more precisely as 2D scatterplots in which the shifts in the expression of gene clusters in infected samples in relation to the uninfected sample are observed along two axes (Figure 4.3).

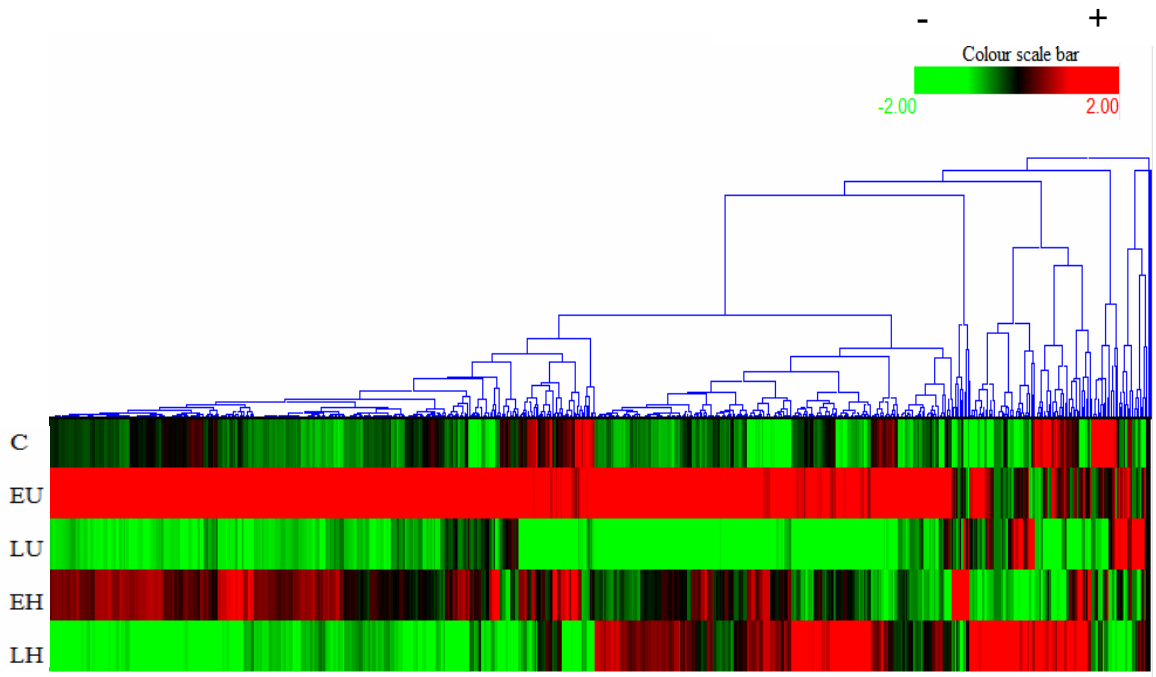
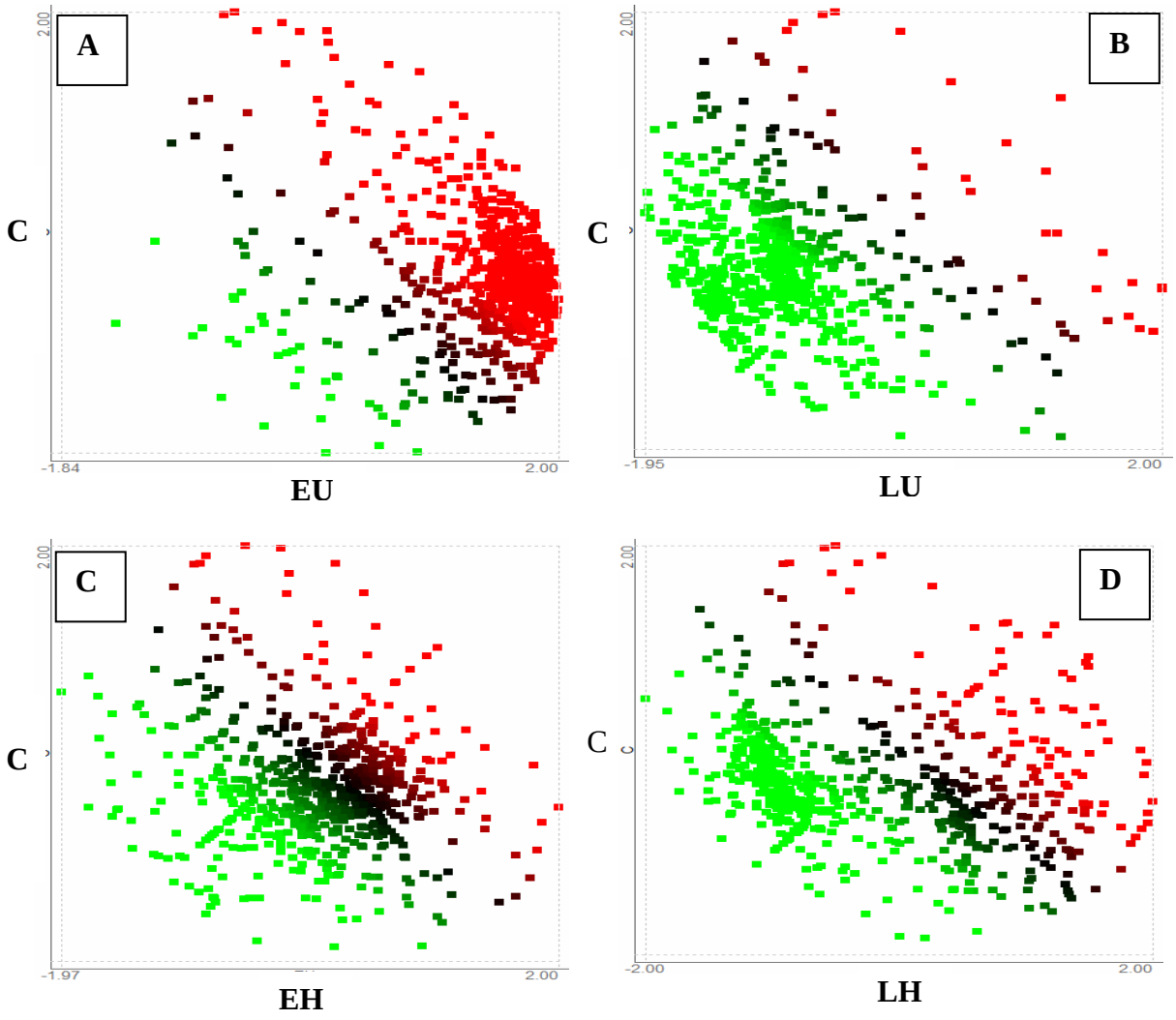


Figure 4.2 Hierarchical cluster of avocado genes obtained from the 454 cDNA mapped reads with changes in their expression profiles in response to *C. gloeosporioides* infection. The dendrogram on top of the colour mosaic display provides a measure of the relatedness of gene expression in each sample. Each gene is represented by a single column of coloured boxes, and each row represents a time point. A bright red colour represents higher than uninfected levels of gene expression in the infected samples, bright green colour represents lower than uninfected levels of gene expression. Black colour represents genes equally expressed in both infected and uninfected. Brown indicates missing or excluded data. The fold change scale bar is shown above the cluster with a ratio from -2 to 2.



Figures 4.3 Pearson's 2 D scatterplots of avocado genes obtained from the 454 cDNA mapped reads with changes in their expression profiles in response to *C. gloeosporioides* infection vs the uninfected sample. The 2D scatterplots show the entire distribution of genes represented in the dendogram and colour mosaic. (A) The distribution of genes expressed in EU vs C, (B) the distribution of genes expressed in LU vs C, (C) the distribution of genes expressed in EH vs C and (D) the distribution of genes expressed in LH vs C. A bright red colour represents high value and a bright green colour represents low value. The middle value has a black colour.

Many sequences obtained from the 454 cDNA mapped reads coded for specific groups of proteins such as: senescence-associated protein genes, cytochrome genes, hypothetical protein genes, genes encoding proteins involved in metabolism, genes encoding plant defence and stress-related proteins etc. Because of this fact, hierarchical clustering analysis was done for each of these groups of genes to observe their expression profile at each time point. For all the following hierarchical clusters (Figures 4.4 – 4.12), each gene is represented by a single column of coloured boxes, and each row represents a time point. A bright red colour represents higher levels of gene expression; bright green colour represents lower levels of gene expression and black colour represents genes equally expressed. Brown indicates missing or excluded data.

4.3.5.2 Senescence-associated protein genes

Figure 4.4 shows that over time, senescence associated protein genes expression profiles in response to *C. gloeosporioides* infection are similar within the harvested and unharvested treatments. Based on the colour mosaic display and 2 D scatterplots, their expression is down regulated in all the unharvested infected samples and is up regulated in the harvested infected samples. This observation is confirmed by the statistical analysis (Table 4.4).

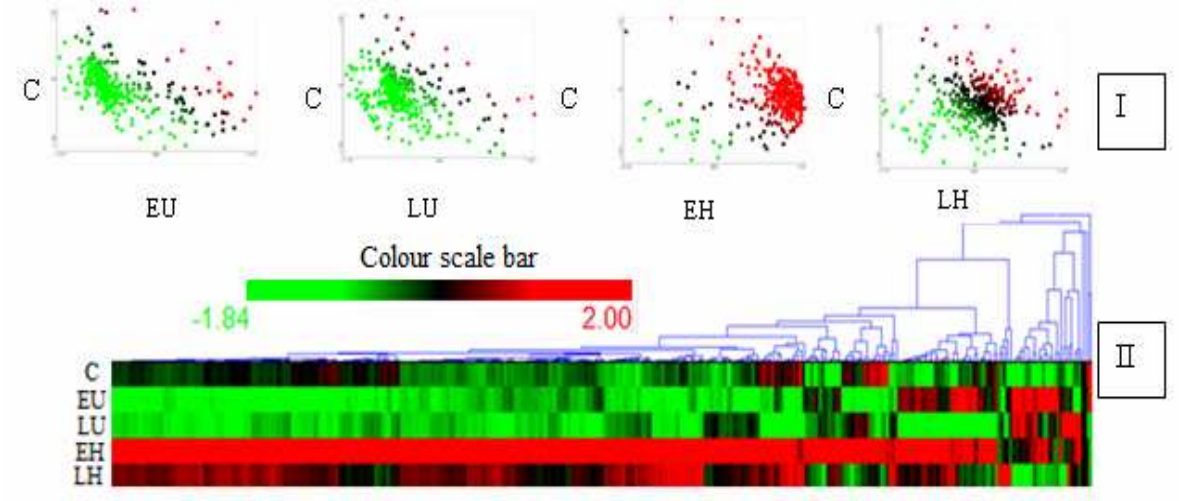


Figure 4.4 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of senescence-associated protein genes. The fold change scale bar is shown above the cluster with a ratio from -1.84 to 2.

4.3.5.3 Cytochrome genes

Genes coding for some cytochrome proteins such as: cytochrome P450 like TBP protein, cytochrome P450 monooxygenase, cytochrome c oxidase and cytochrome f also show variation in their expression following *C. gloeosporioides* infection. In a binary tree (dendogram) each gene corresponds to a terminal node of the binary tree. The results show (Figure 4.5) that the joining points of many substrees are further from the root. Based on the fact that the similarity of the nodes or subtrees is indicated by the distance from the root to a subtree, there is a high similarity between genes coding for cytochrome proteins. In addition the colour mosaic display and 2 D scatterplots show that these genes are highly expressed in both EU and EH infected samples and are significantly down regulated in the LU infected samples compared to the uninfected samples (Figure 4.5). Once again, this observation is confirmed by the statistical analysis (Table 4.4)

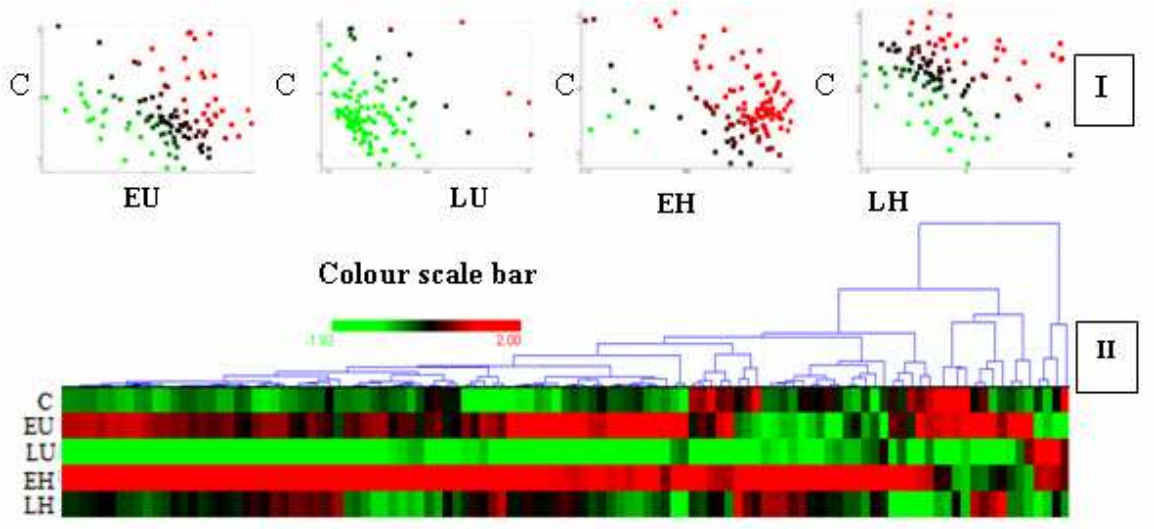


Figure 4.5 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of cytochrome genes. The fold change scale bar is shown above the cluster with a ratio from -1.93 to 2.

4.3.5.4 Hypothetical proteins

Hypothetical proteins were differentially expressed in the infected samples following *C. gloeosporioides* infection (Figure 4.6). In the dendogram many hypothetical proteins are merged together as nodes and the height of the subtree is short due to the similarity at the level of distance measure. Some subtrees are taller indicating that some hypothetical proteins are not so close to each other. The colour mosaic display indicates high expression of many of these genes in EH, and to a lesser extent, EU samples and a significant reduced expression in LU samples (see Table 4.4).

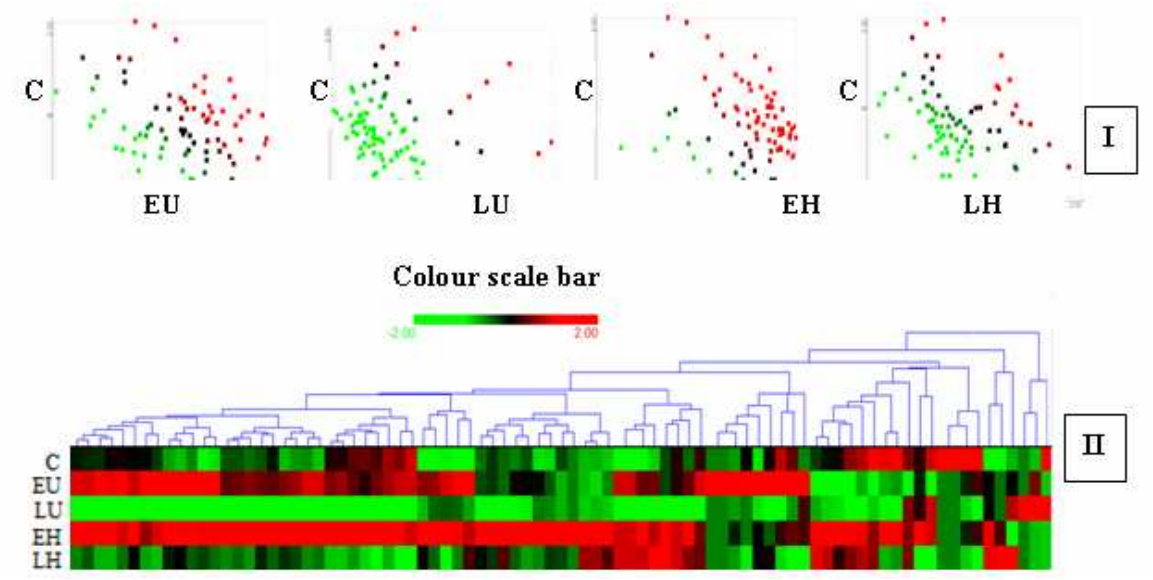


Figure 4.6 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of hypothetical proteins. The fold change scale bar is shown above the cluster with a ratio from -2 to 2.

4.3.5.5 Proteins involved in metabolism

Genes coding for proteins predicted to be involved in metabolism were also differentially expressed following *C. gloeosporioides* infection. The following clusters were differentially expressed in all the infected samples (Figure 4.7): putative RNA helicase, integrase and retrotransposon; cellulose synthase 1 operon protein C precursor and maturase K; fructose-bisphosphate aldolase and proline rich protein; helicase domain-containing protein and ubiquitin.

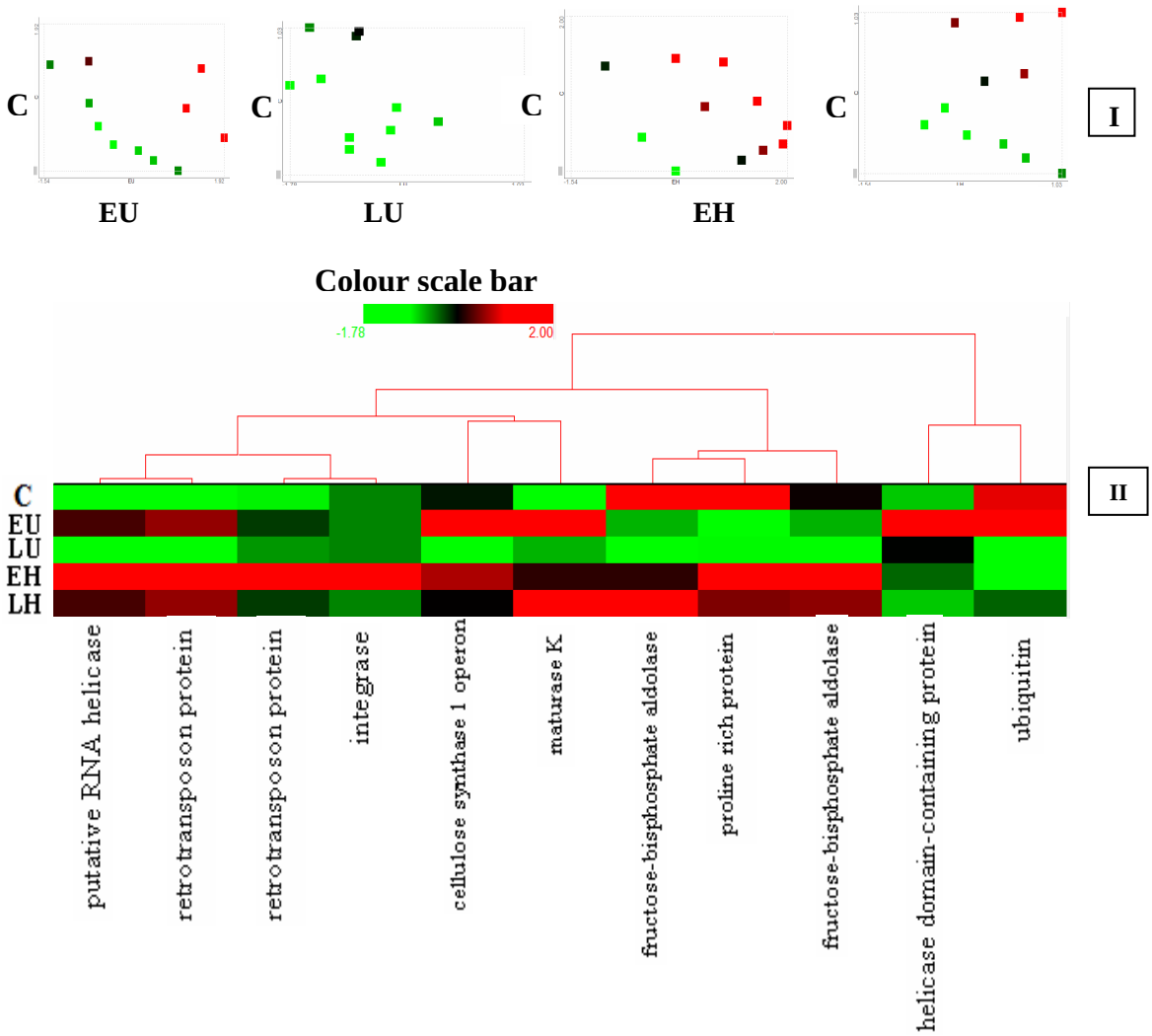


Figure 4.7 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of genes encoding proteins involved in metabolism. The fold change scale bar is shown above the cluster with a ratio from -1.78 to 2.

4.3.5.6 Proteins involved in defence response

Figure 4.8 shows that over time, genes predicted to be involved in defence response to *C. gloeosporioides* attack are differentially expressed. The dendogram shows that some defence genes which are exhibiting similar expression patterns are clustered together such as: endochitinase with endo-1,4-β-glucanase; cell wall associated hydrolase with enolase; catalase with cell wall associated protein. The colour mosaic display and 2 D

scatterplots indicates a significant up regulation of a number of the defence genes in the EH infected samples (see Table 4.4).

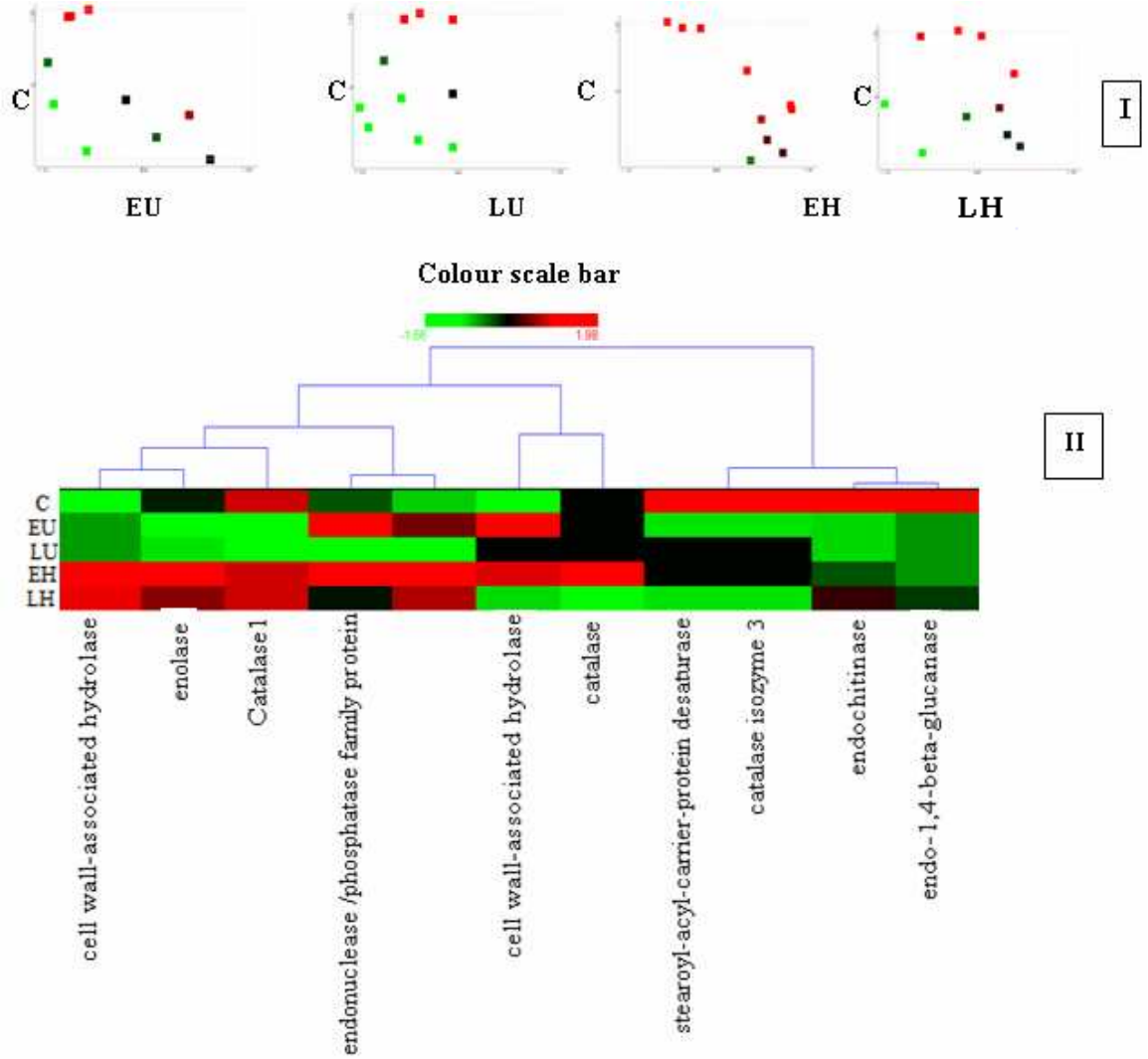


Figure 4.8 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of genes encoding proteins involved in defence response. The fold change scale bar is shown above the cluster with a ratio from -1.66 to 1.98.

4.3.5.7 Genes coding for transcription factors and proteins involved in signal transduction

Data for genes involved in signal transduction and transcriptional activation could be clustered and the binary tree presents the result by using average linkage and Pearson

correlation coefficient. Lower and higher expressions of these genes are observed in the LU and EH infected samples, respectively (Figure 4.9).

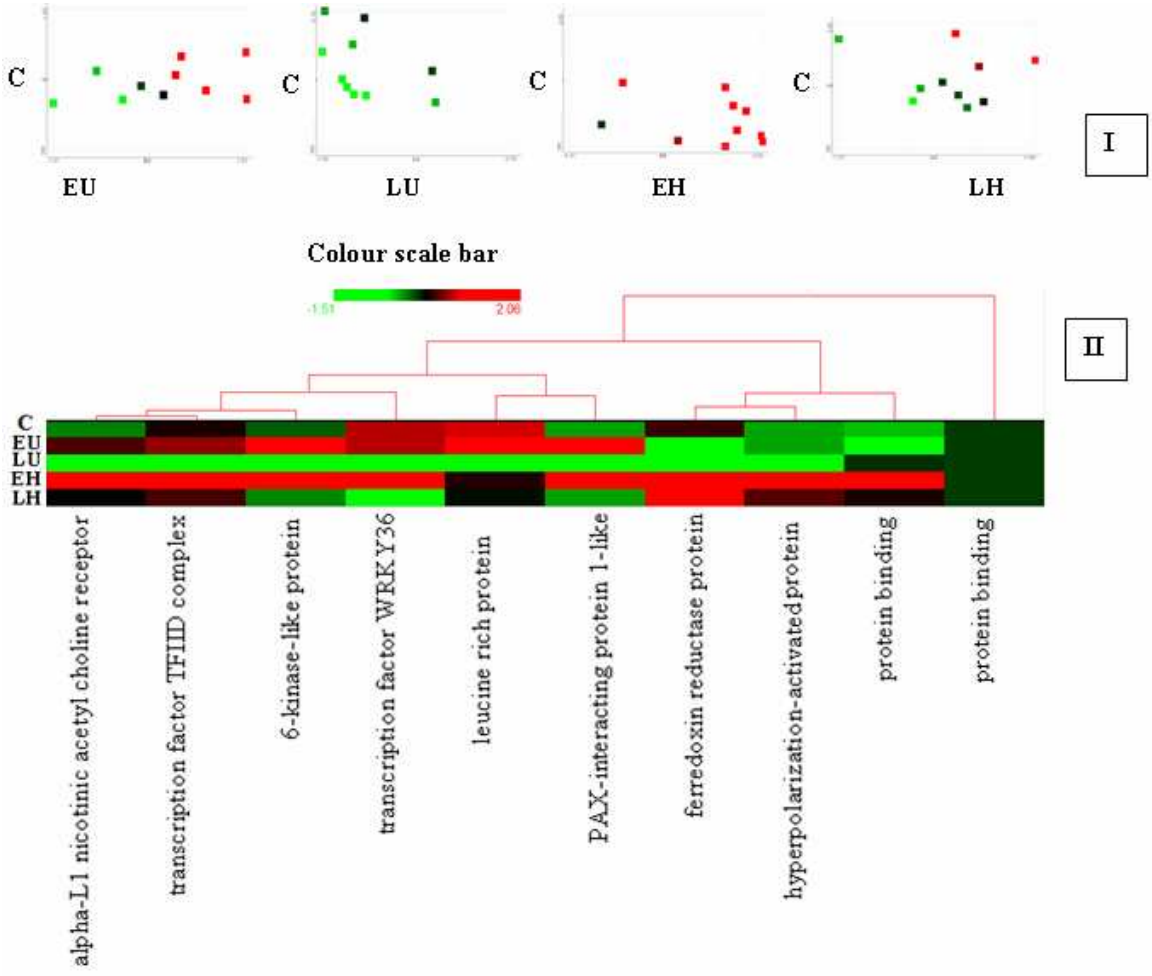


Figure 4.9 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of genes coding for transcription factors and proteins involved in signal transduction. The fold change scale bar is shown above the cluster with a ratio from -1.51 to 2.06.

4.3.5.8 Genes coding for proteins involved in electron transport and stress response

Genes predicted to be involved in electron transport and stress response were differentially expressed in the infected samples following *C. gloeosporioides* infection (Figure 4.10) and formed sequence clusters as seen in the binary tree. An up regulation in their expression

profile is observed in the EU, EH and LH samples and a down regulation in the LU samples.

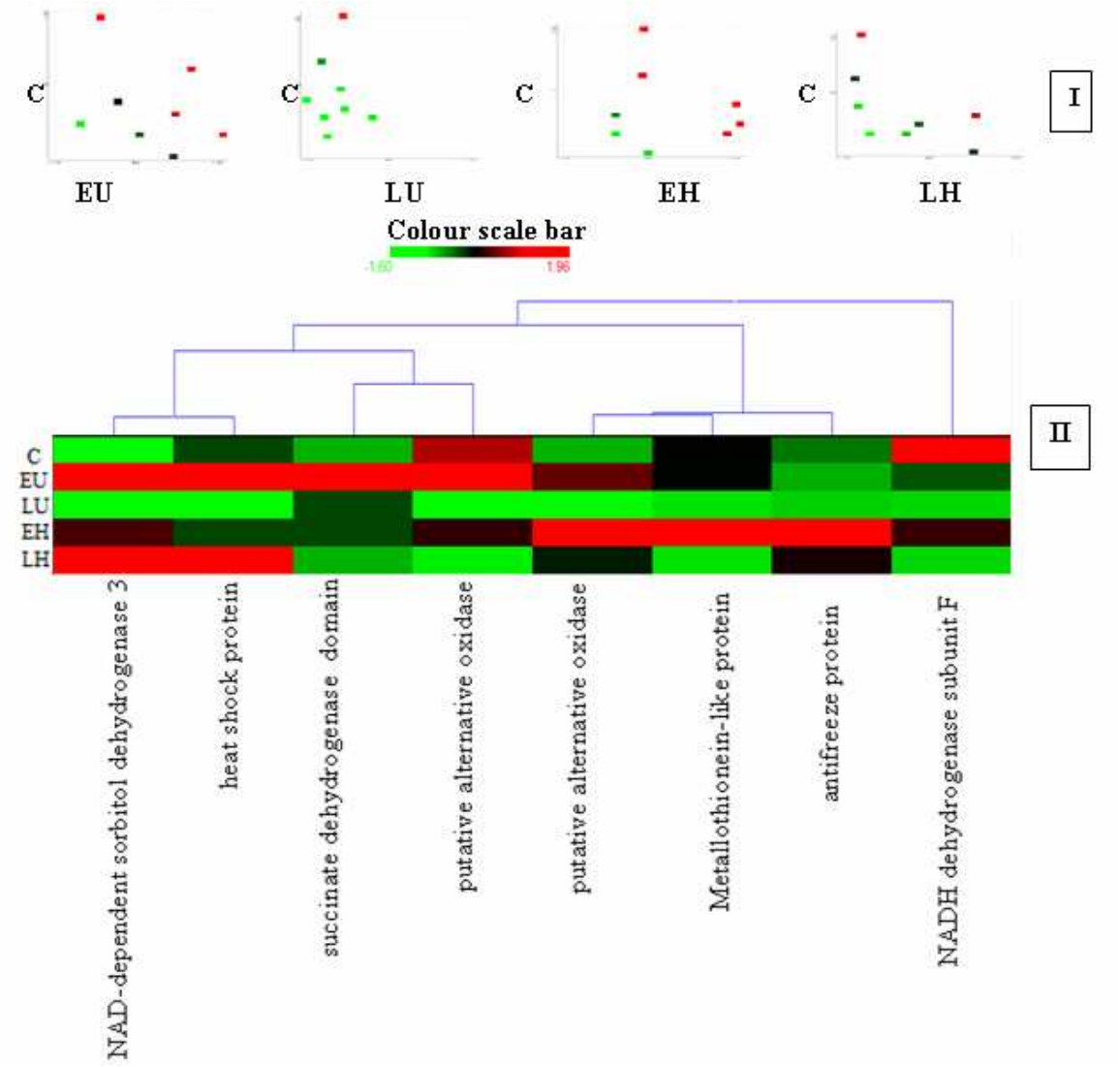


Figure 4.10 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of genes coding for proteins involved in electron transport and stress response. The fold change scale bar is shown above the cluster with a ratio from -1.80 to 1.96.

4.3.5.9 Genes coding for proteins involved in protein synthesis and photosynthesis

Genes of these categories were differentially expressed in the infected samples (Figure 4.11). In the dendogram genes are merged together as nodes and the height of the subtree is short because of the similarity at the level of distance measure. Some subtrees are taller indicating that some genes are not so close to each other. The colour mosaic display indicates a high gene expression in the unharvested and EH infected samples and a slight down regulation in the LH infected samples.

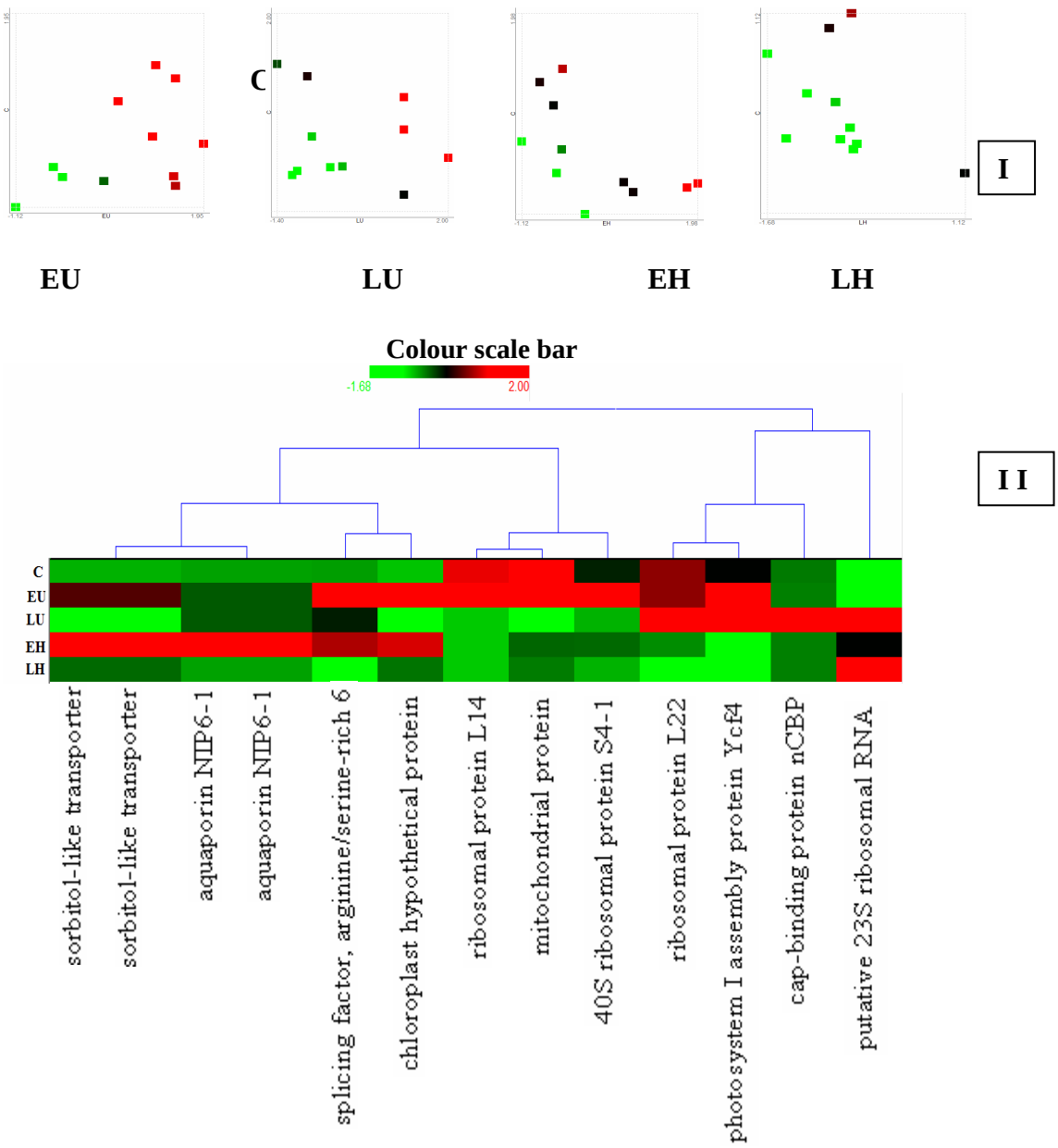


Figure 4.11 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of genes coding for proteins involved in protein synthesis and photosynthesis. The fold change scale bar is shown above the cluster with a ratio from -1.68 to 2.

4.3.6 Genes coding for expressed proteins

Genes coding for protein identified as expressed protein were differentially expressed in the infected samples (Figure 4.12). They were all clustered together as they are exhibiting similar expression patterns as seen in the binary tree, and an up regulation in their expression profile is observed in the EU and EH samples and a down regulation in the LU samples.

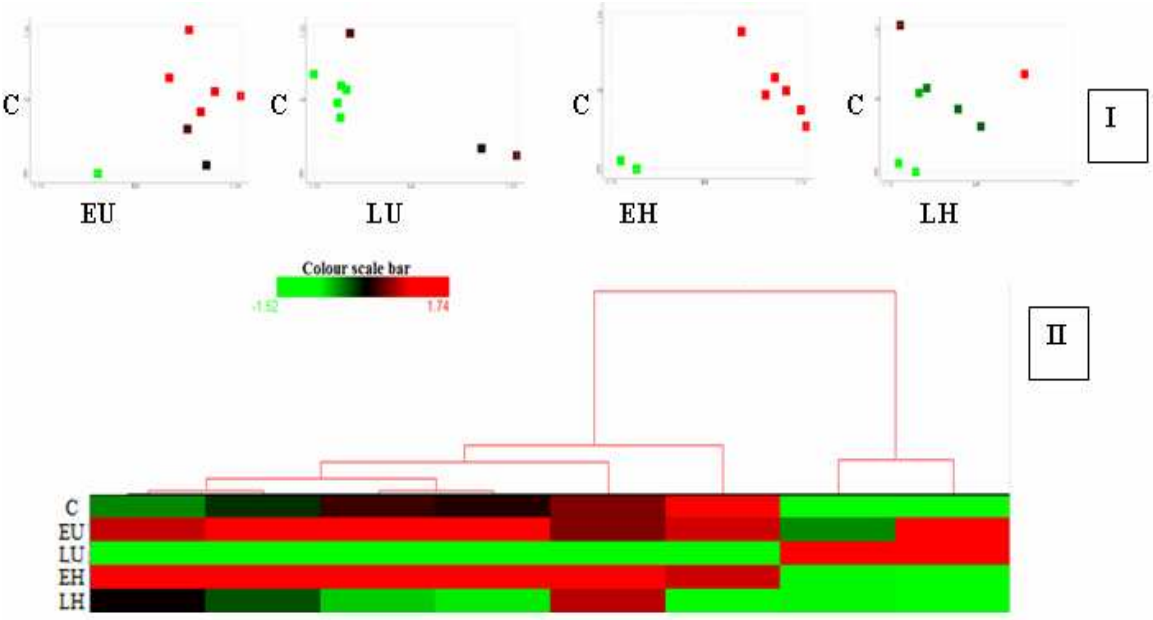


Figure 4.12 Pearson's 2 D scatterplots (I) and Hierarchical cluster (II) of genes coding for expressed proteins. The fold change scale bar is shown above the cluster with a ratio from -1.52 to 1.74.

4.4 DISCUSSION

454 sequencing analysis

Genome sequencing and transcriptome analysis have been dramatically impacted during the last decade by the development and advances in DNA sequencing technologies. Transcriptome analyses at a large scale of many plants have been facilitated using microarrays and serial analysis of gene expression (SAGE) techniques. However, these techniques are effectives for model plants with known genome sequences. Based on the high number of reads generated per run together with the low sequencing error rate in the contigs obtained, 454 sequencing is well adapted to sequence the transcriptome of both model and non-model plants than some conventional methods such as deep EST sequencing using the Sanger method which is time-consuming, labour intensive, expensive and requires cDNA cloning. In addition the elimination of a bacterial cloning step that can bias the composition of the cDNA library is another major advantage of the 454 sequencing of transcriptomes. This approach has been used successfully for analyzing the transcriptome of chestnuts (Barakat *et al*., 2009), maize (Emrich *et al*., 2007) and *Arabidopsis* (Weber *et al*., 2007). We have applied this approach to understand the gene expression changes occurring in avocado fruit in response to *C. gloeosporioides* infection.

In this study, we produced about 215 781 reads from avocado fruit transcriptome in a one-quarter run with the Roche 454 GS FLX Titanium platform. From the observations presented in Chapter Two showing that the infection by *C. gloeosporioides* was effective and the from transcriptome sequencing, it is confirmed that numerous genes and the products of many of these genes are directly or indirectly involved in the interaction between avocado fruit and *C. gloeosporioides.* In addition the BLASTN revealed that many sequences obtained showed similarities at the nucleotide level (Altschul *et al.*, 1990) with

others species belonging to the order of Laurales such as *Cinnamomum camphora, Peumus boldus, Gyrocarpus americanus, Calycanthus occidentalis, Gomortega keule, Cryptocarya meissneriana, Laurus nobilis, Daphnandra micrantha, Hernandia nymphaeifolia*. Complementary DNA sequences generated from the uninfected and infected avocado fruits cover various biological activities and molecular functions indicating that 454 sequencing constitutes a powerful tool for sequencing the transcriptome and gene discovery of non model species such as avocado.

Gene expression profiles

The expression profiles of the expressed genes assessed by quantification during assembly process and later by statistical analysis combined with hierarchical clustering analysis, which are independent methods, revealed the relatedness of gene expression in each sample and showed that many categories of genes were differentially expressed over time. The expression profiles of avocado genes obtained from the 454 cDNA mapped reads (Figure 4.2) show that many groups of avocado genes were up regulated in the early unharvested fruits. Among those up regulated some were also up regulated in the early harvested samples and others in the late harvested samples following the infection. For instance genes coding for senescence associated proteins were up regulated in the early and late harvested infected samples (Figure 4.4). Previously it was demonstrated that senescence associated protein genes encode various proteins such as lipases, proteases, RNases, transcription factors, proteins involved in transportation and antioxidant enzymes (Espinoza *et al*., 2007). Due to the lack of avocado genome, individual avocado senescence associated genes could not be identified. But it can be surmised that some of those genes were expressed in order to trigger avocado defense response mechanism against *C. gloeosporioides* infection. In addition other genes coding for some cytochrome proteins and proteins involved in

signal transduction were also up regulated in the early unharvested and harvested avocado fruits after infection. So, avocado fruits are able to response quickly to *C. gloeosporioides* attack by expressing genes coding for defence related proteins. It can also be observed from the gene expression pattern that some of those genes up regulated were down regulated almost in all the late unharvested fruits and in other part of the late harvested samples (Figure 4.2). This observation could lead to the suggestion that avocado defence related genes are activated immediately following *C. gloeosporioides* attack, but their defensive action does not last for long. This could also be one explanation of why avocado Fuerte varieties are more susceptibles to anthracnose disease. Similar gene expression patterns were obtained in strawberry (*Fragaria ananassa*) exhibiting antrachnose symptoms when infected by *Colletotrichum acutatum* (Casado-Díaz *et al*., 2006).

Thus, avocado fruits react to *Colletotrichum* infection by expressing a large group of defence related genes to fight against the fungal attack. Some of these important groups of genes expressed are discussed below.

Signal transduction genes

Firstly, one of the categories well represented are genes involved in signal transduction such as mitogen-activated protein kinases (MAPK), leucine-rich repeat (LRR) receptor-like protein kinase, salicylic acid-activated MAP kinase, calcium ion binding protein, salicylic acid-binding protein, Pto kinase interactor, receptor-like serine/threonine kinase, signal recognition particle receptor protein and many others. Signal transduction networks allow cells to perceive changes in the extracellular environment and to mount an appropriate response. MAPK are serine/threonine-specific protein kinases that participate in transducing extracellular stimuli to the host genome and would be activated after *C.*

gloeosporioides infection to enable pathogen recognition and to stimulate plant responses. It has been shown that MAPKs in several plant species are activated during plant responses to elicitors or pathogens (Madhani and Fink, 1998; Zhang and Klessig, 2001). Because the defence signalling pathways that lead to the plant-pathogen response are activated after elicitor binding to receptors, it is more likely that LRR receptor-like protein kinase and signal recognition particle receptor protein were expressed in order to mediate the fungus recognition by the plant (Dangl and Jones, 2001). The expression of salicylic acid-binding protein may suggest that salicylic acid, which is involved in the activation of various plant defence responses following pathogen attack, plays an important signalling role following C. *gloeosporioides* infection in avocado (Hammond-Kosack and Parker 2003). Calcium ion binding protein and calcium-dependent protein kinase were also expressed, indicating Ca^{2+} signalling activities in avocado following infection. Previous studies revealed that the fluctuations in cytosolic Ca^{2+} levels that are mediated by Ca^{2+} permeable channels located at the plasma membrane of the plant cell can serve as a regulation of the plant response to pathogen invasion (Bush, 1995; White and Broadley, 2003). In addition many fungi that infect plant tissue do so by penetrating the cell tissue with pectinases. The increasing tissue calcium content increases the concentration of pectins holding cells together, which lead to a greater ability to resist these enzymes (Easterwood**,** 2002). Ca^{2+} can also play a crucial role in the cell wall by determining a strong structural rigidity through cross-links within the pectin polysaccharide matrix (White and Broadley, 2003).

Transcription factors

Transcriptional activation of genes is a crucial part of the plants defence system against pathogens. Several transcription factors such as bZIP proteins, WRKY proteins, zinc finger family protein, ethylene responsive element binding proteins (EREBP), DNA binding

proteins were identified. In many plant species, the transcription factor WRKY and *Myb* genes expressed in avocado, are strongly and quickly up-regulated in response to pathogen attack, wounding or abiotic stresses. Studies demonstrated that in tobacco, multiple WRKY genes are induced after infection with bacteria or tobacco mosaic virus, or treatment with fungal elicitors SA or H_2O_2 (Yoda *et al*., 2002; Takemoto *et al*., 2003). Some studies showed that *Myb* genes are involved in regulation of disease resistance genes (Yang and Klessig, 1996; Vailleau *et al*., 2002). Based on these previous studies it can be predicted that the transcription factors were produced after *C. gloeosporioides* infection in order to activate genes involved in the defence system of avocado fruit.

Genes involved in oxidative burst

Some of the genes differentially expressed following *C. gloeosporioides* infection are genes involved in the oxidative burst such as: glutathione peroxidase, cytochrome C oxidase, catalase isozyme 3, dehydroascorbate reductase and NADH-plastoquinone oxidoreductase. It has been shown that at the early stage of plant's defence response, the oxidative burst occurs which is a rapid and transient production of large amounts of reactive oxygen species (ROS) at the site of infection (Wojtaszek, 1997). Ion fluxes subsequently induce extracellular production of ROS intermediates, such as hydrogen peroxide, superoxide and hydroxyl free radical, catalyzed by a plasma membrane-located NADPH oxidase (Somssich and Hahlbrock, 1998). Extrapolating from the finding of Tenhaken et al. (1995), the ROS produced in the oxidative burst after *C. gloeosporioides* infection could not only protect avocado against the fungus invasion, but could also serve to trigger the overall defensive response system. It is well known that the earliest reactions of plant cells include changes in plasma membrane permeability leading to calcium and proton influx

and potassium and chloride efflux (McDowell and Dangl, 2000). Previous work with soybean infected by *Pseudomonas syringae* pv. *glycinea* demonstrated that accumulation of H_2O_2 from an oxidative burst stimulates a rapid influx of Ca^{2+} into soybean cells, which activates a physiological cell death program. These findings establish a signal function for Ca^{2+} downstream of the oxidative burst in the stimulation of a physiological cell death program in soybean (Levine, 1996).

Genes involved in stress responses

Chaperone protein Dnak (heat shock protein 70) and others known to be involved in stress response during plant interaction with a pathogen were expressed in all the infected samples. During stress, avocado fruit would adapt its metabolism and activate a large variety of physiological and biochemical changes in order to repair damages or to protect their cells from the effects of stress caused by *C. gloeosporioides*. For instance, heat shock proteins 70/ Chaperone proteins (Dnak) families are known to be in charge of protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membranes, and can also serve to assist in protein refolding under stress conditions (Wang *et al*., 2004). Previous studies showed that under biotic and abiotic stress, the transcriptome and proteome sometimes change rapidly and dramatically (Watson *et al*., 2003; Rampitsek and Srinivasan 2006). Based on these observations it can be hypothesized that chaperone proteins (Dnak) families were expressed in avocado to stabilize the proteins and membranes, and to assist in protein refolding under stress condition initiated by *C. gloeosporioides.*

Defence response/resistance genes

Another category of genes differentially expressed in response to *C. gloeosporioides* infection are genes involved in defence response such as β-glucanases, endochitinases and endopeptidase known to have antifungal activity (van Loon and van Strien 1999) and plant aspartic proteinase which also exhibits antimicrobial activity. Other genes expressed like pathogenesis-related protein 6, cinnamoyl-CoA reductase, cytochrome P450 monoxygenases and others are known to be involved in various processes of plant defence against pathogens, such as cell death related to hypersensitivity response, construction of a physical barrier to block the pathogen progression, as well as systemic resistance. We found elongation factor-1 which is involved in controlling the extent of the cell death in the defence response and acetyl co-enzyme A carboxyltransferase involved in the regulation of resistance gene expression (Barakat *et al.*, 2009). Genes which encode proteins involved in lignin biosynthesis, such as cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis (Kawasaki *et al.*, 2006) and cytochrome P450 monoxygenases (Boerjan *et al*., 2003; Barakat *et al*., 2009) were also expressed. It is well established that lignification is a mechanism for disease resistance in plants, which leads to an ultra-structurally modified reinforced cell wall (Walter, 1992; Bhuiyan *et al.,* 2009). This structural modification enables the cell wall to become more resistant to mechanical pressure applied by the fungal appressoria during penetration as well as more water resistant and thus less accessible to cell wall-degrading enzymes (Zeyen *et al*., 2002). The biosynthesis of lignin can be stimulated by various biotic and abiotic stress conditions, such as metabolic stress, pathogen infection, wounding and perturbations in cell wall structure (Tronchet *et al*., 2010). Previous studies in various plants showed an over-expression of genes involved in lignin synthesis during pathogen infection (Sibout *et al*., 2005; Wang *et al*., 2006). Based

on these findings it is likely that lignin biosynthesis and cytochrome P450 were induced in avocado fruit during defence responses to halt *C. gloeosporioides* invasion.

Pathogenesis-related proteins 5 (PR5) are known to possess antifungal activity against a broad spectrum of fungal pathogens and their synthesis is induced by the presence of fungi in the plant (Hu and Reddy, 1997). It was demonstrated that disease resistance to *Botrytis cinerea* was increased in transgenic strawberry plants expressing PR 5 (Schestibratov and Dolgov, 2005). Alpha and beta glucosidase-like protein were also expressed as the result of this fungus infection. According to previous study in plant cells some antibiotic phenolics are stored as inactive bound forms and are converted into biological active antibiotics by plant hydrolyzing enzymes (glycosidases) in response to pathogen invasion (Lattanzio *et al*., 2006). This suggests that glucosidases were expressed in avocado fruit for the activation of some phenolic compounds involved in defence response system.

Genes involved in photosynthesis and plant metabolism

Other identified genes involved in plant metabolism and photosynthesis were also expressed after *C. gloeosporioides* infection. When plants are under stress caused by fungal pathogens, their metabolism of carbohydrates, lipids, proteins and nucleic acid are also affected (Agrios 2005; Baldo *et al*., 2010). Activation of signal transduction networks after pathogen recognition results in reprogramming of cellular metabolism, which leads to a large change in gene activity. For instance the mRNA and protein levels of ribulose-1,5-bisphosphate carboxylase oxygenase, in potato, are drastically reduced by pathogen infection or elicitor treatment (Somssich and Hahlbrock, 1998).The induction of some photosynthetic genes such as: ribulose-1,5-bisphosphate carboxylase/oxygenase, photosystem I assembly protein, photosystem II protein and chloroplast NADH

dehydrogenase, during the interaction between avocado and *C. gloeosporioides* infection may implicate light-sensing mechanisms in the induction of plant disease defence signalling. Previous studies showed that there is a link between plant defence system against pathogen and both light-sensing network and the oxygen-evolving complex in Photosystem II (Abbink *et al*., 2002; Genoud *et al*., 2002). In addition Phytosystem II plays an important role in preventing the accumulation of ROS (Asada, 1999). Another functional category observed in the avocado transcriptomes are genes encoding proteins predicted to play a crucial role in energy production during response to the pathogen infection. For instance, ATP synthase expressed in avocado fruit is an important enzyme that creates energy for the cell to use through the synthesis of adenosine triphosphate (Thilmony *et al*., 2006; Truman *et al*., 2006).

In addition to the role played in energy production by some identified genes encoding proteins predicted to function in photosynthesis, it has been suggested that some of these may have defence functions. Previous study showed that in *Arabidopsis* the oxygen evolving enhancer protein 2 is phosphorylated by a protein complex containing glycine-rich protein 3/wall-associated kinase 1, a pathogenesis related protein required for survival of plants during the pathogen response (Yang *et al*., 2003). Phosphorylated oxygen evolving enhancer protein 2 is thought to modulate formation of $H_2O_2/O_2^{\bullet -}$ which could be involved in some defence processed such as: defence signalling, the induction of defence-related genes, and the regulation of the hypersensitive response (Yang *et al*., 2003).

Flavonol synthase\flavanone 3-hydroxylase an enzyme of the phenylpropanoid metabolic pathway that participates in flavonoid biosynthesis (Turnbull *et al*., 2004) was also expressed in avocado fruit after *C. gloeosporioides* attack. Due to its responsibility for the synthesis of a large range of natural products in plants such as lignans, lignin, flavonoids

and anthocyanins, the phenylpropanoid pathway is considered to be one of the most important metabolic pathways (Verpoorte, 2000).

Genes involved transportation and protein synthesis

Another group of genes identified, includes genes encoding proteins predicted to function in transport processes and protein synthesis. In response to pathogen invasion such as fungi, plants assemble a large array of proteins for their defence. Some of these proteins are: proteins that serve in the recognition of specific pathogen, those involved in signal transduction pathways, defence related proteins, cell wall related proteins/structural protein, protein involved in energy biosynthesis and others proteins implicated directly or indirectly in the defence mechanisms in response to pathogen attack (Agrios, 2005).

Unexpressed genes

Finally, there were gene sequences which were not expressed in infected fruit (Table 3), among which is the gene for limonene synthase, an enzyme which catalyses the stereo-specific cyclization of geranyl diphosphate, the universal C_{10} precursor of the monoterpenes, to form a monocyclic monoterpene, limonene (Ohara *et al*., 2003). Monoterpenes are important constituents of plant essential oils and limonene would likely be an essential contributor to the flavour and fragrance of avocado and its value to the cosmetic industry (Ohara *et al*., 2003). Non expression of this gene by *C. gloeosporioides* infection illustrates an aspect of the economic consequences of the disease to the avocado industry. Malic enzymes are involved in a number of important metabolic processes requiring the conversion of malate to NAD(P)H, pyruvate, and CO_2. In plants, these

enzymes contribute to C_4 photosynthesis, pH balancing mechanisms and fruit ripening processes (Drincovich *et al*., 2001) and repression of the gene indicates the negative effects of anthracnose on essential components of avocado fruit metabolism. It would appear from the Genbank that sequences controlling induction of jasmonic acid (JA) are also not expressed in infected avocado fruits although it has been demonstrated that it mediates resistance to insect pathogen attack and abiotic stress responses to wounding and ozone (Kunkel and Brooks, 2002; Voelckel and Baldwin, 2004). JA also functions in signal transduction between pathogenicity and resistance genes in many plants (Agrios 2005) but it does not appear to be involved in the signal transduction during avocado response to *C. gloeosporioides* attack. However, its non-induction may also be linked to the non-expression of the gene coding for limonene synthase as the production of this enzyme can be stimulated by methyl jasmonate (Ohara *et al*., 2003).Glycosyl transferases also identified are enzymes known to be actively involved in the biosynthesis of carbohydrate moieties of glycoproteins and glycolipids, which serve in various cellular functions (Varki, 1993) and repression of this gene indicates the negative effects of anthracnose on some cellular functions of avocado fruit.

Similar gene expression profiles were obtained in strawberry during response to *C. acutatum* infection (Casado-Díaz *et al*., 2006). In this study, strawberry normalized subtracted libraries of genes up and down regulated were generated after *C. acutatum* infection using SSH method. About 3191 strawberry genes predicted to be involved in various biological processes were identified. Among those genes, 4.5% were identified to be involved in signal transduction (Calcium-dependent protein kinase, LRR protein, MAPK, Serine threonine protein kinase, WD-repeat protein); in transcriptional activation 3.2% (bZIP transcription factor, DNA binding protein EREBP-4, Myb-like transcription

factor 6, WRKY-like transcription factor); in defence response 11.9% (Chitinase 1, Disease-resistant-related protein, Endo-1,4-β-D-glucanase, Pathogenesis-related protein 5-1, Plant peroxidase, Thaumatin-like protein, γ-Thionin); in metabolism 2.1% (cytochrome P450, Glutamine synthetase, Glutathione S-transferase, putative acyl-CoA oxidase, UDP-glucose pyrophosphorylase) and many other genes (38.3 %) with unknown function were also identified (Casado-Díaz *et al*., 2006). These results showed that many strawberry genes expressed in defence response to *C. acutum* attack are similar to those expressed in avocado in response to *C. gloeosporioides* attack.

A similar genomic approach used in this study for avocado gene expression exploration was used to compare the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) in response to a fungus *Cryphonectria parasitica* infection (Barakat *et al*., 2009). Barakat and colleagues (2009) used 454 pyrosequencing to sequence the transcriptome from fungal infected and healthy stem tissues collected from blight-sensitive American chestnut and blight-resistant Chinese chestnut. They produced about a million 454 reads from which 28 890 unigenes were generated from American chestnut and 40 039 unigenes from Chinese chestnut. The size of their data bases was high compared to what we obtained. This finding resulted in the identification of a large number of chestnut genes involved in signal transduction (MAPK), in transcriptional activation (F-box proteins, Myb transcription factor, WRKY), in defence response (Cytochrome P450, Glycosyl hydrolase, Pathogen-responsive α –dioxygenase, Peroxidase) in lignin biosynthesis (4-Coumarate-CoA ligase, Cytochrome P450, Succinyl-CoA ligase, S-adenosylmethionine synthase 3) in the regulation of resistance gene expression (Acetyl-coA carboxyltransferase) and other genes with diverse molecular functions (Barakat *et al*.,

2009). Based on the results obtained in our study and previous studies, 454 sequencing is well adapted for transcriptome studies of non model plant like avocado.

In conclusion, the research outlined here revealed that avocado fruit is able to respond to *C. gloeosporioides* infection by exhibiting a sophisticated molecular system for pathogen recognition and by activating structural and biochemical defence mechanisms. The overall goal was to sequence the whole uninfected and infected avocado transcriptome, then to identify several candidate genes which are differentially expressed as a result of infection. These findings yielded a first insight into some of the genes expressed in this plant-pathogen interaction at the molecular level and could contribute to the design of effective disease management strategies to improve the resistance of avocado varieties to anthracnose disease. For instance, one could develop resistant alleles which could be used in plant breeding to produce more anthracnose resistant Fuerte cultivars. Further analysis of specific defence related genes and qPCR quantification to validate the expression profile of some genes will be presented in the following chapter.

CHAPTER 5

RESISTANCE RESPONSES OF AVOCADO FRUIT TO *COLLETOTRICHUM GLOEOSPORIODES* INFECTION

5.1 INTRODUCTION

Anthracnose of avocado (*Persea americana* Mill.) caused by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. is the most serious devastating disease widely distributed in all avocado growing areas of the world (Bailey *et al.*, 1992; Sreenivasaprasad and Talhinhas, 2005). In South Africa, the incidence of anthracnose is 37% of all cultivars (Sangeetha and Rawal, 2008). In addition, in South Africa, the fungus has been reported on 21 others hosts such as almond, coffee, various citrus varieties, grapevine, mango, papaya, sisal, walnut and several ornamental plants (Swart**,** 1999). Due to their perishable nature, avocado and mango are probably the most susceptible among these plants (Prusky, 1994). Besides anthracnose, *C. gloeosporioides* can also cause dieback, root rot, leaf spot, blossom rot and seedling blight on avocado (Freeman *et al*., 1998). Anthracnose is one of the most widespread forms of decay and the most important disease of avocado (Prusky, 1994). As observed in Chapter 2, *C. gloeosporioides* infects unripe fruits but remains in the quiescent stage and cause no damage until ripening, when extensive decay occurs.

Several studies demonstrated that natural resources of resistance are present in avocado fruit (Prusky and Keen, 1989; Prusky *et al*., 1990; Domergue *et al.*, 2000); as in other plant species, avocado has the ability to respond to pathogen attack and possesses a preformed and/or inducible defence strategy. Unripe avocado fruits are resistant to *C. gloeosporioides* due to the presence of high concentrations of preformed antifungal chemicals such as: 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene (Prusky *et al*., 1982); 1,2,4-trihydroxyheptadec-16-yne; 1,2,4-trihydroxyheptadec-16-ene and 1-acetoxy-2, 4-dihydroxyheptadec-16-ene (Prusky *et al*., 1991b; Adikaram *et al*., 1993). The main action of all these antifungal compounds is to maintain the fungus in a quiescent stage by

inhibiting *C. gloeosporioides* germination and germ tube elongation (Domergue *et al*., 2000; Guyot *et al.* 2005). In order to confirm the antifungal properties of these compounds, Marimani (2011) showed that inoculation of harvested and unharvested Fuerte avocado fruits with *C. gloeosporioides* at approximately 240 days after fruit set causes an increase in the antifungal diene and triene levels, particularly at 2 day post infection. The infection process of *C. gloeosporioides* was more efficient and striking in harvested fruits which contain low antifungal diene and triene contents, than in unharvested fruits which are more resistant to the fungus due to their higher concentration of these compounds, which were shown to inhibit *C. gloeosporioides* germination and germ tube elongation (Marimani, 2011). However the defence systems of avocado against the parasitic attack of *C. gloeosporioides* at the molecular level are not yet elucidated and are poorly understood.

Knowledge of plant susceptibility or resistance to a pathogen attack is based on the interactions between plant disease resistance genes and their corresponding pathogen avirulence genes. In general, when pathogen invasion occurs in plant, a number of metabolic changes are induced within the plant as a defence response. Initially, to confine the spread of the pathogen, a localized resistance reaction, known as the hypersensitive response, is activated in the case of race specific resistance but not always (Kwang-Hyung *et al*., 2004). Meanwhile around the infection site various genes conferring resistance to diseases are induced, which leads to the production of antimicrobial compounds, the phytoalexins, phenolic compounds involved in defence response and various antimicrobial proteins, including pathogenesis-related proteins (PR). Finally systemic acquired resistance can be expressed in the plant as the result of the systemic expression of PR protein genes activation (Puupponen-Pimia, 2001; Agrios, 2005; Plymale *et al.*, 2007), with the

involvement of different molecular and hormonal transduction pathways (Gachomo *et al*., 2003; Hammond-Kosack and Parker, 2003).

Over the years, studies on plant defence-signalling pathways have demonstrated that, depending on the type of parasitic attack, plants are capable of differentially activating distinct defence pathways. Pathogens have the ability to induce expression of defence genes through different signalling pathways that requires plant-signalling molecules such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). Defence signalling is complex and involves an interplay between protein kinase, phospholipids, and defence signalling molecules such as nitric oxide (NO) , reactive oxygen species , SA , JA and ET (Thomma *et al*., 2001; Hammond-Kosack and Parker, 2003). Hydrogen peroxide (H_2O_2) and NO also play a role in cross-talk and convergence points between pathways (Kumar and Klessig, 2000). SA, JA and ET are part of the two major defence signalling pathways known as an SA dependent pathway and an SA-independent pathway that involves JA and ET (Kunkel and Brooks, 2004). Genetic methods are useful to identify signalling molecules and determine their role within the signal transduction networks that control defence mechanisms.

Resistance genes based on their predicted protein structural characteristics can be classified into six classes (Chapter 1) whose products seem to activate a similar range of defence mechanisms. In the plant defence strategy, genes play various biological roles. Some of those genes present in all plants are involved in the passive defence system of the plant by preventing pathogen penetration e.g by forming a thick waxy cuticular layer that protects against penetration. Others genes intervene in non-specific plant defence by producing callose, chitinases, enzymes for oxidative stress protection, glucanases, lignin, phytoalexins,

some antimicrobial secondary metabolites and some phenolic compounds (Glazebrook *et al*., 1997). Other genes are required for race-specific resistance such as major resistance genes that lead to expression of the hypersensitive response and the arrest of pathogen growth (Jørgensen, 1994). Research to identify pathogen-related genes in plants can be used to improve disease resistance in agronomical valuable plants.

Over recent years, many functional R genes conferring resistance to various plant species, against a large spectrum of pathogens such as bacteria, viruses, fungi, nematodes and even insect pathogens with very different lifestyles, have been isolated from different plant species (Crampton, 2006; Van den Berg, 2006; Mafofo, 2008; Baldo *et al*., 2010). Multiple identification and isolation of plant resistance genes have become possible and more accurate due to the development of genomic tools. Identification of R genes can lead to a breakthrough in understanding the molecular mechanisms underlying diseases or other biological progressions. For instance, when comparing the gene expression profiles of a healthy plant to that of an infected plant, individual genes or clusters of genes that play an important role in a particular signalling cascade or in disease aetiology can be identified (Wan *et al,* 2002). In addition, next generation sequencing methods, such as Roche 454, Illumina Solexa and ABI SOLiD technologies can lead to greater unbiased and complete analysis of quantitative and qualitative genetic changes associated with a particular phenotype or in response to pathogen infection (Rounsley *et al*., 2009; Wheeler *et al*., 2008). To date, a comparison of capillary sequencing and next generation sequencing methods (Morozova and Marra, 2008) revealed that Roche 454 sequencing is most widely used for analyzing the transcriptome of non-model organisms than conventional methods such as microarrays, serial analysis of gene expression, or EST analysis generated using capillary sequencing (Barakat *et al*., 2009; Sun *et al*., 2010).

Multiple gene expression profiles in plants are successfully studied today by a sensitive and reliable technique. Real-time polymerase chain reaction analysis is better than other methods available to quantify gene transcripts from plant cells in terms of accuracy, sensitivity, and fast results. It has been used in gene expression profile research as a subsequent tool to evaluate the expression of a number of genes involved in plant defence system. For instance, it has been used when studying the resistance of pearl millet to the biotrophic rust pathogen *Puccinia substriata var indica* to validate the results of the gene expression profiles obtained using SSH and microarray (Crampton, 2006); and also when studying the resistance of banana to *Fusarium oxysporum* (Van den Berg, 2006). In addition, it has been also used in tobacco responding to black shank to confirm the value of some resistance genes generated using SSH (Chacón *et al*., 2009). In this study, we use the sequencing results obtained in Chapter 4 but focus only on the genes predicted to be involved in the plant defence system, to elucidate the resistance mechanisms expressed in avocado fruit under conditions of anthracnose infection. To validate the differential gene expression pattern described in Chapter 4, a subsequent study was done using quantitative real-time PCR to measure the expression of some target genes at a particular time point.

5.2 MATERIALS AND METHODS

5.2.1 Previous preparation prior to sequencing

The preparation of the fungal inoculum, plant materials, inoculation, total RNA and synthesis of double stranded cDNA from total RNA were performed as outlined in Chapter 3 (Materials and methods).

5.2.2 Preparation of cDNA samples for *de novo* transcriptome sequencing with 454 GS-FLX titanium platform and analysis.

Preparation of cDNA samples for *de novo* transcriptome sequencing with 454 GS-FLX Titanium platform and analysis were performed as outlined in Chapter 4 (Materials and methods).

5.2.3 Real Time Reverse Transcriptase -PCR

The expression of catalase and endochitinase was investigated further using the relative quantification real-time PCR method, as described below.

5.2.3.1 Primer design

Eight primers were designed from sequences of cDNA obtained after 454 sequencing using the Integrated DNA Technologies's PrimerQuest Tool (http://eu.idtdna.com/Scitools/Applications/Primerquest/Default.aspx) which incorporates Primer 3 software. The selected genes included those coding for the endogenous control genes (actin and glyceraldehyde 3-phosphate dehydrogenase), catalase, endochitinase, endo-1,4-D-glucanase, CC-NBS resistance protein, pathogenesis related protein 5 and 6.

The primer for the endogenous control gene glyceraldehyde 3-phosphate dehydrogenase was designed from sequences obtained from NCBI. The parameters chosen for each primer were as follows: short amplicons of less than 200 bp, and a Tm of 58- 60.1°C (Table 5.1). The specificity of the primers was first validated by BLASTN. The primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa). The primers pairs were evaluated for efficiency by performing a conventional PCR experiment using cDNA as template with 10 µM of specific primer. Total RNA (2 µg) was reversed transcribed using a RevertAid™ Premium First Strand cDNA Synthesis Kit (Fermentas Life Sciences, South Africa), following the manufacturer's instructions The PCR reactions were carried out as follows: one cycle of 94 °C for 3 min (initial denaturation), 35 cycles of 94 °C for 30 s (denaturation), Tm of each primer for 1 min (annealing) and 72 °C for 2 min (elongation). Final elongation was achieved at 72 °C for 10 min.

Table 5.1 Base composition of primers designed for each different selected gene for qRT-PCR

Putative identity	Primer Product size	Primer Length:	Direction	Primer Sequence (5'-3')	Primer TM (°C)
Actin	166	24	Forward Primer Reverse Primer	AGCTCGCTTATGTGGCTCTTGACT TCTCATGGATTCCAGCAGCTTCCA	60.0
Catalase	162	24	Forward Primer Reverse Primer	TTTGAAGGCATGGATCACATCGGG CTCGAGGCTTCGCCATTAAGTTCT	59.4 58.7
CC-NBS resistance protein	185	24	Forward Primer Reverse Primer	ATCATCCTTGTCTCCATGCGGTCT AAAGCTTTCCAGCTCATTCACGGC	59.8 60.0
Endochitinase	186	24	Forward Primer Reverse Primer	ATCACGTTGTGGCATGACGGTTTG AATACTACGGGCGTGGACCATTCA	60.1 59.9
Endo-1,4-D-glucanase	121	24	Forward Primer Reverse Primer	ACTCTTCCGGAGGACATGCTTTCA TGTATGACATCTTGGCCGGGTTCT	59.9 60.0
GAPDH	186	24	Forward Primer Reverse Primer	AGTGGAGGGTTTGATGACCACAGT ATTTAACGCAGGCAGCACTTTCCC	59.9 60.1
PR-5	171	24	Forward Primer Reverse Primer	TGCAACAGTACTCGTCGGTCTTGA TATCGCTGGTGGACGGGTTTAACA	59.7 60.0
PR- 6	158	24	Forward Primer Reverse Primer	TGCGCGGACTTACAATCAGA AGAGTCCAAAGTGTCGTTCAGCCT	56.3 59.9

5.2.3.2 Quantitative Expression Assays

For the quantification expression assays, catalase and endochitinase were selected along with an actin gene and GADPH as endogenous control or references genes for standardization. A real-time quantification PCR control experiment was performed to examine the linearity of amplification over its dynamic range. A serial dilution (undiluted, 1:10, 1:100, 1:1000, 1:10000) on 5 µl of cDNA and each of the primers sets (10 µM of each primer) for the different genes was used for amplification and the results used to calculate the standard regression curves. Each dilution point on the standard curve was done in duplicate. The standard curve was calculated for each of the selected genes with the following formula: y=mx+b, where b= y-intercept of standard curve line (crossing point) and m=slope of the standard curve line (function of PCR efficiency).

The expression of the selected defence related genes were assessed in two independent biological replicates (cDNA from different fruits) and each biological replicate has its own replicate (technical replicate). The expression of the selected defence related genes were assessed using the Roche Light cycler 1.5 technology. Expression profiles were presented as a ratio for each gene fragment at 0, 1 and 3 d post infection in comparison with the expression of the gene fragments in the calibrator or uninfected control.

Real-time PCR reactions were set up by combining 10 µl of Maxima™ SYBR Green/Fluorescein qPCR Master Mix, 1 µl each of the forward and reverse primers (10 µM), 1 µl of cDNA and nuclease-free water to give a total master mix volume of 20 µl per reaction. Reactions were added to glass capillaries tubes and placed into the LightCycler rotor (Roche Diagnostics). No template control (NTC) reactions contained water as template was used as negative control and 1 µl of cDNA (1:10 dilution) was used as a positive control. The cycling conditions were as follows: initial denaturation for 10 min at 95˚C (hot start) followed by amplification and quantification cycle repeated 40 times each consisting of 15 s denaturing at 95˚C, 30 s annealing at primer specific temperatures (Table 5.1), 30 s primer extension at 72˚C with a single fluorescence measurement. Then a melting curve cycle was obtained by heating to 65˚C for 15 s with a heating rate of 0.1˚C per second and continuous fluorescence measurement, and finally a cooling step at 40˚C for 30 s. LightCycler software (Roche) was employed to calculate crossing points (Ct) for each transcript,Ct being the point at which the fluorescence rises appreciably above the background fluorescence (Pfaffl, 2001).

5.2.3.3 Data analysis

The relative standard curve method was used to quantify the selected genes. Because quantitation should be normalized to an endogenous control, standard curves were prepared

for both the target (catalase and endochitinase) and the endogenous reference (actin and GADPH). For all experimental samples, the amount of target and reference in the samples of interest was calculated using their Ct values and the corresponding standard curve. Then the normalized expression value for each gene was calculated by dividing the average amount of target gene by the average amount of target reference gene. Finally, the relative expression level of the target gene in the samples of interest was determined by dividing the normalized target amounts by the value of the calibrator or (Control). The calibrator then became the 1X sample, and all other quantities were expressed as an n fold difference relative to the calibrator (Applied Biosystems, 2004). The average input of each treatment of the target gene and the reference control and the standard deviation were calculated prior to calculating the normalised values using the statistical analysis software GraphPad inStat 3. Real-time PCR data were statistically compared between treatments at each time point using one-way ANOVA (Kuznetsova *et al*., 2010).

5.3 RESULTS

A cDNA samples prepared from total RNA were sequenced using the 454 GS-FX platform. This single sequencing run produced 215 781 reads from the avocado fruit transcriptome, with an average read length of 252-300 nucleotides (range = 41–562) as presented in Chapter 4.

5.3.1 Defences related genes differentially expressed in unharvested and harvested avocado fruit following *C. gloeosporioides* infection.

After 454 sequencing and assembly of the reads obtained, the cDNA sequences were submitted to BLASTX in order to identify defence putative genes differentially expressed as a results of the infection of *C. gloeosporioides* in unharvested and harvested avocado fruits. We then determined which defence related genes were in common in the transcriptomes generated from uninfected fruits and infected fruits from each time point (EU, EH, LU and LH). Then during the sequence analysis, the reads were also quantified in order to determine the number of copies expressed per time-point. Based on the similarity displayed after comparing the sequences obtained to the non-redundant protein databases BLASTX program, we selected four groups of genes related to defence response in avocado (Table 5.2). The selected groups are genes involved in transcription factors, signal transduction, defence and stress response. All the genes selected after comparing the sequences to the protein databases BLASTX had a very high percentage of coverage, a good percentage of identity and belong to the plant kingdom. But due to the fact that there is little genomic information of avocado in Genbank (genome not yet sequenced) the E-value could not be relied on to make a selection. These reads were expressed in all the

infected samples and some of these reads were also expressed in uninfected samples. Table 5.2 contains the summary of some genes obtained from these reads. We used (0) to indicate that the genes expressed were induced at the particular time point; (1) the gene was expressed at the same level in both uninfected and infected sample at that time point and >1 to indicate that the gene was expressed in both uninfected and infected but the number of copies was up regulated in the infected sample.

The sequences of these selected genes are presented in the appendix. But the sequences results of other genes were not presented due to the high number of sequences obtained after 454 sequencing (70.6 megabases of sequence data generated).

Table 5.2 The unmapped reads from EU, EH, LU and LH samples mapping to the unmapped reads from the control and their putative function.0 means not mapping with the uninfected; 1 means mapped one time in both uninfected and infected, >1 means mapped more than on time (up regulated in the infected samples).

Name	ccession num of simi sequence	Similar sequence from database (Putative function)	E-value	Max Identity %	Number of copies expressed			
					Early Unharvest	Late Unharvest	Early harves	Late harvest
SIGNAL TRANSDUCTION								
Gene001	NP_973603.1	ADP-ribosylation factor GTPase-activating protein AGD10 [Arabidopsis thaliana]	5 e-26	79	11	2	7	2
Gene002	XP_002531638.1	Casein kinase, putative [Ricinus communis]	9 e-18	83	0	2	0	0
Gene003	XP_002880383.1	Kinase family protein [Arabidopsis lyrata subsp. lyrata]	0.094	73	0	0	1	1
Gene004	NP_201509.1	Mitogen-activated protein kinase 19 [Arabidopsis thaliana]	7 e-21	60	1	0	0	0
Gene005	ACR07972.1	Pti1-like S/T protein kinase [Hordeum vulgare subsp. vulgare]	1 e-38	71	1	0	0	0
Gene006	AAR87711.1	Salicylic acid-binding protein 2 [Nicotiana tabacum]	1 e-10	63	0	0	0	1
Gene007	NP_196670.1	Serine-rich protein-like protein [Arabidopsis thaliana]	7 e-11	60	0	0	0	1
Gene008	ADN96595.1	Thioredoxin h [Vitis vinifera]	3 e-08	64	1	0	1	2
TRANSCRIPTION FACTOR								
Gene009	AEF30544.1	Ethylene transcription factor [Castanea sativa]	6 e-06	53	1	0	0	0
Gene010	NP_177591.1	F-box/kelch-repeat protein [Arabidopsis thaliana]	2 e-25	64	1	0	0	0
Gene011	AAS10005.1	MYB transcription factor [Arabidopsis thaliana]	2 e-07	83	0	0	1	0
Gene012	CAD56217.1	Transcription factor EREBP-like protein [Cicer arietinum]	0.069	100	1	1	1	0
STRESS RESPONSE								
Gene013	XP_002891955.1	Early-responsive to dehydration 2 [Arabidopsis lyrata subsp. lyrata]	5 e-32	91	2	0	0	0
Gene014	ADF30255.1	Heat shock protein [Cucumis sativus]	1 e-20	58	1	0	1	0
Gene015	AAF34134.1	High molecular weight heat shock protein [Malus x domestica]	3 e-34	98	0	0	0	1
Gene016	AAL49788.1	Putative heat shock protein 90 [Arabidopsis thaliana]	4 e-13	83	0	0	2	1

TABLE 5.2 (continued)

Name	Accession num of similar sequence	Similar sequence from database (Putative function)	E-value	Max Identity %	Number of copies expressed			
					Early Unharvest	Late Unharvest	Early harvest	Late harvest
STRESS RESPONSE								
Gene017	NP_001077933.1	Ubiquitin fusion degradation 1 [Arabidopsis thaliana]	9.1	93	1	0	0	1
Gene018	NP_191404.2	Universal stress protein (USP) family protein [Arabidopsis thaliana]	8 e-29	86	1	0	3	6
DEFENCE								
Gene019	AAK15049.1	Asparaginyl endopeptidase [Vigna radiata]	2 e-20	66	0	1	0	0
Gene020	NP_172655.1	Aspartic proteinase A1 [Arabidopsis thaliana]	4 e-28	77	0	0	0	1
Gene021	ADB03784.1	Catalase [Ipomoea batatas]	1 e-64	84	0	3	0	3
Gene022	ACF06566.1	Catalase 2 [Elaeis guineensis]	2 e-45	92	0	1	3	1
Gene023	AAD30292.1	Catalase 3 [Raphanus sativus]	5 e-57	76	0	2	2	3
Gene024	ADQ39593.1	Class II chitinase [Malus x domestica]	4 e-06	82	3	0	0	4
Gene025	BAA10929.1	Cytochrome P450 like_TBP [Nicotiana tabacum]	8e-49	63	29	58	45	63
Gene026	ABX79341.1	Cysteine protease [Vitis vinifera]	4 e-46	81	1	0	0	0
Gene027	ABK78689.1	Cysteine proteinase inhibitor [Brassica rapa]	2 e-15	75	0	0	2	2
Gene028	CAB01591.1	Endochitinase [Persea Americana]	2 e-58	77	3	0	0	2
Gene029	ABY58189.1	Endo-1,4-D-glucanase [Persea americana]	6 e-48	100	0	0	0	3
Gene030	ACG44564.1	Endopeptidase Clp [Zea mays]	4 e-28	52	0	0	0	1
Gene031	NP_001105119.1	Legumain-like protease [Zea mays]	1 e-09	66	0	1	0	0
Gene032	XP_002527223.1	Oligopeptidase A, putative [Ricinus communis]	5 e-09	86	0	0	1	0
Gene033	AAF15308.1	Stearoyl-acyl-carrier-protein desaturase [Persea americana]	9 e-20	100	0	0	1	1
Gene034	BAK19068.1	Ubiquitin [Ipomoea nil]	0.33	100	0	0	1	0

5.3.2 Defences related genes induced in unharvested and harvested avocado fruit following *C. gloeosporioides* infection.

Some data from the 454 read sequences of infected avocado fruits during assembly did not map with the 454 read sequences of uninfected avocado fruits. Comparison of these 454 read sequences of infected avocado fruits sequences to BLASTX similarity search against the NCBI protein database allowed function assignment based on the similarities with known function plant protein sequences. Genes obtained from these 454 reads are considered to be induced after *C. gloeosporioides* infection and this chapter focuses on genes involved in signal transduction, transcription, defence and stress responses in order to study the defence mechanism of avocado fruit against anthracnose disease and the defence signalling pathway involved. These groups of gene products in unharvested (Table 5.3 and 5.4) and harvested (Table 5.5 and 5.6) infected avocado fruit during early and late responses are presented. Some of these induced genes involved in signal transduction are calcium-dependent protein kinase, leucine-rich repeat transmembrane protein kinase, mitogen-activated protein kinases (MAPK), salicylic acid-activated MAP kinase, salicylic acid-binding protein 2, salicylic acid-induced protein kinase (SIPK), WD-repeat protein and many others as shown in the tables. Some of genes identified to be involved in transcriptional activation are bZIP transcription factor, ethylene-responsive element binding protein, MYB proteins and WRKY transcription factors. Other induced genes identified, predicted to be involved in stress and defence responses are: universal stress protein family protein, aspartic proteinases, β-1,3-glucanases, β-glucosidases, catalase, cysteine proteinase inhibitor, endochitinase, programmed cell death protein 5, and others presented in the Table 5.3 and 5.4 for the unharvested samples and in Table 5.5 and 5.6 for the harvested samples.

Table 5.3 Summary of genes induced in unharvested avocado fruits during early response (pool of 1, 4 and 24h) to *C. gloeosporioides* infection with their putative function.

Name	Accession Num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		SIGNAL TRANSDUCTION		
Gene0001	XP_002532559.1	Calcium-dependent protein kinase, putative [Ricinus communis]	8e-25	68
Gene0002	XP_002521983.1	Calcium ion binding protein, putative [Ricinus communis]	9e-21	45
Gene0003	XP_002871973.1	Kinase family protein [Arabidopsis lyrata subsp. lyrata]	2e-24	90
Gene0004	NP_172244.2	Leucine-rich repeat transmembrane protein kinase, putative [Arabidopsis thaliana]	3e-09	74
Gene0005	AAF66615.1	LRR receptor-like protein kinase [Nicotiana tabacum]	6e-40	81
Gene0006	BAE46985.1	Mitogen-activated protein kinase [Nicotiana tabacum]	1e-37	91
Gene0007	ABY58272.1	Serine-threonine protein kinase [Persea americana]	4e-11	83
Gene0008	AAQ76042.1	Signal recognition particle receptor protein [Cucumis sativus]	1e-10	78
Gene0009	XP_002518444.1	WD-repeat protein, putative [Ricinus communis]	1e-39	77
		TRANSCRIPTION FACTOR		
Gene0010	ACF60482.1	bZIP transcription factor [Oryza sativa Japonica Group]	1e-08	57
Gene0011	BAD18011.1	MADS-box transcription factor [Asparagus virgatus]	7e-16	76
Gene0012	AAM63665.1	Transcription factor, putative [Arabidopsis thaliana]	2e-33	89
Gene0013	XP_002524838.1	WRKY transcription factor, putative [Ricinus communis]	2e-27	76
		STRESS RESPONSE		
Gene0014	BAJ11784.1	Dehydration responsive protein [Corchorus olitorius]	0.092	61
Gene0015	XP_002869603.1	Early-responsive to dehydration 8 [Arabidopsis lyrata subsp. lyrata]	1e-40	95
Gene0016	XP_002514902.1	Stress associated endoplasmic reticulum protein, putative [Ricinus communis]	2e-09	100
Gene0017	ABD57310.1	Stress-associated protein 1 [Solanum lycopersicum]	6e-17	74
		DEFENCE		
Gene0018	XP_002531635.1	α-glucosidase, putative [Ricinus communis]	1e-17	60
Gene0019	AAK15049.1	Asparaginyl endopeptidase [Vigna radiata]	1e-41	87
Gene0020	AEE78232.1	beta-D-glucan exohydrolase - like protein [Arabidopsis thaliana]	2e-11	52
Gene0021	AAD30291.2	Catalase 2 [Raphanus sativus]	3e-05	70
Gene0022	AAF61733.1	Catalase 3 [Helianthus annuus]	3e-05	75
Gene0023	ABR19829.1	Cysteine proteinase [Elaeis guineensis]	8e-53	90
Gene0024	BAB64929.1	Defensin-like protein [Pyrus pyrifolia]	3e-06	55
Gene0025	AAG51234.1	Disease resistance protein MLO, putative; 5304-2185 [Arabidopsis thaliana]	0.014	41
Gene0026	CAB01591.1	Endochitinase [Persea americana]	6e-49	100
Gene0027	AEE84132.1	Enhanced disease resistance 2 protein [Arabidopsis thaliana]	0.014	51
Gene0028	ACE96388.1	Esterase/lipase/thioesterase [Populus tremula]	2e-30	100
Gene0029	AAZ94162.1	Enzymatic resistance protein [Glycine max]	6e-17	63
Gene0030	AAF97315.1	Lipoxygenase [Arabidopsis thaliana]	4e-08	72
Gene0031	AAM47598.1	NBS/LRR resistance protein-like protein [Capsicum annuum]	9e-10	65
Gene0032	ABA33845.1	Pathogenesis-related protein 6 [Zea diploperennis]	3e-16	59
Gene0033	NP_001154663.1	Ribonuclease III family protein [Arabidopsis thaliana]	8e-06	
Gene0034	ABF96384.1	Serine carboxypeptidase family protein, expressed [Oryza sativa Japonica Group]	7e-11	71

Table 5.4 Summary of genes induced in unharvested avocado fruits during late response (pool of 3, 4, 5 and 7 day post infection) to *C. gloeosporioides* infection with their putative function.

Name	Accession Num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		SIGNAL TRANSDUCTION		
Gene0035	ACG37954.1	Mitogen-activated protein kinase organizer 1 [Zea mays]	2e-16	71
Gene0036	ACM89569.1	Pto kinase interactor [Glycine max]	5e-08	90
		TRANSCRIPTION FACTOR		
Gene0037	ACM49845.1	Ethylene responsive transcription factor 2a [Prunus salicina]	2e-14	68
Gene0038	BAD18011.1	MADS-box transcription factor [Asparagus virgatus]	7e-16	76
Gene0039	AAM63665.1	Transcription factor, putative [Arabidopsis thaliana]	2e-33	89
		STRESS RESPONSE		
Gene0040	AAB84193.1	Dormancy-associated protein [Pisum sativum]	0.58	51
		DEFENCE		
Gene0041	BAJ33502.1	β- glucosidase like protein [Delphinium grandiflorum]	9e-34	61
Gene0042	BAC79443.1	Catalase [Acacia ampliceps]	1e-24	98
Gene0043	AAF61733.1	Catalase 3 [Helianthus annuus]	3e-05	75
Gene0044	XP 002332294.1	CC-NBS resistance protein [Populus trichocarpa]	9e-11	58
Gene0045	NP_567868.1	Endonuclease V family protein [Arabidopsis thaliana]	2e-27	80
Gene0046	CAH59407.1	Endopeptidase 1 [Plantago major]	2e-08	65
Gene0047	ABZ85667.1	LRR-like disease resistance protein [Brassica rapa subsp. pekinensis]	2e-10	76
Gene0048	NP_001154256.1	Metalloendopeptidase [Arabidopsis thaliana]	2.2	54
Gene0049	AAM47598.1	NBS/LRR resistance protein-like protein [Capsicum annuum]	9e-10	65
Gene0050	AR25995.1	Senescence-associated protein, putative [Pyrus communis]	2e-09	91

Table 5.5 Summary of genes induced in harvested avocado fruits during early response (pool of 1, 4 and 24h) to *C. gloeosporioides* infection with their putative function.

Name	Accession Num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		SIGNAL TRANSDUCTION		
Gene0051	ABN10955.2	Auxin response factor 8 [Ipomoea nil]	5e-28	83
Gene0052	XP_002532559.1	Calcium-dependent protein kinase, putative [Ricinus communis]	8e-25	68
Gene0053	XP_002521983.1	Calcium ion binding protein, putative [Ricinus communis]	9e-21	45
Gene0054	ACM89476.1	Leucine-rich repeat family protein / protein kinase family protein [Glycine max]	8e-09	65
Gene0055	ADD62693.1	Mitogen-activated protein kinase kinase [Capsicum annuum]	2e-11	57
Gene0056	NP_188044.1	1-phosphatidylinositol-4-phosphate 5-kinase [Arabidopsis thaliana]	0.060	80
Gene0057	AAL40864.1	Receptor protein kinase-like protein [Capsicum annuum]	2e-14	84
		TRANSCRIPTION FACTOR		
Gene0058	AAS68190.1	Myb transcription factor [Vitis vinifera]	6e-27	89
Gene0059	XP_002524838.1	WRKY transcription factor, putative [Ricinus communis]	2e-27	76
Gene0060	NP_179571.1	Zinc finger (CCCH-type) family protein [Arabidopsis thaliana]	3e-27	77
		STRESS RESPONSE		
Gene0061	ABE79560.1	Chaperone protein dnaK (heat shock protein 70) [Medicago truncatula]	7e-19	53
Gene0062	AAM00365.1	Saline responsive OSSRIII protein [Oryza sativa]	4e-05	77
Gene0063	NP 191404.2	Universal stress protein (USP) family protein [Arabidopsis thaliana]	4e-18	58
Gene0064	BAC84424.1	Water-stress protein-like protein [Oryza sativa Japonica Group]	1e-12	41
		DEFENCE		
Gene0065	ABZ02704.1	Accelerated cell death 1 [Arabidopsis thaliana]	2e-06	60
Gene0066	ABG37021.1	Aspartic protease [Nicotiana tabacum]	4e-27	70
Gene0067	BAB62890.1	Aspartic proteinase 1 [Glycine max]	3e-26	70
Gene0068	CAI39245.1	beta-Amylase [Glycine max]	4e-19	74
Gene0069	NP_191763.3	Catalytic/ hydrolase [Arabidopsis thaliana]	5e-29	70
Gene0070	CAC81812.1	Chitinase, putative [Musa acuminata]	1e-35	86
Gene0071	AAL15885.1	γ-Thionin putative [Castanea sativa]	2e-11	50
Gene0072	XP_002527223.1	Oligopeptidase A, putative [Ricinus communis]	4e-29	66
Gene0073	ADP69173.1	Pathogenesis related protein-5 [Populus tomentosa]	3e-14	86
Gene0074	ACE97327.1	Pectinesterase inhibitor [Populus tremula]	4e-07	69
Gene0075	AAL35364.1	Peroxidase [Capsicum annuum]	2e-09	70
Gene0076	ACG48882.1	Programmed cell death protein 5 [Zea mays]	1e-05	83
Gene0077	AAK59275.1	Thaumatin-like protein [Sambucus nigra]	5e-13	86

Table 5.6 Summary of genes induced in harvested avocado fruits during late response (pool of 3, 4, 5 and 7 day post infection) to *C. gloeosporioides* infection with their putative function

Name	Accession Num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		SIGNAL TRANSDUCTION		
Gene0078	XP_002532559.1	Calcium-dependent protein kinase, putative [Ricinus communis]	8e-25	68
Gene0079	BAE46985.1	Mitogen-activated protein kinase [Nicotiana tabacum]	1e-37	91
Gene0080	ADD62693.1	Mitogen-activated protein kinase kinase [Capsicum annuum]	2e-11	57
Gene0081	ABJ89812.1	Salicylic acid-activated MAP kinase [Nicotiana attenuata]	2e-33	84
Gene0082	BAC53772.1	Salicylic acid-induced protein kinase [Nicotiana benthamiana]	2e-33	84
Gene0083	NP_175758.2	Signal peptidase I family protein [Arabidopsis thaliana]	2e-20	53
Gene0084	AAQ76042.1	Signal recognition particle receptor protein [Cucumis sativus]	1e-10	78
Gene0085	CAA71142.1	SNF1-related protein kinase [Cucumis sativus]	1e-23	83
		TRANSCRIPTION FACTOR		
Gene0086	ADL36656.1	C3HL domain class transcription factor [Malus x domestica]	3e-27	79
Gene0087	ACM49845.1	Ethylene responsive transcription factor 3a [Prunus salicina]	2e-14	68
Gene0088	XP_002877726.1	Myb family transcription factor [Arabidopsis lyrata subsp. lyrata]	7e-39	90
		STRESS RESPONSE		
Gene0089	ABD57310.1	Stress-associated protein 1 [Solanum lycopersicum]	6e-17	74
Gene0090	NP_566406.1	Universal stress protein (USP) family protein [Arabidopsis thaliana]	3e-26	71
		DEFENCE		
Gene0091	AAK58515.1	β-1,3-glucanase-like protein [Olea europaea]	9e-06	75
Gene0092	AEE78232.1	β -D-glucan exohydrolase - like protein [Arabidopsis thaliana]	2e-11	52
Gene0093	BAC79443.1	Catalase [Acacia ampliceps]	1e-24	98
Gene0094	AAF61733.1	Catalase 3 [Helianthus annuus]	3e-05	75
Gene0095	ADQ43720.1	Chitinase I [Casuarina equisetifolia]	3e-35	82
Gene0096	CAB01591.1	Endochitinase [Persea americana]	6e-49	100
Gene0097	ABY58190.1	Endo-1,4-D-glucanase [Persea americana]	4e-71	100
Gene0098	NP_191415.2	Endonuclease/exonuclease/phosphatase family protein [Arabidopsis thaliana]	4e-19	81
Gene0099	CAH59407.1	Endopeptidase 1 [Plantago major]	2e-08	65
Gene00100	ABZ85667.1	LRR-like disease resistance protein [Brassica rapa subsp. pekinensis]	2e-10	76
Gene00101	NP_001154256.1	Metalloendopeptidase [Arabidopsis thaliana]	2.2	54
Gene00102	XP_002519488.1	Multidrug resistance protein 1, 2, putative [Ricinus communis]	2e-19	81
Gene00103	AAM47598.1	NBS/LRR resistance protein-like protein [Capsicum annuum]	9e-10	65
Gene00104	ACE97327.1	Pectinesterase inhibitor [Populus tremula]	4e-07	69

5.3.3 Real Time-PCR

5.3.3.1 Primer design

PCR products from avocado flesh cDNA amplified with defence-related gene primers produced single bands of the desired size between 100-200 bp, depending on the primer sets used confirming that the sequences obtained from the 454 sequencing are those of avocado fruit (Figure 4.1).

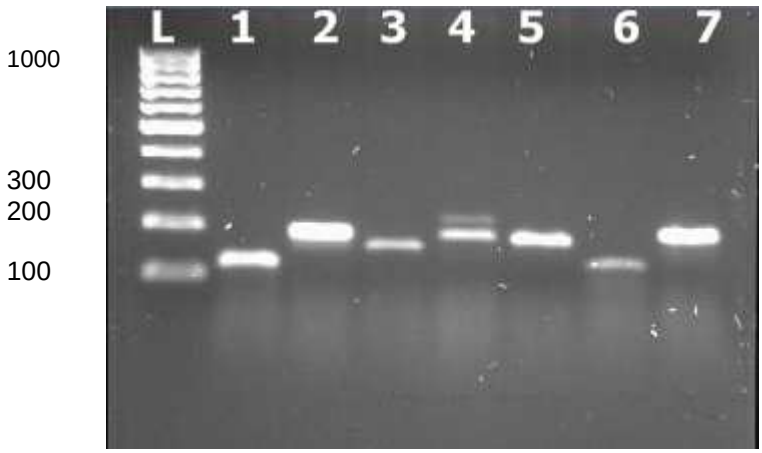


Figure 5.1 PCR products amplified from avocado flesh cDNA using specifics primers separated on 2% non-denaturing agarose gel containing EtBr and photographed under ultraviolet light. L, GeneRuler™ 100 bp DNA Ladder. Lane 1, catalase (162bp); lane 2, endochitinase (186); lane 3, pathogenesis related protein 6 (158); lane 4, CC-NBS resistance protein (185); lane 5, pathogenesis related protein 5 (171 bp); lane 6, endo-1,4-D-glucanase (121) and lane 7, actin gene (166 bp).

5.3.3.2 Quantitative Expression

For verification of the gene expression obtained from the transcriptome sequencing analysis, real time PCR (qPCR) was performed for selected genes. Standard curves were calculated for each of the four genes subjected to qPCR. To generate a standard curve, Ct values/crossing points of different standard dilutions were plotted against the logarithm of

input amount of standard material (Figure 5.2; 5.3 and 5.4). The slope of a standard curve provided an indication of the efficiency of the real-time PCR; and from the slope (S), efficiency was calculated using the following formula: PCR efficiency (%) = $100(10^{(-1/S)} - 1)$. Generally, most amplification reactions do not reach 100% efficiency due to experimental limitations or the inaccuracy of pipetting of known references (Ginzinger, 2002).

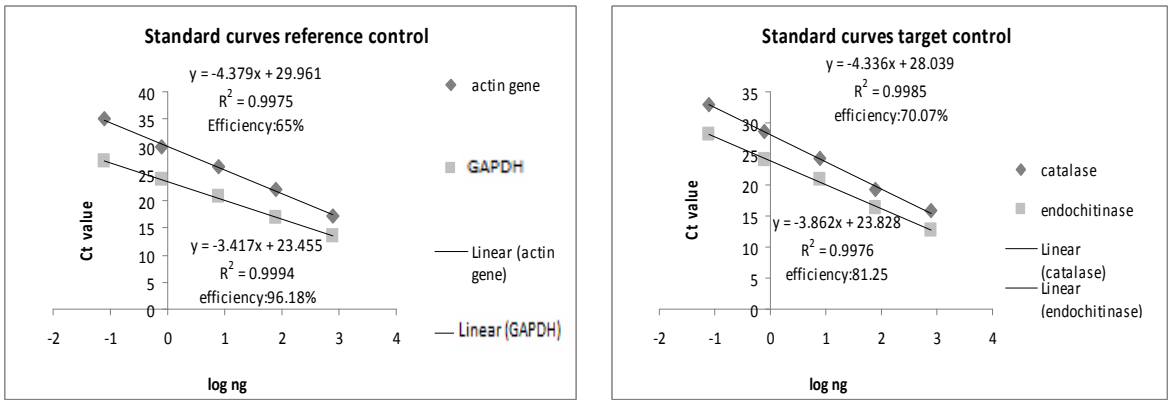


Figure 5.2. Standard curve for each of the control genes (target and reference) used for qPCR. The resulting Ct values for each input amount of cDNA are plotted as a function of the log concentration of input amounts and a linear trendline is fitted to the data.

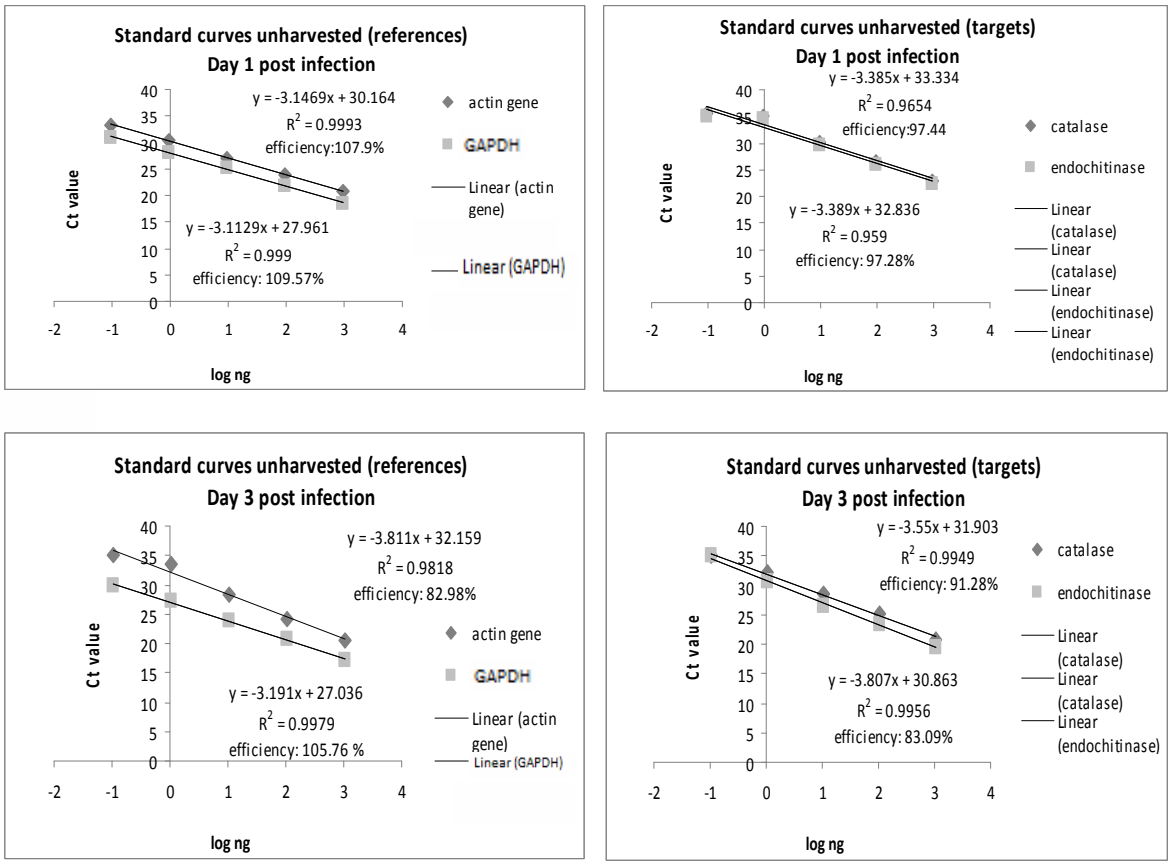


Figure 5.3. Standard curve for each of the genes (target and reference) from unharvested samples used for qPCR. The resulting Ct values for each input amount of cDNA are plotted as a function of the log concentration of input amounts and a linear trendline is fitted to the data.

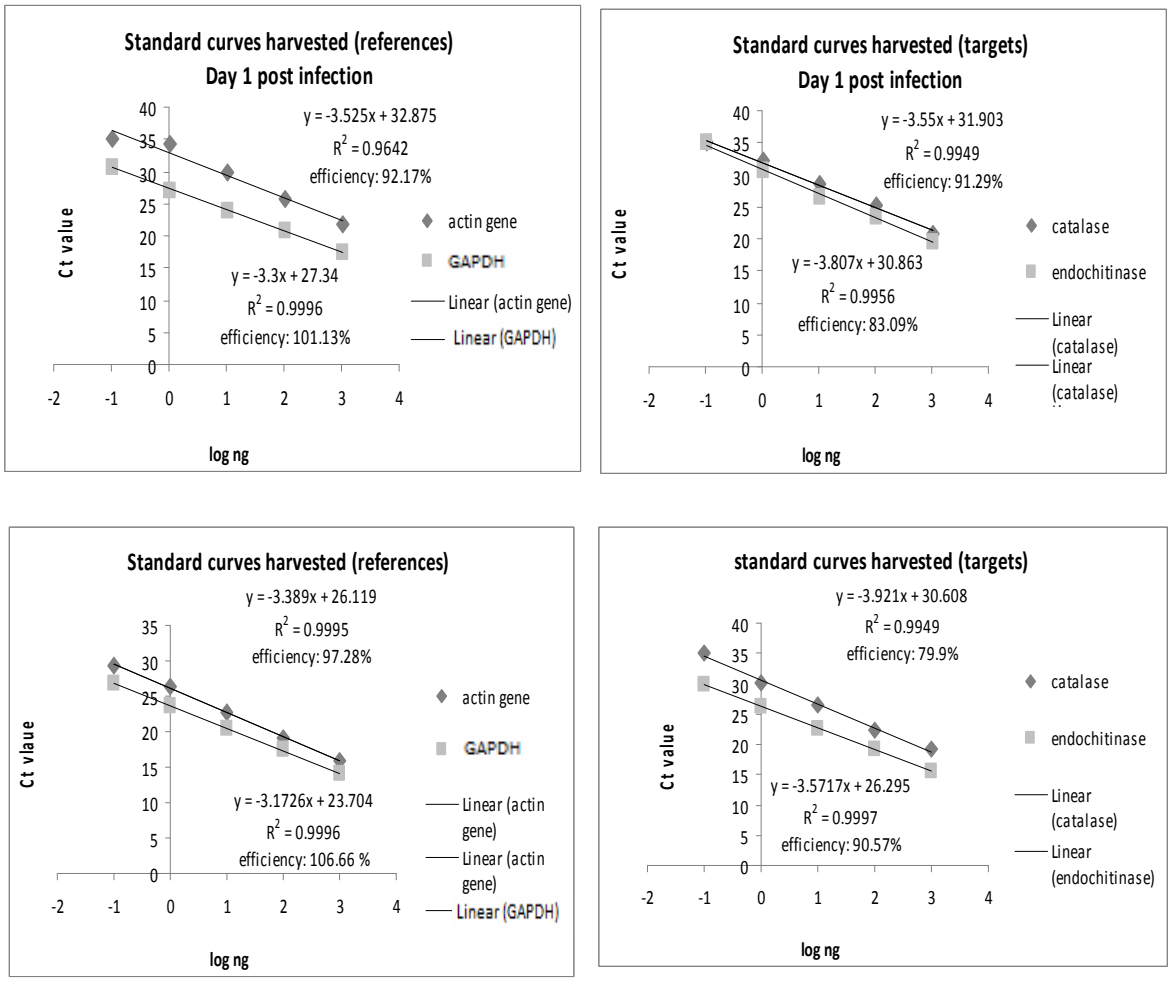


Figure 5.4. Standard curve for each of the genes (target and reference) from harvested samples used for qPCR. The resulting Ct values for each input amount of cDNA are plotted as a function of the log concentration of input amounts and a linear is fitted to the data.

In order to verify the gene expression observed in 454 sequencing analysis, qPCR was performed for selected genes. Genes were selected first on the basis of their putative function in plant defence and their presence revealed by 454 sequencing analysis in at least two time points. Accordingly, the following genes were chosen for qPCR: catalase and endochitinase, which are genes documented to be involved in defence response.

An up regulation was observed in the expression of catalase in unharvested avocado fruits infected with *C. gloeosporioides* at day 1 and 3 post infection (Figure 5.5 A), although the trend was not shown by the expression ratio normalized to actin gene at day 3 post infection. In the harvested avocado fruits, catalase was down regulated at day 1 following infection, but at day 3 an up regulation was observed in the expression of catalase and this pattern was confirmed by both expression ratios (Figure 5.5 B).

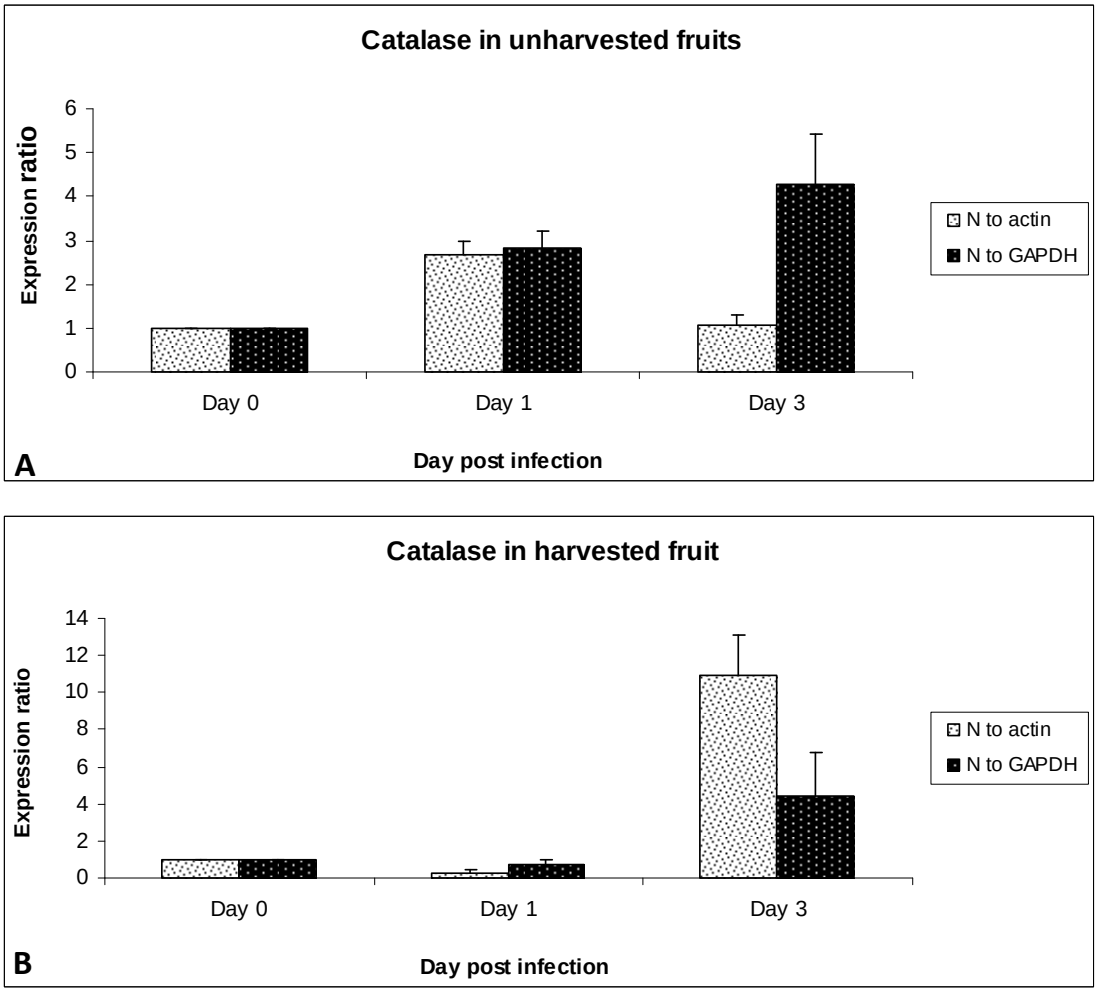
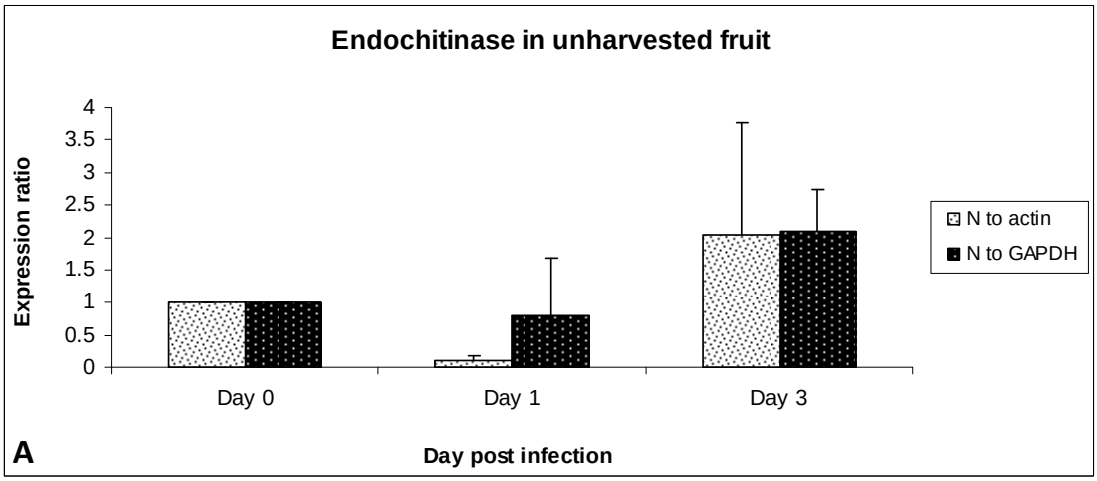


Figure 5.5. Relative gene expression level of catalase in unharvested and harvested avocado at 0, 1 and 3 day following *C. gloeosporioides* infection, quantified using the Roche Light cycler 1.5. Expression is given in terms of a calibrator and error bar is standard error of means, with n= 2 for each data point.

Endochitinase was down regulated in unharvested avocado fruits infected with *C. gloeosporioides* at day 1 (Figure 5.6 A), but at day 3 an up regulation of endochitinase expression was observed and this pattern was confirmed by both expression ratios. A similar pattern was observed for endochitinase expression in harvested avocado at 1 and 3 day following infection (Figure 5.6 B).



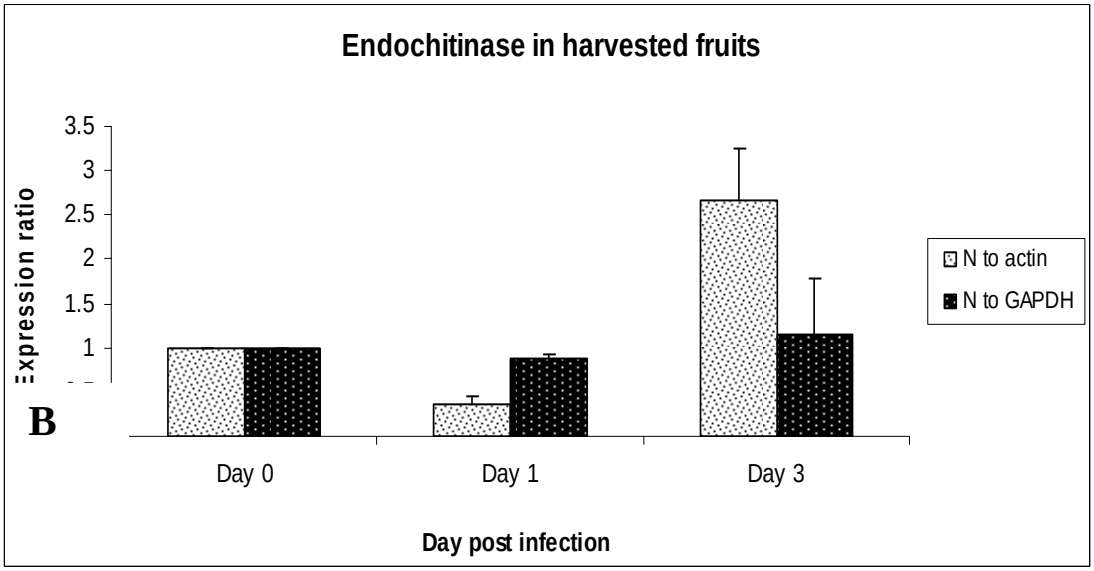


Figure 5.6. Relative gene expression level of endochitinase in unharvested and harvested avocado at 0, 1 and 3 days following *C. gloeosporioides* infection, quantified using the Roche Light cycler 1.5. Expression is given in terms of a calibrator and error bar is standard error of means, with n= 2 for each data point.

5.3.3.3 Gel electrophoresis of the qPCR products

The products obtained from the qPCR of each of the four selected genes, were separated by electrophoresis then visualised on 2 % agarose gel to ensure that single transcripts products were obtained, and to verify LightCycler melting curve analyses that indicated that qPCR reactions were free of primer dimmers (Figure 5.7).

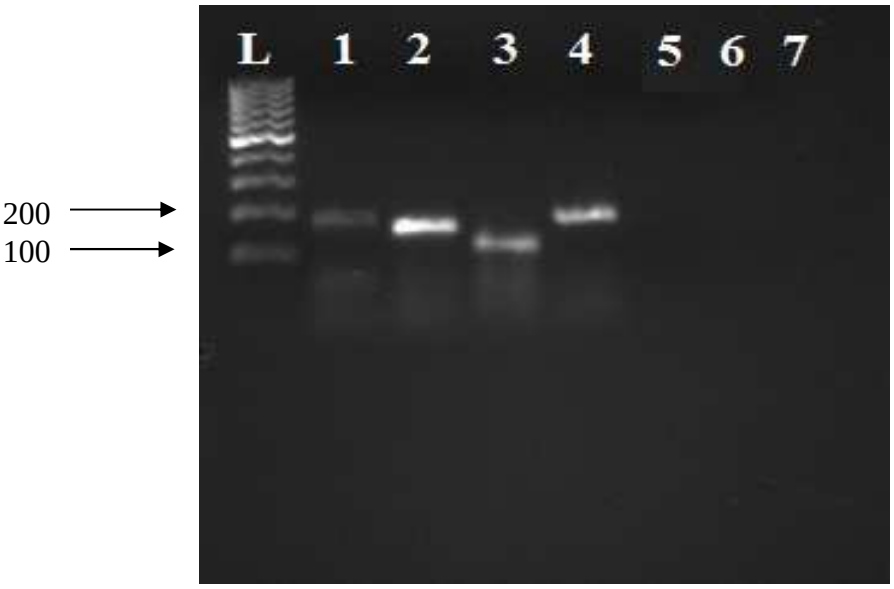


Figure 5.7. qPCR products amplified from harvested infected avocado flesh cDNA using specifics primers separated on 2 % non-denaturing agarose gel containing EtBr and photographed under ultraviolet light. L, GeneRuler™ 100 bp DNA Ladder. Lane 1, actin gene (166bp); lane 2, GAPDH (186); lane 3, catalase (122 bp); lane 4, endochitinase (186); lane 5, water; lane 7, non template control.

5.5 DISCUSSION

Plant disease problems are becoming more extensive because of the development of pathogen resistance to fungicides and the withdrawal of pesticides due to environmental pollution. Plants have evolved their own powerful defence mechanisms to prevent and limit disease on developing fruit. These include biochemical and physical barriers to pathogen invasion, which could be constitutive or inducible in nature (Agrios, 2005). Many investigations have been done to understand innate resistance mechanisms in plants. The parallel focus on molecular biology in the last few years has improved our understanding of plant-pathogen interactions through the identification of a number of endogenous resistance genes and analysis of signalling pathways leading to the hypersensitive response and systemic acquired resistance. This breakthrough has enabled more sophisticated breeding strategies in commercial cultivars to be employed using marker-assisted breeding (Ayliffe and Lagudah, 2004). Within this context this study was carried out to understand the molecular basis of resistance which is activated in pre and harvested avocado fruit during *C. gloeosporioides* infection.

The recognition of a pathogen by a plant triggers rapid defence responses by a number of signal transduction pathways (Rushton and Somssich, 1998; Barakat *et al*., 2009). In this study many genes were identified to be potentially involved in signal transduction and these include mitogen-activated protein kinases (MAPK), salicylic acid-induced protein kinase (SIPK), salicylic acid-activated MAP kinase, salicylic acid-binding protein 2, calcium-dependent protein kinase, leucine-rich repeat transmembrane protein kinase, calcium-dependent protein kinase, WD-repeat protein, amongst others (Tables 5.2; 5.3; 5.4; 5.5 and 5.6). Previous studies revealed that MAPKs are one of the key regulators of the defence

signalling pathways and its activation is one of the earliest responses in plants challenged by pathogens. The MAPK signalling cascade has been reported to be one of the major pathways by which extra cellular stimuli are transduced into intracellular responses (Kovtun *et al*., 2000; Zhang and Klessig, 2001). In agreement with these previous findings, we also identified MAPK, which could be induced as a result of the early response of both unharvested and harvested avocado fruits to *C. gloeosporioides* attack in order to trigger the defence mechanism. It has been also shown that SIPK is involved in plant defence signalling and is induced by pathogens, pathogen-derived elicitors, wounding and oxidative stresses (Samuel and Ellis, 2002). In addition Zhang and Klessig (2001) and others researchers found that activation of two MAPKs, wound-induced protein kinase (WIPK) and SIPK, is one of the earliest responses that occur in tobacco plants challenged with Tobacco Mosaic Virus (TMV) or tobacco suspension cells treated with fungal elicitins or tobacco that has been wounded (Zhang and Klessig, 2001; Sharma *et al*., 2003).

Salicylic acid-binding protein 2 (SABP2), a lipase belonging to the α / β fold hydrolase super family, was identified in harvested avocado fruit some days post infection (Table 5.2). A salicylic acid (SA) receptor is required for the plant immune response reported by Kumar and Klessig (2003). They further proposed that SABP2 belongs to a large class of ligand-stimulated hydrolases involved in stress hormone-mediated signal transduction. Furthermore, SABP2 displays SA-stimulated enzymatic activity and its high affinity with SA indicates that it might be a receptor for SA. This hypothesis is supported by the study done by Gaffney et al (1993), showing that in transgenic tobacco plants effectively silenced for SABP2 and harbouring a bacterial gene encoding salicylate hydroxylase, which converts salicylic acid to catechol, the ability of SA to induce pathogenesis related protein-1 was reduced which resulted in the reduction in local resistance, and inability to induce acquired

resistance against TMV. This loss of SA responsiveness was similar to the phenotype of SA-insensitive systemic acquired resistance (SAR) defective observed in some *Arabidopsis thaliana* mutants by Shah et al (1997). Based on the expression of SIPK and SABP2 in infected avocado fruit (Tables 5.2 and 5.6) and according to the results of previous findings, we propose that SA would have been synthesized to play an important signalling role in the activation of avocado fruit defence responses following *C. gloeosporioides* infection. Many investigations have demonstrated that SA is a key signal for the activation of both local and systemic resistance responses. For instance tobacco and *Arabidopsis* plants failed to develop SAR and display enhanced susceptibility to pathogen infection because they were SA deficient or were unable to accumulate SA after infection (Dong, 2001; Kunkel and Brooks, 2002). SA functions as a secondary signal following pathogen infection to trigger the expression of many pathogen-responsive genes (Rushton and Somssich, 1998). The cell nucleus is a major target of signal transduction, where the terminal signals result to the transcriptional activation of several genes and antimicrobial compounds involved in the plant defence system.

Transcriptional activation of genes is a crucial part of the plants defence mechanism against pathogens. In infected avocado fruit many transcription factors have been differentially expressed during interaction between avocado fruit and *C. gloeosporioides*. Ethylene-responsive element binding protein (EREBP) was identified in infected unharvested avocado fruits during early and late response and in harvested avocado fruit during early response (Table 5.2). EREBP was identified from *Arabidopsis thaliana* and other plants as a homeobox gene that encodes a transcription factor. In plants it is responsible in part for mediating the response to the plant hormone ethylene (Riechmann and Meyerowitz, 1998; Büttner and Singh, 1998). Plant ethylene seems to play a crucial role in different plant

disease resistance pathways. It has been shown that during plant-pathogen interactions the rate of the biosynthesis of ethylene increases rapidly and afterward ethylene induces transcription of some defence related genes such as class β-1,3-glucanase and chitinase class I (Vögeli *et a*l., 1988; Wang *et al*., 2002). The ethylene-responsive element known as the GCC box (AGCCGCC) is commonly found in the promoter region of ethylene-inducible pathogenesis-related protein genes. The GCC box interacts with the ethylene responsive element binding factor which was shown to be the transcription factor that reacts to extracellular signals in order to modulate GCC box-mediated gene expression whether positively or negatively (Ohme-Takagi *et al*., 2000). On the basis of these observations, ethylene seems to be involved in avocado defence system against *C. gloeosporioides*.

Basic region/leucine zipper motif (bZIP) transcription factors were also identified in unharvested avocado fruits during early response (Table 5.3) and MYB proteins in harvested avocado fruits during early and late response (Table 5.5 and 5.6). bZIP transcription factors and MYB proteins were previously reported to play a role in the regulation of pathogens' defence response in plants (Jakoby *et al*., 2002; Vailleau *et al*., 2002). The first evidence for the involvement of such genes in plant-microbe interactions revealed that a tobacco MYB gene is induced in response to (TMV) infection and to bind to a consensus MYB recognition sequence found in a promoter of the defence gene PR1 (Yang and Klessig, 1996). Moreover during the very first steps of the hypersensitive response following *Arabidopsis thaliana* infection by the avirulent strain 147 of *Xanthomonas campestris* pv. *Campestris*, *Arabidopsis thaliana MYB30* was isolated on the basis of its rapid, specific, and transient transcriptional activation. It was also shown that MYB30 increases resistance against a biotrophic fungal pathogen, *Cercospora nicotianae* and different bacterial pathogens (Vailleau *et al*., 2002). Likewise it has been reported that

TGA family of transcription factors which belong to the class of bZIP factors bind to the *as-1 cis* element which are SA-responsive cis elements present in the promoters of immediate early and late SA-inducible genes such as pathogenesis- related genes (Zhou *et al*., 2000; Thurow *et al*., 2005). In addition it was also shown that *Arabidopsis thaliana* bZIP57/OBF4/TGA4 interacts with *Arabidopsis thaliana* ethylene-responsive element binding protein which binds the ethylene response element present in the promoters of various pathogenesis-proteins indicating that these proteins might be involved in SA and ET signalling pathways in response to pathogen infection (Büttner and Singh, 1997). So, due to the presence of salicylic acid-activated MAP kinase, SIPK, SABP2, EREBP and bZIP in infected avocado fruits, it can be proposed that there is a synergistic action between SA and ET pathways in avocado fruit during response to *C. gloeosporioides* infection.

During early response to *C. gloeosporioides* infection in both unharvested and harvested avocado fruits, WRKY transcription factors were also induced (Table 5.3 and 5.5.). WRKY transcription factors have been shown previously to regulate the transcription of a wide range of genes involved in plant defence during early response to a pathogen attack (Moon and Domier, 2005). The WRKY domain, a 60 amino acid region, is defined by the conserved amino-acid sequence WRKYGQK and a zinc-finger-like-motif, and it has been reported that WRKY proteins bind to the W box, which is found in the promoters of various plant defence genes (Maleck *et al*., 2000). A number of studies have shown that WRKY proteins from many plants are rapidly enhanced by a range of pathogens, pathogen elicitors, or treatment SA (Chen and Chen, 2000; Dellagi *et al*., 2000; Eulgem *et al*., 2000; Kim *et al*., 2000; Kalde *et al*., 2003). For instance the expression profiles of *Arabidopsis* WRKY gene showed that 49 out of the 72 *AtWRKY* genes analyzed were differentially regulated in response to SA treatment or infection by a bacterial pathogen (Dong *et al*., 2003). In

accordance to these previous findings, it can be proposed that WRKY proteins may have regulatory functions in avocado fruit response to *C. gloeosporioides* infection.

In this study, accelerated cell death 1 and programmed cell death protein 5 were identified to be expressed in infected harvested avocado fruits during early response (Table 5.5). These genes are known to be involved in the hypersensitive response (HR) (Tanaka *et al*., 2003; Kotb *et al*., 2005). HR is regarded as one of the mechanism, used by plants, to restrict the spread of infection by microbial pathogens to other parts of the plant (Kwang-Hyung *et al*., 2004), and it involves a complex form of programmed cell death as well (Greenberg and Yao, 2004). The early step in the HR is the ion fluxes manifested by an increase in cytosolic calcium which precedes and seems necessary for hypersensitive cell death induced by rust fungi (Xu and Heath, 1998). The presence of calcium-dependent protein kinase and calcium ion binding protein in the infected harvested avocado fruits during early response may have an implication in the HR activation, but further investigations would be needed to ascertain whether HR is involved or not in avocado resistance mechanism to anthracnose disease . At the early stage of a plant's defence response, the oxidative burst occurs leading to a rapid and transient production of large amounts of reactive oxygen species (ROS) at the site of infection (Barna *et al*., 2003). Because of this some antioxidant genes identified in infected avocado fruits such as catalase and peroxidases were activated following the oxidative burst in areas around the site of infection in order to minimise damage of healthy tissue by the ROS (Palatnik *et al*., 2002). It has been shown that during infection of strawberry by *Colletotrichum acutatum*, genes Fahir-1, Falpr10-1, Fapr5-1 and Fapr5-2 coding for hypersensitive response protein were expressed as part of the early mechanism of strawberry defence (Casado-Díaz *et al*., 2006). Besides the role of limiting pathogen invasion in the plant, hypersensitive cell death has been suggested to have additional

contributions to defence. This consists of releasing signals that condition adjacent cells to become responsive to pathogen elicitors and then trigger the systemic resistance throughout the plant (Park, 2005).

Systemic acquired resistance (SAR) is an important broad-spectrum, long-lasting, plant defence response that protects the whole plant against subsequent infection. SAR is characterised by a higher induction of several SAR gene products with direct antimicrobial activity or closely related to classes of antimicrobial proteins. In infected avocado fruits many of these SAR gene products were identified to be expressed in response to *C. gloeosporioides.* According to previous discoveries some of those identified genes are: pathogenesis-related proteins (PR5 and PR 6) and accumulation of defence genes such as β-1-3-glucanases and chitinases (Plymale *et al.*, 2007), genes encoding cysteine-rich proteins related to thaumatin (Linthorst, 1991), defensin (PR12) (Tiryaki and Tunaz, 2004), ribonuclease III family protein (PR10), γ-thionin (PR 13) and peroxidase (PR 9) (Van Loon and Van Strien, 1999). Hu and Reddy (1997) have shown that thaumatin expressed in infected avocado fruit is induced by the presence of fungi and pathogenic molds. In addition Menu-Bouaouiche *et al*. (2003) demonstrated that in some cases their action against pathogens has been related to endo-β-1,3-glucanase activity and to the inhibiting properties of amylase, also identified in this study in harvested infected avocado fruits during early response (Table 5.5). Most pathogenesis-related proteins identified in infected avocado fruits display antimicrobial and antifungal properties related to the destruction of pathogen structures. qPCR revealed that endochitinase was induced in both unharvested and harvested avocado fruits at day 3 following *C. gloeosporioides* infection. Some findings have demonstrated that in most higher fungi, plant β-1,3-glucanases and chitinase attack the components of the cell walls such as β-1,3-glucans and chitin (Van Loon and Van Strien,

1999; Odjakova and Hadjiivanova, 2001; Selitrennikoff, 2001). Antifungal activities *in vitro* of plant β-1,3-glucanases and chitinase have been studied with various bioassays which confirmed the synergistic effects of these two hydrolytic enzymes in tobacco challenged with the fungus *Trichoderma viride* (Stintzi *et al*., 1993). Therefore, in line with theses previous findings, avocado fruit β-1,3-glucanases and chitinase could be a tool in weakening and decomposing of *C. gloeosporioides* cell walls containing β-1,3-glucans and chitin. Other studies revealed that β-1,3-glucanases and chitinase are able to partially degrade fungal cell walls to release oligosaccharides which some have been shown to be perceived by the plant cell as elicitors that lead to the induction of active defence response (Stintzi *et al*., 1993; Edreva, 2005). PR-5 type thaumatin, PR-12 type defensin, γ-thionin (PR 13) (Hammond-Kosack and Jones 2000) identified in avocado have been shown to exhibit antifungal and antibacterial activities, exerting their action at the level of the plasma membrane of the target pathogen. Their plasma membrane-permeabilizing ability leads to the plasmolysis and damage of fungal or bacterial pathogens by inhibiting their growth and development (Broekaert *et al*., 1997). Likewise, Lagrimini et al. (1987) reported earlier that the peroxidase activity of PR-9 also expressed in infected avocado fruit (Table 5.5) could participate to an ultra fortification and rigidification of plant cell wall in response to pathogen infection. Moreover, it was demonstrated that increase in SA levels stimulates the expression of PR proteins (Ward *et al*., 1991; Uknes *et al*., 1992). Ward et al (1991) showed that in tobacco, SA stimulates the expression of the same PR proteins produced after tobacco mosaic virus infection. Uknes et al. (1992) also showed that PR-5 and β-1,3-glucanases are genes regulated by salicylic acid.

Sequence analysis of avocado fruit cDNA responsive to *C. gloeosporioides* infection also revealed genes with homology to genes conferring resistance in various plant species,

against a large spectrum of pathogens such as NBS/LRR resistance protein-like protein, CC-NBS resistance protein, catalase, endopeptidase, aspartic proteinase A1, cysteine protease, cysteine proteinase inhibitor, legumain-like protease, β-glucosidase, β -D-glucan exohydrolase, enhanced disease resistance 2 protein, esterase/lipase/thioesterase, lipoxygenase, pectinesterase inhibitor, disease resistance protein MLO. The genes that encode NBS–LRR proteins constitute the largest and most diverse family of resistance genes in plants (Wroblewski *et al.*, 2007). This family of plant resistance genes are hypothesised to function in a classical gene-for gene interaction in which pathogen elicitors are recognised by the C-terminal LRR receptor region and a hypersensitivity response leading to resistance is activated (Dangl and Jones 2001, Nimchuk *et al*., 2003).

In this study qPCR showed that catalase was up regulated in unharvested avocado fruits infected with *C. gloeosporioides* at day 1 and 3 post infection (Figure 5.5A). In the harvested avocado fruits, catalase was down regulated at day 1 following *C. gloeosporioides* infection, but at day 3 catalase expression was up regulated (Figure 5.5 B). The expression levels of catalase observed using qPCR were similar to the expression observed during quantification (transcript assembly and analysis) using Roche 454 technology. The induction of catalase which degrades H_2O_2 into water and oxygen is most probably related to the oxidative burst which is associated with the antioxidant defence system of plants. It was shown that catalases plays a crucial role in plant defence, aging, senescence and their activity is influenced by other important factors such as SA and nitric oxide (Yang and Poovaiah, 2001). Previously the study of Clark *et al*. (2000) demonstrated that together with ascorbate peroxidase, catalase is involved in the modulation of H_2O_2 which acts downstream of SA as a second messenger for the activation of plant defence responses. Subsequently during the characterisation and expression study of two catalase

genes from peach, it was found that indeed catalase plays an important role in the plant signal transduction pathway which leads to the development of SAR (Bagnoli *et al*., 2004). The induction of catalase in infected avocado fruits may suggest that the oxidative burst was initiated. In addition it can be proposed that catalase was highly expressed in the infected avocado fruits to neutralize the extra level of H_2O_2 which could lead to the damage of healthy cells.

β-glucosidases identified in infected avocado fruits are members of the family 1 glycoside hydrolases which catalyze the hydrolysis of the β-glucosidic bond between two carbohydrate moieties. β-glucosidases were shown by Escamilla-Trevino et al. (2006) to be involved in the formation of intermediates in cell wall lignification. They also play an important role in plant defence by activating some chemical defence compounds such as cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates (Halkier and Gershenzon, 2006). Many plant defence compounds are stored in a non-active glucosylated form and are bioactivated via hydrolysis of the glucosidic linkage catalyzed by β-glucosidases during pathogen attack (Morant *et al*., 2008). For instance it has been shown that β-glucosidases cleaved hydroxamic acid glucoside to release a toxic unstable aglucone which is decomposed to the reactive benzoxaxolinones toxic to invading pathogens (Nikus *et al*., 2001).

Aspartic proteinases, also identified in infected avocado fruits (Tables 5.2 and 5.5), belong to the class of endopeptidases that exhibit antimicrobial activity and are induced by both abiotic and biotic stress (Guevara *et al*., 2002) and have thus been involved in plant defence. A previous study showed that in potato cultivars, aspartic proteinase is induced in response to *Phytophthora infestans* infection (Guevara *et al*., 2005). Plant cysteine

proteases identified in avocado fruits (Table 5.2 and 5.3) were also shown to be involved in signalling pathways and in the response to biotic and abiotic stresses (Grudkowska and Zagdańska, 2005; Salas *et al*., 2008). Cysteine proteinase inhibitor is also induced in a plant's response to the invasion of pathogens in order to irreversibly inactivate the pathogen proteinases. In fact many plant pathogens produce active extracellular enzymes such as polygalacturonases, pectolyases and xylanases that play an important role in pathogenesis. For instance it was demonstrated that an active protease is secreted by the phytopathogenic fungus *Colletotrichum lindemithianum* when grown on plant cell walls or on artificial nutrient medium (Valueva and Mosolov, 2004). Likewise pectinesterase inhibitor identified in harvested avocado fruits during early response (Table5.5) besides their role in the regulation of fruit growth and cell wall extension is probably involved in the fruit defence mechanism against pathogens by inhibiting microbial pathogen pectinesterase/pectin methylesterases (Camardella *et al*., 2001).

In unharvested avocado fruits during early response (Table5.3), sequence analysis also revealed the expression of lipoxygenase which has been reported in several species to be induced during plant-pathogen interaction (Porta and Rocha-Sosa, 2002). Its function in plant defence system seems to be related to the synthesis of a number of compounds involved in signalling functions (Creelman and Mullet, 1997), with antimicrobial activity (Croft *et al*., 1993; Weber *et al*., 1999), or to the development of the hypersensitive response (Rustérucci *et al*., 1999). It was shown in tobacco that lipoxygenases are induced earlier upon infection by *Phytophthora parasitica* var *nicotianae* (Rancé *et al*., 1998).

Many others genes involved in stress response were also expressed in avocado fruits following infection with *C. gloeosporioides*. During stress, avocado fruit adapts its

metabolism and activates a large variety of physiological and biochemical changes to protect its cells.

Responses in unharvested versus harvested fruits

Most of the defence-related genes identified were expressed in unharvested avocado fruits, which could also explain why they developed only minor anthracnose symptoms. This observation was also made by Marimani (2011) who monitored the levels of antifungal diene and triene compounds ((Z, Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene and (Z, Z, E)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene) and observed a rapid increase in these compounds 1-2 days after inoculation of both harvested and unharvested Fuerte fruits with *C. gloeosporioides* 240 days after fruit set, followed by a decline within 7 days to uninoculated control or below control levels; however, levels in unharvested fruit were higher (Marimani 2011). Previous studies (Prusky *et al*., 1990; Prusky *et al*., 1991a; Prusky *et al*., 1991b; Domergue *et al*., 2000) have implicated these preformed antifungal compounds in the resistance of Fuerte avocado to fungal attack in unripe fruit and the development of quiescent infections. The level of these compounds is regulated either by its enhanced synthesis or by the inhibition of its breakdown. In the present study, the enzyme lipoxygenase, which was expressed in unharvested fruits during the early response to the fungal attack, is involved in catalysing the metabolism of the diene which leads to an increase in its levels (Prusky and Keen 1993). In addition, stearoyl-acyl-carrier-protein desaturase expressed in the infected fruits (Table 5.2) is involved in diene biosynthesis primarily by increasing the level of diene precursors, which can be converted into the antifungal diene (Leikin-Frenkel and Prusky, 1998; Madi *et al*., 2003). In the biosynthesis of diene, the desaturation step is also crucial for the creation of the multiple double bonds in the diene and triene compounds (Madi *et al*., 2003). These authors have demonstrated that

induction of desaturases, such as stearoyl-acyl-carrier-protein desaturase and lipid desaturation are an early component of the array of plant responses involved with defence against *C. gloeosporioides.* Our study has confirmed the activation of the enzymes involved with the biosynthesis of antifungal compounds in avocado, at the nucleotide level.

In conclusion, 454 sequencing and analysis of avocado fruits transcriptome and qPCR brought elucidation in the understanding of the molecular basis of defence response mechanisms and signalling networks activated in avocado fruit during *C. gloeosporioides* infection. Results of this study have given information on the genes involved in defence response and some defence signalling molecules such as SA and ET indicating that there is likely to be a synergistic action between SA and ET pathways in avocado fruit during response to *C. gloeosporioides* infection. These findings yielded a first insight into many of the genes expressed in this plant-pathogen interaction at the molecular level and could contribute to the design of effective disease management strategies to improve the resistance of avocado varieties to anthracnose disease.

CHAPTER 6

CONCLUSIONS

6.1 Importance of this study

This final chapter highlights the major results of this investigation and future investigations that could be attempted as a direct result of this study.

The anthracnose pathogen *C. gloeosporioides* is a major cause of disease in the avocado industry, causing significant economic losses, and the fruit of the Fuerte cultivar is particularly susceptible. Identification of genes differentially expressed in avocado during infection with *C. gloeosporioides* represents an important step towards understanding the plants defence responses and would assist in designing appropriate intervention strategies. To date, very little research and resources have been oriented towards understanding mechanisms governing avocado fruit response to fungal parasitic attack. The research outlined in this thesis revealed that avocado fruit is able to respond to *C. gloeosporioides* infection by exhibiting a sophisticated molecular system for pathogen recognition and by activating structural and biochemical defence mechanism. This research represents a very comprehensive analysis of the up- and down-regulation of defence, and other genes involved in aspects of general metabolism, in the avocado fruit when challenged with a fungal parasite. As such it makes a considerable contribution to the genetic data base of non-model but important crop plants and enhanced our understanding of the defence mechanisms and signalling networks involved in plant responses to microbial attack. The ESTs obtained constitute an important resource to scientists involved in understanding the molecular basis of resistance in economically important plants and could contribute to the design of effective alternative disease management strategies at a time when there is powerful movement away from continued use of agrochemicals for disease prevention. For instance, molecular markers for anthracnose resistance genes could be used in plant breeding to produce more anthracnose resistant avocado cultivars. The results of the study

provide a basal gene expression model system which could be used to underpin future gene expression studies involving infection of avocado fruit by other fungal parasites.

6.2 Conclusions

The major conclusions arising from each objective of this study are given as follows:

The first study undertaken was to assess the efficiency of the infection of *C. gloeosporioides* on pre and post-harvest Fuerte avocado fruit and to confirm the identity of the fungus. After fruit inoculation with the fungus, anthracnose symptoms were observed in both unharvested and harvested fruits from day 3 up to day 7 post inoculation. In both experiments, the confocal laser scanning microscope, and the scanning electron microscope showed that ellipsoid spores characteristic of *C. gloeosporioides* were first observed on the inoculated fruit surfaces, followed by fungal hyphae. Major infection symptoms were expressed in harvested fruits contrary to the unharvested fruits which presented few symptoms probably due to their high concentrations of antifungal compounds as suggested in previous studies (Prusky *et al*., 1990; Domergue *et al*., 2000; Marimani, 2011). The upregulation of the genes for stearoyl-acyl-carrier-protein desaturase and lipoxygenase involved in the biosynthesis of the antifungal compounds was observed in harvested fruit which confirms the synthesis of these compounds at the nucleotide level. .

The second objective of this study was to optimize RNA isolation from the skin and flesh of avocado fruit for subsequent complementary DNA (cDNA) transcription. An optimized modified cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol that allowed effective extraction of high-quality total RNA from the skin and flesh of the fruit without the use of phenol was developed. Total RNA obtained from this procedure was of

high quality and was undegraded and was successfully used for cDNA synthesis. The publication arising from this study is a first published report of effective total RNA extraction from avocado fruit.

Thirdly, the next investigation was the first transcriptome analysis of avocado fruit following infection with *C. gloeosporioides.* The whole uninfected and infected avocado fruits transcriptome was sequenced using high-throughput 454 sequencing to determine several candidate genes which are differentially expressed in avocado as a result of *C. gloeosporioides* infection. The single sequencing run produced 215 781 reads from the transcriptome, with an average sequence length of 252-300 nucleotides. A total of 70.6 MB of sequence data were generated and subjected to BLAST searches, from which about 1500 avocado genes encoding proteins functioning in signal transduction, transcriptional control, defence, stress, oxidative burst, metabolism, transportation processes and some genes with unknown functions were identified.

The final investigation of this study focuses on the identification of some avocado defence related genes differentially expressed in avocado during infection with *C. gloeosporioides* which was a crucial step towards understanding disease resistance mechanisms in Fuerte avocado fruit. Salicylic acid and ethylene which are well known defence signalling molecules were identified to be involved in the signalling networks activated in avocado fruit during infection. Subsequently, quantitative real-time PCR was employed to validate the gene expression profiles that had been done using bioinformatics tools. The expression of actin gene, GAPDH, catalase and endochitinase was measured. Then the expression profiles of these candidate resistance genes *C. gloeosporioides* at day 1 and 3 post infection

revealed that they were differentially expressed after infection in avocado fruits when compared to the uninfected sample.

6.3. A model of resistance/susceptibility for Fuerte avocado in response to *C. gloeosporioides* attack

A hypothetical mechanism of how avocado fruit reacts against *C. gloeosporioides* infection is summarized in Figure 6.1, based on the expression of key genes identified and predicted previously to be involved in plant defence response to pathogen attack. Avocado fruits defend themselves against *C. gloeosporioides* by a combination of structural and chemical defences. Avocado defence reaction was initiated by the recognition of *C. gloeosporioides*. The expression of LRR receptor-like protein kinase and LRR transmembrane protein kinase in the infected avocado fruits during early response, show that pathogen-associated molecular factors were released by *C. gloeosporioides* to bind to some host receptors. In addition, signal recognition particle receptor protein, mitogen activated protein kinase, salicylic induce protein kinase and salicylic acid-activated MAP kinase, demonstrated previously to function downstream of receptors or sensors after pathogen recognition, were expressed in the avocado fruits during early and late response (Madhani and Fink, 1998; Dangl and Jones 2001; Zhang and Klessig, 2001). These genes show that in avocado fruits, MAPK cascades form an important link that transduces extracellular stimuli into intracellular responses from extracellular receptors as seen in others plants (Inne, 2001; Zhang and Klessig, 2001). In addition MAPK is also involved in the activation of SIPK during plant response to a pathogen infection. In line with previous studies, successful pathogen recognition also leads to a production of reactive oxygen species (ROS), mainly H_2O_2 (Low and Merida, 1996; Mullineaux *et al*., 2000; Neill *et al*., 2001). Some genes

expressed during early response in infected avocado fruits such as peroxidase are known to be involved in the oxidative burst. So at the early stage of avocado defence response, the production of ROS occurs at the site of infection to activate the overall defensive response system such as lignification or cell wall strengthening, hypersensitive response and defence gene activation as seen in others plants (Wojtaszek, 1997; McDowell and Dangl, 2000). The up regulation of cytochrome P450 monoxygenases involved in the lignin biosynthesis showed that lignification is one of the physical mechanism used by avocado to react against *C. gloeosporioides* infection. In addition ion fluxes also occur following pathogen recognition to activate the defence response. The expression of calcium-dependent protein kinase and calcium ion binding protein in infected avocado fruits during early response show that Ca^{2+} may play a signal function downstream of the oxidative burst in the stimulation of avocado defence response as demonstrated previously in soybean (Levine, 1996).

Salicylic acid-binding protein 2 (SABP2) and ethylene responsive element binding protein expressed in infected avocado fruits are salicylic acid (SA) and ethylene (ET) receptors, respectively, which are required for the plant immune response (Riechmann and Meyerowitz, 1998; Kumar and Klessig, 2003). Knowing the function of SA and ET in the plant defence response, they were synthesized in infected avocado fruit to play an important signalling role in the activation of the defence mechanism as shown previously in other plants (Dong, 2001). ROS occurred at the site of infection in avocado to act synergistically with SA in a signal transduction to trigger and establish the systemic defences (Draper, 1997).

Based on the expression of various genes predicted to encode for transcription factors such as ethylene responsive transcription factor 3a, Myb proteins, WRKY transcription factor, MADS-box transcription factor, C3HL domain class transcription factor and bZIP transcription factor, it could be concluded that transcriptional activation of several defence related genes occurs in avocado fruit following signal transduction. During plant-pathogen interactions ET induces transcription of some defence related genes such as β-1, 3-glucanase and chitinase class I (Vögeli *et a*l., 1988; Wang *et al*., 2002).

Following *C. gloeosporioides* recognition by avocado, signal transduction and transcriptional activation which occurred led to the defence activation which resulted in the production of various avocado defence genes during early and late response to *C. gloeosporioides*. Some of those genes are known to be involved in fungal cell wall degradation such as, β-1, 3-glucanase and chitinase (Edreva, 2005); others in the degradation of pathogen proteins such as: cysteine proteinase inhibitor, pectinesterase inhibitor, cysteine protease, aspartic proteinases, endopeptidase and oligopeptidase A (Guevara *et al*., 2002; Camardella *et al*., 2001; Wroblewski *et al.*, 2007). Other resistant genes identified in infected avocado fruits during early and late response have been shown to exhibit antifungal activities: these include pathogenesis-related proteins (PR5 and PR 6) (Plymale *et al.*, 2007), defensin (PR12) (Tiryaki and Tunaz, 2004), ribonuclease III family protein (PR10), γ-thionin (PR 13) and peroxidase (PR 9) (Van Loon and Van Strien, 1999), NBS/LRR resistance protein-like protein, CC-NBS resistance protein (Wroblewski *et al.*, 2007), catalase (Yang and Poovaiah, 2001), β-glucosidase (Halkier and Gershenzon, 2006), disease resistance protein MLO, enhanced disease resistance 2 protein, esterase/lipase/thioesterase, and lipoxygenase (Rancé *et al*., 1998). SA was shown to be involved in the regulation of some of these defence genes like PR-5 and β-1,3-glucanases

(Uknes *et al*., 1992). SA and ET are involved in the activation of avocado defence responses following *C. gloeosporioides* attack by playing an important signalling role.

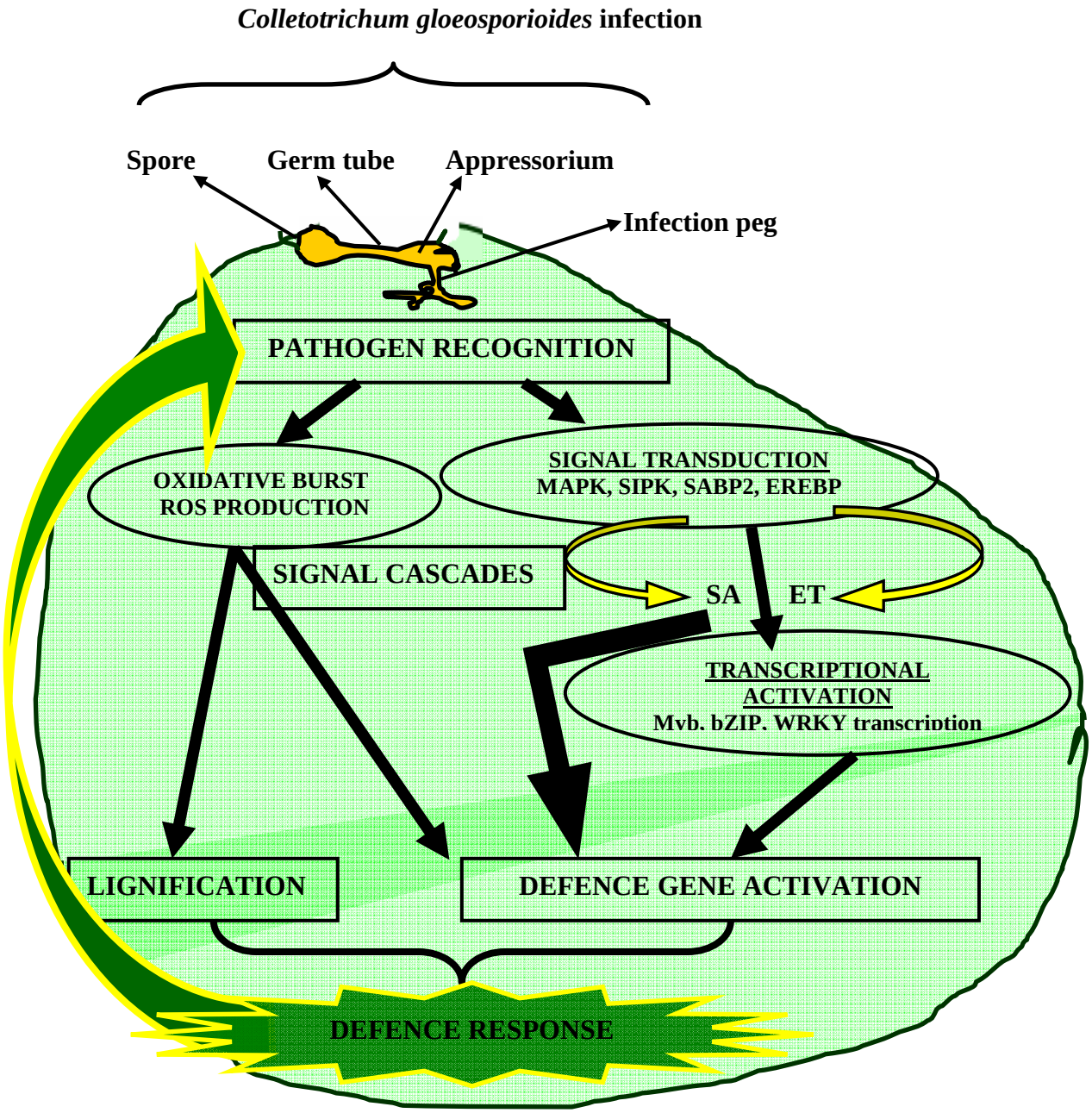


Figure 6.1. Overview of the mechanism response of avocado fruit to *C. gloeosporioides* attack.

6.4 Gene expression in unharvested versus harvested fruit

An important aspect of the experimental design of the study was to include both harvested and unharvested samples. Both unharvested and harvested avocado fruits react quickly to *C. gloeosporioides* attack by expressing a large spectrum of resistance genes most of which were expressed during early response to the fungus in both fruits (Table 6.1). However, there is not a strong overlap in the suites of genes expressed in harvested and unharvested fruits, and in the harvested fruit there is a delay in the induction of a number of important defence genes which suggests that the resistance to *C. gloeosporioides* infection is more efficient in unharvested fruits than in the harvested fruits. The differential induction of defence-related genes in harvested and unharvested fruits is probably linked to different signalling cascades involving differential expression of signal transduction genes in harvested versus unharvested samples (Tables 5.3 – 5.6). It is notable that in harvested fruit, important fungal cell wall-degrading enzymes, such as chitinases and glucanases are only activated during the late response when they are unlikely to have a major retardatory effect on the growth of the fungus: one could speculate that the fungus, unimpeded by inhibitory anti-fungal dienes and trienes, is also able to modulate the expression of host genes coding for enzymes involved in fungal cell wall degradation and so rapidly establish a foothold in host tissue. Based on their high expression in the infected fruits, catalase, class II chitinase, cysteine proteinase, endochitinase and endo-1,4-D-glucanase are the key resistances genes involved in avocados response to *C. gloeosporioides* infection. The biological functions of almost all these cited genes (Table 6.1) were discussed in chapters 4 and 5.

Table 6.1 Resistance genes expressed at a specific time point in the infected avocado fruits after *C. gloeosporioides* infection.

Time Point / Fruits	**Resistances genes expressed**	
	Early response (pool of 1, 4 and 24h)	**Late response** (pool of 3, 4, 5 and 7 dpi)
Unharvested	α-Glucosidase Asparaginyl Endopeptidase Catalase 2 Cysteine proteinase Defensin-like protein Disease resistance protein MLO Endochitinase Enhanced disease resistance 2 protein Esterase/lipase/thioesterase Enzymatic resistance protein Legumain-like protease Lipoxygenase Pathogenesis-related protein 6 Ribonuclease III family protein Serine carboxypeptidase family protein	β- Glucosidase like protein, Catalase CC-NBS resistance protein Endonuclease V family protein
Harvested	Accelerated cell death 1 Aspartic protease Aspartic proteinase 1 β-Amylase Catalase Catalytic/ hydrolase γ-Thionin Oligopeptidase A Pathogenesis related protein-5 Pectinesterase inhibitor Peroxidase Programmed cell death protein 5 Thaumatin-like protein	β-1,3-Glucanase-like protein Catalase Chitinase I,II Endochitinase Endo-1,4-D-glucanase Endonuclease/exonuclease/phosphatase Multidrug resistance protein 1, 2 Pectinesterase inhibitor

6.5 Future work

The present study has confirmed the presence of defence related genes in avocado expressed under C. *gloeosporioides* attack. As such it makes a considerable contribution to the understanding of avocado defence mechanisms. Further investigations could be done to study the cascades involved in this mechanism and the action of SA and ET in order to elucidate the signalling pathways involved in this plant pathogen interaction. From the genetic database obtained, many primers of defence related genes could be designed and used to test the expression profiles of some key defence genes in others avocado cultivars and various cultivar-fungal disease combinations (Cercospora spot or *Phytophthora*). This research has opened the door for a multitude of future molecular investigations such as AFLP-based transcript profiling (cDNA- AFLP) method and microarrays which could be done to study in more detail the expression profiles of some target genes. In addition many molecular markers could be designed from the resistance genes and used via conventional breeding methods or genetic engineering to improve the resistance of avocado cultivars to anthracnose and other fungal diseases.

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8. APPENDICES

Appendix 1. Sequences of selected candidate genes differentially expressed in avocado fruits in response to *C. gloeosporioides* presented in Table 4.1

SIGNAL TRANSDUCTION AND TRANSCRIPTION FACTORS

Gene 00001 leucine rich protein [Arachis hypogaea

AccccAGGgAAaCTCCGAATACAGGCCTAGATCGTTTGTACAGACAGACTTTTTGGGTGCTAAGATCCAAAGTCGAGAGGGAAACAGCCC
AGATCGTACGCTAAGGTCCCTAAGCAATCACTTAGTGGAAAAGGAAGTGATCGAGCGATGACAACCAGGAGGTGGGCTTGGAAGCAGCCA
TCCTTTGAAGAAAGCGTAATAGCTCACTGGTCTAGCTCCATGGCACCGAAAATGTATCAGGGCTCAAGTGATTCACCGAAGCGACGAGAC
CTTGAAAGCTGCTTTTTCAAGTGTCAGTAGCGGGACGTTCTGTCAATCGGGGAAGGTTTTTGGTGACAACACCTGGAGATATCAGAAGTG
AGAATGCTGACATGAGTAACGATAAATCCTGTGAAAAACACGATCGCCTGCCAGTGGAAGGCTTTCTGCGTTCAGTCAATCTACGCAGAG
TGAATCGGTCCCTAAGGAACCCCCGAAAGGGCTGCCGTCCGATGGGTACACGAAAGTGACGAAGTTGCTTTGACTACTGAACCATGCCTG
TCTGTTGGAGCGAATTGGATGATCGGGCCGAGGGCTGCCCCCTCTTCCCCTCGCTCTCCTTTCCCTAATATGAACCTTGAGTCATCAAAG
CCTTTCTGACTCGGCCTGGCCCGGTCGCCCTACGCTACTGGAGCTGAAAAAGGCTAGCGCGCCAGCAAACGAAGGAGCAAGTGTAGGCGA
CCCCAAGGGAGCAAACGAAGGCTGAGAGCCATCTGGCTCGAAAGCCGGAGTGCGCTAGCGCGCACTGGAGTTTTCTTCGCTTGTTCTGGC
CGGAGTGCGCAGGAGCGCACTGGAGTTTTCGGAGCTGCTTGTTGCTTCAAAAGGCGAAACTCTCGTCTTTGGCGACCTAATAACTGACGG
GGCGGACACTTTTTGTTATCTAAAGGCGAAGACTCTGAAGTGGGCGAACACTCGGCCCAGCGGGCGAACATGCCGGGCTCCGGCTCCGCG
CACCTGCTGACACCTTTCGAAGCACTCTTTATTCAACCGCAGTCGTGTTGCGTCACGAGTCTACAAGCCTTTCTCATTTCAGTGCTCGCC
TTTTTAATCTTCAGTAGGGGCCTTTAGTCTTTTGATTAGAGTAGGGGTCGCGAGAGAGCAGAGCGTACCGCCCTGCCATAGTCGCGAGTC
TGTTTATAGTCGCGACTGTTGTCATAGTCAACAAGGTTGAAACTTCCTGGAAAAAACTTCGAATTGGGAGGGCGATCCTCCCGGTGAACT
GACCGTACCCCAAACCGACACAGGTGAACAAGTAGAGTATACTAGGGCGCTTGAGAGAACCATGTCGAAGGAACTCGGCAAAATGACCCC
GTAACTTCGGGAGAAGGGgTGCTCTCCTATCTTTtGATTAGGAAaGCGGCACATACCAGGGGGTAGCGACTGTTtATTAAAAACACAGGA
CtCTGCTAAGTGGTAACACGATGTATAGAGTCTGACA

Gene 00609 transcription factor WRKY36 [Physcomitrella patens subsp. Patens]

AGAAGACCCTGTTGAGCTTGACTCTAGTCCGACTTTGTGAAATGACTTGAGAGGTGTAGGATAAGTGGGAGCCGTCTCGGCGGCGCAAGT
GAAATACCACTACTTTTAACGTTATTTTACTTATTCCGTGAGTCGGAGGCGGGGCATCGCCCCTCCTTTTGGCCCAAGGCCCGCCTCGGC
GGGCCGATCCGGGCGGAAGACATTGTCAGGTGGGGAGTTTGGCT

CYTOCHROME PROTEINS

Gene 00132 cytochrome P450 monooxygenase [Pyrus communis]

AAGACAgcaggACGGTGGTCATGGAAGTCGaaaTCCGCTAAggAGTGTGTAACAACTCACCTGCCGAATCAACTAGCCCCGAAAaTGGAT
GGCGCTGAAGCGCGCGACCCATACCCGGCCGTCGGGGCGATTGCCATGCCCCGATGAGTAGGAGGGCGCGGCGGCTGCTGCAAAACCCGG
GGCGCGAGCCCGGGCGGAGCGGCCGTCGGTGCGGATCTTGGTGGTAGTAGCAAATATTCAAATGAGAACTTTGAAGGCCGAAGAGGGGAA
AGGTTCCATGTGAACGGCACTTGCACATGGGTTAGTCGATCCTAAGGGACGGGGGAAGCCCGTCCGAGAGCGTGCAGCACGCGAGCTCCG
AAAGGGAATCGGGTTAAAATTCCTGAACCGGGACGTGGCGGCTGACGGCAACGTTAGGGAGTCCGGAGACGTCGGCGGGGgCCTCGGGAA
GAGTTATCTTTTCTGTTTAACAGCCTGCCCACCCTGGAAACGGCTCAGCCGGAGGTAGGGTCCAGCGGCTGGAAGAGCACCGCACGTCGC
GTGGTGTCCGGTGCGCCCCCGGCGGCCCTTGAAAATCCGGAGGACCGAGTGCCTCCCACGCCCGGTCGTACTCATAACCGCATCAGGTCT
CCAAGGTGAACAGCCTCTGGTCGATGGAACAATGTAGGCAAGGGAAGTCGGC

Gene 00144 Cytochrome c oxidase subunit 5B, mitochondrial precursor

GCCGCCGCGCCCTCCTACTCATCGGGGCATGGCAATCGCCCCGACGGCCGGGTATGGGTCGCGCGCTTCAGCGCCATCCATTTTCGGGGC
TAGTTGATTCGGCAGGTGAGTTGTTACACACTCCTTAGCGGATTTCGACTTCCATGACCACCGTCCTGCTGTCTTAATCGACCAACACCC
TTTGTGGGTTCTGGGTTAGCGCGCAGTTGGGCACCGTAACCCGGCTTCCGGTTCATCCCGCATCGCCAGTTCTGCTTAACCAAAAATGGC
CCACTTGGAGCTCTCGATTCCGTGGCGCGGCTCAACGGAGCAGCCGCGCCGTCCTACCTATTTAAAGTTTGAGAATAGGTCGAGGGCGTT
GCCGCCCCCGATGCTCTAATCATTGGCTTTACCCGATAGAACTCGCCCGCGGGCTCCAGCTATCCTGAGGGAAACTTCGGAGGGAACCAG
CTACTAGACGGTTCGATTAGTCTTTCGCCCCTATACCCAAGTCAGACGAACGATTTGCACGTCAGTATCGCTGCGGGCCTCCACCAGAGT
TTCCTCTGGCTTCGCCCCGCTCAGGCATAGTTCACCATCTTTCGGGTCCCGACAGGCATGCTCTCACTCGAACCCTTCTCAGGAGATCTA
GGTCGGTCGGCGGTGCACCCCGCTGGGGGGATCCCGCC

Gene 00301 cytochrome P450 like_TBP [Nicotiana tabacum]

GTCAAGCTCAACAGGGTCTTCTTTCCCCGCTGATTCTGCCAAGCCCGTTCCCTTGGCTGTGGTTTCGCTGGATAGTAGACAGGGACAGTG
GGAATCTCGTTAATCCATTCATGCGCGTCACTAATTAGATGACGAGGCATTTGGCTACCTTAAGAGAGTCATAGTTACTCCCGCCGTTTA
CCCGCGCTTGGTTGAATTTCTTCACTTTGACATTCAGAGCACTGGGCAGAAATCACATTGCGTGAGCATCCGCAGGGACCATCGCAATGC
TTTGTTTTAATTAAACAGTCGGATTCCCCTTGTCCGTACCAGTTCTGAGTCGACTGTTCGACGCCCGGGGAAGGCCCCCGAGGGAGCCGT
TCCCGGTCCGTCCCCCGGCCGGCACGCGGCGACCCGCTCTCGCCGCGGGAGCAGCTCGAGCAGTCCGCCGACAGCCGACGGGTTCGGGAC
AGGGACCCCCGTGCCCAGCCCTCAGAGCCAATCCTT

Gene 00475 cytochrome P450-like TBP protein [Lilium longiflorum]

ACTTATCCTACACCTCTCAAGTCATTTCACAAAGTCGGACTAGAGTCAAGCTCAACAGGGTCTTCTTTCCCGCTGATTCTGCCAAGCCGT
TCCCTTGGCTGTGGTTTCGCTGGATAGTAGACAGGGACAGTGGGAATCTCGTTAATCCATTCATGCGCGTCACTAATTAGATGACGAGGC
ATTTGGCTACCTTAAGAGAGTCATAGTTACTCCCGCCGTTTACCCGCGCTTGGTTGAATTTCTTCACTTTGACATTCGGAGCACTGGGCA
GAAATCACATTGCGTGAGCATCCGCAGGGACCATCGCAATGCTTTGTTTT

Gene 00614 cytochrome P450 like_TBP [Nicotiana tabacum]

CCCGGTCGTACTCATAACCGCATCAGGTCTCCAAGGTGAACAGCCTCTGGTCGATGGAACAATGTAGGCAAGGGAAGTCGGCAAAACGGA
TCCGTAACCTCGGGAAAAGGATTGGCTCTGAGGGCTGGGCACGGGGGTCCCTGTCCCGAACCCGTCGGCTGTCGGCGGACTGCTCGAGCT
GCTCCCGNGGCGAGAGCGGGTCGCCGCGTGCC

HYPOTHETICAL PROTEINS

Gene 00082 hypothetical protein [Arabidopsis thaliana]
CAGGTGGGGAGTTTGGCTGGGGCGGCACATCTGTTAAAAGATAACGCAGGTGTCCTAAGATGAGCTCAACGAGAACAGAAATCTCGTGTG
GAACAAAAGGGTAAAAGCTCGTTTGATTCTGATTTCCAGTACGAATACGAACCGTGAAAGCGTGGCCTATCGATCCTTTAGACCTTCGGG
ATTTGAAGCTAGAGGTGTCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTT
CGATGTCGGCTCTTCCTATCATTGTGAAGCAGAATTCACCAAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGGTTTAGAC
CGTCGTGAGACAGGTAGTTTACCCTACTGATGATCGTGCCACGATAGTAATTCAACCTAGTACGAGAGGAACCGTTGATTCGCACAATTG
GTCATCGCGCTTGGTTGAAAAGCCAGTGGCGCGAAGCTACCGTGCGTCGGATTATGACTGAACGCCTCTAAGTCAGAATCCAAGCTAAAC
ACGCGGCGCCCGCGCCCGCCGCCCGCTTGCCGACCCTCAGTAGGGGCCTCTTTGGCCCCCATGGGCCCGTGCCGTTGGTGAAGCCCCCGC
GGCGGACGAGCCGCGACGGGCCGCCTTGAAGATTAATTTCGACCGGACGGCGGGCAGAATCCTTTGCAGACGAATCTGTGCGACACTGGG
GCTGAATCTCAGTGGATCGTGGCAGCAAGGCCACTCTGCCACTTACAAT

Gene 00213 hypothetical protein LOC100382981 [Zea mays]
GGGCCTTGGGACCAAAAGGAGGGGCGATGCCCCGCCTCCGACTCACGGAATAAGTAAAATAACGTTAAAAGTAGTGGTATTTCACTTGCG
CCGCCGAGACGGCTCCCACTTATCCTACACCTCTCAAGTCATTTCACAAAGTCGGACTAGAGTCAAGCTCAACAGGGTCTTCTTTCCCCG
CTGATTCTGCCAAGCCCGTTCCCTTGGCTGTGGTTTCGCTGGATAGTAGACAGGGACAGTGGGAATCTCGTTAATCCATTCATGCGCGTC
ACTAATTAGATGACGAGGCATTTGGCTACCTTAAGAGAGTCATAGTTACTCCCGCCGTTTACCCGCGCTTGGTTGAATTTCTTCACTTTG
ATTCAGAGCACTGGGCAGAAATCACATTGCGTGAGCATCCGCAGGGACCATCGCAATGCTTTGTTTTAATTAAACAGTCGGATTCCCCTT
GTCCGTACCAGTTCTGAGTCGACTGTTCGACGCCCGGGGAAGGCCCCCGAGGGAGCCGTTCCCGGTCCGTCCCCCGGCCGGCACGCGGCG
ACCCGCTCTCGCCGCGGGAGCAGCTCGAGC

Gene 00264 hypothetical protein SORBIDRAFT [Sorghum bicolour]
AGAACCCACAAAGGGTGTTGGTCGATTAAGACAGCAGGACGGTGGTCATGGAAGTCGAAATCCGCTAAGGAGTGTGTAACAACTCACCTG
CCGAATCAACTAGCCCCGAAAATGGATGGCGCTGAAGCGCGCGACCCATACCCGGCCGTCGGGgCGATTGCCATGCCCcGATGAGTAGGA
GGGCGCGGCGGCTGCTGCAAAaCCCGGGGCGCGAGCCCGGGCGGAGCGGCCGTCGGTGCGGATCTTGGTGGTAGTAGCAAATATTCAAAT
GAGAACTTTGAAGGCCGAAGAGGGGAAAGGTTCCATGTGAACGGCACTTGCACATGGGTTAGTCGATCCTAAGGGACGGGGGAAGCCCGT
CCGAGAGCGTGCAGCACGCGAGCTCCGAAAGGGAATCGGGTTAAAAATTCCTGAACCGGGACGTGGCGGCTGACGGCAACGTTAGGGAGT
CCGGAGACGTCGGCGGGGGCCTCGGGAAGAGTTATCTTTCTGTTTAACAGCCTGCCCACCcTGGAAAC

DEFENCE/STRESS

Gene 00654 catalase [Arabidopsis thaliana]
ATTCTCCTGAATGTGAGATTTTGGGTTTGGTTTGAAGGCATGGATCACATCGGGGAACTTCATCCCATCGCGAATGAAGAACACGGGGAA
GTTGTTTCCAACCAGATCGAAATTTCCCTCTCTAGTGTAGAACTTAATGGCGAAGCCTCGAGGGTCTCTGAT

Gene 00509 endo-1,4-beta-glucanase [Malus x domestica]
AaTACGATGgAGgCaGCAGCCAgTgCGGCTGcTGTCTCTGCTGCAACATCAGAGCCTGGATTCTGTGTAGACACTTTGTAAACGTTGCGA
GGTGTATCCATGTCTTCTGGCCTCTCCCAGCATCGGTGATCCGCGTTGGGCTCTCCTACTTGGACATATAAAGAATTGGAAGTGGCTGTA
GAGGCCTTGAGTAGATAATCCGTGCTCCAGCGAAGGGCCGCTCTTGCATTCTCTACCTGCTCCGGCATCAaGCAGCCGAACTCAATGATA
CCCCATGCCAGCATTGTCgtAGTg

Gene 00405 endochitinase [Persea americana]
GGAGCCATAGCTGACTCCAAGCAAGTCACAGTACCTCTTGTAGAAGCCAATGCGGTCTGCCACCTTGTCATTGAATCCCTTGCCGCATTC
GATCCCACCGTTGATGATGTTGGTGATGACCCCATATCCTGGAAGGCGGCCCGCCGCCCTGTCAGCAGCAGAAGGCGTCCATCGACCGGT
GATCACGTTGTGGCATGACGGTTTGGGAGATTGTGGGGTCATCCAGAACCAGAGGGCTGTTTTGAAGGAGATGACTGGATCTGTGGCCAC
AGCATCTGGATTGTTGATAAGATCATAGCCTATGGCTCTTCCTGCTGGACCATAGTTGTAGTTGTATGAAATCTGAATGGTCCACGCCCG
TAGTATTTTT

Gene 00308 heat shock protein [Cucumis sativus]
GCAGAATGGCCAGAGGATAACTTTCCCTGGAGAGGCTGATGAGGCGCCGGACACCGTCACAGGTGATATAGTCTTTGTCCTACAACAGAA
GGAGCACCCCAAGTATAAGCGCAAGGGAGATGATCTATTTGTGGAGCACACCCTGTCTCTAACTGAGGCACTTTGTGGATTCCATTTTGT
ACTGACCCACCTTGATAACAGGCAGCTGCTGATTAAGTCGAATCCTGGGGAGGTTGTCAAGCCAGATCAGTTCAAGGCAATAAACGATGA
AGGTATGCCAATGTACCAGCGGCCCTTCATGAAGGGGAAACTATACATCCATTTCACAGTGGATTTCCCTGATTCACTTACTCCAGACCA
ATGCAAGGCTTTGGAGAAAGTCTTGCCTCCGAGGGCCTCAGCCCAGCTTACGGATATGGAACTGGATGAATGCGAAGAGACGACGCTGCA
TGATGTGAACATTGAGGAAGAGATGAG

Gene 00653 Metallothionein-like protein [Arabidopsis thaliana]
ATTGTGAATTGCAGAGTTAGTGTCGAGAGAGAGAGAATGTCTTGCTGTGGTGGAAACTGTGGCTGTGGATCTGGTTGCAAGTGTGGAAGT
GGATGTGGAGGATGCAAGATGTATCCTGATCTGTCCTTCTCTGGAGAAAGCAGCACCACTGAAAGCCTGATCA

SENESCENCE ASSOCIATED PROTEINS

Gene 00091 putative senescence-associated protein
AAGGCCCGCCTCGGCGGGCCGATCCGGGCGGAAGACATTGTCAGGTGGGGAGTTTGGCTGGGGCGGCACATCTGTTAAAAGATAACGCAG
GTGTCCTAAGATGAGCTCAACGAGAACAGAAATCTCGTGTGGAACAAAAGGGTAAAAGCTCGTTTGATTCTGATTTCCAGTACGAATACG
AACCGTGAAAGCGTGGCCTATCGATCCTTTAGACCTTCGGGATTTGAAGCTAGAGGTGTCAGAAAAGTTACCACAGGGATAACTGGCTTG
TGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATTGTGAAGCAGATTCACCAAGTGTTGGA
TTGTTCACCCACCAATAGGGAACGTGAGCTGGGATTAGACCGTCGTGAGACAGGTTAGTTTACCCTACTGATGATCGTGCCACGATAGTA
ATTCAACCTAGTACGAGAGGAACCGTTGATTCGCACAATTGGTCATCGCGCTTGGTTGAAAGCCAGTGGCGCGAAGCTACCGTGCGTCGG
ATTATGACTGAACGCCTCTAAGTCAGAATCCAAGCTAAACACGCGGCGCTCGCGCCCGCCGCCCGCTTGCCGACCCTCAGTAGGGGCCTC
TTTGGCCCCCATGGGCCCGTGCCGTTGGTGAAGCCCCCGCGGCGGACGAGCCGCGACGGGCCGCCTTGAAGATTAATTTCGACCGGACGG
CGGGCAGAATCCTTTGCAGACGAATCTGTGCG

Gene 00225 senescence-associated protein [Cupressus sempervirens
GAATACGAACCGTGAAAGCGTGGCCTATCGATCCTTTAGACCTTCGGgATTTGAAGCTAGAGGTGTCAGAAAAGTTACCACAGGGATAAC
TGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATTGTGAAGCAGAATTCACCAA
GTGTTGGATTGTTCACCCACCAATAGGGAACgTGAGCTGGGaTTAGACCGTCGTGAGACAGGTtAGTTTtACCcTAcTGATGATCGTGCc
ACGATAGTAaTTCAACcTAGTACGAGAGGAACCGTTGATTCGCACAATTGGTCATCGCGCTTGGTTGAAAGCCAGTGGCGCGAAGCTACC
GTGCGTCGGATTATGACTGAACGCCTCTAAGTCAGAATCCAAGCTAAACACGCGGCGCCcGCGCCCGCCGCCCGCTTGCCGACCCTCAGT
AGGGGCCTCTTTGGCCCCCATGGGCCCGTGCCGTTGGTGAAGCCCCCGCCGGCGGACGAGCCGCGACGGGCCGCCTTGAAGATTAATTTC
GACCGGACGGCGGGCAGAATCC

Gene 00407 putative senescence-associated protein [Pisum sativum]
ACCCACcAATAGGGAACgTGAgCTGGGATTAGACCGTCGTGAGACAGGTTAGTTTACCTACTGATGATCGTGCCACGATAGTAATTCAAC
CTAGTACGAGAGGAACCGTTGATTCGCACAATTGGTCATCGCGCTTGGTTGAAAGCCAGTGGCGCGAAGCTACCGTGCGTCGGATTATGA
CTGAACGCCTCTAAGTCAGAATCCAAGCTAAaCACGCGGCGCTCGCGCCCGCCGCCCGCTTGCCGACCCTCAGTAGGGGCCTCTTTGGCC
CCCATGGGCCCGTGCCGTTGGTGAAGCCCCCGCGGCGGACGAGCCGCGACGGGCCGCCTTGAAGATTAATTTCGACCGGACGGCGGGCAG
AATCCTTT

Gene 00464 putative senescence-associated protein
AAGGATTCTGCCCGCCGTCCGGTCGAAATTAATCTTCAAGGCGGCCCGTCGCGGCTCGTCCGCCGCGGGGGCTTCACCAACGGCACGGGC
CCATGGGGGCCAAAGAGGCCCCTACTGAGGGTCGGCAAGCGGGCGGCGGGCGCGAGCGCCGCGTGTTTAGCTTGGATTCTGACTTAGAGG
CGTTCAGTCAGTAATCCGACGCACGGTAGCTTCGCGCCACTGGCTTTCAACCAAGCGCGATGACCAATTGTGCGAATCAACGGTTCCTCT
CGTACTAGGTTGAATTACTATCGTGGCACGATCATCAGTAGGGTAAAACTAACCT

Gene 00473 putative senescence-associated protein [Lilium longiflorum]
GATTCGGCAGGTGAGTTGTTACACACTCCTAGCGGATTTCGACTTCCATGACCACCGTCCTGCTGTCTTAATCGACCAACACCCTTGTGG
GTTCTGGGTTAGCGCGCAGTTGGGCACCGTAACCCGGCTTCCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGCCCACTTGG
AGCTCTCGATTCCGTGGCGCGGCTCAACGGAGCAGCCGCGCCGTCCTACCTATTTAAAGTTTGAGAATAGGTCGAGGGCGTTGCGCCCCC
GATGCCTCTAATCATTGGCTTTACCCGATAGAACTCGCCCGCGGGCTCCAG

Gene 00282 senescence-associated protein [Lilium longiflorum]
AAaGTTACCACAGGGATAACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCAT
TGTGAAGCAGAATTCACCAAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGGTTTAGACCGTCGTGAGACAGGTTAGTTTt
ACCCTACTGATGATCGTGCCACGATAGTAATTCAACCTAGTACGAGAGGAACCGTTGATTCGCACAATTGGTCATCGCGCTTGGTTGAAA
aGCCAGTGGCGCGAAGCTACCGTGCGTCGGATTATGACTGAACGCCTCTAAGTCAGAATCCAAGCTAAACACGCGGCGccGCGCCCGCCG
CCCGCTTGCCGACCCTCAGTAGGGGCCTCTTTGGCCCCCATGGGCCCGTGCCGTTGGTGAAGCCCCCGCGGCGGACGAGCCGCGACGGGC
CGCCTTGAAGATTAATTTCGACCGGACGGCGGGCAGAATCCTTTG

Gene 00496 putative senescence-associated protein [Pisum sativum]
CAAaGGATTCTGCCCGCCGTCCGGTCGAAATTAATCTTCAAGGCGGCCCGTCGCGGCTCGTCCGCCGCGGGGGCTTCACCAACGGCACGG
GCCCATGGGGGCCAAAGAGGCCCCTACTGAGGGTCGGCAAGCGGGCGGCGGGCGCGAGCGCCGCGTGTTTAGCTTGGATTCTGACTTAGA
GGCGTTCAGTCATAATCCGACGCACGGTAGCTTCGCGCCACTGGCTTTtCAACCAAGCGCGATGACCAATtGTGCGAATCAACGGTTCCT
CTCGTACTAGGTTGAATTACTATCGTGGCACGATCATCAGTa

Gene 00504 senescence-associated protein [Picea abies]
GGAAGACATTGTCAGGTGGGGAGTTTGGCTGGGGCGGCACATCTGTTAAAAGATAACGCAGGTGTCCTAAGATGAGCTCAACGAGAACAG
AAATCTCGTGTGGAACAAAAGGGTAAAAGCTCGTTTGATTCTGATTTCCAGTACGAATACGAACCGTGAAAGCGTGGCCTATCGATCCTT
TAGACCTTCGGGATTTGAAGCTAGAGGTTGTCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTG
CTTTTTGATCCTTCGATGTCGGCTCTTCCTATCAATTCTGATTTCCAGTACGAATACGAACCGTGAAAGCGTGGCCTATCGATCCTTTAG
ACC

Gene 00617 putative senescence-associated protein [Pisum sativum]
GCAAAGGATTCTGCCCGCCGTCCGGTCGAAATTAATCTTCAAGGCGGCCCGTCGCGGCTCGTCCGCCGCGGGGGCTTCACCAACGGCACG
GGCCCATGGGGGCCAAAGAGGCCCCTACTGAGGGTCGGCAAGCGGGCGGCGGGCGCGAGCGCCGCGTGTTAGCTTGGATTCTGACTTAGA
GGCGTTCAGTCATAATCCGACGCACGGTAGC

METABOLISM

Gene 00237 fructose-bisphosphate aldolase [Persea Americana]
ACTGTTCCTTCCAATAGGACATGGTGGTCGTTGAGTGCCTtGTAGATCGCAGCCAGAACCCTCTCTGTTATGTCAGCGCACTTTTCAATG
CTATGAGGCCcATCAACTAGAATCTCAGGCTCAATAATTGGGACGAGTCCATTCTCCTGGCAGATGATGGCACAACGGGCCAACCCATTT
GCACTCTCGTTGATTGCCAACTGAGAAGGTTCAGTGGGGCCGATCTTGAGCACGGCTCTCCACTTGCCAAAACGGGCTCCAGTCGAGTAG
TACTGTTGGCAACGCTGCGCAAGGCCATCAAGGCCTTGGGTGGCGATCTCGCCATTGATGCCAGCAATATCGACATAGCCCGTGTCAACC
TTGATGCCCGGGAGGACACCTCCTTCCTTGAGGATCTCGACAAaGGGCTTTCCAGATGATGACTTCTGGTAGAGGgTCTCCTCAAAGAGG
ATCGCACCACTGAGGTATTGCAGGGCGTTAGGGGTACAAAAGAGGAGCTCACGGAGAGCCCTACGATTGGATTCGACATTCTCTACaTTG
ACGCtGG

PHOTOSYNTHESIS AND CELL STRUCTURE

Gene 00447 Chloroplast hypothetical protein [Zea mays subsp. mays]
CTTCCATTTAGCAGCACCTTAGGATGGCATAGCCAACACATTAATGGCGAGGTTCAAACGAGGAAAGGCTTACGGTGGATACCTAGGCAC
CCAGAGAGGAGGAAGGGCGTAGTAAGCGACGAAATGCTTCGGGAAGTTGAAAATAAGCATAGATCCGGAGATTCCCGAATAGGTCAACCT
TTCGAACTGCTGCTGAATCCATGGGCAGGCAAGAGACAACCTGGCGAACTGAAACATCTTAGTAGCCAGAGGAAAAGAAAGCAAAGCGAT
TTCCGTAGTAGCGGCGAGCGAAATGGGAGCAGCCTAAACCGTGAAACGAGGTTGTGGGAGAGCAATACA

Gene 00665 photosystem I assembly protein Ycf4 [Zea mays]
GTAGCGGTTATGATCGATTCGATAGAAAAGAAGGAATAGTGTGTATTTTTCGTTGGGGATTTCCTGGAATAAATCGTCGCATCTTCCTTC
GATTCCTTATGAGAGAGATTCAATCGATCAGAATGGAAGTTAAAGAGGGTCTTTATCCT

ELECTRON TRANSPORT AND EXPRESSED PROTEINS

Gene 00453 Aquaporin NIP6-1 [Medicago truncatula]
CTTCACAGACTGCGTTCGTTCTCTCGAGCTATCTCTCTGTCTCTGCAACTCCAAGAAGAAGGAAATGGGAGAGGAAGAGAAGAAACCGGA
AGAGAAGAAGGAGGAAGAAGCGAAGAAGCCAGAGGAAGCGAAGAAaGAGGAGGAGAAGAAGGAAGAGAAAGCAGGCGAGGAAGCCAAGGA
GGAGAAGAAGGAGGAAAaGGAAGCTCCTCCTCCTCCGCCTCCTGAAGAGTTCGAATACAGAGTCTACATGCATTGTGAAGCTTGTGCGAA
GAAGGTGAAGCGATCTCTGCGTGGATTCGCAGGAGTGGTAGATGTGATTAcGGATtGCAAGGCAC

Gene 00037 Expressed protein [Arabidopsis lyrata subsp. lyrata]
CCAGAAcccACaaAGGGTGTTGGTCGATTAAGACAGCAGGACGGTGGTCATGGAAGTCGAAATCCGCTAAGGAGTGTGTAACAACTCACC
TGCCGAATCAACTAGCCCCGAAAATGGATGGCGCTGAAGCGCGCGACCCACACCCGGCCGTCGGGGCGATTGCCATGCCCCGATGAGTAG
GAGGGCGCGGCGGCTGCTGCAAAACCTGGGGCGCGAGCCCGGGCGGAGCGGCCGTCGGTGCGGATCTTGGTGGTAGTAGCAAATATTCAA
ATGAGAACTTTGAAGGCCGAAGAGGGGAAAGGTTCCATGTGAACGGCACTTGCACATGGGTTAGTCGATCCTAAGGGACGGGGGAAGCCC
GTCCGAGAGCGTGCAGCACGCGAGCTCCGAAGGGAATCGGGTTAAATTCCTGAACCGGGACGTGGCGGCTGACGGCAACGTTAGGGAGTC
CGGAGACGTCGGCGGGGGCCTCGGGAAGAGTTATCTTTTCTGTTTAACAGCCTGCCCACCCTGGAAACGGCTCAGCCGGAGGTAGGGTCC
AGCGGCTGGAAGAGCACCGCACGTCGCGTGGTGTCCGGTGCGCCCCCGGCGGCCCTTGAAAATCCGGAGGACCGAGTGCCTCCCACGCCC
GGTCGTACTCATAACCGCATCAGGTCTCCAAGGTGAACAGCCTCTGGTCGATGGAACAATGTAGGCAAGGGAAGTCGGCAAAACGGATCC
GTAACCTCGGGAAAAGGATTGGCTCTGAGGGCTGGGCACGGGGGTCCCTGTCCCGAACCCGTCGGCTGTCGGCGGACTGCTCGAGCTGCT
CCCGCGGCGAGAGCGGGTCGCCGCGTGCCGGCCGGGGGACGGACCGGGAACGGCTCCCGTCGGGGGCCTT

Gene 00043 Expressed protein [Arabidopsis lyrata subsp. lyrata]
GCTAAGGAGTGTGTAACAACTCACCTGCCGAATCAACTAGCCCCGAAAATGGATGGCGCTGAAGCGCGCGACCCATACCCGGCCGTCGGG
GCGATTGCCATGCCCCGATGAGTAGGAGGGCGCGCGGCTGCTGCAAAACCCGGGGCGCGAGCCCGGGCGGAGCGGCCGTCGGTGCGGATC
TTGGTGGTAGTAGCAAATATTCAAATGAGAACTTTGAAGGCCGAAGAGGGGAAAGGTTCCATGTGAACGGCACTTGCACATGGGTTAGTC
GATCCTAAGGGACGGGGGAAGCCCGTCCGAGAGCGTGCAGCACGCGAGCTCCGAAAGGGAATCGGGTTAAATTCCTGAACCGGGACGTGG
CGGCTGACGGCAACGTTAGGGAGTCCGGAGACGTCGGCGGGGGCCTCGGGAAGAGTTATCTTTCTGTTTAACAGCCTGCCCACCCTGGAA
ACGGCTCAGCCGGAGgTAGGGTCCAGCGGCTGGAAGAGCACCGCACGTCGCGTGGTGTCCGGTGCGCCCCcGGCGGCCCTTGAAAaTCCG
GAGGACCGAGTGCCTCCCACGCCCGGTCGTACTCATAACCGCATCAGGTCTCCAAGGTGAACAGCCTCTGGTCGATGGAACAATGTAGGC
AAGGGAAGTCGGCAAAACGGATCCGTAACTCGGGAAAAGGATTGGCTCTGAGGGCTGGGCACGGGGGTCCCTGTCCCGAACCCGTCGGCT
GTCGGCGGACTGCTCGAGCTGCTCCCGCGGCGAGAGCGGGTCGCCGCGTGCCGGCCGGGGGACGGACCGGGAACGGCTCCCTCGGGGGCC
TTCCccGGGCgTCGAACaGTCGaCTCAGAACtGgTACGgACAAGGG

Gene 00532 NAD-dependent sorbitol dehydrogenase 3 [Malus x domestica]
CCTTTCCAACAGACAAACGATAATCATCGACATCAACAATAACAATTCGTGGAGCTCCAAAGGCACGGGCTGCGAGCATCATAATAAGGC
CGATAGGACCTGCTCCAACGATCAAAACATTTGTCTCTGGACCCACATTTGCACGACGACAAGCATGAATTCCGACACTCAAGGGCTCAC
ACATAGCCCCTTCCTCCAAGCTAACATTCTCAGGCAGCTTGAAACACAAATCAGCAGGATGCACCACCTGATTTGCAAGAGAACCATTAT
AGGGGGGCTACGAATACGAACCGTGAAAGCGTGGCCTATCGATCCTTTAGACCTTCGG

UNKNOWN FUNCTION

Gene 00086 unknown [Zea mays]
GCTGGGGCGGCACATCTGTTAAAAGATAACGCAGGTGTCCTAAGATGAGCTCAACGAGAACAGAAATCTCGTGTGGAACAAAAGGGTAAA
AGCTCGTTTGATTCTGATTTCCAGTACGAATACGAACCGTGAAAGCGTGGCCTATCGATCCTTTAGACCTTCGGGATTTGAAGCTAGAGG
TGTCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCAGCCAAGCGTTCATAGCGACGTTGCTTTTTGATCCTTCGATGTCGGCTCTTC
CTATCATTGTGAAGCAGAATTCACCAAGTGTTGGATTGTTCACCCACCAATAGGGAACGTGAGCTGGTTAGACCGTCGTGAGACAGGTTA
GTTTTACCCTACTGATGATCGTGCCACGATAGTAATTCAACCTAGTACGAGAGGAACCGTTGATTCGCACAATTGGTCATCGCGCTTGGT
TGAAAAGCCAGTGGCGCGAAGCTACCGTGCGTCGGATTATGACTGAACGCCTCTAAGTCAGAATCCAAGCTAAACACGCGGCGCTCGCGC
CCGCCGCCCGCTTGCCGACCCTCAGTAGGGGCCTCTTTGGCCCCCATGGGCCCGTGCCGTTGGTGAAGCCCCCGCGGCGGACGAGCCGCG
ACGGGCCGCCTTGAAGATTAATTTCGACCGGACGGCGGGCAGAATCCTTTGCAGACGAATCTGTGCGACATGGGGCTGAATCTCAGTGGA
TCGTGGCAGCAAGGCCACTCTGCCACTTACAATACCCCGTCGCG

Appendix 2. Summary of genes induced in avocado fruits in response to *C. gloeosporioides* infection with their putative function.

NAME	Accession num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		DEFENCE		
Gene 001	ABZ02704.1	accelerated cell death 1 [Arabidopsis thaliana]	2e-06	60
Gene 002	XP_002531635.1	alpha-glucosidase, putative [Ricinus communis]	1e-17	60
Gene 003	AAK15049.1	asparaginyl endopeptidase [Vigna radiata]	1e-41	87
Gene 004	ABG37021.1	aspartic protease [Nicotiana tabacum]	4e-27	70
Gene 005	BAB62890.1	aspartic proteinase 1 [Glycine max]	3e-26	70
Gene 006	NP_199124.3	aspartyl protease family protein [Arabidopsis thaliana]	4e-18	56
Gene 007	AAK58515.1	beta-1,3-glucanase-like protein [Olea europaea]	9e-06	75
Gene 008	CAI39245.1	beta-amylase [Glycine max]	4e-19	74
Gene 009	AEE78232.1	beta-D-glucan exohydrolase - like protein [Arabidopsis thaliana]	2e-11	52
Gene 010	BAJ33502.1	beta glucosidase like protein [Delphinium grandiflorum]	9e-34	61
Gene 011	BAC79443.1	catalase [Acacia ampliceps]	1e-24	98
Gene 012	AAD30291.2	catalase 2 [Raphanus sativus]	3e-05	70
Gene 013	AAF61733.1	catalase 3 [Helianthus annuus]	3e-05	75
Gene 014	NP_191763.3	catalytic/ hydrolase [Arabidopsis thaliana]	5e-29	70
Gene 015	XP_002332294.1	CC-NBS resistance protein [Populus trichocarpa]	9e-11	58
Gene 016	CAC81812.1	chitinase, putative [Musa acuminata]	1e-35	86
Gene 017	ADQ43720.1	chitinase I [Casuarina equisetifolia]	3e-35	82
Gene 018	AAT40738.1	chitinase 2 [Nepenthes khasiana]	2e-35	80
Gene 019	BAB82473.1	chitinase 3 [Triticum aestivum]	2e-34	82
Gene 020	ABR19829.1	cysteine proteinase [Elaeis guineensis]	8e-53	90
Gene 021	BAB64929.1	defensin-like protein [Pyrus pyrifolia]	3e-06	55
Gene 022	AAG51234.1	disease resistance protein MLO, putative; 5304-2185 [Arabidopsis thaliana]	0.014	41
Gene 023	AAP45181.2	Disease resistant protein rga3, putative [Solanum bulbocastanum]	0.12	52
Gene 024	ABY58190.1	endo-1,4-D-glucanase [Persea americana]	4e-71	100
Gene 025	CAB01591.1	endochitinase [Persea americana]	6e-49	100
Gene 026	NP_191415.2	endonuclease/exonuclease/phosphatase family protein [Arabidopsis thaliana]	4e-19	81
Gene 027	NP_567868.1	endonuclease V family protein [Arabidopsis thaliana]	2e-27	80
Gene 028	CAH59407.1	endopeptidase 1 [Plantago major]	2e-08	65
Gene 029	AEE84132.1	enhanced disease resistance 2 protein [Arabidopsis thaliana]	0.014	51
Gene 030	AAZ94162.1	enzymatic resistance protein [Glycine max]	6e-17	63
Gene 031	ACE96388.1	esterase/lipase/thioesterase [Populus tremula]	2e-30	100
Gene 032	AAL15885.1	gamma-thionin putative [Castanea sativa]	2e-11	50
Gene 033	ACI25289.1	late blight resistance protein Rpi-pta1 [Solanum stoloniferum]	0.12	52
Gene 034	AAF97315.1	lipoxygenase [Arabidopsis thaliana]	4e-08	72
Gene 035	ABZ85667.1	LRR-like disease resistance protein [Brassica rapa subsp. pekinensis]	2e-10	76
Gene 036	NP_001154256.1	metalloendopeptidase [Arabidopsis thaliana]	2.2	54
Gene 037	XP_002519488.1	multidrug resistance protein 1, 2, putative [Ricinus communis]	2e-19	81
Gene 038	AEE31214.1	multidrug resistance-associated protein 13 [Arabidopsis thaliana]	2e-30	85
Gene 039	AAM47598.1	NBS/LRR resistance protein-like protein [Capsicum annuum]	9e-10	65
Gene 040	XP_002527223.1	oligopeptidase A, putative [Ricinus communis]	4e-29	66
Gene 041	ABX71220.1	osmotin [Piper colubrinum]	3e-14	82
Gene 042	ABA33845.1	pathogenesis-related protein 6 [Zea diploperennis]	3e-16	59
Gene 043	ADP69173.1	pathogenesis related protein-5 [Populus tomentosa]	3e-14	86
Gene 044	ACE97327.1	pectinesterase inhibitor [Populus tremula]	4e-07	69
Gene 045	AAL35364.1	peroxidase [Capsicum annuum]	2e-09	70
Gene 046	ACG48882.1	programmed cell death protein 5 [Zea mays]	1e-05	83
Gene 047	AAM62652.1	protease inhibitor II [Arabidopsis thaliana]	3e-12	66
Gene 048	NP_001154663.1	ribonuclease III family protein [Arabidopsis thaliana]	8e-06	
Gene 049	AAR25995.1	senescence-associated protein, putative [Pyrus communis]	2e-09	91
Gene 050	ABF96384.1	Serine carboxypeptidase family protein, expressed [Oryza sativa Japonica Group]	7e-11	71
Gene 051	AAK59275.1	thaumatin-like protein [Sambucus nigra]	5e-13	86
Gene 052	ADL60501.1	WRKY disease resistance protein [Malus x domestica]	2e-09	81

Appendix 2 (continued)

Name	Accession num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		SIGNAL TRANSDUCTION/CELLULAR COMMUNICATION		
Gene053	ABN10955.2	auxin response factor 8 [Ipomoea nil]	5e-28	83
Gene054	NP_195050.6	1-phosphatidylinositol-4-phosphate 5-kinase/ ATP binding / phosphatidylinos phosphate kinase/ protein binding / zinc ion binding [Arabidopsis thaliana]	5e-13	79
Gene055	ACO40261.1	3-phosphoglycerate kinase [Heteranthelium piliferum]	1e-55	83
Gene056	XP_002532559.1	calcium-dependent protein kinase, putative [Ricinus communis]	8e-25	68
Gene057	XP_002521983.1	calcium ion binding protein, putative [Ricinus communis]	9e-21	45
Gene058	XP_002526485.1	casein kinase II beta chain, putative [Ricinus communis]	3e-37	77
Gene059	XP_002871973.1	kinase family protein [Arabidopsis lyrata subsp. lyrata]	2e-24	90
Gene060	NP_172244.2	leucine-rich repeat transmembrane protein kinase, putative [Arabidopsis thaliana]	3e-09	74
Gene061	ACM89476.1	leucine-rich repeat family protein / protein kinase family protein [Glyc max]	8e-09	65
Gene062	AAF66615.1	LRR receptor-like protein kinase [Nicotiana tabacum]	6e-40	81
Gene063	XP_002517700.1	F-box/LRR-repeat protein, putative [Ricinus communi	5e-47	69
Gene064	XP_002304470.1	F-box family protein [Populus trichocarpa]	7e-17	72
Gene065	BAE46985.1	mitogen-activated protein kinase [Nicotiana tabacum]	1e-37	91
Gene066	ADD62693.1	mitogen-activated protein kinase kinase [Capsicum annuum]	2e-11	57
Gene067	XP_002513833.1	Mitogen-activated protein kinase kinase kinase, putative [Ricinus commun	8e-06	57
Gene068	XP_002533161.1	casein kinase II, alpha chain, putative [Ricinus communis]	4e-33	95
Gene069	NP_201509.1	MAPKKK19; ATP binding / kinase/ protein kinase/ protein serine/threon kinase [Arabidopsis thaliana]	5e-13	71
Gene070	ACG37954.1	mitogen-activated protein kinase organizer 1 [Zea mays]	2e-16	71
Gene071	ACM89569.1	Pto kinase interactor [Glycine max]	5e-08	90
Gene072	NP_001105753.1	Pti1 protein [Zea mays]	7e-07	86
Gene073	XP_002881172.1	pfkB-type carbohydrate kinase family protein [Arabidopsis lyrata subsp. lyrata]	3e-22	55
Gene074	XP_002530817.1	rac gtpase, putative [Ricinus communis]	2e-15	58
Gene075	AAL40864.1	receptor protein kinase-like protein [Capsicum annuum]	2e-14	84
Gene076	ACJ37422.1	receptor-like serine/threonine kinase [Glycine max]	1e-31	72
Gene077	ABJ89812.1	salicylic acid-activated MAP kinase [Nicotiana attenuata]	2e-33	84
Gene078	BAC53772.1	Salicylic acid-induced protein kinase [Nicotiana benthamiana]	2e-33	84
Gene079	ABY58272.1	serine-threonine protein kinase [Persea americana]	4e-11	83
Gene080	BAD67854.1	S-domain receptor-like protein kinase-like [Oryza sativa Japonica Group]	2e-24	75
Gene081	NP_175758.2	signal peptidase I family protein [Arabidopsis thaliana]	2e-20	53
Gene082	AAQ76042.1	signal recognition particle receptor protein [Cucumis sativus]	1e-10	78
Gene083	CAA71142.1	SNF1-related protein kinase [Cucumis sativus]	1e-23	83
Gene084	AAM18133.1	small G-protein ROP3 [Medicago truncatula]	1e-15	58
Gene085	ABQ42149.1	thioredoxin [Sonneratia caseolaris]	3e-04	83
Gene086	XP_002527178.1	WD-repeat protein, putative [Ricinus communis]	1e-39	77
		OXIDATIVE BURST/ ENERGY		
Gene087	BAG30911.1	ascorbate peroxidase [Capsicum chinense]	9e-24	90
Gene088	P49317.1	Catalase isozyme 3 >emb|CAA85426.1| catalase [Nicotiana plumbaginifolia]	5e-34	94
Gene089	YP_567107.1	cytochrome b6 [Vitis vinifera]	3e-60	78
Gene090	AAW30282.1	cytochrome c biogenesis ccmF [Laurus nobilis]	4e-38	98
Gene091	ABY83854.1	Cytochrome C oxidase subunit I [Plantago lanceolata]	1e-43	100
Gene092	YP_004222659.1	cytochrome f [Anthriscus cerefolium]	8e-09	100
Gene093	BAD26579.1	cytochrome P450 like_TBP [Citrullus lanatus]	3e-13	96
Gene094	ABC68408.1	cytochrome P450 monooxygenase CYP89H3 [Glycine max]	2e-45	72
Gene095	XP_002523033.1	dehydroascorbate reductase, putative [Ricinus communis]	3e-33	85
Gene096	AEC09244.1	galactose oxidase/kelch repeat-containing protein [Arabidopsis thaliana]	2e-07	53
Gene097	ABC74528.1	Glucose-6-phosphate dehydrogenase [Populus trichocarpa]	6e-21	96
Gene098	XP_002533075.1	glutathione peroxidase, putative [Ricinus communis]	6e-12	79
Gene099	AAF61392.1	glutathione S-transferase [Persea americana]	1e-13	97
Gene100	ACN59435.1	glyceraldehyde-3-phosphate dehydrogenase [Dimocarpus longan]	1e-18	84
Gene101	CAA48253.1	ketol-acid reductoisomerase [Arabidopsis thaliana]	2e-36	82
Gene102	AAC04245.1	MgATP-energized glutathione S-conjugate pump [Arabidopsis thaliana]	2e-28	87
Gene103	ABQ41114.1	monodehydroascorbate reductase [Vitis vinifera]	4e-15	92

Appendix 2 (continued)

Name	Accession num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		METABOLISM		
Gene 103	ADO64899.2	acetyl-CoA carboxylase carboxyltransferase beta subunit [Theobroma cacao]	1e-19	90
Gene 104	XP_002525341.1	acyl-CoA oxidase, putative [Ricinus communis]	5e-47	81
Gene 105	XP_002532384.1	1-deoxyxylulose-5-phosphate synthase, putative [Ricinus communis]	2e-56	70
Gene 106	XP_002514626.1	Alpha-1,4-glucan-protein synthase [UDP-forming], putative [Ricinus communis]	8e-35	93
Gene 107	ACG42565.1	calcium homeostasis regulator CHoR1 [Zea mays]	8e-17	68
Gene 108	XP_002275562.1	PREDICTED: similar to cinnamoyl-CoA reductase family [Vitis vinifera]	1e-18	50
Gene 109	ACE96393.1	esterase/lipase/thioesterase [Populus tremula]	2e-30	100
Gene 110	XP_002510442.1	glycosyltransferase, putative [Ricinus communis]	2e-07	74
Gene 111	ADR79441.1	glycyl-tRNA synthetase-like protein [Liriodendron tulipifera]	4e-23	94
Gene 112	XP_002519647.1	ornithine aminotransferase, putative [Ricinus communis]	4e-34	81
Gene 113	CAB41092.1	pectate lyase-like protein [Arabidopsis thaliana]	0.001	52
Gene 114	ADN34053.1	starch synthase [Cucumis melo subsp. melo]	1e-41	81
Gene 115	ACO40485.1	terpene synthase [Actinidia deliciosa]	3e-17	58
Gene 116	ACG45528.1	ubiquitin-protein ligase [Zea mays]	2e-17	76
		TRANSCRIPTION FACTOR		
Gene 117	ACF60482.1	bZIP transcription factor [Oryza sativa Japonica Group]	1e-08	57
Gene 118	ADL36656.1	C3HL domain class transcription factor [Malus x domestica]	3e-27	79
Gene 119	ABL97952.1	DNA binding transcription factor [Brassica rapa]	1e-34	92
Gene 120	ACM49845.1	Ethylene responsive transcription factor 3a [Prunus salicina]	2e-14	68
Gene 121	BAD18011.1	MADS-box transcription factor [Asparagus virgatus]	7e-16	76
Gene 122	AAS68190.1	Myb transcription factor [Vitis vinifera]	6e-27	89
Gene 123	XP_002877726.1	Myb family transcription factor [Arabidopsis lyrata subsp. lyrata]	7e-39	90
Gene 124	AAD10237.1	TATA-box binding protein [Phaseolus vulgaris]	3e-06	100
Gene 125	AAM63665.1	transcription factor, putative [Arabidopsis thaliana]	2e-33	89
Gene 126	NP_001152266.1	Basic helix-loop-helix (bHLH) transcription factor [Zea mays]	1e-23	72
Gene 127	XP_002524838.1	WRKY transcription factor, putative [Ricinus communis]	2e-27	76
Gene 128	NP_179571.1	zinc finger (CCCH-type) family protein [Arabidopsis thaliana]	3e-27	77
		TRANSPORTATION		
Gene 129	XP_002331473.1	ABC transporter family protein [Populus trichocarpa]	3e-16	86
Gene 130	CAA04768.1	acyl carrier protein [Fragaria vesca]	4e-23	94
Gene 131	XP_002315914.1	amino acid transporter [Populus trichocarpa]	3e-30	83
Gene 132	XP_002526293.1	GABA-specific permease, putative [Ricinus communis]	2e-30	82
Gene 133	XP_002522620.1	mitochondrial dicarboxylate carrier protein, putative [Ricinu	2e-27	92
Gene 134	ACD46687.1	putative amino acid permease [Triticum aestivum]	6e-20	65
Gene 135	ACD56666.1	putative permease [Gossypium arboreum]	3e-23	74
Gene 136	CAJ29291.1	putative polyol transporter protein 4 [Lotus japonicus]	6e-49	69
Gene 137	NP_177024.1	proton-dependent oligopeptide transport (POT) family protein [Arabidopsis thaliana]	8e-21	83
Gene 138	ABK29439.1	sugar transport protein [Coffea canephora]	2e-52	76
Gene 139	NP_563741.1	transport protein, putative [Arabidopsis thaliana]	4e-06	95
Gene 140	XP_002529245.1	translocon-associated protein, alpha subunit, putative [Ricinus communis]	3e-30	77
		STRESS RESPONSE		
Gene 141	BAJ11784.1	dehydration responsive protein[Corchorus olitorius]	0.092	61
Gene 142	AAB84193.1	dormancy-associated protein [Pisum sativum]	0.58	51
Gene 143	XP_002869603.1	early-responsive to dehydration 8 [Arabidopsis lyrata subsp. lyrata	1e-40	95
Gene 144	ACJ11742.1	Chaperone protein dnaK (heat shock protein 70) [Gossypium hirsutum]	2e-37	100
Gene 145	AAM00365.1	saline responsive OSSRIII protein [Oryza sativa]	4e-05	77
Gene 146	CAC85227.1	salt tolerance protein 1 [Beta vulgaris]	3e-22	63
Gene 147	XP_002514902.1	stress associated endoplasmic reticulum protein, putative [Ricinus communis]	2e-09	100
Gene 148	ABD57310.1	stress-associated protein 1 [Solanum lycopersicum]	6e-17	74
Gene 149	NP_176461.1	stress-inducible protein, putative [Arabidopsis thaliana]	1e-37	77
Gene 150	NP_566406.1	universal stress protein (USP) family protein [Arabidopsis thaliana]	3e-26	71
Gene 151	BAC84424.1	water-stress protein-like protein [Oryza sativa Japonica Group]	1e-12	41

Appendix 2 (continued)

Name	Accession num of similar sequence	Similar Sequence From database (BLASTX) (Putative function)	E-value	Max Identity %
		PROTEIN SYNTHESIS		
Gene 152	XP_002528028.1	elongation factor 1-alpha, putative [Ricinus communis]	3e-44	82
Gene 153	CAA71882.1	Elongation factor 2 [Nicotiana tabacum]	9e-15	94
Gene 154	AAF02837.1	elongation factor EF-2 [Arabidopsis thaliana]	6e-33	83
Gene 155	XP_002513404.1	eukaryotic translation elongation factor, putative [Ricinus communis]	3e-35	89
Gene 156	BAB08857.1	eukaryotic initiation factor 4, eIF4-like protein [Arabidopsis thaliana]	3e-11	70
Gene 157	AEE75261.1	Ribosomal protein S5/Elongation factor G/III/V family protein [Arabidopsis thaliana]	2e-17	69
Gene 158	NP_001105988.1	putative splicing factor [Zea mays]	5e-12	60
Gene 159	ADL64029.1	ribosomal protein S3 [Trimenia moorei]	2e-22	96
Gene 160	YP_004021349.1	ribosomal protein S11 [Theobroma cacao]	5e-11	100
Gene 161	XP_002888287.1	translational activator family protein [Arabidopsis lyrata subsp. lyrata]	1e-13	81
		PHOTOSYNTHESIS		
Gene162	NP_196924.1	3-isopropylmalate dehydrogenase, chloroplast, putative [Arabidopsis thaliana]	1e-32	95
Gene 163	ACG59771.1	chloroplast aspartate aminotransferase [Triticum aestivum]	3e-14	94
Gene 164	AAX53163.1	chloroplast photosynthetic oxygen-evolving protein 33 kDa subunit [Nicotiana benthamiana]	2e-36	75
Gene 165	NP_001105381.1	chloroplast phytoene dehydrogenase, chloroplastic/chromoplastic precursor [Zea mays	1e-24	94
Gene 166	AAL77589.1	chloroplast ribose-5-phosphate isomerase [Spinacia oleracea]	1e-17	64
Gene 167	NP_862765.1	photosystem I assembly protein Ycf4 [Calycanthus floridus var. glaucus]	5e-16	98
Gene 168	YP_001294267.1	photosystem II protein Z [Illicium oligandrum]	3e-12	100
Gene 169	ABB59688.1	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [Umbellularia californica]	2e-49	100
		CELL STRUCTURE AND COMPONENT		
Gene 170	ADN34200.1	annexin [Cucumis melo subsp. melo]	2e-16	66
Gene 171	ACS28251.1	cell division control protein [Nicotiana glutinosa]	2e-33	90
Gene 172	NP_564367.1	integral membrane HRF1 family protein [Arabidopsis thaliana]	1e-20	89
Gene 173	BAD61522.1	polygalacturonase-like [Oryza sativa Japonica Group]	4e-04	51
		REGULATION		
Gene 174	BAB90396.1	ADP-ribosylation factor [Oryza sativa Japonica Group] (regulation)	5e-05	100
Gene 175	ADB93067.1	aquaporin [Jatropha curcas](regulation)	3e-31	87
Gene 176	AAM12952.1	auxin-regulated protein [Zinnia violacea] (regulation)	2e-06	53
Gene 177	XP_002875307.1	regulator of chromosome condensation family protein [Arabidopsis lyrata subsp. lyrata] lyrata subsp. lyrata]	4e-17	82
		FUNCTION: UNCLASSIFIED		
Gene 178	ABA99240.2	amidase, hydantoinase/carbamoylase family protein, expressed [Oryza sativa Japonica Group]	8e-22	85
Gene 179	CBI37545.3	hypothetical protein VITISV_021988 [Vitis vinifera]	7e-13	73%
Gene 180	BAB11623.1	N-carbamyl-L-amino acid amidohydrolase-like protein [Arabidopsis thaliana]	5e-23	90
Gene 181	NP_001152224.1	O-succinylhomoserine sulfhydrylase [Zea mays]	1e-35	84
Gene 182	ADZ75466.1	oxygen evolving enhancer protein 1 [Litchi chinensis]	3e-38	77
Gene 183	XP_002325077.1	predicted protein [Populus trichocarpa]	7e-42	76
Gene 184	CBI35816.3	unnamed protein product [Vitis vinifera]	3e-40	100

Appendix 3 Summary of sequencing and assembly

	C	EU	LU	EH	LH
Number of reads	36024	35648	25046	71820	47243
Reads fully assembled in contigs	25774	29067	21367	46200	31859
Number of contigs	1568	2544	693	1138	1115
Outliers reads	301	178	188	827	500

Control, Early Unharvested, Late Unharvested, Early Harvested and Late Harvested (C, EU, LU, EH and LH respectively).

Appendix 4

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The isolation of high quality RNA from the fruit of avocado (*Persea americana* Mill.)

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Received 6 April 2011; received in revised form 19 April 2011; accepted 21 April 2011

Abstract

The fruit of avocado is high in structural carbohydrates and proteins and also contains polyphenols which interfere with the isolation of high quantity and quality of RNA. The aim of this study was to optimize RNA isolation from avocado fruit for subsequent complementary DNA (cDNA) transcription. We describe an optimized cetyltrimethylammonium bromide (CTAB)-based RNA extraction protocol that allows effective extraction of high-quality total RNA from the skin and flesh of the fruit without the use of phenol. Total RNA obtained from this procedure is of high quality and is undegraded, as assessed by spectrophotometric readings and electrophoresis, and was successfully used for cDNA synthesis. This is a first report of effective RNA extraction from avocado fruit.
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Keywords: Avocado fruit; cDNA; CTAB; Flesh; Polyphenols; Polysaccharides; Proteins; RNA extraction; Skin

Extraction of RNA of high quantity and quality is a preliminary step for many investigations in plant molecular biology such as Northern Blot hybridization, mRNA purification, PCR amplification, cDNA synthesis and cDNA library construction (Hu et al., 2002). However, to isolate RNA from some plant tissues is manifestly difficult due to the presence of high amounts of secondary metabolites, such as polysaccharides and polyphenolic compounds, which can co-precipitate or bind to RNA and result in poor yields (Gasic et al., 2004). Avocado (Persea americana Mill.) produces a climacteric fruit which, in addition to mainly monounsaturated and polyunsaturated oils, contains high levels of proteins and structural polysaccharides (Bergh, 1992; Naveh et al., 2002), and polyphenols (López-Gómez, 2002). In preliminary experiments, we tested four protocols using either phenol or CTAB in the extraction buffer (Hu et al., 2002; López-Gómez and Gómez-Lim, 1992; Pandit et al., 2007; Valderrama-Cháirez et al., 2002) and one commercially available RNA extraction kit (RNeasy Plant Mini Kit, Qiagen) to extract RNA from the skin (exocarp) and flesh (mesocarp) of avocado fruit but the results were unsatisfactory for all the protocols because of the poor quality and quantity of the resulting total RNA. In order to achieve an improvement in both yield and purity of RNA from avocado fruit we used the CTAB/NaC1 method of Chang et al. (1993) and modified it by replacing PVP K 30 with PVP K 40, removing spermidine from the extraction buffer and including a simple polysaccharide precipitation step that does not affect the RNA yield but removes contaminating polysaccharides.

Fruits of avocado (cv. Fuerte.) were collected from the orchards of Roodewal farm near Nelspruit, Mpumalanga Province, South Africa (25°25′54.62″S; 30°56′15.34″E). The skin and flesh tissues (±5 g) were immediately sliced out and frozen in liquid nitrogen, and stored at −80 °C until needed. DEPC-treated water was used for all solutions. Frozen tissues were ground with a mortar and a pestle in the presence of liquid nitrogen. The resulting powder (0.5–1 g) was transferred to

Abbreviations: DEPC, diethyl pyrocarbonate; dNTP, deoxyribonucleotide triphosphate; DTT, dithiothreitol; EtBr, ethidium bromide; EtOH, ethanol; FW, fresh weight; LiCl, lithium chloride; mRNA, messenger RNA; PCR, polymerase chain reaction; PVP, polyvinylpyrrolidone; TAE, tris-acetate-EDTA.

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doi:10.1016/j.sajb.2011.04.009

sterile centrifuge tubes containing 10 ml of pre-warmed (65 °C) sterile extraction buffer (2% CTAB, 2% PVP K 40, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, 2 M NaCl, and 400 µl 2% β-mercaptoethanol added just before use) and incubated at 65 °C for 5–10 min to lyse the cells completely. Following the addition of 15 ml of chloroform:isoamyl alcohol (24:1 [v/v]) tubes were vortexed and centrifuged at 5000 rpm for 20 min at room temperature. After centrifugation, the upper phase was transferred to a new tube with 15 ml of chloroform:isoamyl alcohol, then vortexed and centrifuged again for 20 min at 5000 rpm. After the second centrifugation, the supernatants (12 ml) were transferred to sterile tubes following the addition of 3 ml of 10 M LiCl (1/4-volume). After overnight incubation at −20 °C, the tubes were centrifuged (10,000 rpm, 30 min, 4 °C) and the pellets re-suspended in 700 µl NaCl-sodium dodecylsulphate-Tris-EDTA buffer (1 M NaCl, 0.5% SDS, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA), pre-warmed at 60 °C, and incubated for a few minutes at 60 °C to ensure complete re-suspension of the RNA-pellet in the buffer. The suspension was transferred to a 2 ml microcentrifuge tube and extracted with 700 µl of chloroform:isoamyl alcohol followed by a spin down at 10,000 rpm for 10 min at room temperature. The upper phase (600 µl) was transferred to a 2 ml microcentrifuge tube containing 1.2 ml of 100% EtOH and the RNA was precipitated at −70 °C for 1 h. After incubation for the precipitation of nucleic acids, the tubes were centrifuged (30 min, 13 000 rpm, 4 °C). Nucleic acids were recovered by washing with 1 ml of 70% EtOH (v/v) and centrifuging at 10,000 rpm for 2 min, air dried and re-suspended in 100 µl DEPC water. The RNA yield was measured with a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware USA) and the 260/280 and 260/230 absorption ratios were verified as quality indexes (six replications). The RNA was examined by electrophoresis on 2% agarose/TAE gels containing EtBr in order to assess its integrity. Firstly, one volume of the 2× RNA loading buffer was mixed with one volume of the RNA sample at 70 °C for 10 min. After cooling on ice for 3 min, the samples were loaded and run on the gel in the 1 X TAE electrophoresis buffer at 80 V. Doubled strand cDNA was synthesized starting with the total RNA, using a cDNA Synthesis System Kit (Roche), following the manufacturer's instructions. The final pellet was air dried and dissolved in 50 µl water, PCR Grade. The synthesis of double-stranded cDNA was monitored by running a 2% agarose gel after measuring the yield with a ND-1000 spectrophotometer (NanoDrop, Wilmington, Delaware, USA).

The problem of the RNA co-precipitation was prevented by adding PVP K 40 and β-mercaptoethanol in the extraction buffer as reducing reagents with CTAB as the detergent, and extraction with chloroform–isoamyl alcohol instead of phenol to remove proteins. The problem of polysaccharide contamination was solved by using 2 M NaCl instead of less than 1 M in the extraction buffer and 1.0 M NaCl in the NaCl-sodium dodecylsulphate-Tris-EDTA buffer to dissolve the RNA pellet. The increase of the NaCl concentration in the buffers helps to remove polysaccharides (Fang et al., 1992) and dissolves the CTAB-RNA complex, in order to allow more CTAB and polysaccharides to be removed in the chloroform extraction. In addition the precipitation step overnight at −20 °C instead of 4 °C allows high quality RNA to be recovered.

The flesh and skin yielded 164.67 ± 6.09 µg g^{-1} FW and 86.83 ± 6.01 µg g^{-1} FW RNA respectively and the high quality of the RNA was confirmed firstly by the A260/A280 absorbance ratio which was always above 2.0 (flesh, 2.14 ± 0.01; skin, 2.10 ± 0.01) indicating that RNA was relatively free of protein contamination; and the A260/230 ratio (flesh, 2.27 ± 0.06; skin, 2.07 ± 0.06) which was also higher than 2.0, indicating that RNA was of high purity and without polyphenol and polysaccharide contamination (Logemann et al., 1987; López-Gómez, 2002; Manning, 1991). Similar results were obtained when RNA was extracted from different apple tissues also using an extraction buffer containing CTAB, PVP and β-mercaptoethanol (Gasic et al., 2004). Furthermore, the RNA integrity was confirmed by the sharpness of ribosomal RNA bands visualized by electrophoresis (Fig. 1) which showed distinct 28S and 18S bands, indicating that the RNA was not degraded and was also relatively free of RNases (Fig. 1). Similar results were observed by Valderrama-Cháirez et al. (2002) when they isolated RNA from ripe mango mesocarp. Finally, the RNA quality was tested by cDNA synthesis using the AMV reverse transcriptase with the synthetic oligonucleotide, oligo $(dT)_{15}$ primer. The cDNA was successfully synthesized without any amplification with a good yield (1.12 ± 0.26 µg g^{-1} FW) in a total volume less than 50 µl and

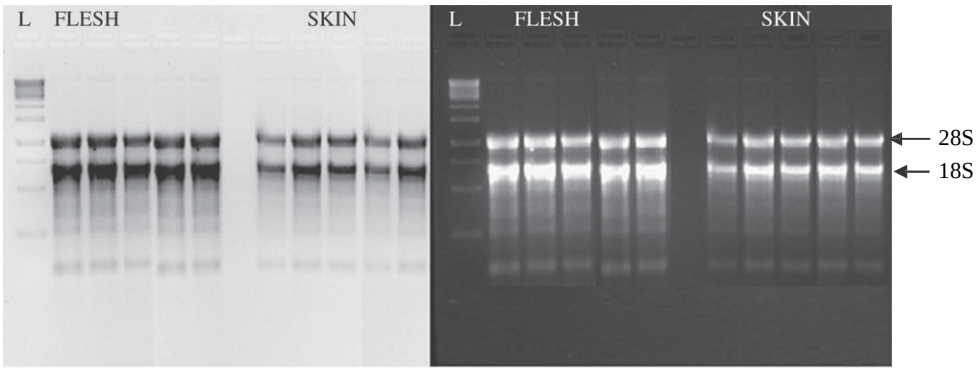

Fig. 1. Total RNA from avocado fruit flesh and skin separated on 2% non-denaturing agarose gel containing EtBr and photographed under ultraviolet light. L, RiboRuler™ High Range RNA Ladder.

the reverse transcription products, resolved on 2% agarose/TAE gel electrophoresis, exhibited clear bands (data not shown). These results demonstrated that total RNA obtained was of sufficient quality to be used for downstream transcriptome analysis. This is a first report of the extraction of high quality RNA from avocado fruit.

Acknowledgements

We acknowledge financial support from the National Research Foundation, South Africa (ICD2007052500003: 2008–2011), the University of the Witwatersrand Postgraduate Merit Award, the Bradlow Bursary and the Oppenheimer Memorial Trust. We thank Ms Farhahna Van Schalk for valuable technical advice.

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Edited by E Balazs

Appendix 5

J Phytopathol
Ó 2012 Blackwell Verlag GmbH
doi: 10.1111/j.1439-0434.2012.01925.x
Journal of Phytopathology

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454 Sequencing for the Identification of Genes Differentially Expressed in Avocado Fruit (cv. Fuerte) Infected by Colletotrichum gloeosporioides

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Received December 6, 2011; accepted April 18, 2012

Keywords: Persea americana, RNA extraction, Colletotrichum gloeosporioides, gene expression, defence responses, 454 sequencing

Abstract

The anthracnose pathogen, Colletotrichum gloeosporioides (Penz.) Penz. & Sacc., is a major cause of disease in the avocado industry, causing significant economic losses, and infects all cultivars. In South Africa, cvs Fuerte and Hass are the most widely grown. Identification of genes differentially expressed in avocado during infection with the fungus represents an important step towards understanding the plants defence responses and would assist in designing appropriate intervention strategies. In this study, 454 sequencing and analysis of the transcriptome of infected cv. Fuerte avocado fruits were performed using the Roche 454 GS FLX Titanium platform. cDNA libraries enriched for differentially expressed genes were constructed from unharvested and harvested avocado fruit tissues collected after 1, 4 and 24 h postinfection (early response) and after 3, 4, 5 and 7 days postinfection (late response), then sequenced. RT-PCR was used to validate the sequencing results. The single sequencing run produced 215 781 reads from the transcriptome with an average sequence length of 252–300 nucleotides. A total of 70.6 megabases of sequence data were generated and subjected to BLAST searches from which 639 genes encoding proteins functioning in metabolism, signal transduction, transcriptional control, defence, stress, transportation processes and some genes with unknown functions were identified. Avocado is able to respond to C. gloeosporioides infection by exhibiting a sophisticated molecular system for pathogen recognition and by activating structural and biochemical defence mechanisms. This study represents the first transcriptome analysis of avocado fruit following infection with C. gloeosporioides, and the findings are discussed in relation to the known or putative functions of the gene products.

Introduction

Avocado fruit (Persea americana Mill.) is one of the most important sources of human nutrition, and in South Africa, production is concentrated mainly in the warm subtropical areas of the Limpopo and Mpumalanga provinces in the north east of the country. Approximately 12 400 ha are currently utilized for avocado production in South Africa, and total production ranges between 85 000 and 100 000 tons per annum with 50% of the production being exported, mostly to Europe (Donkin 2007). Most of the orchards are planted with cvs Fuerte and Hass (74%) (Donkin 2007). Avocado, like many other crops, is susceptible to attack by filamentous fungi of the genus Colletotrichum, pathogens that cause anthracnose disease. Anthracnose is the most severe postharvest disease of avocado fruit (Pernezny et al. 2000) and affects all cultivars planted in South Africa (Witney 2002), but cv. Fuerte is the most susceptible (Darvas and Kotze 1981). The disease reduces avocado fruit shelf life and negatively affects fruit quality, taste and marketability (Freeman et al. 1998; Pernezny et al. 2000).

The elucidation of gene expression profiles in the infected tissue may bring more understanding of how avocado fruit reacts at the molecular level to Colletotrichum attack and could, therefore, contribute to the development of strategies to improve its production and storage to reduce the effects of the pathogen. Over the last few years, next-generation sequencing technologies have been used as powerful approaches for discovering new genes and analysing gene expression profiles in plant tissues (Mardis 2008). The sequencing of cDNA instead of genomic DNA focuses analysis on the transcribed portion of the genome which reduces the size of the sequencing target space. Many applications have been elucidated through transcriptome sequencing, such as: gene expression profiling, genome annotation and rearrangement detection to non-coding RNA discovery and quantification (Morozova and Marra 2008). On the basis of the high number of reads generated per run together with the low sequencing error rate in the contigs obtained, 454

sequencing is well adapted to sequence the transcriptome of both model and non-model plants (Barakat et al. 2009).

Avocado is a sub-tropical fruit-tree with 24 chromosomes (2n = 24) and a haploid small genome size of approximately 907 Mbp, just six times greater than that of Arabidopsis thaliana (Arumuganathan and Earle 1991). To date, the avocado genome has not been published, and a limited number of ESTs have been sequenced, annotated and published on NCBI (<http://www.ncbi.nlm.nih.gov/nucest?term=persea americana>). In this study, we focused on comparing the cDNA libraries generated from healthy avocado fruit and fruit infected with C. gloeosporioides, using the Roche 454 GS FLX Titanium Platform. Because C. gloeosporioides causes quiescent infections in unripe fruit maintained by high concentrations of preformed antifungal compounds (Prusky et al.1990, 1991a,b; Domergue et al. 2000), it was necessary to include both pre- and postharvest fruits in the experimental design. This comparison enabled us to identify a large number of differentially expressed genes. The strategy undertaken here does not require prior sequence knowledge or genome reference and relies exclusively on publicly available software and basic scripting tools. To the best of our knowledge, this is the first attempt to discover differentially expressed candidate genes in avocado fruit following infection with C. gloeosporioides.

Materials and Methods

Preparation of fungal inoculum

The strain of C. gloeosporioides used was isolated from an anthracnose lesion on a cv. Fuerte avocado fruit picked in an organic orchard in Mpmulanga province (Giovanelli 2008). The fungus was cultured on 5% (w/v) Malt Extract Agar (Merck). Spore suspensions (conidia) of the fungus were prepared as described previously by Prusky et al. (1990).

Plant materials and inoculation

The experimental design included unharvested (inoculated and uninoculated) and harvested (inoculated and uninoculated) cv. Fuerte avocado fruits from Roodewal farm, Nelspruit, South Africa (25°25¢54.62¢¢S 30°56¢15.34¢¢E). The age of the fruit was c. 240 days after fruit set, and fruit was collected 2 weeks before the beginning of the normal crop harvest. For in planta inoculations, the fruits were first surface-sterilized by immersion in 1% sodium hypochlorite for 10 min followed by rinsing in sterile deionized water three times and left to air dry. Afterwards the fruits were wiped with 70% ethanol (Merck), allowed to air dry (Giovanelli 2008), wounded and inoculated with 100 ll of C. gloeosporioides spore suspension ($1 \cdot 10^7$ spores/ml) using a sterilized needle of 2 mm length and 1 mm thick. The inoculated fruits were covered with a clean, sterile plastic bag in which sterile deionized water was sprayed to maintain humidity. For the postharvest inoculation, after the sterilization steps, the fruits were wounded and inoculated with 100 ll of the spore suspension and incubated at 25°C in aluminium trays covered with aluminium foil; humidity was maintained by placing a petri dish filled with sterile deionized water at the centre of each tray (Kwang-Hyung et al. 2004). The control fruits for both unharvested and harvested treatments were treated similarly to infected fruits and were inoculated with 100 ll of sterile deionized water. The harvested control fruit was placed in a separate tray to prevent the spread of infection.

Observation of Colletotrichum gloeosporioides infection process on inoculated avocado fruits

The infection process of C. gloeosporioides was observed by scanning electron microscopy (SEM) of harvested and unharvested fruits using a method described by Palhano et al. (2004).

Total RNA extraction

Total RNA was extracted from the mesocarp of avocado fruit (1 g) as described by Djami-Tchatchou and Straker (2011).

Synthesis of double-stranded cDNA from total RNA

Doubled-stranded cDNA was synthesized starting with the total RNA (20 lg), using a cDNA Synthesis System Kit (Roche, Mannheim, Germany), following the manufacturerÕs instructions. Then, cDNA of three biological replicates of each infected sample collected after 1, 4 and 24 h was pooled together for the early response studies for early unharvested (EU) and early harvested (EH) treatments, and the same was carried out with the infected samples collected after 3, 4, 5 and 7 days postinfection for late unharvested (LU) and late harvested (LH) treatments for the late response studies.

454 Library construction and sequencing

cDNA of the control and infected samples of each time point was used to construct a 454 library following the supplierÕs instructions (Roche Diagnostics, Mannheim, Germany). The sample preparation was carried out prior to sequencing, using the GS FLX Titanium Sequencing Kit XLR70 in combination with the matching GS FLX Titanium PicoTiterPlate Kit 70 · 75 following the supplierÕs instructions (Roche Diagnostics). Sequencing using the 454 Genome Sequencer -FLX titanium system (Roche) followed and then the individual samples had to be separated based on their individual tags.

Transcript assembly and analysis

The data from the 454 read sequences of each sample were assembled into contigs using the proprietary Roche 454 Newbler Assembler software. Reads from each library were assembled separately following a combined assembly of all data from all time points to yield contigs that correspond to transcripts. The cDNA sequences were annotated using clc Workbench software (CLC bio, Cambridge, MA, USA) and

BLAST [Basic Local Alignment Search Tool (Altschul et al. 1990)]. Similarities at the nucleotide level were identified using blastn, and protein homologies were identified using the non-redundant protein databases blastx (Altschul et al. 1990). Each gene was classified into a functional category based on the putative function played by the gene product. During the assembly process, there were other reads that did not map with other reads at each time point in both uninfected and infected samples. These unmapped reads also represent avocado genes and/or parts of the avocado genes. The function assignment of these genes was carried out based on homology after comparing their sequences to the non-redundant protein databases blastx program (data not shown). Sequences have been lodged with GenBank.

Hierarchical clustering of gene expression data

The expression profiles of the genes obtained from the mapped read were measured by hierarchical clustering algorithm using Hierarchical Clustering Explorer 3.0 software (http://www.cs.umd.edu/hcil/) that is visualization software for interactive exploration of multidimensional datasets. The hierarchical clustering algorithm was based on the combination of the average linkage analysis (unweighted pair group method with arithmetic mean) and Pearson's correlation coefficient (Tang et al. 2001). All the computational details used are described in the cluster manual available at http://www.cs.umd.edu/hcil/multi-cluster/hce3-manual/hce3_manual.html).

Reverse transcription PCR

Reverse Transcription PCR was carried out to validate the results of the sequencing. Total RNA (2 lg) was reversed transcribed using a RevertAidÔ Premium First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, South Africa), following the manufacturerÕs instructions. Seven primers were designed from sequences of cDNA obtained after 454 sequencing using the Integrated DNA TechnologiesÕs PrimerQuest Tool (http://eu.idtdna.com/Scitools/Application/Primerquest/Default.aspx) which incorporates Primer3 software to test the expression of seven target genes. The PCR experiments using cDNA as template with 10 lm of specific primer were carried out as follow: one cycle of 94℃ for 3 min (initial denaturation), 35 cycles of 94℃ for 30 s (denaturation), Tm of each primer for 1 min (annealing) and 72℃ for 2 min (elongation). Final elongation was achieved at 72℃ for 10 min.

Results

Observation of Colletotrichum gloeosporioides infection process on avocado fruits

In harvested fruits, symptoms of anthracnose had developed by day 3 postinoculation. These symptoms were characterized by black fruit rot and spots, as well as white mycelial growth that developed on the wounded inoculated and adjacent uninoculated areas. Scanning electron micrographs of avocado fruit showed an increased number of spores that resulted from a successful hyphal colonization of the fruit surfaces (Fig. 1b). At 4 and 7 days postinfection in

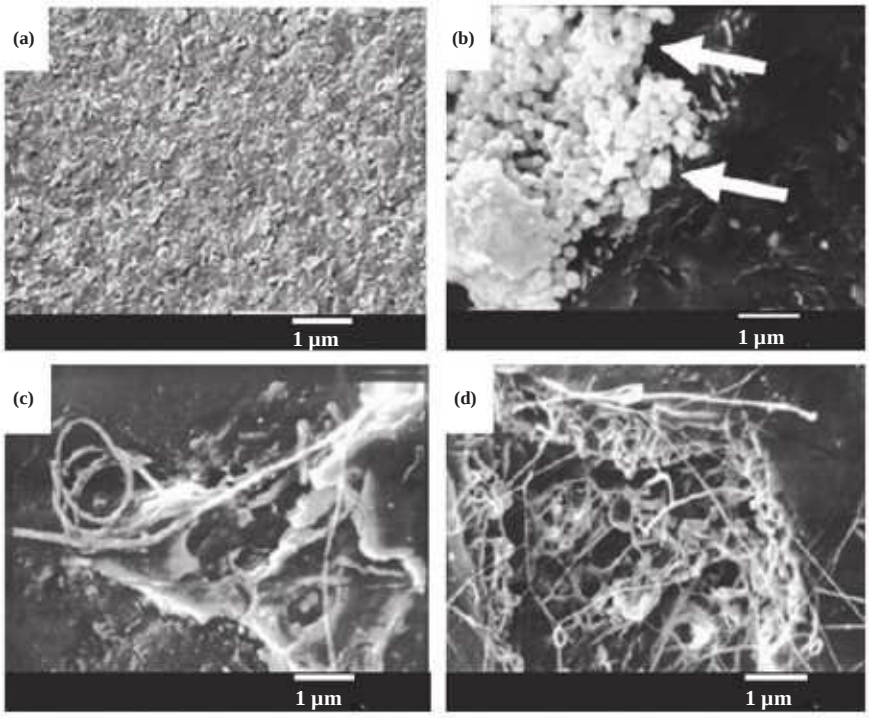

Fig. 1 Scanning electron micrographs of avocado fruit infected with Colletotrichum gloeosporioides. (a) Uninfected sample. (b) An increased number of C. gloeosporioides spores shown by the arrows on the surface of inoculated fruit at 4 dpi. (c,d) Severe tissue destruction and damage with some hyphae protruding out of the lenticels at 4 and 7 dpi

harvested fruit, severe tissue destruction and damage were observed with some hyphae protruding out of the lenticels, which resulted in cell wall and cuticle destruction (Fig. 1c,d). In general, unharvested fruits showed delayed, minor symptom development with little sporulation, compared with harvested fruits (data not shown). Figure 1a shows an uninfected sample with no symptom development.

Sequencing and de novo assembly

The single sequencing run produced 215 781 reads from avocado fruit transcriptome, with an average sequence length of 252–300 nucleotides. A total of 70.6 megabases (MB) of sequence data were generated resulting in the assembly of approximately 1500 contigs (Table 1); more specifically, 11.4 MB of healthy transcriptome sequence, 11.5 MB of EU transcriptome sequence, 8.3 MB of LU transcriptome sequence, 23.9 MB of EH transcriptome sequence and 15.5 MB of LH transcriptome sequence.

Sequences analysis

The comparison between the healthy and infected transcriptomes enabled us to identify a large number of candidate pathogen response genes. We first determined how many times a gene was represented in each of the libraries based on the number of reads for each unigene count. Putative functions of each of the genes were determined by comparing their sequence with other sequences present in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The selection criteria of each gene were based on the high percentage of maximum identity (range of the percentage = 51–100%) and its inclusion in the plant kingdom. During the assembly process, there are certain reads called mapped reads that align to other reads at each time point. Of the 709 genes obtained from these reads expressed in both uninfected and infected samples, 639 showed similarity to previously known plant gene sequences, and 70 had no significant similarity to plant genes in the database. The 639 sequences exhibiting homology to plant genes could be classified based on the function of their protein products: 358 had homology to senescence-associated protein genes, 114 had homology to cytochrome genes (cytochrome P450-like TBP protein, cytochrome P450 monooxygenase, cytochrome c oxidase and cytochrome f), 102 had homology to hypothetical protein genes, 18 showed similarities to genes encoding proteins involved in metabolism, 14 showed homology to genes encoding plant defence and stress-related proteins, nine showed homology to transcription factor and cellular communication genes, nine had homology to expressed protein genes, eight had homology to genes coding for proteins involved in photosynthesis and cell structure, and seven showed homology to genes of proteins involved in electron transport. Of the 639 cDNA sequences exhibiting differential expression in response to C. gloeosporioides infection, some sequences were selected to show their expression per time point (Table 2). The following are notable for their likely role in wound or defence responses. Leucine-rich protein was up-regulated in the EU sample and down regulated in LU and harvested samples. Cytochrome P450 monooxygenase involved in lignification and senescence-associated protein were up-regulated in all the infected samples. Stress-related protein, such as heat shock protein, was up-regulated during early response in unharvested fruit and during late response in harvested fruit. Transcription factor WRKY36 was up-regulated during early response in harvested fruit and down regulated during late response in both unharvested and harvested fruits. Catalase showed a slight up regulation in the EH response and down regulation in the LH response (Table 2).

During the assembly process, there were reads from the infected samples that did not map with the reads from the uninfected samples. These reads also represent genes and/or parts of the genes obtained and are considered to be induced after C. gloeosporioides infection in the infected samples. These genes were classified according to their putative function after comparing their sequences to the non-redundant protein databases blastx program. On the basis of the protein homology in GenBank, of the 218 induced genes, the largest set of these genes (24%) was assigned to defence, followed by the second largest group (17%) assigned to unclassified genes. Of the other genes, 15.6% were found to have significant homology to genes encoding proteins involved in signal transduction, 7.8% had homology to genes involved in oxidative burst and energy, 6.4% had homology to genes involved in metabolism, 6.4% had homology to genes involved in protein synthesis, 5.5% showed homology to transcription factors, 5.5% showed similarity to genes involved in transportation, and 5% showed similarity to stress-related protein genes. Finally, 3.7% of the genes had homology to genes for proteins involved in photosynthesis while genes involved in regulation and cell structure constituted the smallest group, comprising 1.8% of the sequences. Some of these induced genes are presented in Table 2. Mitogen-activated protein kinase (MAPK) and leucine-rich repeat receptor-like protein kinase were expressed in all the infected samples. Salicylic acid-binding protein was expressed in all the infected harvested samples. Calcium-dependent protein kinase and calcium ion-binding protein were expressed in both

Table 1
Summary of sequencing and assembly

	C	EU	LU	EH	LH
Number of reads	36 024	35 648	25 046	71 820	47 243
Reads fully assembled in contigs	25 774	29 067	21 367	46 200	31 859
Number of contigs	1568	2544	693	1138	1115
Outliers reads	301	178	188	827	500

C, control; EU, early unharvested; LU, late unharvested; EH, early harvested; LH, late harvested.

Table 2
Summary of some selected candidate genes differentially expressed in avocado fruits in response to Colletotrichum gloeosporioides infection with their different expression per time point (reads mapped to the individual transcripts for every time point)

Name	Similar sequence from database BLASTn (accession number)	Max identity %	Similar sequence from database BLASTx (accession number)	E-value	Putative function	Copies per time point expressed				
						Cont	EU	LU	EH	LH
gene00001	Cinnamomum camphora large subunit ribosomal gene (DQ008772.1|)	99	Leucine-rich protein [Arachis hypogaea] (ABH09320.1)	4e-04	Signal transduction	107	145	41	88	83
gene00609	Daphnandra micrantha 26S ribosomal RNA gene |DQ008629.1)	96	Transcription factor WRKY36 [Physcomitrella patens subsp. Patens (XP_001775684.1)	8.7	Transcription factor	7	7	4	9	3
gene00308	Laurus nobilis 26S ribosomal RNA gene (DQ008626.1)	100	Heat shock protein [Cucumis sativus] (ADF30255.1)	7e-79	Stress-related protein	2	3	1	2	3
gene00144	Cryptocarya meissneriana 26S ribosomal RNA gene (DQ008627.1)	98	Cytochrome c oxidase subunit 5B, mitochondrial precursor (ACN10266.1)	7e-05	Oxidation	8	19	9	22	6
gene00132	Gomortega keule 26S ribosomal RNA gene (AY095460.1)	98	Cytochrome P450 monooxygenase [Pyrus communis] (AAR25996.1)	2e-06	Lignification	12	40	20	49	30
gene00654	Cananga odorata large subunit ribosomal RNA gene (DQ008784.1)	100	Catalase [Arabidopsis thaliana] (CAA45564.1)	4e-24	Defence	1	1	1	2	0
gene00237	Arabidopsis thaliana clone 34690 mRNA, (AY087376.1)	72	Fructose-bisphosphate aldolase [Persea americana] (CAB77243.2)	2e-81 9e-90	Metabolism	7	4	1	13	9
gene00307	Persea americana stearoyl-acyl-carrier-protein desaturase mRNA, (|AF116861.1)	99	Stearoyl-acyl-carrier-protein desaturase [Persea americana] (AAF15308.1)		Metabolism	4	1	2	2	1
gene00665	Liriodendron tulipifera chloroplast, complete genome (DQ899947.1)	95	Photosystem I assembly protein Ycf4 [Zea mays] (NP_043035.1)	1e-18	Photosynthe-sis	1	2	2	0	0
gene00082	Peumus boldus 26S ribosomal RNA gene (AY095466.1)	96	Hypothetical protein [Arabid-opsis thaliana] (BAF01964.1)	2e-48	Unknown function	126	207	178	262	167
gene00091	Calycanthus occidentalis 26S ribosomal RNA gene (AY095454.1)	95	Putative senescence-associated protein [Trichosanthes dioica] (ABN50032.1)	6e-30	Unclassified function	135	187	184	349	236
gene00086	Gyrocarpus americanus 26S ribosomal RNA gene (DQ008624.1)	100	Unknown [Zea mays] (ACR36970.1)	1e-45	Unknown function	68	165	127	139	91

C, control; EU, early unharvested; LU, late unharvested; EH, early harvested; LH, late harvested.

unharvested and harvested samples during early response but only in the harvested infected samples during late response to C. gloeosporioides infection. DnaK-like chaperone protein (heat shock protein 70) was expressed in all the infected samples. Many other genes predicted to be involved in plant defence response were also expressed in infected samples such as endo-1,4-d-glucanase expressed in EU and LH samples; endochitinase expressed in EU and in all harvested samples. Endopeptidase was expressed in both unharvested and harvested samples during late response, and aspartic proteinase was expressed only in the harvested samples. Among proteins predicted to be involved in proteins synthesis, elongations factor 1 was expressed in all the infected samples, and acetyl-CoA carboxylase carboxyltransferase, involved in metabolism, was expressed only in all the infected unharvested samples (Table 2).

Finally, during the assembly, other reads from the uninfected sample did not map with the reads from the infected samples. Because genes obtained from these reads are only expressed in the uninfected samples, they were considered to be repressed in the infected samples after C. gloeosporioides infection. These unassembled reads likely correspond to transcripts as well, but in very low copy number. The function assignment of these genes was carried out based on homology after comparing their sequences to the non-redundant protein databases blastx program (Table 3). Some of those genes expressed only in uninfected avocado fruit are common plant regulatory factor 6, (R)-limonene synthase, cytosolic NADP-malic enzyme and Jasmonic acid (JA) known to be involved in signal transduction (Voelckel and Baldwin 2004).

Hierarchical cluster analysis

Hierarchical clustering analysis was used to group together into clusters, genes with similarity in the expression patterns in both uninfected and infected avocado fruits (Fig. 2). In hierarchical clustering explorer, a black colour represents higher levels of gene expression, whereas a white colour represents lower levels of gene expression. Shades of grey represent the middle value or represent genes equally

Table 3
Summary of some selected candidate genes expressed in infected avocado fruits in response to Colletotrichum gloeosporioides infection with (1) for expressed and (0) not expressed

Name	Accession number of similar sequence	Similar sequence from database BLASTx (accession number)	E-value	Max identity %	Expression EU	LU	EH	LH
Signal transduction								
GSKRSVY03G674	XP_002532559.1	Calcium-dependent protein kinase, putative [Ricinus communis]	8e-25	68	1	0	1	1
GSKRSVY03G674	XP_002521983.1	Calcium ion-binding protein, putative [Ricinus communis]	9e-21	45	1	0	1	1
GSKRSVY03HAFMN	XP_002880383.1	Kinase family protein [Arabidopsis lyrata subsp. lyrata]	0.094	73	1	1	1	1
GSKRSVY03HESC7	AAF66615.1	LRR receptor-like protein kinase [Nicotiana tabacum]	3e-33	75	1	1	1	1
GSKRSVY03GBQJL	NP_201509.1	Mitogen-activated protein kinase kinase kinase 19 [Arabidopsis thaliana]	7e-21	60	1	1	1	1
GSKRSVY03GQ80Y	AAQ76042.1	Signal recognition particle receptor protein [Cucumis sativus]	1e-10	78	1	0	1	1
GSKRSVY03F8UTL	AAR87711.1	Salicylic acid-binding protein 2 [Nicotiana tabacum]	1e-10	63	0	0	1	1
Transcription factor								
GSKRSVY03GM222	NP_001152266.1	Basic helix-loop-helix (bHLH) transcription factor [Zea mays]	1e-23	72	1	0	0	0
GSKRSVY03FTAOQ	ACF60482.1	bZIP transcription factor [Oryza sativa Japonica Group]	1e-08	57	1	0	0	0
GSKRSVY03GK39R	AAS68190.1	Myb transcription factor [Vitis vinifera]	6e-27	89	1	0	1	1
GSKRSVY03GXVL4	AAM63665.1	Transcription factor, putative [Arabidopsis thaliana]	2e-33	89	1	1	1	1
GSKRSVY03GNKZW	AAD10237.1	TATA-box binding protein [Phaseolus vulgaris]	3e-06	100	0	0	0	1
GSKRSVY03GSUTC	CAD56217.1	Transcription factor EREBP-like protein [Cicer arietinum]	0.069	100	1	1	1	1
Oxidative burst								
GSKRSVY03GYLF9	ACO37154.1	ACC oxidase [Stenocereus stellatus]	4e-21	88	0	0	1	0
GSKRSVY03GI3OU	XP_002533075.1	Glutathione peroxidase, putative [Ricinus communis]	6e-12	79	1	0	0	0
GSKRSVY03G8YH8	AAF61392.1	Glutathione S-transferase [Persea americana]	8e-43	73	1	1	1	0
GSKRSVY03GTJHX	ACG39782.1	NADH-cytochrome b5 reductase [Zea mays]	9e-35	90	1	1	1	1
GSKRSVY03FT4FZ	BAD83480.2	NADH dehydrogenase subunit 4 [Nicotiana tabacum]	2e-16	100	1	1	0	0
GSKRSVY03F44AG	YP_784442.1	NADH-plastoquinone oxidoreductase subunit 1 [Drimys granadensis]	6e-23	100	1	1	0	1
Stress								
GSKRSVY03GPM0Z	ABE79560.1	Chaperone protein dnak (heat shock protein 70) [Medicago truncatula]	7e-19	53	1	1	1	1
GSKRSVY03GFJSV	XP_002514902.1	Stress associated endoplasmic reticulum protein, putative [Ricinus communis]	2e-09	100	1	0	0	1
GSKRSVY03GF2U4NP	192977.2	Stress-inducible protein, putative [Arabidopsis thaliana]	2e-22	77	1	0	1	1
GSKRSVY03GOQET	NP_191404.2	Universal stress protein family protein [Arabidopsis thaliana]	8e-29	86	1	0	1	1
Defence								
GSKRSVY03F3ZMXAAK	15049.1	Asparaginyl endopeptidase [Vigna radiata]	2e-20	66	1	1	0	0
GSKRSVY03GCUDV	NP_172655.1	Aspartic proteinase A1 [Arabidopsis thaliana]	4e-28	77	0	0	1	1
GSKRSVY03G5UE1AAD	30292.1	Catalase 3 [Raphanus sativus]	5e-57	76	1	1	1	1
GSKRSVY03FQV2BBAB	82473.1	Chitinase 3 [Triticum aestivum]	2e-34	82	0	1	0	0
GSKRSVY03HB7OOADQ	39593.1	Class II chitinase [Malus · domestica]	4e-06	82	1	0	0	1
GSKRSVY03GX18BABX	79341.1	Cysteine protease [Vitis vinifera]	4e-46	81	1	1	1	0
GSKRSVY03GNVEE	ABK78689.1	Cysteine proteinase inhibitor [Brassica rapa]	2e-15	75	0	1	1	1
SKRSVY03GNNXLCAB	01591.1	Endochitinase [Persea Americana]	2e-58	77	1	0	1	1
GSKRSVY03FYVXX	ABY58189.1	Endo-1,4-d-glucanase [Persea americana]	6e-48	100	1	0	0	1
GSKRSVY03F2JKPACG	44564.1	Endopeptidase Clp [Zea mays]	4e-28	52	0	1	0	1
GSKRSVY03GOA0N	AEE84132.1	Enhanced disease resistance 2 protein [Arabidopsis thaliana]	0.014	51	1	0	0	0
GSKRSVY03GC6XGAAF	97315.1	Lipoxygenase [Arabidopsis thaliana]	4e-08	72	1	0	0	0
GSKRSVY03FTET8XP	002527223.1	Oligopeptidase A, putative [Ricinus communis]	5e-09	86	0	0	1	0
GSKRSVY03GJCY8ABA	33845.1	Pathogenesis-related protein 6 [Zea diploperennis]	3e-16	59	1	0	0	1
Metabolism／photosynthesis								
GSKRSVY03GZDGLABI	18045.1							
GSKRSVY03GP4T5ADO	64899.1							
		Acetyl co-A carboxylase [Strombosia grandifolia]	4e-10	81	1	1	0	0
		Acetyl-CoA carboxylase carboxyltransferase beta subunit [Theobroma cacao]	1e-19	90	1	1	0	0
GSKRSVY03F9QFM	YP_004021302.1	ATP synthase CF1 alpha subunit [Theobroma cacao]	6e-90	99	0	0	1	0
GSKRSVY03G37BH	ACG42565.1	Calcium homoeostasis regulator CHoR1 [Zea mays]	8e-17	68	1	0	0	0
GSKRSVY03GALC8	ACG59771.1	Chloroplast aspartate aminotransferase [Triticum aestivum]	3e-14	94	0	0	1	0
GSKRSVY03FU0FO	AAL77589.1	Chloroplast ribose-5-phosphate isomerase [Spinacia oleracea]	1e-17	64	1	0	0	0

Table 3
Continued

Name	Accession number of similar sequence	Similar sequence from database BLASTx (accession number)	E-value	Max identity %	Expression EU	LU	EH	LH
GSKRSVY03F5V9Q	XP_002532986.1	Flavonol synthase/flavanone 3-hydroxylase, putative [Ricinus communis]	1e-04	65	1	0	0	0
GSKRSVY03GMBAO	CAP12013.1	Photosystem II protein Z [Coffea myrtifolia]	2e-06	94	1	0	1	1
GSKRSVY03HHL3Q	AAF15308.1	Stearoyl-acyl-carrier-protein desaturase [Persea americana]	9e-20	100	0	0	1	1
GSKRSVY03F83NGACO	40485.1	Terpene synthase [Actinidia deliciosa]	3e-17	58	1	0	0	0
GSKRSVY03G18AN	ACG45528.1	Ubiquitin-protein ligase [Zea mays]	2e-17	76	1	1	1	0
Protein synthesis/regulation								
GSKRSVY03G8FX4	BAB90396.1	ADP-ribosylation factor [Oryza sativa Japonica Group]	5e-05	100	0	0	1	0
GSKRSVY03GSJKM	ADB93067.1	Aquaporin [Jatropha curcas]	3e-31	87	1	0	0	0
GSKRSVY03FWAU0	AAM12952.1	Auxin-regulated protein [Zinnia violacea]	2e-06	53	1	0	1	1
GSKRSVY03GUM9Q	XP_002528028.1	Elongation factor 1-alpha, putative [Ricinus communis]	9e-51	100	1	1	1	1
GSKRSVY03G7MD1	CAA71882.1	Elongation factor 2 [Nicotiana tabacum]	9e-15	94	0	0	1	0
GSKRSVY03G7F0Q	XP_002513404.1	Eukaryotic translation elongation factor, putative [Ricinus communis]	3e-35	89	0	0	1	0
GSKRSVY03GX76M	AAZ75913.1	Ribosomal protein L16 [Coffea humilis]	0.014	100	1	1	0	0
GSKRSVY03GFXL2	BAD83474.2	Ribosomal protein S3 [Nicotiana tabacum]	0.001	100	1	0	1	0
Transportation								
GSKRSVY03GIDYK	CAB41144.1	H+-transporting ATPase-like protein [Arabidopsis thaliana]	3e-23	81	0	0	0	1
GSKRSVY03FW9PQ	XP_002526521.1	Peptide transporter, putative [Ricinus communis]	2e-15	62	0	1	0	1
GSKRSVY03FMG9J	XP_002526529.1	Protein transport protein sec23, putative [Ricinus communis]	7e-11	89	0	0	1	1
Cell structure and component								
GSKRSVY03F6K47ADN	34200.1	Annexin [Cucumis melo subsp. melo]	2e-16	70	1	0	0	0
GSKRSVY03GI44TACS	28251.1	Cell division control protein [Nicotiana glutinosa]	2e-33	90	1	1	1	1
GSKRSVY03GAQ1Y	NP_564367.1	Integral membrane HRF1 family protein [Arabidopsis thaliana]	1e-20	89	0	0	1	0

EH, early harvested; LH, late harvested; LU, late unharvested.

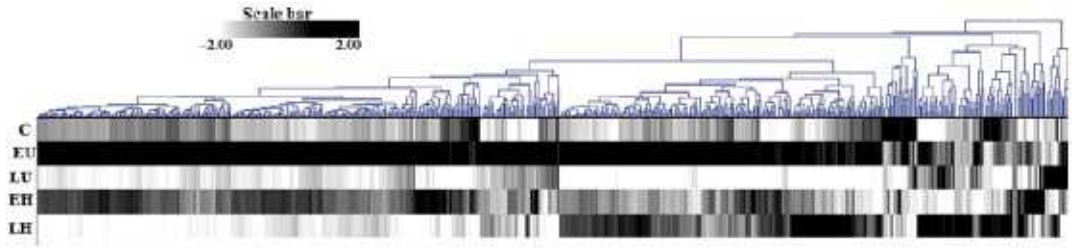

Fig. 2 Hierarchical cluster of avocado genes obtained from the 454 cDNA mapped reads with changes in their expression profiles in response to Colletotrichum gloeosporioides infection. The dendrogram on top lists the samples studied and provides a measure of the relatedness of gene expression in each sample. Each gene is represented by a single column of coloured boxes, and each row represents a time point. Black represents higher levels of gene expression; white represents lower levels of gene expression. Grey colours represent genes equally expressed. The fold change scale bar is shown above the cluster with ratios from)2 to 2

expressed. The result shows that the joining points of many subtrees are farther from the root. On the basis of the fact that the similarity of the nodes or subtrees is indicated by the distance from the root to a subtree, there is a high similarity between each group of genes (Eisen et al. 1998; Tang et al. 2001). In general, based on the colour mosaic display, the expression of most genes was up-regulated in the EU samples and down regulated in a part of the LU- and LH-infected samples. In the EH and some LH samples, the expression of some genes did not change when compared with the uninfected samples.

The details of the separated hierarchical clustering analysis (data shown in Supporting Information) of each group of genes obtained from the 454 cDNA mapped reads showed that senescence-associated genes were down regulated in all the unharvested infected samples and up-regulated in the harvested infected samples. Genes coding for some cytochrome proteins were highly expressed in both EU- and EH-infected samples and were significantly down regulated in the LU-infected samples compared with the uninfected samples. The expression of hypothetical proteins was high and down regulated in LU- and EH-infected samples, respectively. Proteins involved in metabolism were differentially expressed in all the infected samples as well, and an up regulation of the defence genes was noticed in the EH-infected samples. Lower and higher

expressions of genes coding for transcription factors and proteins involved in signal transduction were observed respectively in the LU- and EH-infected samples during C. gloeosporioides attack. Genes coding for proteins involved in electron transport and stress response exhibited similar expression patterns, and an up regulation in their expression profile was observed in the EU, EH, LH samples and a down regulation in the LU samples. Finally, in the unharvested and EH-infected samples, a high gene expression and a slight down regulation in the LH-infected samples were observed for genes predicted to be involved in protein synthesis and photosynthesis.

Reverse transcription PCR

PCR products from avocado flesh cDNA amplified with defence-related gene primers produced single bands of between 100 and 200 bp, depending on the primer sets used confirming that the sequences obtained from the 454 sequencing are those of avocado fruit (Fig. 3).

Discussion

The infection process by C. gloeosporioides was successful as shown by SEM observation (Fig. 1). We produced approximately 215 781 reads from avocado fruit transcriptome in a one-quarter run with the Roche 454 GS FLX Titanium platform. From the anthracnose symptoms exhibited by the fruits and the transcriptome sequencing, it is clear that numerous genes and the products of many of these genes are directly or indirectly involved in the interaction between avocado fruit and C. gloeosporioides. In addition, the blastn revealed that many sequences obtained showed similarities at the nucleotide level (Altschul et al. 1990) with other species belonging to the order Laurales such as Cinnamomum camphora, Peumus boldus, Gyrocarpus americanus, Calycanthus occidentalis and Gomortega keule. cDNA sequences generated from the uninfected and infected fruits cover various biological activities and molecular functions indicating that 454 sequencing constitutes a powerful tool for sequencing the transcriptome and gene discovery of non-model species such as avocado. The expression profiles of the genes measured by quantification during assembly process and hierarchical clustering analysis, which are two independent methods, revealed that many categories of genes were differentially expressed in avocado fruits following C. gloeosporioides infection.

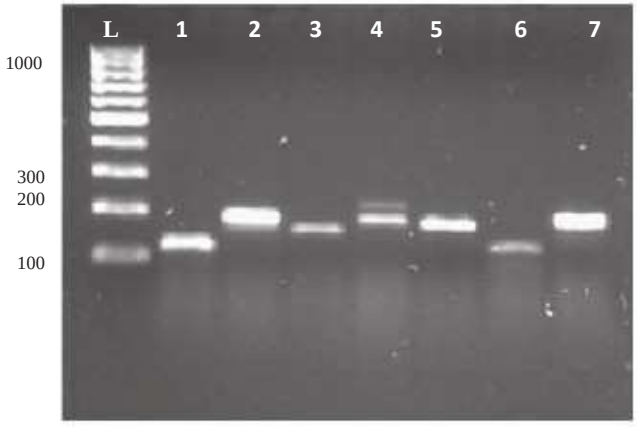

Fig. 3 PCR products amplified from avocado flesh cDNA using specifics primers separated on 2% non-denaturing agarose gel containing EtBr and photographed under ultraviolet light. L, GeneRulerÔ 100 bp DNA Ladder. Lane 1, catalase (162 bp). Lane 2, endochitinase (186 bp). Lane 3, pathogenesis-related protein 6 (158 bp). Lane 4, CC-NBS resistance protein (185 bp). Lane 5, pathogenesis-related protein 5 (171 bp). Lane 6, endo-1,4-d-glucanase (121 bp). Lane 7, actin gene (166 bp)

Signal transduction genes

Mitogen-activated protein kinases are serine/threonine-specific protein kinases that participate in transducing extracellular stimuli to the host genome and would be activated after C. gloeosporioides infection to enable pathogen recognition and to stimulate plant responses. It has been shown that MAPKs in several plant species are activated during plant responses to elicitors or pathogens (Zhang and Klessig 2001). Because the defence signalling pathways that lead to the plant–pathogen response are activated after elicitor binding to receptors, it is more probably that LRR receptor-like protein kinase and signal recognition particle receptor protein were expressed to mediate the fungus recognition by the plant (Dangl and Jones 2001). The expression of salicylic acid-binding protein may suggest that salicylic acid, which is involved in the activation of various plant defence responses following pathogen attack, plays an important signalling role following C. gloeosporioides infection in avocado (Hammond-Kosack and Parker 2003). Calcium ion-binding protein and calcium-dependent protein kinase were also expressed, indicating Ca^{2+} signalling activities in avocado following infection. Previous studies revealed that the fluctuations in cytosolic Ca^{2+} levels that are mediated by Ca^{2+} permeable channels located at the plasma membrane of the plant cell can serve as a regulation of the plant response to pathogen invasion (White and Broadley 2003).

Transcription factors

In many plant species, the transcription factor WRKY and Myb genes expressed in avocado are strongly and quickly up-regulated in response to pathogen attack, wounding or abiotic stresses. Studies demonstrated that in tobacco, multiple WRKY genes are induced after infection with bacteria or tobacco mosaic virus, or treatment with fungal elicitors SA or H_2O_2 (Takemoto et al. 2003).

Genes involved in stress responses

DnaK-like chaperone protein (heat shock protein 70) and others presented in the results known to be involved in stress response during plant interaction with a pathogen were expressed in all the infected samples. During stress, avocado fruit would adapt its metabolism and activate a large variety of physiological and biochemical changes to repair damages or to

protect their cells from the effects of stress caused by C. gloeosporioides. For instance, heat shock proteins 70/DnaK-like chaperone protein families are known to be in charge of protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membranes and can also serve to assist in protein refolding under stress conditions (Wang et al. 2004). Previous studies showed that under biotic and abiotic stress, the transcriptome and proteome sometimes change rapidly and dramatically (Watson et al. 2003; Rampitsch and Srinivasan 2006). On the basis of this fact, it could be suggested that DnaK-like chaperone protein families were expressed in avocado to stabilize the proteins and membranes and to assist in protein refolding under stress condition initiated by C. gloeosporioides.

Defence response/resistance genes

Another category of genes differentially expressed in response to C. gloeosporioides infection are genes involved in defence response such as b-glucanases, endochitinases and endopeptidase known to have antifungal activity (Van Loon and Van Strien 1999) and plant aspartic proteinase that exhibit antimicrobial activity. Other genes expressed are known to be involved in various processes of plant defence against pathogens, such as cell death related to hypersensitivity response, construction of a physical barrier to block the pathogen progression, as well as systemic resistance. We identified elongation factor 1 that is involved in controlling the extent of the cell death in the defence response and acetyl co-enzyme A carboxyltransferase involved in the regulation of resistance gene expression (Barakat et al. 2009). Genes that encode proteins involved in lignin biosynthesis, such as cinnamoyl-CoA reductase, a key enzyme in lignin biosynthesis (Kawasaki et al. 2006) and cytochrome P450 monoxygenases (Barakat et al. 2009), were also expressed. It is well established that lignification is a mechanism for disease resistance in plants, which leads to an ultra-structurally modified reinforced cell wall (Bhuiyan et al. 2009).

Genes involved in photosynthesis and plant metabolism

Other identified genes involved in plant metabolism and photosynthesis were also expressed after C. gloeosporioides infection. When plants are under stress caused by fungal pathogens, their metabolism of carbohydrates, lipids, proteins and nucleic acid are also affected (Agrios 2005; Baldo et al. 2010). Activation of signal transduction network after pathogen recognition results in reprogramming of cellular metabolism, which leads to a large change in gene activity. For instance, the mRNA and protein levels of ribulose-1,5-bisphosphate carboxylase oxygenase, in potato, are drastically reduced by pathogen infection or elicitor treatment (Somssich and Hahlbrock 1998). The induction of some photosynthetic genes such as ribulose-1,5-bisphosphate carboxylase/oxygenase, photosystem I assembly protein, photosystem II protein and chloroplast NADH dehydrogenase, during the interaction between avocado and C. gloeosporioides infection, may implicate light-sensing mechanisms in the induction of plant disease defence signalling. Previous studies showed that there is a link between plant defences system against pathogen and both light-sensing network and the oxygen-evolving complex in Photosystem II (Abbink et al. 2002; Genoud et al. 2002). Another functional category observed in the avocado transcriptomes are genes encoding proteins predicted to function in oxidation processes. Such genes may play a crucial role in energy production during response to the pathogen infection. For instance, ATP synthase expressed in avocado fruit is an important enzyme that creates energy for the cell to use through the synthesis of adenosine triphosphate (Thilmony et al. 2006).

Unexpressed genes

Finally, there were gene sequences that were not expressed in infected fruit (Table 4), among which is the gene for limonene synthase, an enzyme that catalyses the stereo-specific cyclization of geranyl diphosphate, the universal C_{10} precursor of the monoterpenes, to form a monocyclic monoterpene, limonene (Ohara et al. 2003). Monoterpenes are important constituents of plant essential oils, and limonene would likely be an essential contributor to the flavour and fragrance of avocado and its value to the cosmetic industry (Ohara et al. 2003). Non-expression of this gene in infected samples due to C. gloeosporioides infection illustrates an aspect of the economic consequences of the disease to the avocado industry. Malic enzymes are involved in a number of important metabolic processes requiring the conversion of malate to NAD(P)H, pyruvate and CO_2. In plants, these enzymes contribute to C_4 photosynthesis, pH-balancing mechanisms and fruit-ripening processes (Drincovich et al. 2001), and repression of the gene indicates the negative effects of anthracnose on essential components of avocado fruit metabolism. It would appear from Genbank that sequences controlling induction of JA are also not expressed in infected avocado fruits although it has been demonstrated that it mediates resistance to insect pathogen attack and abiotic stress responses to wounding and ozone (Kunkel and Books 2002; Voelckel and Baldwin 2004). JA also functions in signal transduction between pathogenicity and resistance genes in many plants (Agrios 2005), but it does not appear to be involved in signal transduction during avocado response to C. gloeosporioides attack.

Responses in unharvested vs. harvested fruits

Most of the defence-related genes identified were expressed in unharvested avocado fruits, which could also explain why they developed only minor anthracnose symptoms. This observation was also made by Marimani (2011) who monitored the levels of antifungal diene and triene compounds [(Z, Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene and (Z, Z, E)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-5,12,15-triene] and observed a rapid increase in these compounds

Table 4
Summary of genes expressed only in the uninfected avocado fruits with their putative function

Name	Accession num of similar sequence	Similar sequence from database (BLAST X)	Putative function	E-value	Max identity %
GSKRSVY03GJAYX	AAM21683.1	1-aminocyclopropane-1-carboxylate synthase 1 [Persea americana]	Lyase (metabolism)	1e-27	96
GSKRSVY03G4BFJ	XP_002534067.1	Calcium-activated outward-rectifying potassium channel, putative [Ricinus communis]	Ion transport	1e-23	83
GSKRSVY03F9L77	CAC00657.1	Common plant regulatory factor 6 [Petroselinum crispum]	Regulation	2e-06	70
GSKRSVY03GK9XD	ABB86962.1	Cytosolic NADP-malic enzyme [Malus · domestica]	Oxidation	2e-21	95
GSKRSVY03HEJ9S	NP_001150497.1	Early nodulin 93 [Zea mays]	Cell structure	3e-08	73
GSKRSVY03GW9XX	ACN38307.1	Eukaryotic translation initiation factor 4e [Carica papaya]	Protein synthesis	1e-39	72
GSKRSVY03F39EF	AAF04915.1	Jasmonic acid 2 [Solanum lycopersicum]	Signal transduction	1e-38	66
GSKRSVY03FPBYD	ACG35116.1	Legumin-like protein [Zea mays]	Nutrient Reservoir Activity(metabolism)	3e-31	74
GSKRSVY03G4MFH	XP_002533356.1	(R)-limonene synthase [Ricinus communis]	Lyase (metabolism)	3.8	66
GSKRSVY03G7EPV	XP_002521801.1	Patellin-3, putative [Ricinus communis]	Transport/cell Division	6e-27	82
GSKRSVY03G27MX	YP_740231.1	Photosystem II phosphoprotein [Liriodendron tulipifera]	Photosynthesis	8e-43	97
GSKRSVY03F0YJ9	CAB77245.1	Putative seed imbibition protein [Persea americana]	Protease (defence)	2e-50	90
GSKRSVY03G55F0	NP_001147686.1	Seven-transmembrane-domain protein 1 [Zea mays]	G protein-coupled receptors (signal transduction)	7e-11	60

1–2 days after inoculation of both harvested and unharvested Fuerte fruits with C. gloeosporioides 240 days after fruit set, followed by a decline within 7 days to uninoculated control or below control levels; however, levels in unharvested fruit were higher (Marimani 2011). Previous studies (Prusky et al.1990, 1991a,b; Domergue et al. 2000) have implicated these preformed antifungal compounds in the resistance of Fuerte avocado to fungal attack in unripe fruit and the development of quiescent infections. The level of these compounds is regulated either by its enhanced synthesis or by the inhibition of its breakdown. In this study (Table 3), the enzyme lipoxygenase was expressed in unharvested fruits during the early response to the fungal attack. A previous study revealed that lipoxygenase is involved in catalysing the metabolism of the diene that leads to an increase in its levels (Prusky and Keen 1995). In addition, stearoyl-acyl-carrier-protein desaturase (Table 2) expressed in the infected fruits is involved in diene biosynthesis primarily by increasing the level of diene precursors, which can be converted into the antifungal diene (Leikin-Frenkel and Prusky 1998; Madi et al. 2003). In the biosynthesis of diene, the desaturation step is also crucial for the creation of the multiple double bonds in the diene and triene compounds (Madi et al. 2003). These authors have demonstrated that induction of desaturases, such as stearoyl-acyl-carrier-protein desaturase and lipid desaturation, is an early component of the array of plant responses involved with defence against C. gloeosporioides. Our study has confirmed the activation of the enzymes involved with the biosynthesis of antifungal compounds in avocado, at the nucleotide level.

In conclusion, our research provides a first comprehensive survey of the biological response of avocado fruit to C. gloeosporioides infection at the transcriptome and molecular level. In addition, it demonstrates that the plant has a sophisticated molecular system for pathogen recognition and activation of structural and biochemical defence mechanisms. These findings could contribute to the design of effective disease management strategies to improve the resistance of avocado varieties to anthracnose disease. For instance, one could develop molecular markers for anthracnose resistance genes that could be used in plant breeding to produce more anthracnose-resistant Fuerte cultivars. However, in a transcriptome analysis of this nature, it is essential that the sequence reads represent the true expression level as the PCR step in the library generation can falsify the reads. Additional quantification is required using a method such as qPCR. For verification of the gene expression obtained from the transcriptome sequencing analysis, real-time PCR (qPCR) was performed for selected genes. Genes were selected firstly on the basis of their putative function in plant defence and their presence revealed by 454 sequencing analysis in at least two time points. The defence-related genes, catalase and endochitinase, were chosen for qPCR, and these data will be presented in a sequel paper that concentrates on expression of avocado fruit genes specifically related to defence and resistance.

Acknowledgements

We acknowledge financial support from the National Research Foundation, South Africa (ICD2007052500003: 2008–2011), the University of the Witwatersrand Postgraduate Merit Award, the Bradlow Award and the Oppenheimer Memorial Trust. We thank

Mr Don Taylor, the owner of Roodewal farm, and Mr Musa Marimani for technical assistance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Hierarchical functional gene clusters obtained from the 454 cDNA mapped reads. A = senescence associated proteins, B = cytochromes,C = hypotheticalproteins,D = proteins involved in metabolism.

Figure S2. Hierarchical functional gene clusters obtained from the 454 cDNA mapped reads. E = proteins involved in defence response, F = transcription factors and proteins involved in signal transduction, G = proteins involved in electron transport and stress response, H = proteins involved in protein synthesis and photosynthesis.

Table S1. Primer sequences used in this work.

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